J Physiol (2003), **552.3**, *pp.* 869–880
DOI: 10.1113/jphysiol.2003.046920

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Journal of Physiology

Oestrogen effects on urine concentrating response in young women

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Oestrogen lowers the plasma osmotic threshold for arginine vasopressin (AVP) release but without commensurate changes in renal concentrating response, suggesting oestrogen (OE_2) may lower renal sensitivity to AVP. Ten women (23 ± 1 years) received a gonadotropin releasing hormone analogue (GnRHa), leuprolide acetate, to suppress OE_2 for 35 days, and then added OE_2 (two patches each delivering 0.1 mg day^{-1}) on days 32–35. On days 28 and 35 we tested blood and renal water and sodium (Na^+) regulation during stepwise 60 min AVP infusions (10, 35, 100, 150 and 200 μu (kg body weight)$^{-1}$ Pitressin). Plasma OE_2 concentration increased from 19 ± 4 to 152 ± 3 pg ml^{-1} and plasma progesterone concentration was unchanged (1.0 ± 0.4 and 0.7 ± 0.1 ng ml^{-1}) for GnRHa and OE_2 administration, respectively. Standard log plots of plasma AVP concentration ($[AVP]_P$) *vs.* urine osmolality (Osm_U) were fitted to a sigmoidal curve, and EC_{50} was determined by non-linear regression curve fitting of concentration–response data. Osm_U rose exponentially during AVP infusions, but hormone treatments did not affect EC_{50} (3.3 ± 0.07 and 3.1 ± 0.6 pg ml^{-1}, for GnRHa and OE_2, respectively). However, the urine osmolality increase was greater within the physiological range (~2.5–3.4 pg ml^{-1} $[AVP]_P$) during OE_2 treatment. Throughout most of the AVP infusion, the rate of clearance of AVP from plasma (PCR_{AVP}) was increased during OE_2 (45.5 ml (kg body weight)$^{-1}$ min^{-1}) compared to GnRHa administration (33.1 ml (kg body weight)$^{-1}$ min^{-1}; mean for the 100–200 μu (kg body weight)$^{-1}$ infusion rates). The rate of renal free water clearance (C_{H_2O}) was similar between hormone treatments. Sodium excretion fell during OE_2 administration due to greater distal tubular sodium reabsorption. Despite more rapid PCR_{AVP}, renal concentrating response to graded AVP infusions was unaffected by oestrogen treatment suggesting oestrogen does not affect overall renal sensitivity to AVP. However, OE_2 may increase renal fluid retention within a physiological range of AVP.

(Received 13 May 2003; accepted after revision 8 August 2003; first published online 8 August 2003)
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In pre-menopausal women, the rate of progression of renal disease is slower than that in men of similar age (Neugarten *et al.* 2000; Dubey & Jackson, 2001). This sex difference is no longer apparent in menopausal women and oestrogen administration delays the onset of renal disease in older women (Silbiger & Neugarten, 1995; Neugarten *et al.* 2000). Moreover, oestrogen modulates whole body water and sodium at rest, and during perturbations of normal body fluid homeostasis such as dehydration, sodium loading and hyponatraemia (Ayus & Arieff, 1996; Calzone *et al.* 2001; Stachenfeld & Keefe, 2002). While these findings suggest an important function for oestrogen in renal water and sodium regulation, specific oestrogen actions in the kidney still need clarification.

A number of studies have demonstrated that the osmotic threshold for arginine vasopressin (AVP) release is reduced when oestrogen is elevated (Spruce *et al.* 1985; Trigoso *et al.* 1996; Stachenfeld *et al.* 1998*a*,*b*, 1999; Calzone *et al.* 2001; Stachenfeld & Keefe, 2002). Despite the earlier AVP release, the rate of renal free water clearance (C_{H_2O}) during osmotic stimulation appears to be unaffected, indicating that oestrogen may lower renal tubular sensitivity to AVP. We have observed no oestrogen effect on C_{H_2O} responses to osmotic stimulation in young women despite greater $[AVP]_P$ response (Calzone *et al.* 2001; Stachenfeld & Keefe, 2002). In addition, although osmotic secretion of AVP is greater in the mid-luteal phase of the menstrual cycle (Forsling *et al.* 1981; Calzone *et al.* 2001; Stachenfeld & Keefe, 2002) (i.e. where oestrogen and progesterone are relatively high), this luteal phase increase is usually not accompanied by greater water retention (Gaebelein & Senay, 1982; Fortney *et al.* 1988, 1994; Calzone *et al.* 2001). Moreover, there have been a number of reports of diabetes insipidus in pregnant women (Barron *et al.* 1984; Sills *et al.* 1995). Finally, oestrogen

attenuates the antidiuretic action of AVP in the rat (Carlberg *et al.* 1984; Wang *et al.* 1995), and may modulate AVP action in the collecting duct at the receptor level (Stumpf *et al.* 1980; Hatano *et al.* 1988; Dubey & Jackson, 2001).

The direct effects of oestrogen on AVP actions in the kidney are difficult to define in young women. Progesterone also affects body water and sodium regulation (Myles & Funder, 1996; Stachenfeld *et al.* 1998*a*, 1999) but there is no time in the normal menstrual cycle when progesterone is elevated without concomitant elevations in oestrogen. In the present investigation we suppressed endogenous production of oestrogen and progesterone in young women using the gonadotropin releasing hormone agonist (GnRHa) leuprolide acetate followed by controlled oestrogen administration. During treatment with both GnRHa alone and GnRHa with concomitant oestrogen, we administered graded infusions of synthetic AVP and measured increases in urine osmolality to test the hypothesis that oestrogen would attenuate the urine-concentrating response compared to GnRHa alone. That is, oestrogen would reduce renal sensitivity to AVP. In addition, we determined renal blood flow and renal prostaglandins under both conditions to determine whether oestrogen effects on renal concentrating response to AVP stimulation are due to either of these mechanisms. Oestrogen effects on renal sodium regulation were also determined.

METHODS

We recruited 10 healthy non-smoking women (aged 23 ± 1 years), with no contraindications to GnRHa or oestrogen administration, to participate in these experiments. All subjects were interviewed about their medical history, reported no abnormal reproductive history and reported a history of regular (26–32 day) menstrual cycles. Subjects also provided written confirmation of a negative Papanicolaou smear and normal physical examination within 1 year of being admitted to the study. They gave written informed consent to participate in the study, which conformed with the guidelines contained in the Declaration of Helsinki, and had prior approval by the Human Investigation Committee of Yale University School of Medicine.

Experimental design

To suppress reproductive function for the duration of the study, the subjects received the GnRHa leuprolide acetate each day for 5 weeks. For 4 days at the end of the fifth week, the women received oestrogen. Experimental protocols were performed at the end of the fourth (GnRHa administration alone) and fifth (GnRHa administration with hormone) weeks. This design permitted within-subject comparisons concerning oestrogen effects on renal concentration response to AVP, as well as renal blood flow, and sodium and fluid regulation.

