The influence of 17β-estradiol on annexin 1 expression in the anterior pituitary of the female rat and in a folliculo-stellate cell line

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Abstract

Annexin 1 (ANXA1) is a Ca^{2+}- and phospholipid-binding protein that plays an important role as a mediator of glucocorticoid action in the host-defence and neuroendocrine systems. Sex differences in hypothalamo–pituitary–adrenal (HPA) axis activity are well documented and a number of studies have demonstrated that gonadal steroids act as regulators of HPA activity. The aim of this study was to investigate the effect of ovariectomy and 17β-estradiol replacement, and estrous cycle stage, on anterior pituitary ANXA1 content. The amount of anterior pituitary ANXA1 determined by western blotting varied with estrous cycle stage with a peak at estrus declining to a trough at proestrus. Ovariectomy resulted in a significant ($P<0.05$) decrease in anterior pituitary ANXA1 content.

Administration of 17β-estradiol (1 µg/100 g) significantly ($P<0.01$) increased anterior pituitary ANXA1 expression in the ovariectomized animals. In contrast, there was no change in pituitary ANXA1 content in response to 17β-estradiol in adrenalectomized and adrenalectomized/ovariectomized rats. Treatment of TtT/GF cells, a folliculo-stellate cell line, with 17β-estradiol (1·8–180 nM) increased ANXA1 mRNA expression and increased the amount of ANXA1 protein externalized in response to a dexamethasone stimulus. These results indicate that 17β-estradiol stimulates ANXA1 expression in the anterior pituitary and *in vivo* an adrenal factor contributes to the mechanism of action.

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Introduction

Sex differences in hypothalamo–pituitary–adrenal axis (HPA) activity are well documented and a number of studies have demonstrated that gonadal steroids act as regulators of HPA activity (Viau & Meaney 1991, Freeman 1994, Carey *et al.* 1995). Changes in plasma corticosterone are associated with estrous cycle stage in rodents such that peak adrenocorticotrophic hormone (ACTH) and corticosterone levels are highest in the afternoon of proestrus (Carey *et al.* 1995), the time of maximal estradiol secretion (Freeman 1994). In addition, the stress responsiveness of the female HPA varies with the estrous cycle, with increased corticosterone release in response to stress occuring at proestrus (Viau & Meaney 1991). Sexual dimorphism in corticosterone secretion is well established: female rodents display elevated corticosterone secretion in basal and some stress conditions relative to males (Critchlow *et al.* 1963). Furthermore, estrogen exerts stimulatory effects on stress-induced ACTH and glucocorticoid release, whereas testosterone acts to inhibit HPA activity (Seale *et al.* 2004). In addition, it is now evident that exposure to gonadal steroids influences HPA programming during perinatal development (Seale *et al.* 2005) and is responsible for the changes in the HPA responsiveness at puberty (Viau *et al.* 2005).

Annexin 1 (ANXA1) is a member of the annexin family of Ca^{2+}- and phospholipid-binding proteins (Moss & Morgan 2004) that has been demonstrated to act as a mediator of glucocorticoid action in the inflammation and in the control of anterior pituitary hormone release (John *et al.* 2004). Functional studies have demonstrated a key role for ANXA1 in mediating certain acute, non-transcriptional inhibitory actions of the glucocorticoids on the release of ACTH (Taylor *et al.* 1995, 1997) and other pituitary hormones (reviewed in John *et al.* 2004). The effect of glucocorticoids in several tissues, including the anterior pituitary, is to cause externalization of ANXA1 from the cytoplasm to the outer cell surface where it is retained by a Ca^{2+}-dependent mechanism (Chapman *et al.* 2002) and to induce ANXA1 synthesis (Philip *et al.* 2001). ANXA1 is expressed strongly in the S100-positive folliculo-stellate cells (FS cells) in the anterior pituitary (Traverso *et al.* 1999) and *in vivo* almost total loss of FS cell ANXA1 immunoreactivity occurs in adrenalectomized rats which is replenished upon glucocorticoid replacement (Ozawa *et al.* 2002). Functional and binding studies suggest that the glucocorticoid-induced translocation of ANXA1 via the ATP-binding cassette transporter ABCA1 (Chapman *et al.* 2003, Omer *et al.* 2006) is an important mechanism which enables the protein to access binding sites on the surface of the endocrine cells and thereby exert paracrine regulation on the release of ACTH and other pituitary hormones via surface binding proteins (Christian *et al.* 1997).

The role of ANXA1 has so far been studied almost exclusively in male rodents and it is not known how ANXA1

varies with the estrous cycle of the rat and the female sex estrogen status. Our recent findings demonstrate that ANXA1 null mice exhibit sexual dimorphisms in HPA function and inflammatory responses (Hannon *et al.* 2003, Morris *et al.* 2006). The occurrence of gender-based differences in the susceptibility to a number of disease models in the rodent (Schuurs & Verheul 1990, Homo-Delarche *et al.* 1991, Whitacre *et al.* 1998) has been attributed to complex interactions between the differential HPA axis activity and the immune system of male and female rats. In human subjects, a strong correlation exists between serum cortisol concentrations and ANXA1 expression in peripheral blood leukocytes (Mulla *et al.* 2005). Mulla *et al.* (2005) proposed that changes in leukocyte ANXA1 expression in human subjects may serve as an index of tissue sensitivity to glucocorticoids, and that such changes might contribute to autoimmune and inflammatory diseases associated with glucocorticoid dysregulation. It is therefore possible that gonadal regulation of ANXA1 expression may be of physiological significance in contributing to the etiology of the sexual dimorphisms in neuroendocrine and host-defence function and susceptibility to disease. In the present study, we have investigated the hypothesis that 17β-estradiol regulates ANXA1 expression in the anterior pituitary. We now report (1) the effects of ovariectomy and 17β-estradiol replacement in female rats, and (2) the effects of 17β-estradiol in a FS cell line, on ANXA1 expression.

