Estradiol regulates AQP2 expression in the collecting duct – a novel inhibitory role for estrogen receptor alpha

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Abstract

While there is evidence that sex hormones influence multiple systems involved in salt and water homeostasis, the question of whether sex hormones regulate aquaporin 2 (AQP2) and thus water handling by the collecting duct has been largely ignored. Accordingly, the present study investigated AQP2 expression, localization and renal water handling in intact and ovariectomized (OVX) female rats, with and without estradiol or progesterone replacement.

OVX resulted in a significant increase in urine osmolality and increase of p256-AQP2 in the renal cortex at 7 days post-OVX, as well as induced body weight changes. Relative to OVX alone, estradiol repletion produced a significant increase in urine output, normalized urinary osmolality and reduced both total AQP2 (protein and mRNA) and p256-AQP2 expression, whereas progesterone repletion had little effect. Direct effects of estradiol on AQP2 mRNA and protein levels were further tested in vitro using the mpkCCD principal cell line. Estradiol treatment of mpkCCD cells reduced AQP2 at both the mRNA and protein level in the absence of deamino-8-ᴅ-AVP (dDAVP), and significantly blunted the dDAVP-induced increase in AQP2 at the protein level only. We determined that mpkCCD and native mouse collecting ducts express both estrogen receptor (ER)α and ERβ, and that female mice lacking ERα displayed significant increases of AQP2 protein compared to WT littermates, implicating ERα in mediating the inhibitory effect of estradiol on AQP2 expression.

These findings suggest that changes in estradiol levels, such as during menopause or following reproductive surgeries, may contribute to dysregulation of water homeostasis in women.

Keywords: AQP2, estrogen, αER KO mice
Introduction

The kidney plays a central role in the maintenance of body fluid homeostasis, filtering approximately 180 L of plasma per day and reabsorbing all but a tiny fraction to maintain salt and water balance. The terminal portion of the nephron, the collecting duct, is a key site of water reabsorption (reviewed in (32)). Permeability of the collecting duct to water, and thus its ability to passively reabsorb water from the tubular lumen into the hypertonic interstitium of the kidney, is achieved via insertion of aquaporin 2 (AQP2) water channels into the apical membrane of collecting duct principal cells (reviewed in (32)). Trafficking and insertion of AQP2 into the apical membrane is tightly regulated by the hormone arginine vasopressin (AVP), acting via the V2 receptor (V2R) (5). The intracellular signalling cascade of AVP increases cAMP and protein kinase-dependent phosphorylation of AQP2 at e.g. Ser-256 (p256-AQP2) which is critical for trafficking to the apical membrane (28; 29; 31; 45). This mechanism allows concentration of the urine and conservation of body water in response to dehydration as sensed centrally by osmoreceptors.

Our current understanding of the influence of sex and sex hormones on water homeostasis is far from complete and the literature abounds with conflicting findings, likely due to the complexity of the system. Several studies suggest that sex hormones can influence the threshold of osmoreceptors, and either enhance or attenuate AVP release (reviewed in (36)). In addition to fluid retention experienced during pregnancy and the luteal phase of the menstrual cycle, a number of studies in humans have reported that women are more sensitive than men to the anti-diuretic effects of AVP (15; 38) which may underlie their increased risk of developing hyponatremia, for example in response to administration of the AVP analog desmopressin (20). Liu and colleagues (26) recently reported that whole kidney V2 receptor expression is approximately 2-fold higher in female rats compared to male rats, providing a possible explanation of the enhanced sensitivity of females
to agonists of the V2 receptor. Regardless, the molecular mechanisms underlying sex differences in renal V2 receptor expression and sensitivity are currently unknown.

While the studies discussed above have focused on AVP and the V2 receptor, relatively less is known regarding potential sex-hormonal effects on their downstream target in the collecting duct, AQP2. It has been demonstrated that inner medullary AQP2 is increased in pregnant rats suggesting that AQP2 might play a role in water retention related to pregnancy (33). Furthermore, expression of AQP2 by endometrial cells has been shown to correlate with estrogen levels in women (6), and a recent study identified an estrogen response element in the AQP2 gene promoter (46), providing a molecular basis for sex hormone regulation of AQP2. A small number of studies have also reported that the kidney expresses both estrogen receptor α (ERα) and estrogen receptor β (ERβ) (6; 17; 23; 44), although which of these receptors, and which of the ERβ splice variants, specifically localize to the collecting duct is not known.

The present study was designed to investigate if sex hormones, specifically estradiol and progesterone, can influence the expression and trafficking of AQP2. Particularly, the studies examined whether ovariectomy (OVX) affected renal water handling in female rats, and investigated whether estradiol or progesterone replacement affected expression of AQP2 and the phosphorylated form p256-AQP2 in renal cortex and inner medulla (IM). To further clarify the role of estradiol in collecting duct the expression of AQP2, ERα and ERβ in the collecting duct was also investigated, and AQP2 levels measured in mpkCCD cells in response to estradiol, and in mice lacking ERα (termed αER knockout mice).
Methods

Preparation of intact and ovariectomized rats

The rat protocols were approved by the board of the Institute of Clinical Medicine, Aarhus University, according to the license for use of experimental animals issued by the Danish Ministry of Food, Agriculture and Fisheries. Studies were performed on female Munich-Wistar rats initially weighing 225 ± 20 grams (Møllegaard Breeding Centre, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water. During the entire experimental period, rats were kept in individual cages, with a 12:12-h light-dark cycle, a temperature of 21±2°C, and a humidity of 55±2%. After a period of acclimation, rats were anesthetized with servoflurane (Abbott Scandinavia), placed on a heating table to maintain body temperature at 37°C during surgery and subjected to OVX. Through an abdominal midline incision the ovaries were exposed. The fallopian tubes were ligated and bilateral OVX performed. An additional group of rats underwent a sham operation (abdominal midline incision and ovarian exposure without ligation of fallopian tubes or removal of ovaries) and served as a control group. Further, vaginal smears were examined in all the rats to monitor ovarian cycle and the rats with intact ovaries displayed consistent 4-day estrous cycles. Rats were allocated to the following protocols:

Protocol 1: 1) OVX (n = 11): OVX was performed and the rats were treated with subcutaneous sesame oil as vehicle for 7 days after OVX. 2) OVX+E (n = 11): OVX was performed and the rats were treated with subcutaneous estradiol (Sigma, Copenhagen, Denmark) in sesame oil (25 µg/kg/day) for 7 days after OVX. 3) OVX+P (n = 11): OVX was performed and the rats were treated with subcutaneous progesterone (Sigma, Copenhagen, Denmark) in sesame oil (10 mg/kg/day) for 7 days after OVX. 4) Sham-operated controls were prepared in parallel (n = 11). After 7 days, the kidneys were prepared for semiquantitative immunoblotting and
immunohistochemistry (IHC). Protocol 2 is the same as Protocol 1 but rats were treated for 14 days, rather than 7 days.

**Clearance studies**

The rats were maintained in metabolic cages, allowing daily quantitative urine collections and measurements of food and water intake. Blood was collected from the abdominal aorta at the time of sacrifice. Urine volume and osmolality, and plasma osmolality were measured with a vapor pressure osmometer (Osmomat 030, Gonotec, Berlin, Germany).

**Serum progesterone and 17β-estradiol**

Serum was also collected in order to measure 17β-estradiol and progesterone levels. Serum progesterone and 17β-estradiol levels were measured by competitive immunoassay using direct chemiluminescent technology (ACS: Centaur, Chiron Diagnostics).

**αER knockout mice**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. αER knockout mice were bred in-house from founders that were a kind gift of Dr. Kenneth Korach (NIH). The mice were created on the C57BL/6 background employing Cre/lox technology to globally-delete exon 3 using sox2-driven Cre, preventing expression of ERα66 as well as all known ERα splice variants in the entire body (14). Kidneys of female αER knockout and control mice (C57BL/6 background) were rapidly removed following deep anesthesia with ketamine/xylazine followed by thoracotomy, and processed for immunoblotting or immunohistochemistry as described below.

**Cell culture**

MpkCCD cells were cultured as previously described (11). Cells were seeded at a density of \(1.5 \times 10^5\) cells/cm\(^2\) on semipermeable filters (pore size: 0.4 \(\mu\)M, Transwell, Corning Costar,
Cambridge, MA) and cultured for 8 days. Unless stated otherwise, cells were exposed to 1 nM of the stable AVP analog deamino-8-d-AVP (dDAVP) at the basolateral side for 4 days to induce AQP2 expression (22). Estradiol (Sigma, Copenhagen, Denmark) was used at a concentration of 50 nM and added to both sides of the filters for 24 h.

**Protein isolation for immunoblotting**

Rat renal tissue (cortex + outer medulla (C+OM) and inner medulla (IM)) was homogenised in dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2) containing the following protease inhibitors: 8.5 µM leupeptin (serine and cysteine protease inhibitor, Sigma Aldrich, USA) and 0.4 mM Pefabloc (serine protease inhibitor, Roche A/S, Denmark). The tissue was homogenised for 30 sec at 1250 rpm by an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Germany) and then centrifuged at 4500 G at 4°C for 15 min. Mouse renal tissue was homogenized in a pH 7.4 buffer containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10% glycerol plus protease inhibitors, and centrifuged at 10,000 rpm for 5 min. MpkCCD cells were collected and lysed using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Vedbaek, Denmark). Cell suspension were sonicated and centrifuged for 1000 g for two min at room temperature. Gel samples were prepared from the supernatant in Laemmli sample buffer containing 2% SDS. The total protein concentration of the homogenate was measured using a Pierce BCA protein assay kit (Roche, Denmark).

**Electrophoresis and immunoblotting**

Protein samples were run on 12% polyacrylamide minigels (Bio-Rad Mini Protean II). Proteins were transferred to a nitrocellulose membrane (Hybond ECL RPN 3032D, Amersham Pharmacia Biotech, UK) or PVDF membrane (mouse renal tissue; Immobilon-FL, Millipore, USA). Afterwards the blots were blocked with 5% non-fat dry milk or Li-Cor Odyssey Blocking buffer
(mouse tissue; Li-Cor Biosciences, USA) in PBS-T (80 mM Na$_2$HPO$_4$, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, 0.1 Tween 20, adjusted to pH 7.4). After washing with PBS-T the blots were incubated with primary antibodies overnight at 4°C. Antigen-antibody complex was visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P448, diluted 1:3000, DAKO, Denmark) using enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, UK). For mouse tissue, immunoreactivity was visualized using fluorescently tagged secondary antibodies (Rockland Immunochemical, USA and Li-Cor Biosciences for IRDYE800 conjugates; Molecular Probes, USA for Alexa Fluor 680 conjugates) and a Li-Cor Odyssey infrared imaging system. Immunolabeling controls were performed using peptide-absorbed antibody.

**Immunohistochemistry**

Fixed rat kidneys were dehydrated in graded ethanol and left overnight in xylene. The tissue was embedded in paraffin wax, cut into 2 μm thick sections on a rotary microtome (Leica). Sections were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase was blocked in 35% H$_2$O$_2$ in methanol. To retrieve antigens, sections were boiled in TEG-buffer with 10 mM Tris, 0.5 mM EGTA, at pH 9. Aldehydes were quenched in 50 mM NH$_4$Cl in PBS, and the sections were blocked in 1% BSA, 0.2% gelatin, 0.05% Saponine in PBS. Then, sections were incubated with primary antibody diluted in in PBS with 0.1% BSA, 0.3% Triton X-100 overnight at 4°C. For immunofluorescence staining, fluorophore-tagged secondary antibodies were applied (see below). Where indicated, Topro3 (Invitrogen) was applied as a nuclear stain.