GnRH analogue (leuprolide acetate). This analogue demonstrates greater receptor binding and decreased degradation than endogenous GnRH, and acts as a potent inhibitor of gonadotropin secretion. When leuprolide acetate is given continuously, it down-regulates the hypothalamic–pituitary–ovarian axis, with internalization and uncoupling of the GnRH receptors at the pituitary level. Following an initial stimulation, chronic administration suppresses steroidogenesis via follicular stimulating hormone (FSH) secretion, leading to low or undetectable oestrogen and progesterone concentrations within 14 days. The GnRHa administration began 2–7 days following the subject's luteinizing hormone peak. This peak precedes ovulation, usually on day 12–14 of a 28 day menstrual cycle, which was determined individually by the use of ovulation prediction kits (OvuQuick, Quidel Corp., San Diego, CA, USA). Some of the subjects ($n = 4$) were already taking oral contraceptives, so they ceased taking the pills and began GnRHa administration in the third week of the pill regimen. The subjects self-administered daily subcutaneous injections of the GnRHa (0.5 mg day^{-1}; Lupron, TAP Pharmaceuticals Inc., Deerfield, IL, USA) after training by qualified medical personnel. This method of GnRHa administration was chosen because it is easily discontinued in the event of uncomfortable side effects, such as headaches or vasomotor symptoms ('hot flashes'), and the suppression of the hypothalamic–pituitary–ovarian axis is reversed upon cessation of drug therapy.

Oestrogen add-back. For oestrogen treatment, the subjects received 17β-oestradiol administered using two transdermal patches delivering 0.1 mg day^{-1} each (Vivelle; CIBA Pharmaceuticals, Summit, NJ, USA) for 4 days (Stachenfeld *et al.* 2001*a*).

Study protocol

Subjects self-administered an oral dose of 300 mg of lithium carbonate at 22.00 h on the night before each experiment, which provided a near-constant plasma level of lithium by the next morning (Thomsen, 1984, 1990). For each experiment, the subjects arrived at the laboratory at approximately 07.00 h, after having eaten a light (~300 kcal) breakfast, having drunk 10 ml (kg body weight)$^{-1}$ of water, and having refrained from alcohol and caffeinated beverages for the previous 12 h. Upon reporting to the laboratory the subjects voided their bladders and were weighed to the nearest 10 g on a beam balance. The subjects were then seated in a semi-recumbent position for a 60 min control period in an environmental chamber (27 °C, 30 % relative humidity) to ensure a steady state in plasma volume and constituents. During this control period, a 22 gauge teflon intravenous catheter was placed in an antecubital or forearm vein in each arm with a heparin block (20 u ml^{-1} in 0.9 % saline) to maintain catheter patency. A blood pressure cuff was positioned for automatic readings by a sphygnomanometric device (Colin Medical Instruments, Komaki, Japan) to monitor changes in blood pressure. A three-lead electrocardiogram (Colin Medical Instruments) provided continuous heart rate monitoring.

At the end of the control period, a baseline blood sample was taken, and a urine sample collected. Following the urine sample, a bolus of para-aminohippurate (PAH) was infused (10 mg kg^{-1}, ~2–2.5 ml). The PAH bolus was followed by infusions of synthetic AVP (Pitressin, Parke-Davis, Morris Plains, NJ, USA) in normal saline for serial 60-min periods. Vasopressin was infused at varying concentrations (0.01 μu ml^{-1} kg^{-1} for the first two hours, and 1 μu ml^{-1} kg^{-1} for the final three hours), and at varying rates (0.1, 0.35, 0.1, 0.15, and 0.20 ml min^{-1}) to achieve the following infusion rates: 10, 35, 100, 150, 200 μu Pitressin min^{-1} (kg body weight)$^{-1}$. This rate of infusion was chosen to increase plasma levels of AVP to ~15 pg ml^{-1}, which is not associated with an increase in systolic, diastolic or mean arterial pressure in normal subjects (Moses & Steciak, 1986). The PAH infusions continued

(10.25 mg PAH (kg body weight)$^{-1}$ at the rate of 1 ml min^{-1} in sterile 0.9 % saline) using a Harvard constant infusion syringe pump throughout the experimental protocol. Blood samples were obtained every 30 min, and urine collected every 60 min for determination of renal clearances and osmoregulatory function. The subjects drank 100 ml tap water immediately following each urine sample. Blood pressure and heart rate were recorded every 15 min throughout the infusion.

All blood samples were analysed for haematocrit (Hct), haemoglobin (Hb), total protein (TP), osmolality (Osm_P), and plasma concentrations of creatinine ($[Cr]_P$), arginine vasopressin ($[AVP]_P$) and serum concentrations of sodium ($[Na^+]_S$) and potassium ($[K^+]_S$). Hourly blood samples were also analysed for plasma concentrations of PAH ($[PAH]_P$) and lithium ($[Li^+]_P$). Blood samples at baseline, and after 120 and 300 min were analysed for plasma renin activity (PRA). The baseline blood sample was also analysed for concentrations of oestrogen ($[OE_2]_P$) and progesterone ($[P4]_P$). Volume, osmolality (Osm_U), and concentrations of sodium ($[Na^+]_U$), potassium ($[K^+]_U$), lithium ($[Li^+]_U$), PAH ($[PAH]_U$), renal prostaglandin E2 ($[PGE2]_U$) and creatinine ($[Cr]_U$) were measured from all urine samples.

Blood and urine analysis

Blood samples were separated into aliquots. One aliquot was immediately analysed in triplicate for haemoglobin and haematocrit by cyanomethaemoglobin assay and micro-haematocrit, respectively. A second aliquot was transferred to a heparinized tube to be analysed for Osm_P and $[Cr]_P$. A third aliquot, for the determination of $[Na^+]_S$ and $[K^+]_S$, was placed into a tube without anticoagulant. The remaining aliquots were placed into tubes containing EDTA for analysis of $[AVP]_P$ and PRA. The tubes were centrifuged at 4 °C and the plasma was taken off. After centrifugation, the EDTA samples were frozen immediately at –70 °C until analysis. For the determination of $[Li^+]_P$, plasma was deproteinized by addition of equal amounts (1 ml) of 0.3 N $ZnSO_4$ and 0.3 N $BaOH_2$. The supernatant (1.5 ml) was dried with a vacuum centrifuge (Speed Vac, Savant, Holbrook, NY, USA) for 1 h, followed by the addition of 100 μl ethanol, and then allowed to evaporate overnight. The pellet was then resuspended with 100 μl of a 140:5:0 mM Na^+: K^+: Li^+ solution, and the $[Li^+]_P$ of the reconstituted solution was measured by flame photometry (model 943, Instrumentation Laboratory, Lexington, MA, USA). $[Li^+]_P$ was determined from a standard curve that was prepared for each experiment using the subject's own plasma drawn on the first day of the experiment (i.e. when samples for $[E_2]_P$ and $[P4]_P$ were drawn), and spiked with graded concentrations of LiCl. For the determination of $[PAH]_P$, 500 μl of distilled water was added to a 125 μl plasma sample. Then 750 μl of sodium nitrate, 400 μl of ammonium sulfamate, and 400 μl of *N*-(1-naphthyl) ethylenediamine dihydrochroloride was added to the sample in sequence, and the absorbance measured at 560 nm. Urine Li^+ concentrations were determined with the same methods used for the plasma, without the deproteinization. Urine PAH concentration was determined following 40–120-fold dilution depending upon overall urine concentration, and was determined using the same assay that was used for the blood samples.