Materials and Methods

Drugs

The following drugs were used: 17β-estradiol, dexamethasone sodium phosphate, and corticotropin releasing factor (CRF). 17β-estradiol (E_2) was dissolved directly in sesame oil for *in vivo* s.c. injection. Dexamethasone (Faulding Pharmaceutical plc, Leamington Spa, Warks, UK) and corticotrophin-releasing hormone (CRH; Bachem, Heidelberg, Germany) used in *in vitro* experiments were diluted directly into the incubation medium. All drugs and reagents were from Sigma Chemical Co. unless otherwise stated.

Animals

Adult female Sprague–Dawley rats (200 g) were purchased from Harlan Olac (Bicester, Oxon, UK) and were allowed to acclimatize for 2 weeks in the animal holding facility before experimentation. The Universities Federation for Animal Welfare (UFAW) Handbook on the Care and Management of Laboratory Animals was followed and the study was carried out under license in accordance with the UK Animals (Scientific Procedures) Act 1986. The rats were housed in groups of five per cage in a quiet room with 14 h light:10 h darkness and temperature maintained at 20–21 °C; food was available *ad libitum*. All experiments were started between 0800 and 1000 h

to avoid changes associated with the circadian rhythm. For estrous cycle studies, rats were smeared daily for 30 days between 0800 and 1000 h. The smears were collected on glass slides, stained with toluidine blue, and examined under a light microscope. The cycle stage was estimated and only rats observed to be cycling regularly were chosen for study ($n = 6$ rats used per cycle stage). Animals were killed by stunning and decapitation and the anterior pituitary glands removed. Trunk blood was collected and placed in chilled heparinized tubes for centrifugal separation (2000 *g*, 10 min) of plasma for subsequent assay of corticosterone. The uteri were weighed postmortem and in all cases weights were found to correlate with the stage of the estrous cycle.

Rat ovariectomy and treatment with 17β-estradiol

Adult female Sprague–Dawley rats (200 g) were anesthetized with Hypnorm/Hypnovel (1:1:2 dilution in water, 3 ml/kg ip; Janssen Pharmaceuticals, Oxford, UK) and subsequently either sham-operated (control) or bilaterally ovariectomized (OVX). In further experiments, subgroups of rats were bilaterally adrenalectomized (ADX), OVX, or bilaterally ADX and OVX. The animals were allowed to recover postoperatively for 7 days, and on day 8 after surgery groups of rats ($n = 6$ rats) received a s.c. injection of vehicle (sesame oil), or 17β-estradiol (1 μg/100 g body weight), 24 and 12 h before sacrifice on day 9. The concentration and regimen of 17β-estradiol treatment has been reported in the literature to produce physiological concentrations of plasma 17β-estradiol (163 pg/ml) in the Sprague–Dawley rats (Young *et al.* 2001). Animals were killed by stunning and decapitation and the anterior pituitary glands removed. Trunk blood was collected and placed in chilled heparinized tubes for centrifugal separation (2000 *g*, 10 min) of plasma for subsequent assay of ACTH and corticosterone. Uteri were also removed and weighed.

Detection of ANXA1 by SDS-PAGE and western blot analysis

ANXA1 was extracted from pituitary tissue and TtT/GF cells by sonication (25 Hz, 20 s, Soniprep 150, MSE, Crawley, UK) on ice in EDTA (10 mM) containing Triton X-100 (1% v/v) as described previously (Chapman *et al.* 2003). The protein content of each sample was determined using the Pierce BCA protein assay (Pierce, Macclesfield, Cheshire, UK). The samples were then analyzed for ANXA1 by SDS-PAGE and subsequent western blotting using a well-characterized polyclonal anti-ANXA1 antibody (raised in sheep against the full-length human recombinant ANXA1 and diluted in the ratio of 1:10 000; Chapman *et al.* 2003) as a probe. Normalization was conducted by gently striping the blots (0·2 M glycine (pH 2·8), 1 h, room temperature) and re-blocking and re-probing with a mouse β-actin antibody (1:10 000, 1 h, room temperature) and mouse secondary antibody (1:5000, 1 h, room temperature; both AbCam, Cambridge, UK). Intensity values were normalized relative to control values. The blots were scanned using a flatbed scanner (HP Scanjet 5200 with Adobe Photodeluxe Business

Edition v1.1) and the band intensity analyzed using the TINA software program (TINA version 2.10, Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