Kidneys of female C57BL/6 mice underwent processing for immunofluorescent staining of estrogen receptors and AQP2 (as a marker for collecting ducts) as previously described (17). Slides were visualized at 630x using a Leica Confocal microscope (Leica TCS SP5, Leica Microsystems). To confirm localization and expression of estrogen receptors in collecting ducts, mouse inner medullary collecting ducts were isolated as previously described (16).
Isolated kidney tubules preparation

Cortical collecting ducts were isolated using immunomagnetic separation by preparing a collagenase-digested cortical tubular suspension (as per Vesey et al (40) with minor modifications), labeling principal cells with biotinylated anti-AQP2 antibodies (SC-9882 biotinylated using DSB-X™ Biotin Protein Labeling kit, Life Technologies, Eugene, OR according to the manufacturer's directions), then selectively retrieving labeled collecting duct fragments with streptavidin-coated magnetic beads (Dynabeads FlowComp Flexi kit, Life Technologies AS, Norway). AQP1 detection in this preparation was reduced compared to whole cortex but not absent, indicating depletion of proximal tubules but some residual contamination (data not shown). To obtain a proximal tubule-enriched preparation for comparison, retrograde aortic perfusion of the kidneys post-mortem with Dynabeads Protein G (Life Technologies AS, Norway) was performed to allow magnetic depletion of glomeruli from the subsequently prepared collagenase-digested cortical tubular suspension, and proximal tubules obtained using the Percoll density gradient centrifugation method, with the distal tubular fraction also included for comparison (Vinay et al (41) with minor modifications). Glomerular contamination of the proximal tubular preparation was minimal, as assessed by Western blot for synaptopodin (sc-21536, Santa Cruz Biotechnology; data not shown), and AQP2 was undetectable, verifying a lack of contamination with collecting ducts (data not shown). Isolated cortical and inner medullary collecting ducts, and proximal tubules then underwent immunoblot analysis for estrogen receptors (as described above), with uterine tissue serving as a positive control.

Confocal microscopy and image processing for semiquantitative analysis of AQP2 in rats

Fluorescence imaging was performed on a Leica DM IRE2 inverted confocal microscope using a Leica TCS SP2 laser and an HCX PC APO CS 63x/1.32 NA oil objective. For the semiquantitative analysis, microscope settings (PMT offset and gain, sampling period and
averaging) were kept similar for all rats (4 rats in each group). Dynamic range was adjusted, so the sections with most intense fluorescent signal only had a few saturated pixels. From each rat, five images of cortical collecting duct tubular segments labeled with AQP2 and pSer256-AQP2 were acquired. Confocal microscopic images were analyzed with Image J software to evaluate the total pixel intensity of each image in relation to the total area of labeled CCD tubules, thus providing semiquantitative data of both AQP2 and pSer256-AQP2 labeling abundance per CCD tubule. The measurements were adjusted to the cell size in order to take variations in cell morphology into account. Multiple regions of interest (five per each image), representing single cross sections through labeled CCD cells (25 cells per rat), were selected randomly and the distribution of apical fluorescent intensity was measured. Results are shown as normalized apical fluorescent intensity relative to control and mean values ± SEM are calculated per group (n = 4 rats per group). Data was analyzed using GraphPad Prism Software. Statistical comparison was performed by one-way ANOVA followed by post hoc analysis with t-test and Bonferroni correction. P values < 0.05 were considered significant.

**Primary and secondary antibodies**

For semiquantitative immunoblotting and immunofluorescence, we used specific antibodies to AQPs, which had been well characterized in previous studies: AQP-2 (H7661) (30), pAQP2Ser256 (KO407) (5). C-terminal specific rabbit anti-ERα (SC-542), rabbit anti-ERβ (SC-8974) and goat anti-AQP-2 (SC-9882) from Santa Cruz Biotechnology (Santa Cruz, CA); an N-terminal specific rabbit anti-ERμ (04-820) from EMD Millipore (Billerica, MA); mouse anti-α-actin (A1978) from Sigma (St. Louis, MO). Rabbit anti-ERα36 (1:150 IF) was a kind gift of Dr. Zhao Yi Wang (Creighton University (42)). Secondary antibodies used were Alexa Fluorophore AF-488 donkey anti-rabbit IgG (A21206; Invitrogen; Grand Island, NY), AF-594 donkey anti-goat
IgG (A11058; Invitrogen), AF-790 goat anti-rabbit IgG, AF-680 goat anti-mouse IgG and IRDye800CW donkey anti-goat IgG (Rockland Immunochemicals Inc., Gilbertsville, PA).

