Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pharmacology, Biochemistry and Behavior 76 (2003) 535–542
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Oestrogen receptor-β and neurohypophysial hormones: functional interaction and neuroanatomical localisation

M.L. Forsling[a,*], I. Kalló[a,b], D.E. Hartley[a], L. Heinze[a], R. Ladek[a], C.W. Coen[a], S.E. File[a]

[a]*Neuroendocrine and Psychopharmacology Research Laboratories, Centre for Neuroscience, King's College London, Guy's Campus, London, SE1 1UL, UK*

[b]*Department of Neurobiology, Institute of Experimental Medicine, Budapest, Hungary*

Received 17 July 2003; received in revised form 9 September 2003; accepted 9 September 2003

Abstract

Oestrogens affect fluid balance, influencing both ingestive behaviour and renal excretion. The renal effects are partly due to altered release of vasopressin and oxytocin. This study was designed to explore the role of oestrogen receptor-β (ERβ) in neurohypophysial hormonal function. Following dietary administration, soya isoflavones reach the brain in sufficient concentration to activate ERβ, but not oestrogen receptor-α (ERα). ERβ function was therefore manipulated by feeding rat diets differing in soya isoflavone content. Fluid balance and neurohypophysial hormone release were measured in male rats maintained for 14 days on a soya isoflavone-free diet or one containing 150 μg/g genistein + daidzein. Food and water intake, body weight, urine flow, osmolality and sodium concentrations were determined daily. After 14 days, plasma and urine osmolality and sodium, vasopressin and oxytocin concentrations were determined. There was no significant difference in weight gain between the two groups or in their excretion of sodium and water or plasma sodium and plasma oxytocin. However, plasma vasopressin was significantly lower in the iso-free group. Double-label immunocytochemistry was used to assess colocalisation of ERβ with the neurohypophysial hormones in male rats. Cell nuclei showing ERβ immunoreactivity were abundant in the posterior magnocellular paraventricular nucleus (PVNpm) and in the supraoptic nucleus (SON). Vasopressin-immunoreactive neurones were similarly distributed, forming the core of the PVNpm and the ventral portion of the SON; majority were positive for ERβ. Cells with oxytocin immunoreactivity were located mainly at the periphery of the PVNpm and in the dorsal SON; only approximately a quarter of these cells showed ERβ immunoreactivity. Thus, the difference in the effects of the soya diet on vasopressin and oxytocin release may be related to the ERβ-activating properties of this diet and to the preponderance of this receptor in vasopressin as opposed to oxytocin cells.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Oestrogen receptor-β; Oxytocin; Paraventricular nucleus; Soya isoflavone; Supraoptic nucleus; Vasopressin

1. Introduction

Recently, there has been considerable scientific interest in the role of soya in the numerous aspects of human health including breast cancer (Dai et al., 2001; Wu et al., 1996; Hirose et al., 1995; Lee et al., 1991), prostate cancer (Strom et al., 1999; Jacobsen et al., 1998; Stephens, 1997; Aldercreutz, 1995) and cardiovascular diseases (Clarkson, 2002; Gardner et al., 2001). Some studies have reported beneficial effects of soya isoflavones with respect to bone density (Amonkar and Mody, 2002; Kritz-Silverstein and Goodman-Gruen, 2002; Messina and Messina, 2000; Scheiber et al., 2001; Scambia et al., 2000), while others have reported no significant effects (Murkies et al., 1995; Hsu et al., 2001). The alleviation of menopausal symptoms has also been reported (Davis, 2001; Knight et al., 2001; Murkies et al., 1995; Scambia et al., 2000; Faure et al., 2002; Han et al., 2002). In general, however, the effects specifically due to soya isoflavones are modest in degree, although assessment of these effects has been hampered by the design of some of the studies (for a detailed review, see Albertazzi and Purdue, 2002).

Two different oestrogen receptors [oestrogen receptor-β (ERβ) and oestrogen receptor-α (ERα)] have been identified in the brain (Kuiper et al., 1996), and soya isoflavones, such as genistein and daidzein, have a higher affinity for

* Corresponding author. Guy's King's and St Thomas' School of Medicine, 2-38A Neuroendocrine Labs, New Hunt's House, Guy's Campus, London Bridge, London SE1 1UL, UK. Fax: +44-20-7848-6194.
E-mail address: mary.forsling@kcl.ac.uk (M.L. Forsling).

0091-3057/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2003.09.013

ERβ than for ERα (Kuiper et al., 1998). Following dietary administration, these isoflavones can reach the brain in concentrations that may be sufficient to activate ERβ but are unlikely to activate ERα (Lephart et al., 2000). Evidence that dietary administration of soya isoflavones can affect brain function comes from findings that they modulate brain neurotrophins (File et al., 2003; Pan et al., 1999a,b), decrease calcium-binding proteins at certain sites in the brain of male rats (Lephart et al., 2000), increase anxiety and corticosterone and vasopressin hormone release in male rats (Hartley et al., 2003). In addition, they have been shown to improve radial arm-maze performance in ovariectomised female rats (Pan et al., 2000) and to improve cognition in young adults (File et al., 2003) and postmenopausal women.

Oestrogen modulates vasopressin release in both man (Stachenfeld et al., 1998; Ekstrom et al., 1992) and animals (Sladek et al., 2000; Hartley et al., 1999a,b), with both basal and stimulated release being affected. Gonadal steroids also influence oxytocin release with progesterone playing a key role (Antonijevic et al., 2000). However, our previous study (Hartley et al., 2003) found that only the former was changed following a relatively short period of dietary administration of soya. One purpose of Experiment 1 was therefore to determine whether this important difference could be replicated. Our previous study involved young male rats (mean body weight 175 ± 4 g) and administration of a modified diet for a period of 18 days, whilst the current study used mature male rats (mean body weight 344 ± 6 g) and a shorter dietary modification period of 14 days.