Secretion of ACTH in vitro by anterior pituitary segments

Rat anterior pituitary segments from (1) OVX- and 17β-estradiol-treated OVX females or (2) proestrus and estrus females were distributed randomly (1 segment/well) in the wells of 24-well tissue culture plates (Costar, Cambridge, MA, USA) and incubated at 37 °C for 90 min in 1 ml incubation medium (1% (vol/vol) aprotonin (Bayer Corp., Saffron Walden, Essex, UK) and 1% (vol/vol) penicillin/-streptomycin in oxygenated Earles' Balanced Salt Solution (pH 7·4); phenol red-free) under a humidified atmosphere saturated with 95% O_2, 5% CO_2. The medium was changed after 1 and 1·5 h. The segments were then incubated for a further 1 h in medium containing CRH (10 nM); controls were exposed to an equal volume (1 ml) of medium alone. Where appropriate dexamethasone (0·1 μM) was included in the medium throughout both the pre- and final incubation periods. Medium from the final incubation was collected and stored in aliquots (300 μl, −20 °C) for subsequent measurement of immunoreactive (ir) ACTH.

ACTH ELISA and corticosterone RIA

ACTH was determined in duplicate by use of a commercially available ELISA (IDS Ltd, Boldon Colliery, Tyne and Wear, UK) according to the manufacturer's instructions. The assay sensitivity was 0·5 pg/ml and the inter- and intraassay coefficients of variation were 5·8 and 3·1% respectively. Corticosterone was determined in duplicate by the use of a commercially available RIA (IDS Ltd) according to the manufacturer's instructions. The minimum detection level of the assay was 0·39 ng/ml and the intra- and interassay coefficients of variation were 10·4 and 12·4% respectively. Dilution of samples provided a parallel displacement to the standards in both assays.

TtT/GF cell incubations

FS-like (TtT/GF) cells were maintained as described previously (Tierney *et al.* 2003) in a humidified incubator at 37 °C, 5% CO_2 and cultured in Dulbecco's modified Eagle medium (DMEM) DMEM-Hams-F12 medium (Invitrogen Ltd.), enriched with 15% (v/v) fetal calf serum (PAA Laboratories Ltd., Yeovil, Somerset, UK), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere. Plates (24 well) containing approximately 50 000 cells/well were allowed to reach 80% confluence. The cells were then incubated either in incubation medium alone (1% (vol/vol) aprotonin (Bayer Corp.) and 1% (vol/vol) penicillin/strepto-mycin in oxygenated Earles' Balanced Salt Solution (pH 7·4) phenol red-free) or with graded concentrations of 17β-estradiol (0·18–1800 nM) for 24 h. The duration of treatment of 24 h

was initially selected as previous ANXA1 promoter-driven luciferase reporter gene studies in TtT/GF cells have demonstrated increased luciferase expression in 24 h (Solito *et al.* 2003). In further time-course experiments, TtT/GF cells were exposed to 17β-estradiol (18 nM) for 3, 6, 12, or 24 h. In addition, TtT/GF cells were incubated for 24 h either in medium alone, 17β-estradiol (18 nM) alone, dexamethasone (0·1 μM) alone, or co-treated with 17β-estradiol (18 nM) and dexamethasone (0·1 μM). At the end of each incubation, period cells were processed for western blot analysis of total ANXA1 expression. In order to investigate the effects of 17β-estradiol pretreatment on dexamethasone-induced externalization of ANXA1, control- and 17β-estradiol (18 nM, 24 h)-treated groups were subsequently incubated for 3 h either in incubation medium alone or with 0·1 μM dexamethasone sodium phosphate. The duration of 3-h treatment with dexamethasone is well established to stimulate ANXA1 externalization (Taylor *et al.* 1997, Chapman *et al.* 2003). At the end of the incubation period, cells were processed for western blot analysis of cell surface and intracellular ANXA1 expression. Cell surface ANXA1 was removed from the outer cell membranes by washing the tissue gently for 2 min in a solution containing 1 mM EDTA in PBS (0·05 M (pH 7·4); Oxoid Chemicals Ltd., Basingstoke, Hants, UK) which, by chelating Ca^{2+}, releases ANXA1 into the medium from Ca^{2+}-dependent cell surface binding sites.

Detection of estrogen receptor (ER)α and ERβ in TtT/GF cells by immunofluorescence microscopy

For immunofluorescence experiments, TtT/GF cells were seeded into eight-well glass chamber slides (Nalge Nunc International, Cambridge, UK) at a density of 5000 cells/well. The cells were either incubated in medium alone or stimulated with 180 nM estradiol for 24 h, then fixed in 3% paraformal-dehyde, 0·05% glutaraldehyde (5 min), and permeabilized with 0·2% Triton X–100 (in PBS at room temperature, 5 min). Non-specific antibody binding sites were blocked with block buffer (3% BSA in PBS; PBS–BSA) for 30 min. The expression of ERα was detected with a mouse monoclonal anti-ERα (prediluted, used neat; Dako, Ely, Cambs, UK) and ERβ with a rabbit polyclonal ERβ antibody (1:100; Oncogene, Notting-ham, UK) overnight at 4 °C in PBS–BSA. Immunoreacted sections were then washed with PBS and incubated for 1 h at room temperature with fluorescein-conjugated anti–mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The chambers were rinsed with PBS and the nuclei counter-stained for 5 min with TOPRO-3 (Molecular Probes, Eugene, OR, USA). Slides were mounted in Vectashield mounting medium (Vector Laboratories) and examined using a titanium crystal sapphire (TCS) confocal microscope (Leica Corp. Microsystems, Wetzlar GmbH, Germany). Non-specific staining and background were assessed by substitution of non-immune sera for primary antisera and incubation with ERα and ERβ antibodies previously absorbed with an excess

of the respective receptor protein (ERα, Dako; ERβ, Oncogene).