**RNA extraction and PCR**

For total RNA extraction, mpkCCD cells were lysed in TRIzol (Invitrogen, Taastrup, Denmark) and treated with chloroform according to manufacturer’s instructions. Total RNA from rat tissue was isolated using the Machery-Nagel’s Nucleospin® RNA II kit (AH Diagnostics, Aarhus, Denmark). cDNA synthesis was performed with Fermentas RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Burlington, Ontario) according to the manufacturer’s instructions. PCR was performed using 100 ng cDNA as a template for PCR amplification. We used Maxima® Hot Star PCR Master Mix according to the manufacturer’s instruction (Thermo Scientific, Slangerup, Denmark). GAPDH and β-actin were used as an internal control. Reaction products were separated on agarose gels and imaged. Nested primers were used to amplify standards and kidney cDNA samples: AQP2: sense 5’-CTT CCT TCG AGC TGC CTT C-3’; antisense 5’-CAT TGT TGT GGA GAG CAT TGA C-3’; Genebank acc. NM_009699.2. V2R: sense 5’-TGT GTT GCT CAT GCT GCT GGC TAG CCT TA-3’; antisense 5’-TCA GGA GGG TGT ATC CTT CAT CAA AGA GGA-3’. Genebank acc. NM_019136.1. ERα: sense 5’-ACC AT T GAC AAG AAC CGG AG-3’; antisense 5’-ACC ATT GAC AAG AAC CGG AG-3’. Genebank acc. NM_007956.4, 170 bp. ERβ: sense 5’-GAA GCT GGC TGA CAA GGA AC-3’; antisense 5’-AAC GAG GTC CTC CCT CAT CG-3’. Genebank acc. NM_029771.3, 252 bp. β-actin: Sense 5’-CTG TGG TGG TGA AGC TGT AG-3’, antisense 5’-TCA TGC CAT CCT GCG TCT-3’. Genebank acc. NM_031144. GAPDH: sense 5’-TAA AGG GCA TCC TGG GCT ACA CT-3’; antisense 5’-TTA CTC CTT GGA GGC CAT GTA GG-3’; Genebank acc. M32599.1. AQP2 mRNA in the renal cortex of αER knockout and control mice was
assessed by quantitative RT-PCR using commercially available primers (QuantiTect Primer Assays, Qiagen, Valencia, CA), with β-actin serving as a control, as previously described (3).

cAMP measurement

MpkCCD cells were grown with or without dDAVP during the last 4 days, with or without 50 nM estradiol for 24 h, with the last 30 min in combination with 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, Sigma, Copenhagen, Denmark). After lysis, intracellular cAMP levels were measured using a cAMP enzyme immunoassay kit (Sigma, Copenhagen, Denmark) according to the manufacturer’s instructions. Results were related to a standard curve on the measurement of cAMP solutions. All measurements were performed in triplicate.

Statistics

Values are presented as means ± SEM. Statistical comparison of 2 experimental groups was performed by unpaired student’s t-test. When several groups were compared this was done by one-way ANOVA followed by post hoc analysis with t-test and Bonferroni correction. P values < 0.05 were considered significant.
Results

Effect of estradiol and progesterone on body weight, food and water intake in OVX rats

All animals survived after surgery. To evaluate the success of OVX and hormone replacement in the rats, serum estradiol and progesterone levels were measured. At both 7 and 14 days post-OVX, serum estradiol and progesterone levels were significantly reduced compared to sham operated control rats, and this decrease was reversed by estradiol and progesterone replacement (Table 1).

To investigate the effect of estradiol and progesterone in OVX rats on whole body turnover of water and food metabolism, rats were placed in metabolic cages and body weight, food and water intake as well as urine output were compared between the different groups. Bodyweight of OVX rats increased over the course of the experiment, becoming statistically greater than that of sham operated rats by day 7 (Fig. 1A). This increased weight gain in the OVX group was accompanied by significantly greater (both absolute and when normalized to body weight) food intake compared with sham operated rats, as measured at days 7 and 14 (Fig. 1B). Estradiol replacement prevented the OVX-induced weight gain and normalized food intake, whereas body weight and food intake were not significantly affected by progesterone treatment. Neither OVX nor estradiol or progesterone administration had any significant effect on absolute nor relative (body weight normalized) water intake, which was similar in all 4 groups (Fig. 1C). There was no significant difference in kidney weight between the groups (data not shown).

Effect of estradiol and progesterone on urine output and osmolality in OVX rats

Urine volume (absolute) and urine output (normalized to body weight) were significantly increased in the estradiol-treated OVX rats compared to untreated OVX rats after 2 and 7 days (Fig. 2A). Urine osmolality was increased in the OVX rats compared to sham-operated control rats after 2 and 7 days (Fig. 2B). Estradiol treatment of OVX rats reduces urine osmolality compared to
untreated OVX rats after 7 days (Fig. 2B). Progesterone replacement had no significant effect on urine output and osmolality in response to OVX. To analyze whether the OVX rats exhibit water retention we measured plasma osmolality and plasma sodium levels. However, our data showed no difference in plasma osmolality or sodium among the four groups (data not shown). Furthermore, there was no significant difference between the 4 groups in urine output or osmolality at 14 days after OVX (Fig. 2A-B). Together, these data demonstrate that estradiol might play a role in the regulation of renal water reabsorption.

**Effect of estradiol and progesterone on AQP2 and p256-AQP2 expression in OVX rats**

Since AQP2 regulates renal water reabsorption across the collecting duct epithelium, we examined the effect of estradiol and progesterone on the expression of AQP2 and pSer256-AQP2 (p256-AQP2) after 7 (Fig. 3) and 14 days (Fig. 4) OVX. Cortical AQP2 mRNA (Table 2) and protein levels (Fig. 3A-B) showed a tendency to increase in the OVX group compared with sham, although this did not reach statistical significance. However, estradiol administration decreased AQP2 mRNA and protein levels in OVX rats at 7 days whereas progesterone had no effect (Table 2 and Fig. 3A-B). Cortical p256-AQP2 protein levels were increased in response to 7 days OVX and this was attenuated by estradiol administration (Fig. 3A and C). In IM, there was no significant change in AQP2 and p256-AQP2 among the four groups (Fig. 3D-F). Furthermore, the expression of vasopressin receptor (V2R) was measured in cortex and IM in response to 7 days OVX. However, no change in V2R expression was detected among the four groups (Table 2). At 14 days post-OVX, no differences in total AQP2 or p256-AQP2 protein levels were observed among the four groups (Fig. 4A-F).
Effect of estradiol and progesterone on AQP2 and p256-AQP2 trafficking in OVX rats

To further investigate whether female sex hormones affect trafficking of AQP2 and p256-AQP2 after 7 days OVX, we performed confocal laser microscopy analysis. This approach was used to analyze and quantify total AQP2 and p256-AQP2 localization at the apical membrane in the cortical collecting duct principal cells. Immunohistochemical reactivity against AQP2 and p256-AQP2 was analyzed in the principal cells of cortical collecting duct (Fig. 5). These experiments demonstrated a mostly apical distribution of both total AQP2 and p256-AQP2 in sham operated rats as well as untreated and progesterone treated OVX rats (Fig. 5A and C). However, a relatively lower apical abundance of AQP2 and p256-AQP2 was observed in OVX rats treated with estradiol compared to the other three experimental groups. Quantification of the apical fluorescent intensity showed a significant decrease in both AQP2 and p256-AQP2 distribution in estradiol-treated OVX rats compared to untreated OVX rats (Fig. 5B and D). Taken together this indicates that estradiol plays an important role for the regulation of both expression and trafficking of AQP2 and p256-AQP2 in rats.