Plasma and urine sodium and potassium concentrations were measured by flame photometry (model 943). Plasma and urine osmolalities were measured by freezing point depression (Advanced Instruments 3DII, Needham Heights, MA, USA), TP by refractometry, and $[Cr]_P$ was determined by colourimetric assay (Sigma Diagnostic Products). Plasma [AVP], PRA, $[E_2]_P$, $[P4]_P$ and $[PGE2]_U$ were measured by radioimmunoassay. Plasma [AVP] was determined after extraction from plasma by the methods described by Freund *et al.* (Freund *et al.* 1987, 1991) on octadecylsilane cartridges (SEP-PAK C_{18}, Waters Associates, Marlborough, MA, USA). Extracted samples were assayed using a disequlibrium assay with the extracts incubated with the antiserum at 4 °C for 72 h followed by the addition of ^{125}I-arginine vasopressin (New England Nuclear, Boston, MA, USA). Bovine serum albumin-coated charcoal was used for separation of free and antibody-bound labelled AVP. This assay is highly specific for AVP, with the antiserum prepared against a lysine vasopressin–thyroglobin conjugate and has a sensitivity of 0.6 pg ml^{-1}. Extraction recovery of AVP was determined using plasma spiked with a known concentration of AVP (Peninusula Laboratories, Belmont, CA, USA). The recovery sample was extracted and analysed along with the subjects' samples. The extraction recovery was 87 %.

Intra- and interassay coefficients of variation for the midrange standards were, respectively, as follows: $[AVP]_P$ (10.3 pg ml^{-1}) 14.8 % and 16.4 % and PRA (3.6 ng ANG I ml^{-1}h^{-1}) 10.3 % and 9.7 % (Diasorin, Stillwater, MN, USA). Samples for the following were each run in a single-assay kit, with intra-assay coefficients of variation for the midrange standards for $[E_2]_P$ (61 pg ml^{-1}) of 13.0 % (Diagnostic Products, Los Angeles, CA, USA), for $[P4]_P$ (2.1 ng ml^{-1}) of 5.2 % (Diagnostic Products), and for $[PGE2]_U$ (23.7 pg ml^{-1}) of 3.7 % (Amersham, Chicago, IL, USA).

Calculations

Changes in plasma volume were estimated from changes in Hct and Hb concentration from the baseline sample according to the equation:

$$\%\Delta PV = 100\{([Hb_b]/[Hb_a])(1 - Hct_a \times 10^{-2})/(1 - Hct_b \times 10^{-2})\} - 100,$$

where subscripts a and b denote measurements at time a and pre-AVP infusion, respectively (Greenleaf, 1979). Similarly, these changes in Hct and Hb were used to estimate percent changes in plasma volume between baseline samples of GnRHa *versus* GnRHa with oestrogen (Stachenfeld *et al.* 1999, 2001*a*).

Body water handling was determined through the assessment of overall fluid balance and the renal clearance of free water, osmolality, and sodium concentration. The following equations were used to calculate renal function: glomerular filtration rate (GFR) was estimated from creatinine clearance, and effective renal plasma flow (ERPF) was determined using renal PAH clearance $(C_{PAH}) = U_V[PAH]_U/[PAH]_P$ and ERPF = $C_{PAH}/((1 - Hct)/100)$, where U_V represents urine flow rate. The use of C_{PAH} was valid in our subjects, because mean $[PAH]_P$ was 0.46 and 0.42 mg % for the GnRHa and oestrogen treatments, respectively. These low levels of $[PAH]_P$ resulted in PAH secretion ration rates of ~6.0 mg min^{-1}, well below the transport maximum for PAH of ~80 mg min^{-1}. Renal clearance of Li^+ (C_{Li^+}) was used to approximate proximal renal tubular Na^+ handling because filtered Li^+ is absorbed almost exclusively by the proximal tubule in the same proportion as Na^+ and water (Thomsen, 1984, 1990). Urine Na^+ excretion ($U_{Na^+}V$) was calculated as the product of $[Na^+]_U$ and volume (ml min^{-1}); fractional excretion of Na^+ $(FE_{Na^+}) = (U_V[Na^+]_U/GFR[Na^+]_f)100$, where $[Na^+]_f$ represents the Donnan factor for cations multiplied by $[Na^+]_S$; fractional excretion of water $(FE_{H_2O}) = (U_V/GFR) \times 100$; filtered sodium load $(FL_{Na^+}) = [Na^+]_S \times 1.05 \times$ Donnan factor $\times$ GFR; proximal

Table 1. Blood responses at baseline and in response to infusions of synthetic arginine vasopressin (AVP) during administration of gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen treatment

	AVP infusion rate (μu kg^{-1} min^{-1})					
	0	10	35	100	150	200
Hct (%)						
GnRHa†	39.3 ± 0.8	38.2 ± 0.8	38.0 ± 1.0	37.2 ± 1.0	37.0 ± 0.9	36.5 ± 0.8
Oestrogen	37.7 ± 1.0*	36.8 ± 1.0*	36.4 ± 1.0*	35.8 ± 1.0*	35.4 ± 1.0*	35.1 ± 0.9*
Hb (g)						
GnRHa†	11.1 ± 0.3	10.8 ± 0.3	10.6 ± 0.3	10.4 ± 0.3	10.4 ± 0.3	10.4 ± 0.3
Oestrogen§	10.5 ± 0.3*	10.6 ± 0.4	10.3 ± 0.4	10.2 ± 0.4	10.1 ± 0.4	10.0 ± 0.4
PV (%)						
GnRHa	—	5.7 ± 0.7	8.0 ± 1.0	10.8 ± 1.0	11.4 ± 0.8	11.8 ± 0.8
Oestrogen	—	5.8 ± 1.0	6.9 ± 1.4	10.0 ± 1.4	10.6 ± 1.2	10.8 ± 1.4
Osm_P (mosmol (kg H_2O)$^{-1}$)						
GnRHa†	280 ± 2	276 ± 1	275 ± 1	274 ± 2	274 ± 1	274 ± 1
Oestrogen§	277 ± 2*	274 ± 2	274 ± 2	274 ± 2	273 ± 2	275 ± 1
TP (g)						
GnRHa†	6.9 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1
Oestrogen§	6.8 ± 0.1	6.5 ± 0.1	6.4 ± 0.1	6.3 ± 0.1	6.4 ± 0.1	6.4 ± 0.1
$[Na^+]_S$ (mequiv l^{-1})						
GnRHa†	137.2 ± 0.9	135.2 ± 0.7	134.9 ± 0.6	135. ± 0.7	135.2 ± 0.7	135.5 ± 0.5
Oestrogen§	135.6 ± 0.8*	134.3 ± 0.6	134.3 ± 0.8	134.1 ± 0.8	134.2 ± 0.8	134.5 ± 0.8
$[K^+]_S$ (mequiv l^{-1})						
GnRHa	3.72 ± 0.09	3.88 ± 0.14	3.93 ± 0.08	3.93 ± 0.09	3.83 ± 0.08	3.83 ± 0.04
Oestrogen	3.74 ± 0.08	3.83 ± 0.09	3.78 ± 0.08	3.77 ± 0.08	3.76 ± 0.06	3.86 ± 0.07
$[AVP]_P$ (pg ml^{-1})						
GnRHa†	1.4 ± 0.1	4.1 ± 0.9	5.2 ± 0.8	7.6 ± 1.2	10.6 ± 1.1	11.1 ± 0.5
Oestrogen§	1.2 ± 0.2	2.6 ± 0.6	4.0 ± 0.7*	5.8 ± 0.9*	7.9 ± 1.0*	9.9 ± 0.4*