Real-time PCR

Total RNA was isolated from pituitary tissue or TtT/GF cells using the RNeasy kit (Qiagen) following the manufacturer's instructions and treated with DNA-free DNAse (Ambion, Witney, UK) to remove traces of genomic DNA contamination. Three micrograms of tRNA from each sample were subsequently reverse transcribed using 1 μl Moloney murine leukemia virus reverse transcriptase (Clontech), 2 μl 10 mM dNTP, 2 μl 100 mM dithiothreitol in a total volume of 20 μl. To measure cDNA levels, a threshold cycle (Ct) was selected within the exponential phase of the amplification for all standards and samples. Standards were generated by serial dilutions of a cDNA pool from rat pituitary. A standard curve was generated by plotting standards against Ct values and sample values were read from this standard curve. ANXA1 expression was then detected and quantified by real-time PCR (QPCR) in 25 μl reactions containing 12·5 μl Absolute QPCR mix (ABgene, Epsom, Surrey, UK), 0·4 μM of each of the ANXA1 primers (For primer 5′ATACAGATGCC-AGGGCTTTGTATGA3′; Rev primer 5′TGGGATGTCT-AGTTTCCACCACACA3′), and 0·25 μM ANXA1 FAM/TAMRA-dual-labeled probe (Sigma-Genosys) in a 96-well plate format. The QPCR assay was performed in an ABI PRISM 7700 (Applied Biosystems, Warrington, Cheshire, UK) and cycled as follows: initial enzyme activation at 95 °C for 15 min then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The assays were normalized relative to an endogenous control, 18S rRNA (primers and probes from Applied Biosystems) and the results expressed as the amount of ANXA1 mRNA/18SRNA.

Data analysis

Semi-quantitative measures of ANXA1 protein expression were made by comparisons of western blot band optical densities (arbitrary units) to give a relative numerical guide to the ratios of the band intensities normalized to actin and their variance. Responses to steroids were calculated as a percentage of the corresponding vehicle control and expressed as the mean ± S.E.M. ($n = 3$ gels); statistical comparisons between the normally distributed data from groups were made by ANOVA. Each of the studies was repeated thrice, and in all instances the data profile was similar. For hormone release studies, preliminary analysis by the Shapiro and the Wilkes test confirmed that the data were normally distributed. Subsequent analysis was done by ANOVA with *post hoc* comparisons by Scheffe's test. Since the rate of basal ACTH release *in vitro* varied between experiments, statistical comparisons were made within experiments only. Differences were considered significant if P was < 0·05.

Results

Effect of ovariectomy and 17β-estradiol replacement on anterior pituitary ANXA1 expression

In order to investigate the regulation of anterior pituitary ANXA1 content by 17β-estradiol *in vivo*, female rats were sham-operated or ovariectomized and treated with either vehicle or 17β-estradiol (1 μg/100 g body weight (BW); 24 h) and anterior pituitary ANXA1 measured by western blot analysis. ANXA1 was readily detectable in female anterior pituitary tissue by western blot analysis as a 37 K molecular band which corresponded to the native biologically active species of the protein. Western blot analysis demonstrated a significant ($P < 0·01$) reduction in anterior pituitary ANXA1 total content following ovariectomy compared with sham-operated (Fig. 1A: OVX shown in western blot lanes 1 and 2 compared with sham-operated shown in lanes 3 and 4; densitometry shown in Fig. 1C). 17β-Estradiol treatment of OVX females for 24 h caused a significant ($P < 0·01$) increase in anterior pituitary ANXA1 content (Fig. 1A, 17β-estradiol-treated OVX lanes 5 and 6 versus vehicle-treated OVX lanes 1 and 2; Fig. 1C). Similarly, 17β-estradiol treatment of sham-operated females for 24 h also caused a significant ($P < 0·01$) increase in anterior pituitary ANXA1 content (Fig. 1A, 17β-estradiol-treated sham-operated lanes 7 and 8 versus vehicle-treated sham-operated lanes 3 and 4; Fig. 1C). No change was observed in the amount of β-actin detected (Fig. 1B). Circulating corticosterone was significantly reduced ($P < 0·05$) in OVX compared with sham-operated female rats (Fig. 1D) and in OVX animals corticosterone was significantly increased ($P < 0·01$) in response to 17β-estradiol (Fig. 1D). No significant difference in circulating corticosterone was measured in sham-operated animals treated with 17β-estradiol (Fig. 1D) compared with vehicle. The basal corticosterone levels were high in these experiments and it is possible that the animals were stressed during the testing conditions. Surgery and treatments were indicated to have been effective as the uterine weights of the OVX rats were significantly ($P < 0·01$) lower than sham-operated, and 17β-estradiol treatment of OVX rats caused a significant increase ($P < 0·05$) in uterine weight such that there was no significant difference compared with the sham-operated uterine weights (Fig. 1E).