Effect of estradiol on mpkCCD cells

Estrogen receptors are present in key osmosensitive regions of the brain as well as on vasopressin-releasing neurons (36), and estradiol has been shown to alter the osmotic threshold for AVP release in women (37; 38), and increase basal plasma AVP in post-menopausal women (37). Accordingly, we utilized the mpkCCD cortical collecting duct cell line to further investigate whether estradiol has direct effects on collecting duct AQP2 expression. We determined the effect of estradiol exposure on the AQP2 and p256-AQP2 abundance in mpkCCD cells incubated with and without 1 nM dDAVP for 24 h. Estradiol decreased AQP2 protein expression without dDAVP stimulation, but p256-AQP2 was not significantly reduced (Fig. 6A). Likewise, total AQP2 but not p256-AQP2 was significantly reduced in response to estradiol treatment when mpkCCD cells were
stimulated with dDAVP (Fig. 6A). To further investigate whether estradiol regulates AQP2 via genomic or non-genomic pathways in mpkCCD cells, we measured AQP2 mRNA levels and observed that estradiol alone decreased AQP2 mRNA level, but this effect was lost in the presence of dAVP. Then interestingly, estradiol alone increased cAMP, as did dAVP to a greater magnitude, with no additive effect of estradiol (Fig. 6C). Expression of ERα, ERβ and GRER1 in both mpkCCD cells and rat renal cortex was confirmed by PCR (Fig. 6D). Taken together, these data support a direct, inhibitory effect of estradiol on AQP2 gene transcription, but suggest that estrogen may also exert additional inhibitory effects on AQP2 protein abundance in the presence of AVP.

**Localization of estrogen receptors in the kidney**

Membrane-targeted splice variants of ERα are now known to exist in both humans and rodents, and could potentially contribute to both inhibitory genomic and non-genomic estrogen signaling (42). Accordingly, we sought to further identify which ERα splice variants are present in the mpkCCD cell lines. We used a combination of confocal immunofluorescent microscopy of kidney sections from female mice and Western blot analysis of mpkCCD cells and microdissected collecting ducts from both male and female mice (Fig. 7). An antibody directed against the N-terminus of ERα (thus recognizing the full-length ERα66 protein) revealed only faint positive staining of epithelial cells including collecting ducts in the cortex and inner medulla (Fig. 7A). Western blot analysis confirmed these findings with essentially undetectable ERα66 and only a small amount of ERα46 found in isolated inner medullary collecting ducts using an antibody that recognizes the C-terminus of these splice variants. More prominent staining of epithelial cells was observed with an antibody specific for the ERα36 splice variant, with cortical and inner medullary collecting ducts showing positive expression by both immunofluorescent co-localization with AQP2 and by Western blot (Fig. 7B). ERβ expression was also observed in the collecting ducts as
well as proximal tubules (Fig. 7C). MpkCCD cells showed faint positive expression of the ERα splice variants and ERβ by Western blot (Fig. 7). These data indicate that the predominant forms of ERα expressed in the collecting ducts of mice are the splice variants ERα36 and ERα46, along with expression of ERβ. These data are consistent with transcripts of ERα, ERβ and GPER1 being detected in both the mpkCCD cells and in rat renal cortex (Fig. 6D), supporting that the receptors are present at both the transcript and protein level.

**Increased AQP2 protein expression in αER KO mice**

To further investigate which estrogen receptors contribute to the regulation of AQP2 in vivo, we compared AQP2 expression in αER KO mice (lacking all ERα splice variants) and control mice. Our data revealed significantly increased protein levels of AQP2 in cortex, outer and inner medulla of αER KO mice compared to control mice (Fig. 8), indicating that estradiol’s inhibitory effect on AQP2 expression might be mediated through ERα. Similar to effects seen in dDAVP-treated mpkCCD cells, renal cortical AQP2 mRNA (WT, 1.04 ± 0.13 vs αER KO, 1.14 ± 0.20, n=6-7) was not significantly different between αER KO and control mice.
Discussion

The main finding of the present study was that estradiol administration attenuated the increased AQP2 and p256-AQP2 expression and trafficking in cortical collecting duct principal cells as well as reducing water retention in rats subjected to 7 days OVX. Furthermore, results showed that estradiol reduced both AQP2 mRNA and protein expression in cortical collecting duct mpkCCD cells despite increased cAMP production. Using αER KO mice we demonstrated increased AQP2 protein expression indicating that estradiol’s inhibitory effect on AQP2 might be mediated through ERα. Overall, the results of the present study support the view that estradiol exerts direct inhibitory effects on collecting duct AQP2 expression, and that this may involve both transcriptional and non-genomic effects.