Hct, haematocrit; Hb, blood haemoglobin concentration; PV, plasma volume (percent change from baseline); Osm_P, plasma osmolality; TP, total protein; $[AVP]_P$, plasma concentration of arginine vasopressin; $[Na^+]_S$ and $[K^+]_S$, serum concentrations of sodium and potassium. *Significant hormone effect (ANOVA). †Significantly difference over time within GnRHa. §Significantly difference over time within oestrogen. Differences were considered statistically significant at $P < 0.05$. Data are expressed as means ± S.E.M.

fractional Na^+ reabsorption (PFR_{Na^+}) = (1 − C_{Li^+}/GFR) × 100; and distal fractional sodium reabsorption (DFR_{Na^+}) = (1 − C_{Na^+}/C_{Li^+}) × 100; fractional reabsorption of water in the proximal tubules (PFR_{H_2O}) = 1 − C_{Li^+}/GFR) × 100; and distal fractional water reabsorption (DFR_{H_2O}) = (1 − U_V)/C_{Li^+}) × 100. Distal fractional sodium and water reabsorptions described here are the percentages of sodium or water absorbed relative to the total sodium or water delivered to the distal tubule. The plasma clearance rate of AVP (PCR_{AVP}) at any given infusion rate was determined by dividing the infusion rate by the plasma concentration of AVP.

Data analysis

To determine overall urine renal concentrating sensitivity to AVP in individual subjects, standard log plots of $[AVP]_P$ *versus* urine osmolality were fitted to a sigmoidal curve (Siggaard-Andersen *et al.* 1984; Vago *et al.* 1995; Dodson & Rhoden, 2001). EC_{50} and log EC_{50} were determined by non-linear regression curve fitting of concentration–response data fitted to the equation $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + (EC_{50}/X)^n)$, where Y_{min} and Y_{max} are the minimal and maximal responses, respectively, X is the AVP concentration, and n is the Hill slope (GraphPad Prism, San Diego, CA, USA).

Statistics. Data are expressed as means ± S.E.M. The variables over time (control tests and oestrogen intervention tests) were analysed by conditions (oestrogen *versus* GnRHa alone), using ANOVA for repeated measures. When significant differences were found, orthogonal contrasts tested differences between specific means related to the hypothesis of interest. Paired *t* tests were used to determine differences in EC_{50} and log EC_{50} between treatments. Differences were considered statistically significant when $P < 0.05$ (SPSS, SPSS Inc., Chicago, IL, USA).

Sample size calculation. Expected Osm_U differences between the GnRHa and oestrogen treatments were derived from data

collected in our laboratory from subjects at rest (Calzone, 2001). In an earlier study administration of OE_2 and/or P4 increased Osm_U by 148 mosmol (kg H_2O)$^{-1}$ with an estimated pooled standard deviation for the group of 22 mosmol (kg H_2O)$^{-1}$ during hypertonic saline infusion.

The desired statistical test is two-sided at an alpha level of 0.05 with 80 % power to detect a difference. Based on our previous work, 80 % power is sufficient to detect a significant alteration in Osm_U. For a two-sided test, $Z(\alpha) = 1.96$, and for 80 % power, $Z(\beta) = 0.84$. The formula for calculating sample size for continuous response variables is (Colton, 1974):

$$N = 2[(Z(\alpha) + Z(\beta))^2 (s)^2 / (d)^2].$$

Substituting the values, the calculated sample size is eight subjects.

RESULTS

Eight of ten subjects reported occasional vasomotor symptoms during GnRHa treatment and some of these symptoms were relieved during oestrogen administration. Mild breast tenderness was also reported during the initial period of GnRHa administration. The subjects reported no other adverse effects due to the GnRHa or hormone administration, and the side effects did not lead to subject attrition.

Prior to beginning the study, subjects' weight and height were 69.6 ± 4.1 kg and 166 ± 3 cm, respectively. Subjects' weight was unaffected by either the GnRHa (69.9 ± 3.9 kg) or oestrogen (70.0 ± 3.9 kg) treatment. Pre-infusion $[E_2]_P$ and $[P4]_P$ during treatment with GnRHa alone were 19 ± 4 pg ml^{-1} and 0.4 ± 0.1 ng ml^{-1}, respectively; $[E_2]_P$ increased to 152 ± 31 pg ml^{-1} during GnRHa with oestrogen treatment ($P < 0.05$) with no change in $[P4]_P$ (0.7 ± 0.1 ng ml^{-1}). These data demonstrate a high compliance with, and effectiveness of, the GnRHa/oestrogen add-back protocol.

There were small, but consistent reductions in Hct and Hb (Table 1) suggesting a small increase in plasma volume due to oestrogen treatments (~7.9 %). In addition, plasma osmolality fell by 3 ± 1 mosmol (kg H_2O)$^{-1}$ during oestrogen administration ($P < 0.05$), as did $[Na^+]_S$ (Δ 1.6 ± 0.7 mequiv, Table 1). Baseline renal excretory variables were unaffected by oestrogen administration, except FE_{Na^+} was reduced (Figs 1 and 2, Table 2, $P < 0.05$). Blood pressure was unaffected by oestrogen administration (Table 2). Pre-infusion PRA was reduced during oestrogen treatment (Fig. 3, $P < 0.05$). Finally, oestrogen treatment did not alter pre-infusion renal excretion of prostaglandin E_2 (PGE_2, Fig. 3).