Oestrogen also affects fluid balance through an effect on ingestive behaviour (Forsling and Peysner, 1988), as well as an effect on the kidney, either directly or through altered renal responsiveness to renally active hormones (Boyce et al., 2001; Zhou et al., 1992). To date, the influence of dietary soya on fluid balance has not been reported and this was a second purpose for the study. The site at which oestrogens affect posterior pituitary hormone release has been unclear. Oestrogen modulates vasopressin release in response to both hypovolaemia and elevated plasma sodium (Hartley et al., 1999a,b); since these stimuli involve very different pathways, it appears likely that oestrogen acts directly on the magnocellular neurones producing the hormone. However, while radioactive oestradiol-binding studies have shown oestradiol uptake in rat and mouse neurophysin-ir cells (Rhodes et al., 1981; Sar and Stumpf, 1980), immunoreactivity for oestrogen receptors was not apparent in the supraoptic (SON) or paraventricular (PVN) nuclei of the rat (Simonian and Herbison, 1997). However, Shughrue et al. (1997, 1996) and Li et al. (1997) demonstrated that messenger RNA (mRNA) for ERβ and ERβ-like immunoreactivity are found in the PVN and SON.

The purpose of Experiment 2 was to determine whether the anatomical distribution of receptors could explain the different effects of the soya diet on the release of vasopressin and oxytocin. The presence of ERβ mRNA or immunoreactivity in the SON and PVN of rats has been reported (Hrabovszky et al., 1998; Alves et al., 1998, Isgor et al., 2003), but these studies did not provide quantitative data on the incidence of colocalisation with magnocellular vasopressin and oxytocin neurones.

The interest in the use of soya in the postmenopausal women is likely to grow following the early termination of one aspect of the Women's Health Initiative study, which examined a randomised controlled primary prevention trial on conjugated equine oestrogen and medroxyprogesterone acetate (Rossouw et al., 2002). There is therefore an urgent need for scientific investigation of the physiological effects of soya isoflavones.

2. Methods

2.1. Animals

The studies were performed in accordance with the Animals (Scientific Procedures) Act, 1986. Male hooded Lister rats (Charles River Margate, Kent, UK) were housed under controlled lighting conditions (12:12-h light/dark cycle; lights on at 07:00 h) with constant temperature (22–24 °C) and humidity (40–50%) and were allowed food and water ad libitum. In Experiment 1, 16 rats were initially maintained on our standard laboratory maintenance diet [Rat and Mouse No. 1 maintenance diet (R&M1); Special Diet Services Witham Essex, UK]. This diet contained approximately 150 μg of isoflavones (genistein + daidzein) per gram of diet (Odum et al., 2001). The rats were then randomly allocated (eight per group) either to a diet containing no soya isoflavones (iso-free diet; 2016-Teklad Global, Harlan UK, Bicester, Oxon, UK) or to a diet with 150 μg genistein + daidzein per gram of diet (iso-150 diet; 2018 Teklad Global/Teklad 9607 TRM Harlan UK; the specific measurements of the isoflavone contents of the two diets were made by Dr Tobin, Harlan Teklad). The protein content of the diets were matched with the iso-150 diet deriving protein from soyabean meal and the iso-free diet, not containing soyabean meal, deriving protein from the other major grain ingredients. The sodium contents of the iso-150 diet and the iso-free diet were 0.25% and 0.23%, respectively. The rats were maintained on these diets for 14 days. In Experiment 2, 10 rats were maintained on the standard R&M1 diet.

2.1.1. Experiment 1

2.1.1.1. Measurement of fluid balance, osmolality and electrolytes. All rats were housed in pairs and weighed daily Monday–Friday in the morning (08:00–09:00 h) and evening (17:00–18:00 h). At the same time, food and water

intakes were recorded. Individual rats were studied in metabolism cages (NKP Dartford, Kent, UK) for a maximum of 12 h with intervals of at least 3 days between each period of observation. Whilst in the metabolism cages, rats had access to preweighed water and food in excess of requirements. Urine volumes were measured on rats under observation in the metabolism cages with samples taken for determination of osmolality and electrolytes. After 14 days of observations, blood was obtained from animals by decapitation at 09:00 h for determination of plasma and urine osmolality and sodium and plasma oxytocin and vasopressin concentrations. The sodium concentrations were measured by flame photometry (410C Corning, Halstead, Essex, UK), with osmolalities being determined by vapour pressure osmometry (Wescor Vapour Pressure Osmometer, UT, USA).

2.1.1.2. Hormone determinations. Oxytocin and vasopressin were extracted from plasma using C18 Sep-Pak columns (Waters Associates Northwick, Middx, UK) as previously described (Forsling, 1985). Vasopressin was determined using the First International Standard for vasopressin (77/501). The lower limit of detection was 0.1 pmol/l and the intra- and interassay coefficients of variation 7.5% and 11.6%, respectively, at 2.5 pmol/l. The oxytocin assay employed the Fourth International Standard (76/575) and had a lower limit of detection of 0.1 pmol/l and the intra- and interassay coefficients of variation 5.1% and 8.9%, respectively, at 2.5 pmol/l.