Our data documented that estradiol contributed importantly to the alterations of AQP2 and p256-AQP2 protein level and trafficking in cortical collecting duct principal cells 7 days after OVX. Interestingly, cortical AQP2 mRNA levels in OVX rats were also reduced in response to estradiol administration. Consistent with these findings, decreased urine output and increased urine osmolality were attenuated by estradiol treatment 7 days after OVX supporting the view that estradiol might be responsible for changes in renal water balance via regulation of AQP2. These data, suggesting a short-term loss of estradiol allows for enhanced water reabsorption via AQP2, fit well with reports of increased risk of women developing hypervolemic hyponatremic encephalopathy (2), particularly following reproductive surgeries, maneuvers associated with abrupt decreases of plasma estradiol levels (1).

Interestingly, by day 14 there were no longer differences between groups in urine output, urine osmolality or AQP2 levels. Consistent with our data, it has previously been demonstrated that after 14 days OVX there was no detectable difference in urinary output compared with sham
operated or estrogen treated OVX rats (19). The difference at day 7 versus day 14 is not entirely clear, but one possible explanation is that changes in AVP may contribute. AVP plays an important role in regulating renal water clearance and AQP2, and studies have demonstrated that plasma AVP concentrations vary over the rat oestrous cycle (8), but also vary in OVX rats (34). Peysner and Forsling (34) have studied the effect of OVX on AVP release for a period of up to 14 days. They showed that AVP levels in OVX rats were significant increased at day 7 compared to day 1. However, on day 14 plasma AVP levels was reduced and almost at the same level as at day 1. This could indicate that over time and under influence of AVP, the OVX rats have reached a new equilibrium of water balance by day 14. One might speculate that central changes in osmosensing or AVP release might be involved, but this would require further investigation and is beyond the scope of the current study.

A recent study has demonstrated that sex hormones influence the expression of proximal tubule AQP1 (13). To our knowledge, no studies have investigated the regulation of AQP2 in the kidney in OVX rats in response to sex hormone administration. However, a few studies concerning hormonal regulation of AQP2 implicate a role for AQP2 in water movement in other organs. Kim et al found AQP2 protein level to be regulated by estrogen in the urinary bladder in rats subjected to OVX (21). Furthermore, Jablonski et al. demonstrated that estrogen played a role in the regulation of AQP2 expression in the uterus in OVX mice (18). These studies indicate that estrogen might play an important role for the regulation of AQP2 expression which might change water movement in different organ systems.

Using an in vitro system to avoid potentially confounding systemic hormonal effects, we demonstrated that estradiol directly reduced both AQP2 mRNA and protein expression in cortical collecting duct mpkCCD cells, despite increased cAMP production, suggesting that estradiol may regulate AQP2 expression via an estrogen response element (ERE) in the AQP2 promoter. A
functional ERE has been detected in the promoter of AQP2 in endometrial carcinoma (EC) cells which mediates that regulation of AQP2 expression by estradiol in normal endometrium as well as in EC cells (46).

Precisely how the estrogen response element in the AQP2 gene may or may not contribute to collecting duct AQP2 expression may be somewhat complex, given that estrogen receptors bind to their cognate ERE as a dimer. Current information on the localization of estrogen receptors within the kidney, and in particular of splice variants of the receptors, is limited. Expression of all three ERα isoforms, including ERα66, ERα46 and ERα36 was observed primarily in the cytoplasmic fraction of renal cortex from rats (25). Wells and colleagues (44) were able to visualize 66 kDa and 46 kDa ERα protein expression in a wide range of cell types in the rat kidney via immunohistochemistry, they commented that they were unable to do so for ERβ and concluded that it was less abundant. Similarly, Grimont et al (9) reported that they failed to detect ERβ mRNA in the mouse kidney, but it is not clear whether expression may have simply been very low relative to the authors’ expectations, and no positive control data were provided for their primers. Our data show the presence of ERβ in the rat renal cortex, at least at the transcriptional level with our mouse data further demonstrating ERβ expression in the kidney at the protein level, with uterus included as a positive control. Irsik and colleagues (17) demonstrated that both ERα66 and ERα36 co-localized with AQP2 in cortical collecting duct in female mice. ERβ immunostaining was primarily present in mesangial cells and podocytes of female mice (17). Our findings expand on these studies to demonstrate that the splice variants ERα46 and ERα36, as well as ERβ are expressed in both female and male inner medullary collecting ducts from mice as well as in mpkCCD cells. In our study, expression of ERα66 in the collecting duct was relatively less convincing and perhaps only present at relatively low levels.
It is unclear which ERα splice variant could be involved in the regulation of AQP2 in collecting duct cells. ERα36, which includes the DNA binding domain, has a unique C-terminus and lacks transcriptional activation domains (42), can act as a dominant negative by inhibiting ERα66 and ERα transactivation in the nucleus (43). Likewise, ERα46 can inhibit transcription through the AF1 transactivation domain of ERα66 (7). Taken together, this suggests that ERα36 as well as ERα46 can modulate the estrogenic effects of ERα66, or potentially ERβ since the receptors dimerize, in tissue expressing these receptors. Since we have demonstrated expression of both ERα36 and ERα46 in collecting duct cells one might speculate that estradiol can exert an inhibitory effect on AQP2 gene transcription via either ERα36 or ERα46 which is then revealed by OVX.

Our studies also revealed apparent inhibitory effects of estradiol and ERα on AQP2 protein abundance that could not be fully explained by effects on AQP2 mRNA. In addition to classical genomic effects, it is now recognized that estrogens can exert rapid, non-transcriptional effects on cells. ERα36 possesses three myristoylation sites that aid in targeting it to the plasma membrane (42), and it can signal non-genomically via the MAPK pathway (43). Interestingly, p38-MAPK activity has been implicated in polyubiquitination of AQP2 (27). Whether estradiol enhances polyubiquitination of AQP2, a potent signal for degradation of AQP2 (24) merits future investigation but is beyond the scope of the current study.