Effect of adrenalectomy and combined adrenalectomy and ovariectomy on the stimulation of anterior pituitary ANXA1 expression by 17β-estradiol

In order to examine the potential role of the adrenal in the regulatory effects of 17β-estradiol on ANXA1 expression in OVX rats, the effect of adrenalectomy was investigated. Administration of 17β-estradiol (1 μg/100 g) to OVX animals induced a significant ($P < 0·05$) increase in anterior pituitary ANXA1 mRNA (Fig. 2A) and protein (Fig. 2B) compared with vehicle-treated controls. As expected, adrenalectomy

A

← 37 K

1   2   3   4   5   6   7   8

lane number

B

← 42 K

1   2   3   4   5   6   7   8

lane number

C

D

E
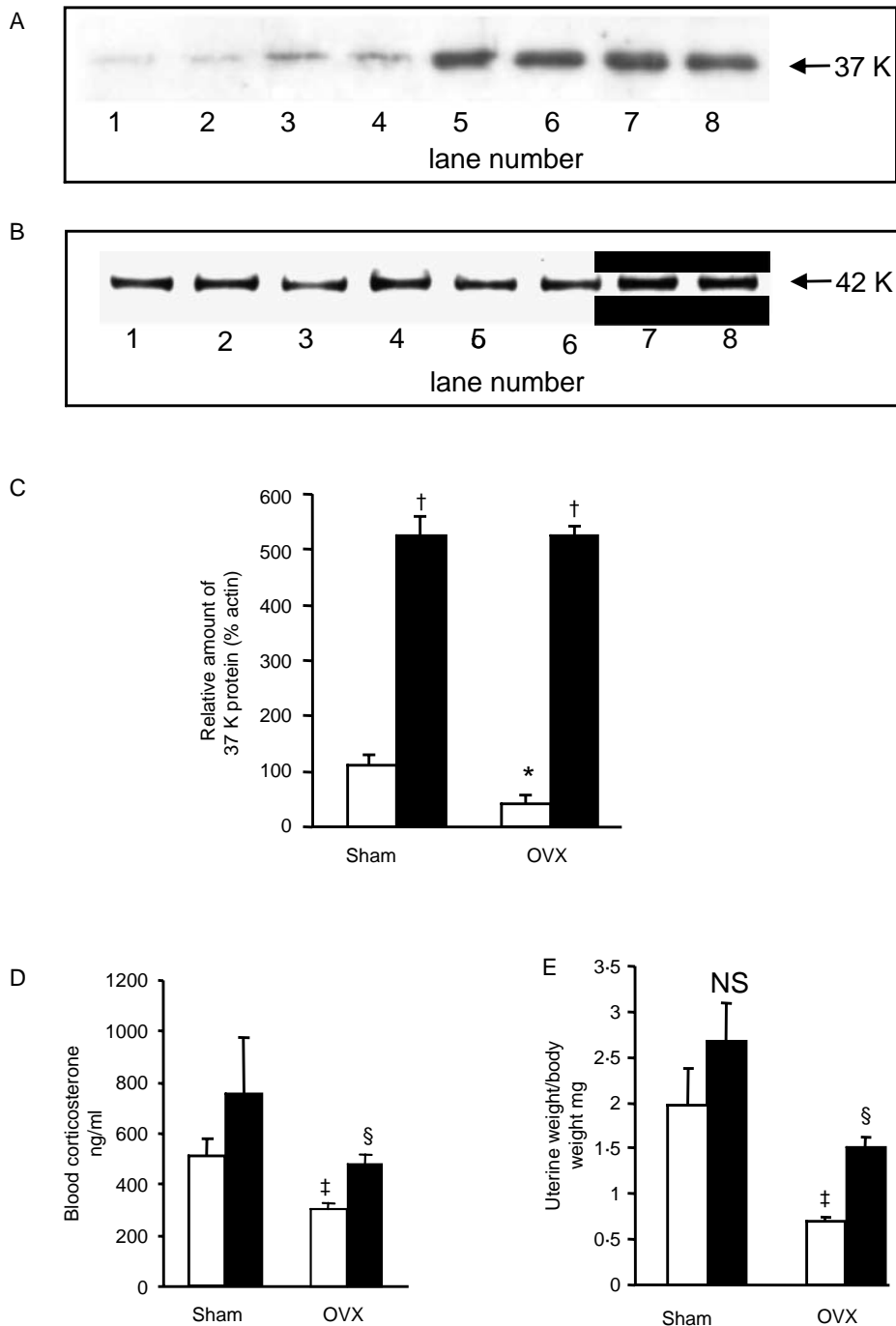
Figure 1 Western blots showing the effect of ovariectomy and replacement 17β-estradiol (E$_2$) treatment for 24 h on pituitary ANXA1 content; (A) shows ANXA1, (B) shows β-actin loading control. Vehicle-treated OVX, lanes 1 and 2; vehicle-treated sham-operated, lanes 3 and 4; E$_2$-treated OVX, lanes 5 and 6; E$_2$-treated sham-operated, lanes 7 and 8. (C) Histogram showing integrated densitometry data; open columns, vehicle-treated; and filled columns, E$_2$-treated. Values are expressed as means±s.e.m., $n=6$ animals. *$P<0·01$ versus sham-operated; [†]$P<0·01$ versus respective vehicle-treated, ANOVA. (D and E) The effect of ovariectomy and replacement with E$_2$ for 24 h on (D) serum corticosterone and (E) uterine weights in female rats. Open columns, vehicle-treated; filled columns, E$_2$-treated. Values are expressed as means±s.e.m., $n=6$ animals. [‡]$P<0·05$ versus sham vehicle; [§]$P<0·01$ versus OVX vehicle; NS, not significant versus sham vehicle; ANOVA plus Scheffe's test.
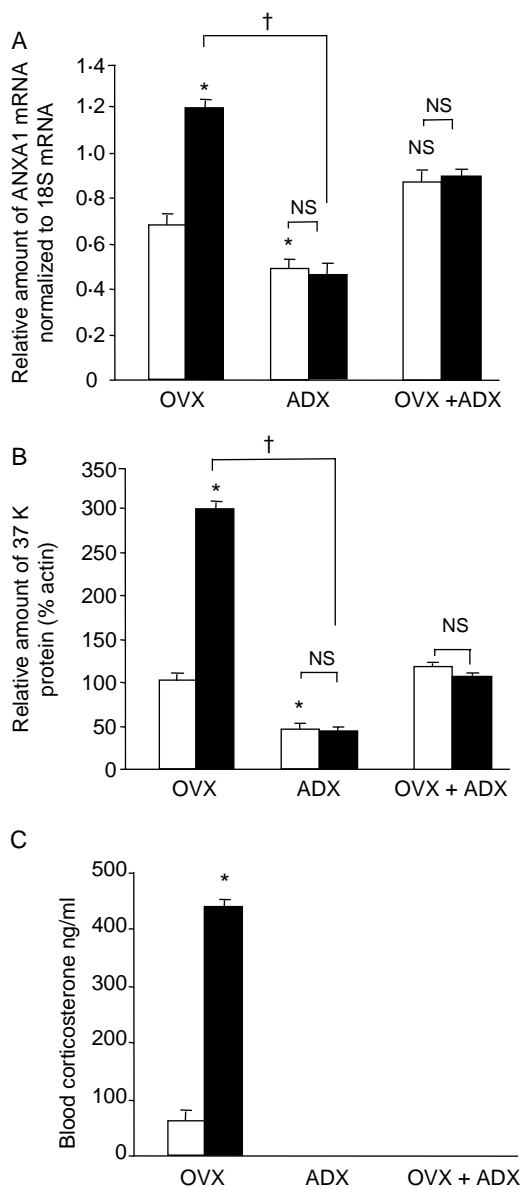
Figure 2 Histograms illustrating the effect of OVX, ADX, and OVX/ADX and 17β-estradiol treatment in female rats on (A) anterior pituitary ANXA1 mRNA, measured by real-time PCR, (B) anterior pituitary ANXA1 protein, measured by western blot analysis, and (C) blood corticosterone. Open columns, vehicle-treated; filled columns, 17β-estradiol-treated. Values are expressed as means ± S.E.M., $n = 6$ animals. NS, not significant versus corresponding vehicle control; *$P < 0.05$ versus vehicle-treated OVX, †$P < 0.05$ versus 17β-estradiol-treated OVX; ANOVA plus Scheffe's test.