2.1.2. Experiment 2

2.1.2.1. Brain tissue collection and preparation. Rats ($n=5$) were perfused transcardially (25 ml/min; under pentobarbital anaesthesia, 80 mg/kg) with a fixative containing 4% acrolein plus 2% PFA in PBS (50 ml) followed by 2% PFA in PBS (150 ml). Brains were removed, postfixed overnight in 2% PFA, cut in the coronal plane (from bregma—0.46, to bregma—2.45; Swanson, 1992) with a freezing microtome (30 μm) and collected into a series of six wells; thus, each well contained consecutive sections separated by 150 μm rostrocaudally.

2.1.2.2. Immunohistochemistry double labelling. Sections to be stained for ERβ and oxytocin or ERβ and vasopressin were pretreated with solutions including 1% sodium borohydride (20 min), 0.5% Triton X-100 (60 min), 0.5% H_2O_2 (10 min) and 2% normal donkey serum (30 min). Sections were incubated for 72 h at 4 °C in Z8P rabbit antimouse ERβ polyclonal antibody (Zymed Labs, South San Francisco, CA, USA; 0.05–0.1 mg/ml).

ERβ-IR was visualised by using the ABC method; biotinylated goat antirabbit antibody (Vector Labs, Peterborough, UK) was used at 1:1000 for 2 h and the Vectastain ABC Elite kit (Vector Labs) at 1:3000 for 1 h. Nickel-enhanced diaminobenzidine (Ni-DAB) was used as the chromogen (0.05% DAB, 0.15% nickel-ammonium sulphate in 0.1 M Tris buffer pH 7.6 with 0.005% H_2O_2). The Ni-DAB reaction product was amplified by employing silver–gold intensification (SGI). Tissue argyrophylia was suppressed with 8% thioglycolic acid for 30 min prior to incubation in the primary antibody. After the Ni-DAB reaction, sections were transferred into 2% sodium acetate and left overnight prior to the silver intensification and gold toning, which were carried out according to a previously described protocol (Dobo et al., 1996; Liposits et al., 1984).

For the subsequent identification of immunoreactivity for oxytocin or vasopressin, sections were incubated in mouse anti-oxytocin-associated neurophysin (PS-38, gift from Dr. H. Gainer) or rabbit antivasopressin (#64717, ICN Biomedicals, Aurora, OH, USA). Previous studies have demonstrated that double-label peroxidase-based immunohistochemistry can be successfully carried out using primary antibodies from the same species when the antigens are in different subcellular compartments and the first reaction product is silver–gold intensified (Liposits et al., 1986).

The secondary antibodies (Vector Labs) employed for visualising oxytocin or vasopressin were biotinylated horse antimouse IgG (1:1000) or biotinylated goat antirabbit IgG (1:1000), respectively. The sections were reacted with DAB alone for these cytoplasmic markers.

2.1.2.3. Evaluation. After development, sections were mounted, cover slipped and analysed for double labelling in the SON. Counting of single- and double-labelled cells was carried out at x100 magnification; this magnification level allowed us to distinguish the black silver grains in the nucleus from the dark brown DAB reaction product often present in the perinuclear region of an immunoreactive cell. Immunoreactive cells were counted in both left and right SON of every sixth section throughout the rostrocaudal extent of the hypothalamus.

2.1.2.4. Statistical analyses. The body weights, food and water intake and urinary excretion were analysed using two-way split plot analyses of variance with dietary treatment as the independent factor and days as the repeated measure. The plasma neurohypophysial concentrations, sodium and osmolality data were analysed using one-way analyses of variance. All values presented are the mean ± S.E.M. A value of $P<.05$ was selected as indicative of a significant difference.

3. Results

3.1. Experiment 1

3.1.1. Body weight, food and water intake

All animals in Experiment 1 showed a significant increase in body weight over the 14-day period [days,

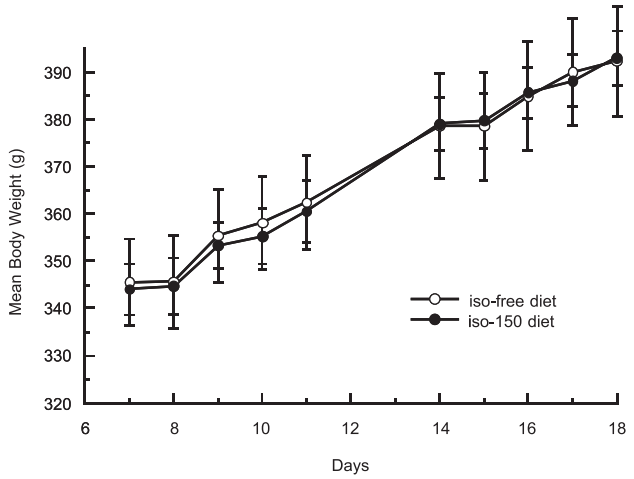

Fig 1. Mean (± S.E.M.) body weight (g) of male rats fed a diet containing 150 μg/g soya isoflavone (iso-150) or a diet containing no soya isoflavones (iso-free) over an observation period of 2 weeks.

$F(9,126)= 83.5$; $P<.001$; Fig. 1]. The rate of increase in body weight did not differ between the two dietary groups [Days × Treatment interaction, $F(9,126)= 0.9$; NS; Fig. 1]. There were no differences between the two dietary groups in their food and water intake [$F(9,54)=1.1$ and 2.2; both NS; see Table 1].