A controversial mediator of non-genomic actions of estrogen, GPER1 (also known as GPR30) is reportedly expressed in the inner medulla of both rats and mice (12). Our data indicate that GPER1 is expressed at the transcriptional level by both mpkCCD cells and rat renal cortex, the latter observation being consistent with a previous publication in rats (25). Studies in several cell types indicate that estradiol can increase cAMP production (10; 39), although the receptor involved
has not been identified. Activation of GPER1 can increase cAMP production (35), and it is 
therefore tempting to speculate that activation of GPER1 might underlie the increase in cAMP 
observed in mpkCCD cells in response to estrogen alone. Whether GPER1 might mediate an effect 
of estrogen on AQP2 levels in vivo by such a mechanism is unknown. A recent study, by Cheng 
and colleagues (4) reported that in mice, GPER1 is predominantly expressed by cells in the 
proximal tubules, distal convoluted tubules and the loop of Henle, but that there was no overlap 
between AQP2 positive cells and GPER1 positive cells. Accordingly, the potential contribution of 
GPER1 to AQP2 regulation in vivo remains unclear and is beyond the scope of the current study.

In conclusion, the results of this study show a novel inhibitory role of estradiol on collecting 
duct AQP2 expression, and further suggest that activation of ERα underlies this effect. These 
findings significantly add to the current understanding of the many levels at which sex hormones 
influence salt and water homeostasis. In terms of the broader implication of our findings on water 
homeostasis in women, our data suggested that changes in sex hormones over the menstrual cycle 
may affect renal water handling and decreased levels female sex hormones might play a role in the 
dysregulation of renal water handling related to peri-menopause.

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Disclosure

The authors declare no conflicts of interest.
Reference List


Figure Legends

**Figure 1:** Effects of estradiol and progesterone on body weight, food and water intake in OVX rats:
A) Time course of body weight changes in sham operated female rats (red line), ovariectomized (OVX) rats (black line), and OVX rats treated with estradiol (blue line) or progesterone (green line). The animals were, in all cases, fed ad libitum. B) Absolute and relative (corrected for body weight) food intake one day before OVX (-1) and 2, 7, or 14 days post-OVX or sham surgery. C) Absolute and relative water intake one day before OVX (-1) and 2, 7, or 14 days post-OVX or sham surgery. Data are means ± SEM of 7 rats in each group. *P < 0.05 OVX compared with sham operated rats. #P < 0.05 treated OVX group compared with untreated OVX rats.

**Figure 2:** Effects of estradiol and progesterone on urine output and osmolality in OVX rats: Urine volume (absolute) A), urine output (corrected for body weight) B) and urinary osmolality C) measured one day before OVX (-1) and 2, 7, or 14 days post-OVX or sham surgery. Data are means ± SEM of 7 rats in each group. *P < 0.05 OVX compared with sham operated rats. #P < 0.05 treated OVX group compared with untreated OVX rats.

**Figure 3:** Effects of estradiol and progesterone on AQP2 and p256-AQP2 expression in kidney cortex and inner medulla after 7 days: Kidney samples were fractionated into cortex and inner medulla from the four experimental rat groups after 7 days treatment. A and D) Immunoblotting performed for protein level of AQP2, p256-AQP2 and β-actin in kidney cortex and inner medulla samples, respectively. B and C) Densitometric analysis of protein band intensity for AQP2 and p256-AQP2 relative to β-actin in renal cortex. E and F) Densitometric analysis of protein band intensity for AQP2 and p256-AQP2 relative to β-actin in inner medulla. Data are means ± SEM of 7 rats in each group. *P < 0.05 OVX compared with sham operated rats. #P < 0.05 treated OVX group compared with untreated OVX rats.
Figure 4: Effects of estradiol and progesterone on AQP2 and p256-AQP2 expression in kidney cortex and inner medulla after 14 days: Kidney samples were fractionated into cortex and inner medulla from the four experimental rat groups after 14 days treatment. A and D) Immunoblotting performed for protein level of AQP2, p256-AQP2 and β-actin in kidney cortex and inner medulla samples, respectively. B and C) Densitometric analysis of protein band intensity for AQP2 and p256-AQP2 relative to β-actin in renal cortex. D and F) Densitometric analysis of protein band intensity for AQP2 and p256-AQP2 relative to β-actin in inner medulla. Data are means ± SEM of 7 rats in each group.

Figure 5: Immunohistochemical staining of AQP2 and p256-AQP2 in kidney cortex 7 days post-OVX or sham surgery: Images show representative laser-scanning confocal microscopy of AQP2 (A) and p256-AQP2 (C) in cortical collecting ducts. Each image was acquired using the same microscopy settings, and representative images are shown for sham, OVX, OVX + estradiol and OVX + progesterone experimental groups, respectively. B and D) A number of images were captured from all the experimental animals and single cell quantification was carried out to determine apical abundance of AQP2 and p256-AQP2. Graph bars represent the mean fluorescent intensity in arbitrary units (AU) ± SEM. Less apical abundance of both AQP2 and p256-AQP2 were observed after estradiol treatment. #P < 0.05 OVX compared with estradiol treated OVX rats.

Figure 6: Effect of estradiol on AQP2 and p256-AQP2 expression in mpkCCD cells: Confluent mpkCCD cells grown on filters were preincubated for 4 days in the absence or presence of 1 nM dDAVP and then incubated for another 24 h without hormones or with 50 nM estradiol and/or 1 nM dDAVP. A) Representative immunoblots and densitometric quantification of AQP2 and p256-AQP2 in cell lysates (non-glycosylated band only; normalized for β-actin). Data are means ± SEM of 9 samples in each group for both AQP2 and p256-AQP2 immunoblots. B) Quantification of
AQP2 mRNA expression. C) mpkCCD cells were treated as described, however, during the last 30 min, 3-isobutyl-1-methylxanthine (IMBX) was added. Cells were lysed and cAMP production was measured. Data are means ± SEM of 6 samples in each group. D) RT-PCR analysis of G protein-coupled estrogen receptor (GPER1), ERα and ERβ in mpkCCD cells and in rat renal cortex. *P < 0.05 compared with dDAVP stimulated mpkCCD cells. #P < 0.05 compared with estradiol stimulated mpkCCD cells.