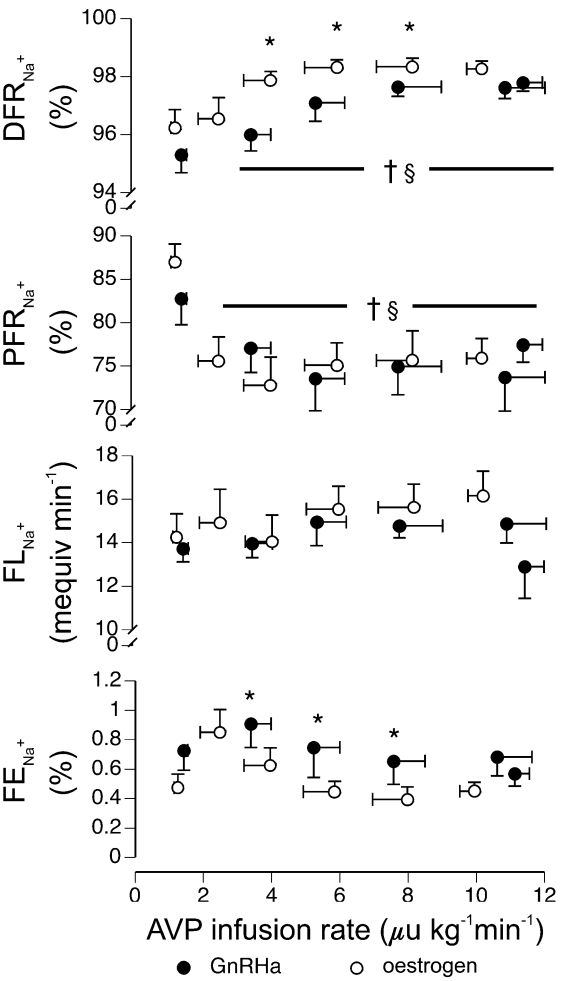

Figure 1. Renal sodium regulation responses to graded infusions of synthetic arginine vasopressin infusion

Fractional reabsorption of sodium (FE_{Na^+}) (*D*), total filtered load of sodium (FL_{Na^+}) (*C*) and fractional sodium reabsorption in proximal (PFR_{Na^+}) (*B*) and distal (DFR_{Na^+}) (*A*) tubules at baseline and during infusion at five different rates of synthetic arginine vasopressin (AVP) during treatment with gonadotropin-releasing hormone analogue (GnRHa) alone and with oestrogen. Variables are shown as functions of plasma AVP concentration. * $P < 0.05$, GnRHa alone *versus* oestrogen treatment. † $P < 0.05$, compared with baseline within GnRHa only treatment. § $P < 0.05$, compared with baseline within oestrogen treatment.

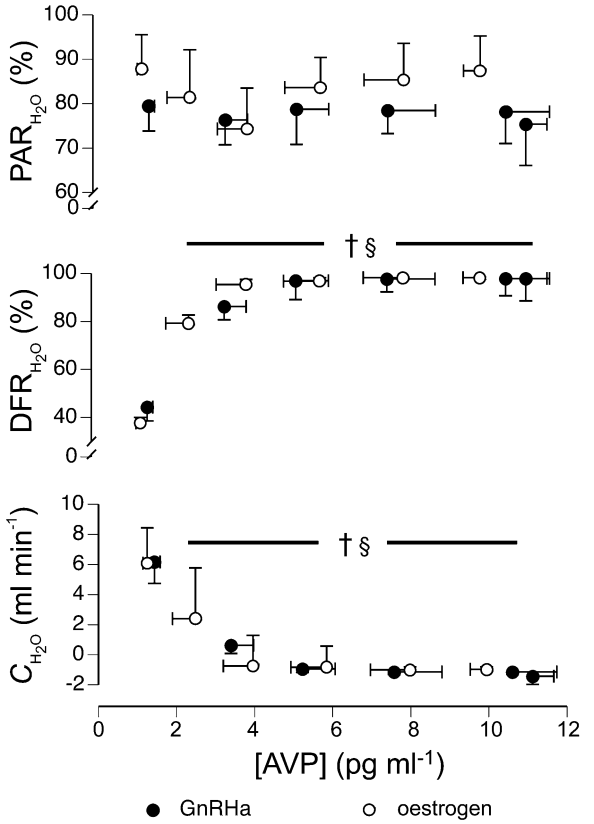

Figure 2. Renal water regulation

Renal free water clearance (C_{H_2O}) (*C*), and proximal (PFR_{H_2O}) (*A*) and distal fractional (DFR_{H_2O}) (*B*) water reabsorption at baseline and during infusion at five different rates of synthetic arginine vasopressin (AVP) during treatment with gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen. Variables are shown as functions of plasma AVP concentration. * $P < 0.05$, GnRHa alone *versus* oestrogen treatment. † $P < 0.05$, compared with baseline within GnRHa only treatment. § $P < 0.05$, compared with baseline within oestrogen treatment.

During AVP infusion, $[AVP]_P$ increased under both GnRHa alone and oestrogen administration conditions at all infusion rates (Table 1). However, starting at the infusion rate of equal to or greater than 35 μu kg^{-1} min^{-1}, $[AVP]_P$ was lower during oestrogen administration compared to GnRHa alone, and remained lower for the rest of the infusion rates (Table 1, $P < 0.05$), and PCR_{AVP} was greater for all infusion rates greater than 35 μu kg^{-1}min^{-1} (Fig. 4). Urine osmolality increased during the AVP infusion under both conditions, but was slightly lower during oestrogen administration, thus EC_{50} and log EC_{50} were unaffected by the oestrogen treatment (Fig. 5 and Table 3, $P < 0.05$). However, at the infusion rate of 35 μu kg^{-1} min^{-1}, when $[AVP]_P$ was 3.4 and 2.5 pg ml^{-1} for GnRHa and oestrogen, respectively, the urine osmolality response for a given $[AVP]_P$ was enhanced in the oestrogen *versus* the GnRHa condition (Fig. 5). We found this response to be apparent in most renal excretory variables, with an enhanced response to the increase in $[AVP]_P$ at these particular concentrations (Figs 1 and 2 and Table 2, $P < 0.05$). Although PFR_{Na^+} was reduced, DFR_{Na^+} increased, leading to lower FE_{Na^+} during oestrogen treatment over the course of the infusion. As expected, C_{H_2O} was drastically reduced and DFR_{H_2O} drastically increased in response to AVP infusions, but these responses were unaffected by hormone treatment (Fig. 2, $P < 0.05$). Starting at the infusion rate of 35 μu kg^{-1} min^{-1}, ERPF was unchanged, and was similar between GnRHa alone and oestrogen administration (Table 2, $P < 0.05$). Finally, PRA remained elevated during oestrogen administration compared to GnRHa, although PRA fell in response to the AVP infusion under both conditions (Fig. 3, $P < 0.05$). Blood pressure was not affected by the AVP infusion or the different hormone treatments (Table 2).

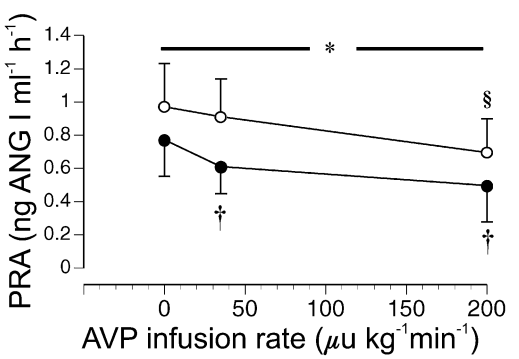

Figure 3. Plasma renin activity

Plasma renin activity (PRA) at baseline and during infusion at five different rates of synthetic arginine vasopressin (AVP) during treatment with gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen. * $P < 0.05$, GnRHa alone *versus* oestrogen treatment. † $P < 0.05$, compared with baseline within GnRHa only treatment. § $P < 0.05$, compared with baseline within oestrogen treatment.