resulted in a significant decrease ($P < 0.05$) in anterior pituitary ANXA1 mRNA (Fig. 2A) and protein (Fig. 2B) compared with OVX. Administration of 17β-estradiol to ADX and ADX/OVX females did not alter the expression of ANXA1 mRNA or protein (Fig. 2A and B). Measurement of corticosterone (Fig. 2C) demonstrated that concentrations

were significantly ($P < 0.05$) elevated in 17β-estradiol-treated OVX animals compared with vehicle-treated OVX and confirmed the effectiveness of ADX.

### Western blot analysis of ANXA1 expression during the estrous cycle

ANXA1 was measured by western blot analysis in anterior pituitary glands from female rats in each estrous cycle stage in order to investigate alterations in ANXA1 protein content in the estrous cycle. Figure 3 illustrates the effect of the estrous cycle on anterior pituitary ANXA1 content. Typical western blots are shown in Fig. 3A–D and densitometry data in Fig. 3E–F. The amount of ANXA1 in anterior pituitary (Fig. 3A and E) began to reduce at metestrus > diestrus and was significantly reduced by approximately a fivefold reduction at proestrus ($P < 0.01$ versus estrus; Fig. 3A proestrus lanes 7 and 8 versus estrus lanes 1 and 2). However, no change was observed in the amount of β-actin detected (Fig. 3B). The ANXA1 content of male anterior pituitary was intermediate to that of proestrus and estrus females (Fig. 3C and E) and no difference was observed in β-actin levels (Fig.3D). Measurement of corticosterone demonstrated highest circulating concentrations at proestrus and lowest concentrations at estrus as previously reported (Fig. 3F; Carey *et al.* 1995).