3.1.2. Fluid balance

The two dietary groups did not differ in their sodium intake [$F(9,54)=1.1$], sodium excretion [$F(1,18)=0.01$; NS], urine osmolality [$F(1,18)=0.5$, NS] or urine flow rate [$F(1,18)=2.8$; NS], see Table 1.

3.1.3. Plasma osmolality and neurohypophysial hormone concentrations

The two dietary groups did not differ in their plasma osmolality [$F(1,14)=0.2$; NS; Table 1]. The vasopressin concentration in the iso-free group was significantly lower than in the iso-150 group [$F(1,14)=7.3$; $P<.05$; see Fig. 2]. However, there was no difference between the groups in their plasma oxytocin concentrations [$F(1,14)=1.1$; NS; Table 1].

Table 1
Mean ± S.E.M. for fluid balance parameters and oxytocin concentrations in male rats fed a diet containing no soya isoflavones (iso-free) or a diet containing 150 μg/g total isoflavone (iso-150)

	iso-free ($n=8$)	iso-150 ($n=8$)
Daily food intake (g)	5.9 ± 0.6	6.8 ± 0.7
Daily water intake (g)	8.6 ± 0.3	9.4 ± 0.2
Daily sodium intake (mmol/100 g body weight)	0.45 ± 0.01	0.46 ± 0.01
Daily sodium excretion (mmol/100 g body weight)	0.44 ± 0.01	0.44 ± 0.01
Daily urine flow (ml)	2.0 ± 0.1	1.9 ± 0.1
Urine osmolality (mosmol/kg)	2759 ± 126	2743 ± 107
Plasma oxytocin (pmol/l)	16.6 ± 2.7	13.3 ± 1.8

There were no significant differences between the two groups for any of these parameters.

3.2. Neuroanatomical distribution of ERβ and the neurohypophysial hormones

3.2.1. Double-labelling study

As illustrated in Fig. 3, cell nuclei showing ERβ immunoreactivity were found to be abundant in the posterior magnocellular subnucleus of the PVN (pm) and ventral SON. Magnocellular vasopressin-immunoreactive neurones showed a similar pattern of distribution forming the core of

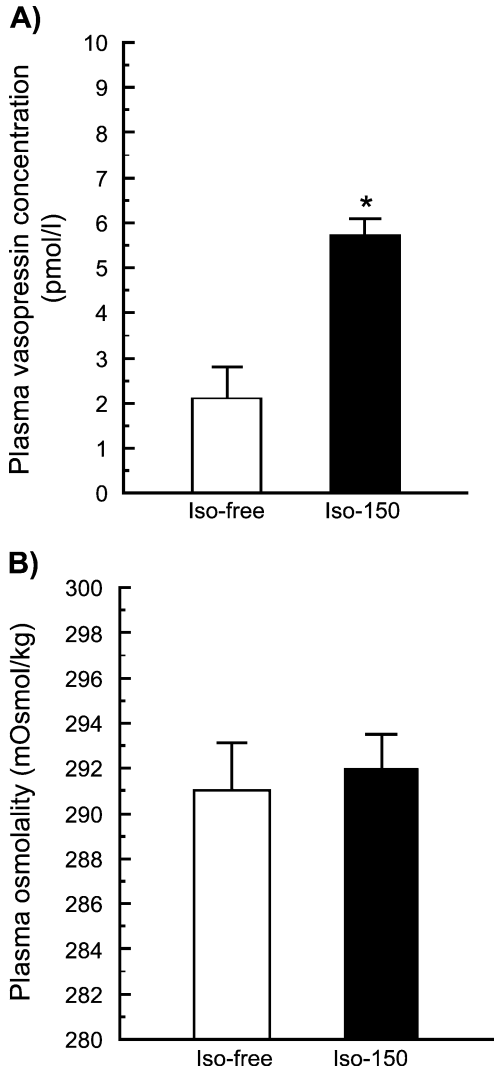

Fig 2. (A) Plasma vasopressin concentrations and (B) plasma osmolality in male rats fed a diet containing no soya isoflavones (iso-free) or a diet containing approximately 150 μg/g total isoflavone (iso-150). The values shown are the mean ± S.E.M. ($n=8$ per group). * $P<.05$ (ANOVA) significantly higher plasma vasopressin concentration in he iso-150 group.