Table 2. Renal and cardiac responses at baseline and in response to infusions of synthetic arginine vasopressin (AVP) during administration of gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen treatment

	AVP infusion rate (μu kg^{-1} min^{-1})					
	0	10	35	100	150	200
GFR (ml min^{-1})						
GnRHa	95 ± 4	98 ± 5	106 ± 7	104 ± 4	105 ± 7	97 ± 11
Oestrogen	100 ± 8	106 ± 11	100 ± 9	100 ± 7	109 ± 7	114 ± 7
U_v (ml min^{-1})						
GnRHa†	8.1 ± 1.5	2.6 ± 0.5†	0.8 ± 0.2†	0.6 ± 0.1†	0.6 ± 0.1†	0.5 ± 0.1†
Oestrogen	7.6 ± 1.0	4.5 ± 0.3*§	0.9 ± 0.3§	0.7 ± 0.2§	0.5 ± 0.1§	0.5 ± 0.1§
Osm_U (mosmol (kg H_2O)$^{-1}$)						
GnRHa	110 ± 39	309 ± 73†	686 ± 69†	808 ± 39†	834 ± 41†	844 ± 37†
Oestrogen	54 ± 6	136 ± 25§	674 ± 81§	782 ± 78§	861 ± 29§	833 ± 47§
C_{Osm} (ml min^{-1})						
GnRHa	1.9 ± 0.2	1.9 ± 0.2	1.7 ± 0.4	1.8 ± 0.3	1.8 ± 0.3	2.2 ± 0.9
Oestrogen	1.5 ± 0.3	2.0 ± 0.3	1.7 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
$U_{Na}V$ (mequiv)						
GnRHa	4.4 ± 0.8	7.2 ± 1.2	5.8 ± 1.4	5.8 ± 1.4	6.3 ± 1.4	5.2 ± 1.2
Oestrogen	2.6 ± 0.3	7.2 ± 1.1	4.8 ± 0.9	3.6 ± 0.5	3.6 ± 0.7	4.8 ± 1.0
$U_K V$ (mequiv)						
GnRHa	1.9 ± 0.5	2.1 ± 0.5	2.1 ± 0.6	2.1 ± 0.7	3.2 ± 0.5	3.5 ± 0.5
Oestrogen	1.0 ± 0.1	2.4 ± 0.4	2.2 ± 0.4	2.5 ± 0.4	2.9 ± 0.5	3.6 ± 0.6
SBP (mmHg)						
GnRHa	135 ± 5	132 ± 4	130 ± 4	132 ± 5	131 ± 4	133 ± 4
Oestrogen	133 ± 5	129 ± 5	127 ± 4	129 ± 4	130 ± 5	128 ± 5
DBP (mmHg)						
GnRHa	75 ± 5	67 ± 3	66 ± 3	69 ± 4	67 ± 4	66 ± 5
Oestrogen	75 ± 5	64 ± 5	62 ± 5	66 ± 5	64 ± 4	67 ± 4
HR (beats min^{-1})						
GnRHa	70 ± 3	68 ± 2	67 ± 3	67 ± 3	63 ± 3	66 ± 3
Oestrogen	74 ± 4	69 ± 3	67 ± 3	67 ± 3	66 ± 2	67 ± 3
ERPF (ml min^{-1})						
GnRHa	—	—	2420 ± 240	2480 ± 215	2266 ± 103	2084 ± 212
Oestrogen	—	—	2306 ± 283	2494 ± 296	2607 ± 347	2565 ± 269

GFR, glomerular filtration rate (estimated from creatinine clearance); U_v, urine flow; C_{Osm}, osmotic clearance; $U_{Na}V$ and $U_K V$, urine sodium and potassium excretions; SBP and DBP, systolic and diastolic blood pressures; HR, heart rate; and ERPF, effective renal plasma flow. *Significant hormone effect (ANOVA). †Significantly different from baseline within GnRHa. §Significantly different from baseline within oestrogen. Differences were considered statistically significant at $P < 0.05$. Data are expressed as means ± S.E.M.

Table 3. Determinants of renal concentrating response (urine osmolality, Osm_U) to graded infusions of arginine vasopressin (AVP)

EC_{50} (pg ml^{-1})		Log EC_{50} (pg ml^{-1})		Maximum (mosmol (kg H_2O)$^{-1}$)	
GnRHa	Oestrogen	GnRHa	Oestrogen	GnRHa	Oestrogen
3.3 ± 0.7	3.1 ± 0.6	0.47 ± 0.07	0.43 ± 0.07	857 ± 40	860 ± 28

EC_{50} and log EC_{50} represent the plasma concentration of AVP and log [AVP] when Osm_U was at 50 % of maximal response, and are indicators of renal sensitivity to AVP. Maximum indicates maximal response of Osm_U to the maximum infusion rate of AVP. Data are presented as means ± S.E.M.

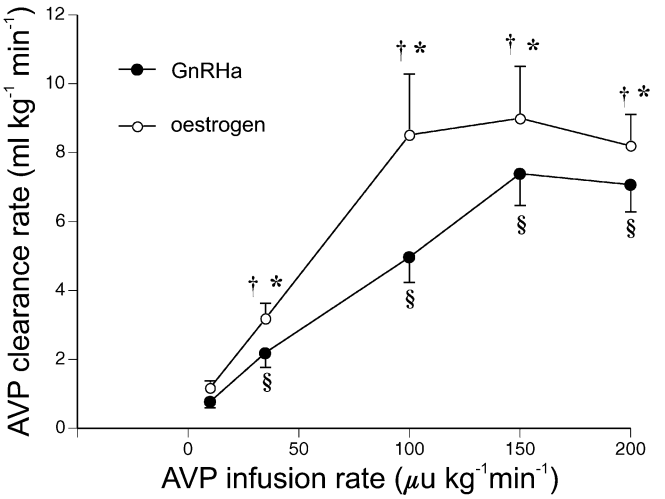

Figure 4. Plasma clearance rate of arginine vasopressin

Plasma arginine vasopressin (AVP) clearance rate (plasma AVP concentration/AVP infusion rate) at baseline and during infusion at five different rates of synthetic arginine vasopressin (AVP) during treatment with gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen. * $P < 0.05$, GnRHa alone *versus* oestrogen treatment.