Regulation of ANXA1 by 17β-estradiol in TtT/GF cells

In order to explore the possibility of direct actions of 17β-estradiol on ANXA1 expression in FS cells, the effects of graded concentrations of 17β-estradiol (0·18–1800 nM) on ANXA1 mRNA and protein content in the TtT/GF FS cell line were investigated. Initial immunofluorescence microscopy studies localized the subcellular distribution of ERα and ERβ in TtT/GF cells (Fig. 4). In control- and 17β-estradiol-treated (24 h, 180 nM) conditions, strong immunofluorescent staining for ERα (Fig. 4A–F) and ERβ (Fig. 4G–L) was localized to the nucleus with weaker immunofluorescence extended throughout the cytoplasm. No non-specific immunolabeling was detected in absorbed antibody controls (Fig. 4M and N). The effects of incubating TtT/GF cells for 24 h with increasing concentrations of 17β-estradiol (0·18–1800 nM) on the amount of ANXA1 mRNA and protein are shown in Fig. 5. Real-time PCR assay of ANXA1 mRNA in TtT/GF cells confirmed that the positive control stimulus dexamethasone (0·1 μM, 24 h) produced a significant ($P < 0.05$) increase in ANXA1 mRNA and revealed that 17β-estradiol (1·8–180 nM, 24 h) caused a concentration-dependent increase in ANXA1 mRNA (Fig. 5A). In contrast, western blot analysis demonstrated no significant effect of graded concentrations of 17β-estradiol (0·18–1800 nM, 24 h; Fig. 5B and C) and no significant effect of 17β-estradiol (18 nM) treatment for various periods (0–24 h; Fig. 5D) on ANXA1 protein content in TtT/GF cells. However, pretreatment of TtT/GF cells