**Figure 7: Localization of estrogen receptors in the mouse kidney:** A-C) Green fluorescence represents ERα detected with an N Terminal-specific antibody, ERα36 and ERβ, respectively in cortex and inner medulla. Red fluorescence represents AQP2 for co-localization with collecting duct. Nuclei are stained blue with DAPI. Scale bar = 20 µM. Shown next to the respective immunofluorescent images are representative immunoblots for the ERα splice variants, ERα66, ERα46 and ERα36 expression as well as for the ERβ expression in mouse cortex, proximal tubules isolated by Percoll density gradient centrifugation (Prox. T.), cortical collecting ducts isolated by immunomagnetic separation (CCD), and the distal tubule fraction (Dist. T.) from the Percoll gradient separation of cortical tubular suspensions, as well as mpkCCD cells and female and male inner medulla collecting ducts (IMCD). MW = molecular weight marker.

**Figure 8: Increased AQP2 expression in female αER KO mice:** Kidneys were dissected into cortex, outer and inner medulla and immunoblotting for AQP2 was performed. Densitometric analysis was carried out to measure protein band intensity for AQP2 relative to β-actin. Representative blots are shown for each group and kidney region. Data are means ± SEM of 6-7 mice in each group. *P < 0.05 WT compared with αER KO mice.
Table 1: Serum concentration for estradiol and progesterone

<table>
<thead>
<tr>
<th></th>
<th>Day 7 Estradiol (pg/ml)</th>
<th>Day 7 Progesterone (ng/ml)</th>
<th>Day 14 Estradiol (pg/ml)</th>
<th>Day 14 Progesterone (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>SHAM</td>
<td>24.5 ± 4.7</td>
<td>12.4 ± 4.0</td>
<td>24.5 ± 1.6</td>
<td>15.7 ± 5.7</td>
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<tr>
<td>OVX</td>
<td>13.6 ± 0.8*</td>
<td>1.7 ± 0.2*</td>
<td>13.6 ± 0.3*</td>
<td>2.0 ± 0.3*</td>
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<tr>
<td>OVX + Estradiol</td>
<td>20.7 ± 1.7*</td>
<td>6.3 ± 2.8</td>
<td>21.4 ± 1.4*</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>OVX + Progesterone</td>
<td>17.7 ± 1.1</td>
<td>33.3 ± 4.4*</td>
<td>16.6 ± 0.7</td>
<td>22.7 ± 2.9*</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM. *P < 0.05 when OVX compared to SHAM. #P < 0.05 when OVX compared to OVX + Estradiol. $P < 0.05 when OVX compared to OVX + Progesterone.
Table 2: mRNA levels of AQP2 and vasopressin V2 receptor after 7 days OVX

<table>
<thead>
<tr>
<th></th>
<th>Day 7 AQP2 in cortex</th>
<th>Day 7 AQP2 in medulla</th>
<th>Day 7 V2R in cortex</th>
<th>Day 7 V2R in medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>1.00 ± 0.19</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.20</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>OVX</td>
<td>1.36 ± 0.19</td>
<td>1.04 ± 0.08</td>
<td>1.06 ± 0.13</td>
<td>0.95 ± 0.06</td>
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<tr>
<td>OVX + Estradiol</td>
<td>0.80 ± 0.06*</td>
<td>0.93 ± 0.05</td>
<td>0.85 ± 0.14</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>OVX + Progesterone</td>
<td>1.11 ± 0.10</td>
<td>0.98 ± 0.09</td>
<td>0.99 ± 0.04</td>
<td>0.89 ± 0.07</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM. #: P < 0.05 when OVX compared to OVX + Estradiol.
Figure 1

A

![Graph showing body weight changes](image)

B

![Graph showing food intake changes](image)

C

![Graph showing water intake changes](image)
Figure 2

A

- Days: -1, 2, 7, 14
- Y-axis: Urine volume (ml/day)
- X-axis: Days
- Bars represent Sham, OVX, OVX + Estradiol, OVX + Progesterone

B

- Days: -1, 2, 7, 14
- Y-axis: Urine Output (μl/min/kg)
- X-axis: Days
- Bars represent Sham, OVX, OVX + Estradiol, OVX + Progesterone

C

- Days: -1, 2, 7, 14
- Y-axis: Urine osmolality (mOsmol/kg H2O)
- X-axis: Days
- Bars represent Sham, OVX, OVX + Estradiol, OVX + Progesterone

* and # symbols indicate significant differences.
Figure 3

(AQP2/β-actin ratio)

Sham
OVX
OVX + Estradiol
OVX + Progesterone

Cortex
Inner medulla
Figure 5

A

Sham  OVX  OVX + Estradiol  OVX + Progesterone

B

Normalized fluorescent intensity relative to control (AU)

C

Sham  OVX  OVX + Estradiol  OVX + Progesterone

D

Normalized fluorescent intensity relative to control (AU)
Figure 6

A

AQP2
p256-AQP2
B-actin

B

AQP2 mRNA expression

C

cAMP production (pmol/mg protein)

D

mpkCCD Cortex

GREP1 ERα ERβ GREP1 ERα ERβ
Figure 7

A

ERα N Terminal Ab  AQP-2  Overlay

Cortex  Inner Medulla

B

ERα36 Ab  AQP-2  Overlay

Cortex  Inner Medulla

C

ERβ Ab  AQP-2  Overlay

Cortex  Inner Medulla
Figure 8

AQP2

β-actin

![Graph showing AQP2 and β-actin protein levels in different regions with WT and αER KO conditions.](image-url)