DISCUSSION

Administration of a GnRH agonist for 4 weeks reduced plasma 17β-oestradiol and progesterone concentrations in healthy young women to postmenopausal levels. Four days of transdermal oestradiol administration added to the GnRHa increased plasma 17β-oestradiol levels by almost eightfold. This design eliminated other potential confounders such as GnRH and progesterone, so successfully isolated oestrogen effects on renal sensitivity to changes in $[AVP]_P$. The similar EC_{50} of the Osm_U–$[AVP]_P$ response curve between GnRHa alone and oestrogen administration indicated no oestrogen effect on AVP-mediated renal concentrating response overall. However, oestrogen administration attenuated the $[AVP]_P$ rise in response to the AVP infusions indicating an increase in plasma AVP clearance rate. Due to the greater PCR_{AVP}, the urine osmolality response was greater to increases in $[AVP]_P$ over the normal physiological range in which AVP has increasing concentrating effects in the tubules. Finally, oestrogen administration increased sodium distal tubular sodium reabsorption.

The plasma clearance rate for AVP was more rapid during oestrogen treatment at all infusion rates greater than 10 μu kg^{-1} min^{-1} (Fig. 4). Unfortunately, our data do not indicate the cause of the lower PCR_{AVP}, which may have been due to a variety of different mechanisms, including more rapid glomerular clearance and/or greater AVP metabolism (Claybaugh & Sato, 1985). The AVP molecule is small enough to be cleared via glomerular filtration, although how oestrogen impacts upon urine excretion of AVP is not known. During hypertonic saline infusion, women increase urinary AVP excretion to a greater extent

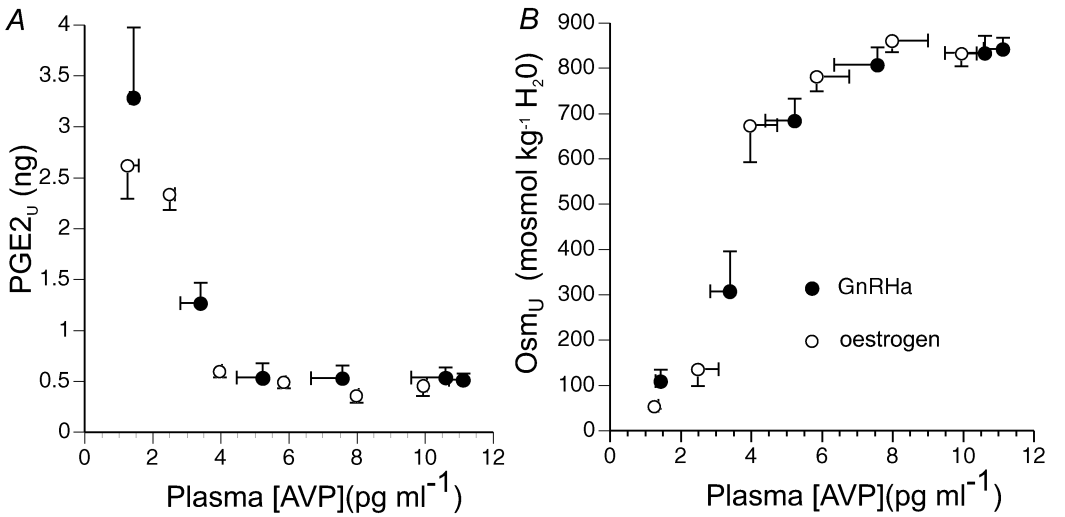

Figure 5. Renal prostaglandin and concentrating response

A, renal excretion of prostaglandin E2 ($PGE2_U$) and (*B*) urine osmolality (Osm_U) as functions of plasma AVP concentrations during treatment with gonadotropin-releasing hormone analogue (GnRHa) alone and together with oestrogen.

than men (Merkelbach *et al.* 1975; Vallotton *et al.* 1983), and ethnyl oestradiol administered to men leads to increases in urinary AVP excretion similar to that of women (Vallotton *et al.* 1983) suggesting that oestrogen increases AVP clearance by the kidney. The greater AVP excretion in urine may also be a reflection of greater $[AVP]_P$ (Moses & Steciak, 1986), but we found that men increased $[AVP]_P$ responses to a greater extent than women during hypertonic saline infusion (Stachenfeld *et al.* 2001*b*). In any event, urinary AVP excretion is difficult to interpret because the level of protein binding influences to a great extent whether or not AVP is filtered by the kidney (Claybaugh & Sato, 1985), and oestrogens can have profound effects on protein binding (De Feo, 1996). While the primary effect of oestrogen on protein binding and metabolism are due to first pass effects during oral administration, it is possible that transdermal oestrogen can have more subtle effects once it reaches the portal circulation.

As such, the more rapid PCR_{AVP} during oestrogen treatment may be a function of increased AVP metabolism, which takes place primarily in the liver, intestine and kidney (Claybaugh & Sato, 1985; Share *et al.* 1985; Claybaugh & Uyehara, 1993), and is also affected by the extent of AVP binding to proteins or to other hormones (Share *et al.* 1985). Studies in animals would tend to suggest that oestrogen does not directly affect AVP metabolism (Crofton *et al.* 1986; Wang *et al.* 1995), but oestrogen effects on AVP protein binding (De Feo, 1996) would certainly impact its metabolism. Moreover, AVP is cleared (not filtered) by the kidney via a specific vasopressin receptor that is inhibited by V_2 receptor antagonists (Keeler *et al.* 1991), and this receptor is sensitive to oestradiol (Wang *et al.* 1993; Dubey & Jackson, 2001).

Despite the more rapid PCR_{AVP}, the $Osm_U - [AVP]_P$ relationship was unaffected by oestrogen treatment. Our findings do not support earlier studies in rats showing that oestrogen inhibits AVP actions on C_{H_2O} in the renal tubules (Wang *et al.* 1994, 1995). Moreover, a close look at Fig. 5 indicates that at the $[AVP]_P$ of ~2.5 – 4.0 pg ml^{-1}, the urine concentrating responses (i.e. the increase in Osm_U) were greater during oestrogen treatment relative to GnRHa treatment, directly conflicting with the animal studies. These findings are important because the more rapid rise in Osm_U during oestrogen took place in a most relevant portion of the curve, that is, in the portion of the curve in which $[AVP]_P$ is still within 'normal' range for rest and exercise in humans. These findings should be followed up with steady-state infusions of the synthetic AVP at a number of steady-state infusion rates within the 10–50 μu kg^{-1} min^{-1} range. The present study is the first of this kind to be conducted in humans, suggesting the data from rats in the earlier studies may not be applicable to humans.

Species differences do not account for the consistent reports of greater AVP responses to osmotic stimulation (Calzone *et al.* 2001; Stachenfeld & Keefe, 2002). One possible explanation is that renal responsiveness to AVP during oestrogen treatment did occur in our subjects, but the greater PCR_{AVP} prevented excess water retention. During AVP infusions to dogs, Weitzman & Fisher (1978) found a strong correlation ($r = 0.993$) between PCR_{AVP} and Osm_U, indicating that those animals that removed AVP from the plasma more efficiently were the animals best at concentrating their urine. If the same relationship was true in our subjects, perhaps a lower renal sensitivity to AVP during oestrogen treatment balanced the more rapid AVP removal from the plasma, leading to variable changes in renal concentrating response.