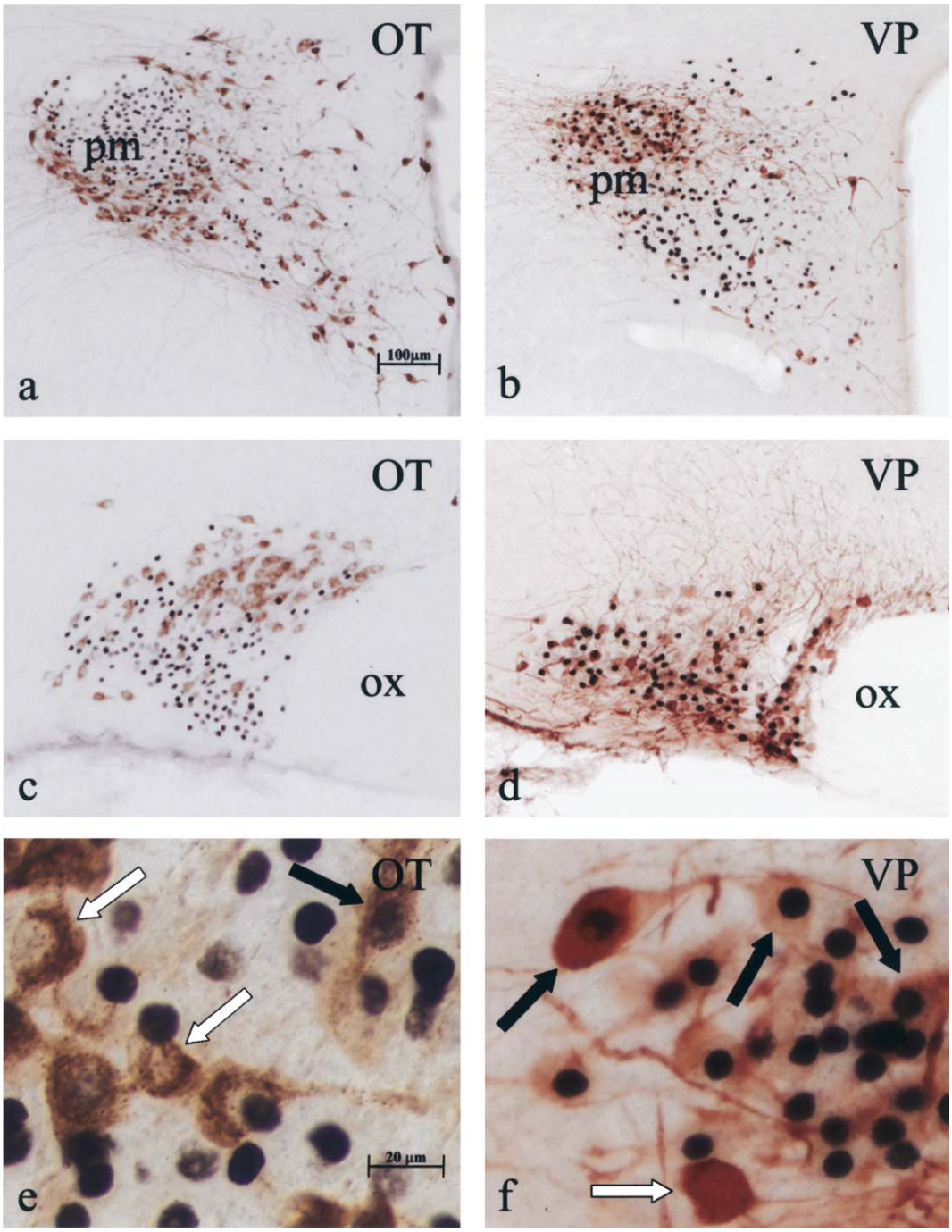

Fig 3. Simultaneous detection of oestrogen receptor-β (ERβ) and oxytocin (OT) or vasopressin (VP) immunoreactivity in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) of intact male rats. ERβ immunoreactive cell nuclei (black dots) are abundant in the posterior magnocellular subnucleus (pm) of the PVN (a, b) and the SON (c, d), regions known to project to the posterior lobe of the pituitary gland. OT-immunoreactive cells (labelled by the grey diaminobenzidine reaction product) are located mainly at the periphery of the pm (a) and in the dorsal portion of the SON (c). In contrast, VP-immunoreactive neurones form the core of the pm (b) and the ventral portion of the SON (d). In the SON, the incidence of ERβ is low in OT cells (black arrow) (e) but more abundant (black arrows) in VP cells (f). White arrows indicate OT (e) or VP (f) cells that are immunonegative for ERβ.

the pm (Fig. 3b) and the ventral portion of the SON, regions known to project to the posterior lobe of the pituitary gland; most of these vasopressinergic neurones were positive for ERβ. In contrast, magnocellular cells displaying oxytocin immunoreactivity were located mainly at the periphery of the pm and the dorsal portion of the SON. While nuclei immunolabelled for ERβ were found in a minority (25%) of oxytocin cells in the SON, this signal

Table 2
Quantification of vasopressin, oxytocin and oestrogen receptor β (ERβ) immunoreactivities and their coexpression in the SON

Analysis of sections immunostained	Percentage of oxytocin or vasopressin cells with ERβ nuclei	Oxytocin or vasopressin cells counted	ERβ nuclei counted
Oxytocin + ERβ	25.0 ± 3.3	624 ± 74	1393 ± 94
Vasopressin + ERβ	62.6 ± 2.6	1896 ± 39	1292 ± 70

Cells (mean ± S.E.M.) immunoreactive for ERβ, oxytocin or vasopressin were counted bilaterally in every sixth section throughout the rostrocaudal extent of the SON ($n = 5$ rats).

was detected in the majority (63%) of supraoptic vasopressin cells (Table 2).

4. Discussion

Experiment 1 found that the soya diet was without any effect on body weight or fluid balance. Thus, the two dietary groups did not differ in urine flow, sodium intake or excretion, plasma sodium or in plasma and urine osmolalities. Whilst we cannot identify the role of particular soya isoflavones from our study, in an in vitro study, Somponpun and Sladek (2002) found that genistein could mimic the effect of oestrogen on NMDA-stimulated vasopressin release from the hypothalamus. Given that fluid balance was unaffected despite differences in vasopressin concentrations, it would appear that renal responsiveness could have been affected. Oestrogen has been shown to affect renal responsiveness in the rat (Eckert et al., 1999). Furthermore, ERβ mRNA has been demonstrated in renal tissue of both rat (Kuiper et al., 1997) and man (Brandenberger et al., 1997).

An important consideration when investigating dietary isoflavones is that the major circulating isoflavone metabolite in rats fed daidzein is equol, a very physiologically active nonsteroidal oestrogen that binds both to ERα and ERβ (Rowland et al., 2003). However, neuroanatomical localisation studies (Shughrue et al., 1997, 1996; Kuiper et al., 1997) have demonstrated that in the SON and PVN of the hypothalamus, ERβ alone is expressed. It is therefore unlikely that the presence of equol would play a significant role in the modulation of the neurohypophysial hormones by its interaction with ERα.