An interesting parallel between our present findings and those of Weitzman & Fisher (1978) is that when the increase in urine osmolality was plotted as a function of the logarithm of $[AVP]_P$ in the dogs, this relationship was linear only to the point where the increase in AVP plasma clearance rate was also linear. A close look at Figs 4 and 5 indicates that this was also the case in the present investigation, with the PCR_{AVP} and Osm_U both showing earlier plateaus during oestrogen treatment. Studies using graded AVP infusions in rats have indicated that the linear component of the PCR_{AVP} response is a function of receptor-mediated (Keeler *et al.* 1991) biologically active AVP (cleared through both GFR *and* uptake by the distal tubules) (Shade & Share, 1976; Weitzman & Fisher, 1978), and is parallel to AVP actions on C_{H_2O} in the kidney (Shade & Share, 1976). In contrast, a plateau in PCR_{AVP} during graded AVP infusions suggests the clearance of a non-biologically active component of AVP (filtered solely by GFR) (Shade & Share, 1976). Thus because the PCR_{AVP} response reached an earlier plateau during oestrogen treatment, the level of biologically active AVP available to the kidney tubules may have been similar under the GnRHa alone and oestrogen treatments, even though $[AVP]_P$ was lower during the oestrogen administration. Moreover, it is in this linear portion of the curve that Osm_U was greater under the oestrogen condition.

Our findings support earlier data indicating that oestrogen administration increases sodium retention in the distal tubules, which may have important clinical implications with respect to fluid balance, orthostatic hypotension and hypertension during pregnancy or other high oestrogen states. Our study does not clarify the mechanism for these oestrogen-related effects in the kidney, but does indicate that the changes in sodium retention take place in the distal tubule. Oestrogen may alter sodium regulation via direct effects on renal distal tubules (Verlander *et al.* 1998). As stated earlier, both α and β oestrogen receptors have been found in the kidney tubules in a number of animal models (Dubey & Jackson, 2001) and in humans

(Mosselman *et al.* 1996; Dubey & Jackson, 2001), and oestradiol increases sodium uptake by both the proximal and distal tubule luminal membranes of rabbit kidneys (Brunette & Leclerc, 2001).

Despite the greater PRA, oestrogen effects on sodium reabsorption in the tubule are generally thought to be independent of the renin–angiotensin–aldosterone system (RAAS) (Verlander *et al.* 1998; Brunette & Leclerc, 2001). PRA typically increases concomitantly with increases in $[E_2]_P$ because oestrogen can stimulate the RAAS by enhancing angiotensinogen synthesis, inhibiting angiotensin-converting enzyme activity and augmenting plasma and tissue levels of renin (Hollenberg *et al.* 1976; Kuroski De Bold, 1999). However, the increase in renin associated with oestrogen administration does not necessarily indicate a change in circulating angiotensin II or aldosterone (Hollenberg *et al.* 1976; Kuroski De Bold, 1999; Calzone *et al.* 2001; Stachenfeld & Keefe, 2002). Moreover, because circulating angiotensinogen is synthesized primarily in the liver, increases in plasma angiotensin II and aldosterone may only be a consequence of oral oestrogen administration. In addition, the greater PRA during oestrogen did not seem to induce a measurable change in ERPF. Finally, the oestrogen-related increases in distal tubule sodium reabsorption in the present study may also have been the result of alterations in renal tubule aldosterone levels, changes in aldosterone-metabolism or protein binding, or mediated through effects on the RAAS actions within the kidney (Hollenberg *et al.* 1976; Kuroski De Bold, 1999).

Oestrogen may also have an indirect impact upon distal tubule sodium via effects on glucocorticoids or glucocorticoid receptors within the tubules (Farman *et al.* 1991). Physiological doses of oestrogen increase plasma glucocorticoids in humans (Mahajan *et al.* 1978) and rats (Burgess & Handa, 1992), which may in turn potentially activate mineralocorticoid receptors. Thus, elevation of plasma glucocorticoids could also indirectly have caused changes in sodium retention during oestrogen treatment. Finally, atrial natriuretic peptide may also play a role in the oestrogen-related changes in sodium regulation because ANP antagonizes renin and aldosterone actions in the kidney (Brenner, 1990) and oestrogen may promote ANP inhibition (Mulay *et al.* 1993).

While earlier studies indicated that AVP inhibits renal prostaglandin E_2 in men (Berl *et al.* 1977; Dixey *et al.* 1986), results have been conflicting in the few studies examining oestrogen effects on renal PGE_2 in women (Mountquin *et al.* 1984; Stratton *et al.* 1986; Farker *et al.* 1997). We found no evidence to suggest that oestrogen modulates the AVP–PGE_2 relationship. Consistent with our findings, urinary PGE_2 excretion is unchanged over the menstrual cycle (Stratton *et al.* 1986; Farker *et al.* 1997), but is significantly elevated in postmenopausal women (Farker *et al.* 1997) suggesting that age, rather than oestrogen status, is responsible for the greater PGE_2. On the other hand, urinary PGE_2 is increased in women following ovarian stimulation with gonadotropins, and is positively correlated with oestrogen and negatively correlated with progesterone concentrations in the blood during oral contraceptive administration. (Mountquin *et al.* 1984). Oral oestradiol, due to first pass effects in the liver and resulting changes in albumin synthesis and release, may induce changes in PGE_2 (Mountquin *et al.* 1984), suggesting we may not have seen renal PGE_2 effects because we used transdermal patches for oestrogen administration. It is also possible that effects of oestrogen on the AVP–PGE_2 relationship only occur when oestrogen is at a pharmacologically high level such as that induced by oral contraceptive administration and ovarian stimulation.

This study is the first to isolate oestrogen effects on $[AVP]_P$ and renal concentrating response to AVP in young women by suppressing reproductive function and adding back oestrogen in a controlled dose. We found a more rapid AVP plasma clearance rate, although where the greater clearance is taking place in the body cannot be determined from our data. This study was an attempt to explain earlier data indicating that oestrogen lowers the osmotic threshold for AVP release, but does not cause increases in renal water-concentrating response. Oestrogen did not alter renal tubular free water reabsorption in response to AVP overall, but did increase urine osmolality at the lower end of the Osm_U–$[AVP]_P$ curve and may indicate that the changes in AVP seen with oestrogen administration in earlier studies were of insufficient magnitude or duration to lead to changes in water retention. Alternatively there may be intrarenal compensatory changes that prevent excess water retention in the presence of greater plasma oestrogen. Our findings support earlier data showing that the primary changes in fluid retention during oestrogen treatment were more closely related to increases in sodium reabsorption in the distal tubule. Finally, oestrogen does not appear to have an impact upon renal blood flow or renal PGE_2. More studies using steady-state infusions of synthetic AVP to induce $[AVP]_P$ at the lower end of the Osm_U–$[AVP]_P$ curve are warranted.

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Acknowledgements

We gratefully acknowledge the technical support of Jodi Crimmins, Wendy Calzone and Nana Asafu-Agyei and the cooperation of the volunteer subjects. This work is supported by The National Institutes of Health, RO1 no. HL62240–01A1.