The results of Experiment 1 replicate those of our previous study (Hartley et al., 2003), in which we found that 18 days' administration of diets differing in soya isoflavone concentrations resulted in significantly different vasopressin, but not oxytocin, concentrations. It also demonstrates that the change in vasopressin can occur after only 14 days of dietary modification, although it is not known how rapidly these changes occur. Our two studies used different ages of rat (young adults vs. mature adults), but in neither case did the administration of the soya diet result in any differences in gain in body weight or daily food or water intake. This again is an important contrast with the effects of oestrogen (Butera, 1996).

The soya diets used in Experiment 1 were selected from those employed by Odum et al. (2001) for a study of the effect of rodent diets on sexual development. Recently, increasing attention has been paid to the composition of commercially available diets. Brown and Setchell (2001) have shown that some widely used diets formulated with soya protein result in very high steady state serum isoflavone concentrations. The high isoflavone content of such diets may, for example, interferes with responses to exogenously administered test compounds (Thigpen et al., 1999) and with the sexual development of the animals (Odum et al., 2001). Our results, together with those of Hartley et al. (2003), show that hormonal levels can be altered; thus, the soya content of various diets could be an important source of interlaboratory variability in both endocrinological and behavioural studies.

Although vasopressin release was influenced by soya isoflavones in the diet, there was no significant effect on oxytocin. Studies on ovariectomised female rats have demonstrated mRNA for ERβ in hypothalamic neurones immunoreactive for oxytocin, but these were largely found in the caudal portion of the PVN (Hrabovszky et al., 1998). Other studies on ovariectomised rats found that ERβ is only associated with oxytocinergic neurones in the parvicellular subdivisions of the PVN, those divisions forming descending projections to the autonomic brainstem or projecting to the median eminence (Alves et al., 1998). Stimulation of these neurones would not affect circulating concentrations of the hormone.

Experiment 2 provides an analysis of the colocalisation of ERβ with oxytocin or vasopressin in the SON of the male rat. The results indicate that 63% of the vasopressin neurones in the SON display ERβ immunoreactivity; this immunoreactivity is found in only 25% of the oxytocin neurones. The present study focused on male animals; two previous studies on female rats indicated greater expression of ERβ mRNA (Hrabovszky et al., 1998) and ERβ immunoreactivity (Alves et al., 1998) in AVP as opposed to oxytocin containing cells of the SON. Given the sexually dimorphic development of the SON (Paula-Barbosa et al., 1993; Madeira et al., 1993), it may be important to undertake quantitative comparisons of these parameters between male and female rats. In this context, it should be noted that Ishunina et al. (2000) found a considerably higher incidence of ERβ immunoreactivity in SON vasopressin neurones in premenopausal women compared with age-matched men; the incidence of ERα immunoreactivity in those neurones was lower than that of ERβ immunoreactivity in premenopausal women but higher in men.

Studies on transfected cells have shown that ERα and ERβ display differential regulation of the AVP promoter (Shapiro et al., 2000). In cells with ERα, oestrogen stimulated luciferase activity; oestrogen also increased promoter activity in cells with low ERβ concentrations. However, in the presence

of high receptor concentrations, ERβ-mediated transcription was inhibited by oestrogen (Shapiro et al., 2000). Further investigations will be required to establish how such observations relate to the present findings.

In summary, soya isoflavones, like endogenous oestrogens, can influence neurohypophysial function, enhancing vasopressin release. No significant effect on oxytocin release was demonstrated, which may be related to the lower incidence of ERβ immunoreactivity in oxytocin neurones compared with vasopressin neurones.

Acknowledgements

This study was supported by grants from the Dunhill Medical Trust and the Wellcome Trust and by the European Union SOCRATES student exchange programme. The authors are grateful to Dr H. Gainer for the kind donation of the PS-38 antibody and Dr Tobin of Harlan Teklad for the detailed calculations of the isoflavone content of our diet.

References

Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect 1995;103(Suppl 7):103–12.

Albertazzi P, Purdue DW. The nature and utility of the phytoestrogens: a review of the evidence. Maturitas 2002;42:173–85.

Alves SE, Lopez V, McEwen BS, Weiland NG. Differential colocalization of estrogen receptor beta (ERβ) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. Proc Natl Acad Sci U S A 1998;95:3281–6.

Amonkar MM, Mody R. Developing profiles of postmenopausal women being prescribed estrogen therapy to prevent osteoporosis. J Commun Health 2002;27:335–50.

Antonijevic IA, Russell JA, Bicknell RJ, Leng G, Douglas AJ. Effect of progesterone on the activation of neurones of the supraoptic nucleus during parturition. J Reprod Fertil 2000;120:367–76.

Boyce N, Ward J, Rosser M, Hale J, Thompson E, Som T, et al. The effect of reproductive status on the renal response to desmopressin (DDAVP) in normal women. J Physiol 2001;531:161.

Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffem RB. Nucleotide tissue distribution of estrogen receptors alpha (ER-α) and beta (ER-β) mRNA in the midgestational human fetus. J Clin Endocrinol Metab 1997;82:3509–12.

Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Lab Invest 2001;81:735–47.

Butera PC. CNS steroids and the control of feeding. In: Stone TW, editor. CNS neurotransmitters and neuromodulators neuroactive steroids. New York: CRC Press; 1996. p. 211–35.

Clarkson TB. Soy phytoestrogens and cardiovascular disease. J Nut 2002;132:566S–9S.

Dai Q, Shu XO, Jin F, Potter JD, Kushi LH, Teas J, et al. Population-based case-control study of soyfood intake and breast cancer risk in Shanghai. Br J Cancer 2001;85:372–8.

Davis SR. Phytoestrogen therapy for menopausal symptoms? BMJ 2001;323:354–5.

Dobo E, Dudas B, Kallo I, Liposits Zs. Novel immunohistochemical procedures for simultaneous detection of neuropeptides in human brain. Neurobiology 1996;4:139.

Eckert T, Forsling ML, Schwarzberg H. The effect of combined oestrogen and progesterone replacement on the renal responses to oxytocin and vasopressin in ovariectomized rats Eur J Endocrinol 1999;141:297–302.

Ekstrom P, Akerlund M, Forsling ML, Kindahl H, Laudanski T, Mrugacz G. Stimulation of vasopressin release in women with primary dysmenorrhoea and after oral contraceptive treatment-effect on uterine contractility. Br J Obstet Gynaecol 1992;99:680–4.

Faure ED, Chantre P, Mares P. Effects of a standardized soy extract on hot flushes: a multicenter, double-blind, randomized, placebo-controlled study. Menopause 2002;9:329–34.

File SE, Hartley DE, Alom N, Rattray M. Soya phytoestrogens change cortical and hippocampal expression of BDNF mRNA in male rats. Neurosci Lett 2003;27(338):135–8.

Forsling ML. Measurement of plasma vasopressin in body fluids. In: Baylis PH, Padfield PD, editors. Posterior pituitary. New York: Marcel Dekker; 1985. p. 161–2.

Forsling ML, Peysner K. Pituitary and plasma vasopressin concentrations and fluid balance throughout the oestrous cycle of the rat. J Endocrinol 1988;117:397–402.

Gardner CD, Newell KA, Cherin R, Haskell WL. The effect of soy protein with or without isoflavones relative to milk protein on plasma lipids in hypercholesterolemic postmenopausal women. Am J Clin Nutr 2001;73:728–35.

Han KK, Soares Jr JM, Haidar MA, de Lima GR, Baracat EC. Benefits of soy isoflavone therapeutic regimen on menopausal symptoms. Obstet Gynecol 2002;99:389–94.

Hartley DE, Dickson SL, Forsling ML. The influence of oestradiol replacement on plasma vasopressin concentrations and Fos protein expression in the supraoptic nucleus following hypovolaemia in the ovariectomised rat. J Physiol 1999a;518:107.

Hartley DE, Dickson SL, Forsling ML. AVP release and Fos protein expression in the rat SON on infusion of hypertonic saline following ovariectomy and oestrogen replacement. J Endocrinol 1999b;163:51 [Supplement].

Hartley DE, Edwards JE, Spiller CE, Alom N, Tucci S, Seth P, et al. The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat. Psychopharmacology (Berl.) 2003;167:46–53.

Hirose K, Tajima K, Hamajima N, Inoue M, Takezaki T, Kuroishi T, et al. A large-scale, hospital-based case-control study of risk factors of breast cancer according to menopausal status. Jpn J Cancer Res 1995;86:146–54.

Hrabovszky E, Kalló I, Hajszan T, Shughrue PJ, Merchenthaler I, Liposits Z. Expression of estrogen receptor-β messenger ribonucleic acid in oxytocin and vasopressin neurons of the rat supraoptic and paraventricular nuclei. Endocrinology 1998;139:2600–4.

Hsu CS, Shen WW, Hsueh YM, Yeh SL. Soy isoflavone supplementation in postmenopausal women. Effects on plasma lipids, antioxidant enzyme activities and bone density. J Reprod Med 2001;46:221–6.

Isgor C, Shieh KR, Akil H, Watson SJ. Colocalization of estrogen beta-receptor messenger RNA with orphanin FQ, vasopressin and oxytocin in the rat hypothalamic paraventricular and supraoptic nuclei. Anat Embryol (Berl) 2003;206:461–9.

Ishunina TA, Kruijver FP, Balesar R, Swaab DF. Differential expression of estrogen receptor alpha and beta immunoreactivity in the human supraoptic nucleus in relation to sex and aging. J Clin Endocrinol Metab 2000;85:3283–91.

Jacobsen BK, Knutsen SF, Fraser GE. Does high soy milk intake reduce prostate cancer incidence? Adventist Health Study (United States) Cancer Causes Control 1998;9:553–7.

Knight DC, Howes JB, Eden JA, Howes LG. Effects on menopausal symptoms and acceptability of isoflavone-containing soy powder dietary supplementation. Climacteric 2001;4:13–8.

Kritz-Silverstein D, Goodman-Gruen DL. Usual dietary isoflavone intake, bone mineral density, and bone metabolism in postmenopausal women. J Women's Health Gend-Based Med 2002;11:69–78.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustaffsson JA. Clon-

ing of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996;93:5925–30.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.

Kuiper GG, Lemmen JG, Carlsson B, Corton J.C, Safe SH, vander Saag T, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–63.

Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE. Dietary effects on breast-cancer risk in Singapore. Lancet 1991;337:1197–200.

Lephart ED, Thompson JM, Setchell KD, Adlercreutz H, Weber KS. Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. Brain Res 2000;859:123–31.

Li X, Schwartz PE, Rissman EF. Distribution of estrogen receptor-β-like immunoreactivity in rat forebrain. Neuroendocrinology 1997;66:63–7.

Liposits Z, Setalo G, Flerko B. Application of the silver-gold intensified 3,3′-diaminobenzidine chromogen to the light and electron microscopic detection of the luteinizing hormone-releasing hormone system of the rat brain. Neuroscience 1984;13:513–25.

Liposits Z, Sherman D, Phelix C, Paull WK. A combined light and electron microscopic immunocytochemical method for the simultaneous localization of multiple tissue antigens. Tyrosine hydroxylase immunoreactive innervation of corticotropin releasing factor synthesizing neurons in the paraventricular nucleus of the rat. Histochemistry 1986;85:95–106.

Madeira MD, Sousa N, Cadete-Leite A, Lieberman AR, Paula-Barbosa MM. The supraoptic nucleus of the adult rat hypothalamus displays marked sexual dimorphism which is dependent on body weight. Neuroscience 1993;52:497–513.

Messina M, Messina V. Soyfoods, soybean isoflavones, and bone health: a brief overview. J Renal Nutr 2000;10:63–8 [April].

Murkies AL, Lombard C, Strauss BJ, Wilcox G, Burger HG, Morton MS. Dietary flour supplementation decreases post-menopausal hot flushes: effect of soy and wheat. Maturitas 1995;21:189–95.

Odum J, Tinwell H, Jones K, Van Miller JP, Joiner RL, Tobin G, et al. Effect of rodent diets on the sexual development of the rat. Toxicol Sci 2001;61:115–27.

Pan Y, Anthony M, Clarkson TB. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proc Soc Exp Biol Med 1999a;221:118–25.

Pan Y, Anthony M, Clarkson TB. Evidence for up-regulation of brain-derived neurotrophic factor mRNA by soy phytoestrogens in the frontal cortex of retired breeder female rats. Neurosci Lett 1999b;261:17–20.

Pan Y, Anthony M, Watson S, Clarkson TB. Soy phytoestrogens improve radial arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17beta-estradiol treatment. Menopause 2000;7:230–5.

Paula-Barbosa MM, Sousa N, Madeira MD. Ultrastructural evidence of sexual dimorphism in supraoptic neurons: a morphometric study. J Neurocytol 1993;22:697–706.

Rhodes CH, Morrell JI, Pfaff DW. Distribution of estrogen-concentrating, neurophysin-containing magnocellular neurons in the rat hypothalamus as demonstrated by a technique combining steroid autoradiography and immunohistology in the same tissue. Neuroendocrinology 1981;33:18–20.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Writing group for the women's health initiative investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. JAMA 2002;288:321–33.

Rowland I, Faughnan M, Hoey L, Wahala K, Williamson G, Cassidy A. Bioavailability of phyto-oestrogens Br J Nutr 2003;89(Suppl. 1): S45–58.

Sar M, Stumpf WE. Simultaneous localization of [^{3}H] estradiol and neurophysin I or arginine vasopressin in hypothalamic neurons demonstrated by a combined technique of dry-mount autoradiography and immunohistochemistry. Neurosci Lett 1980;17:179–84.

Scambia G, Mango D, Signorile PG, Anselmi Angeli RA, Palena C, Gallo D, et al. Clinical effects of a standardized soy extract in postmenopausal women: a pilot study. Menopause 2000;7:105–11.

Scheiber MD, Liu JH, Subbiah MT, Rebar RW, Setchell KD. Dietary inclusion of whole soy foods results in significant reductions in clinical risk factors for osteoporosis and cardiovascular disease in normal postmenopausal women. Menopause 2001;8:384–92.

Shapiro RA, Xu C, Dorsa DM. Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor alpha and beta. Endocrinology 2000;141:4056–64.

Shughrue PJ, Komm B, Merchenthaler I. The distribution of estrogen receptor-β mRNA in the rat hypothalamus. Steroids 1996;61:678–81.

Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-α and-β mRNA in the rat central nervous system. J Comp Neurol 1997;388:507–25.

Simonian SX, Herbison AE. Differential expression of estrogen receptor α and β immunoreactivity by oxytocin neurons of rat paraventricular nucleus. J Neuroendocrinol 1997;11:9–11.

Sladek CD, Swenson KL, Kapoor R, Sidorowicz HE. The role of steroid hormones in the regulation of vasopressin and oxytocin release and mRNA expression in hypothalamo-neurohypophysial explants from the rat. Exp Physiol 2000;85:171S–7S.

Somponpun S, Sladek CD. Role of estrogen receptor-β in regulation of vasopressin and oxytocin release in vitro. Endocrinology 2002;143:2899–904.

Stachenfeld NS, DiPietro L, Palter SF, Nadel ER. Estrogen influences osmotic secretion of AVP and body water balance in post-menopausal women. Am J Physiol 1998;274:R187–95.

Stephens FO. Phytoestrogens and prostate cancer: possible preventive role. Med J Aust 1997;167:138–40.

Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC, et al. Phytoestrogen intake and prostate cancer: a case-control study using a new database. Nutr Cancer 1999;33:20–5.

Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, et al. Phytoestrogen content of purified, open-and closed-formula laboratory animal diets. Lab Anim Sci 1999;49:530–6.

Wu AH, Ziegler RG, Horn Ross PL, Nomura AM, West DW, Kolonel LN, et al. Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiol Biomark Prev 1996;5:901–6.

Zhou Y, Windle RJ, Forsling ML. The effect of ovariectomy and oestrogen replacement on the renal actions of vasopressin in the rat. J Physiol 1992;452:318.