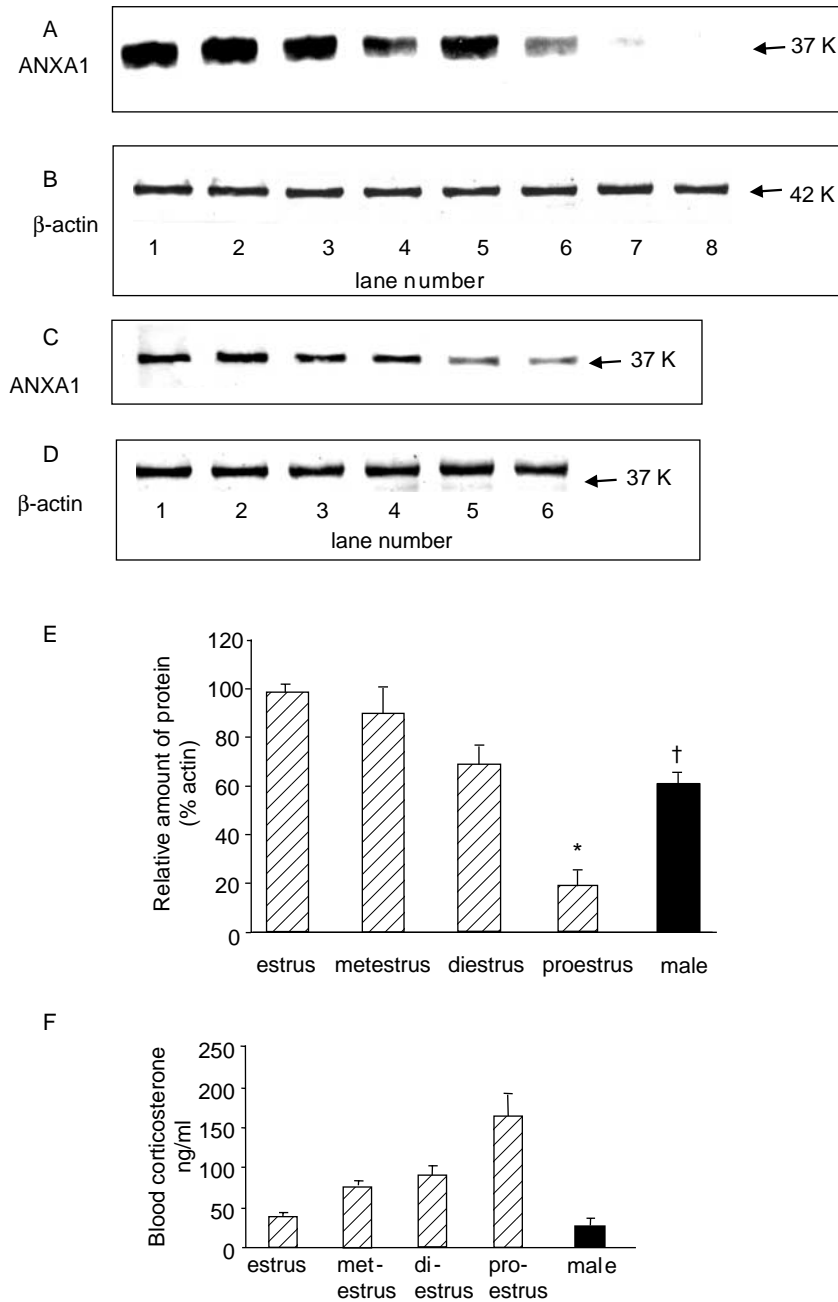
Figure 3 Western blots showing anterior pituitary ANXA1 tissue content at estrus, metestrus, diestrus, and proestrus in female rats; (A) shows ANXA1, (B) shows β-actin. Estrus, lanes 1 and 2; metestrus, lanes 3 and 4; diestrus, lanes 5 and 6; proestrus, lanes 7 and 8. (C) and (D) Comparison of anterior pituitary (C) ANXA1 tissue content and (D) β-actin control in male rats and proestrus and estrus female rats. Estrus, lanes 1 and 2; males, lanes 3 and 4; proestrus, lanes 5 and 6. Histograms showing (E) integrated densitometry data and (F) blood corticosterone measured at estrus, metestrus, diestrus, and proestrus in female rats, and in male rats. Values are expressed as means±S.E.M., $n=6$ animals. *$P<0.01$, †$P<0.05$ versus estrus; ANOVA plus Scheffe's test.
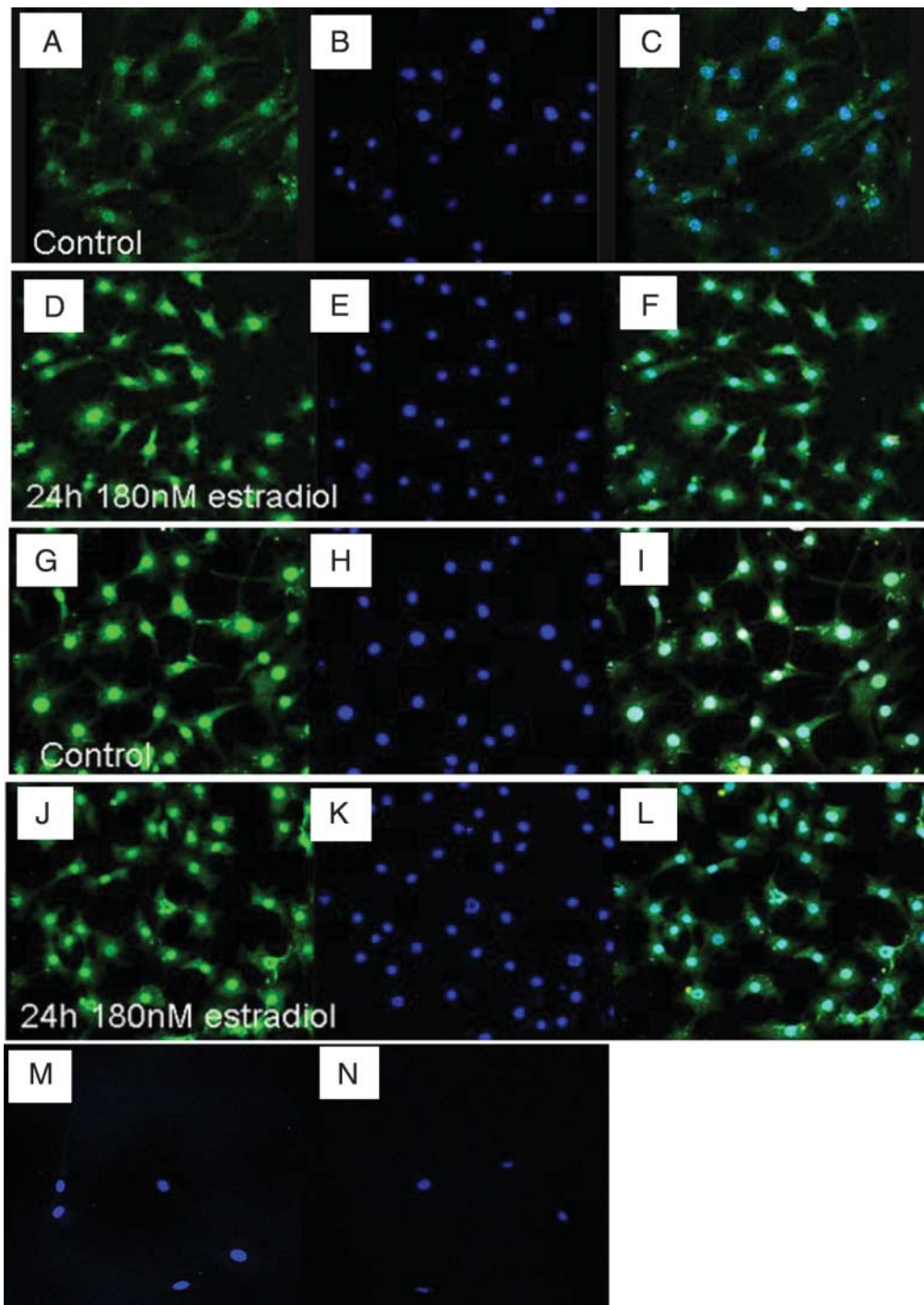
Figure 4 Localization of ERα (A–F) and ERβ (G–L) receptors in control- and 17β-estradiol (E$_2$; 180 nM, 24 h)-treated TtT/GF cells by immunofluorescence microscopy. Figures show fluorescence images of labeling of: A and D, ERα; G and J, ERβ; B, E, H and K, TO-PRO3 nuclear counterstain; C, F, I, L overlay of corresponding ER and nuclear counterstain images. ERα, control (A–C), E$_2$-treated (D–F); ERβ, control (G–I), E$_2$-treated (J–L). (M) ERα preabsorbed antibody negative control, (N) ERβ preabsorbed antibody negative control. Magnification, ×250.
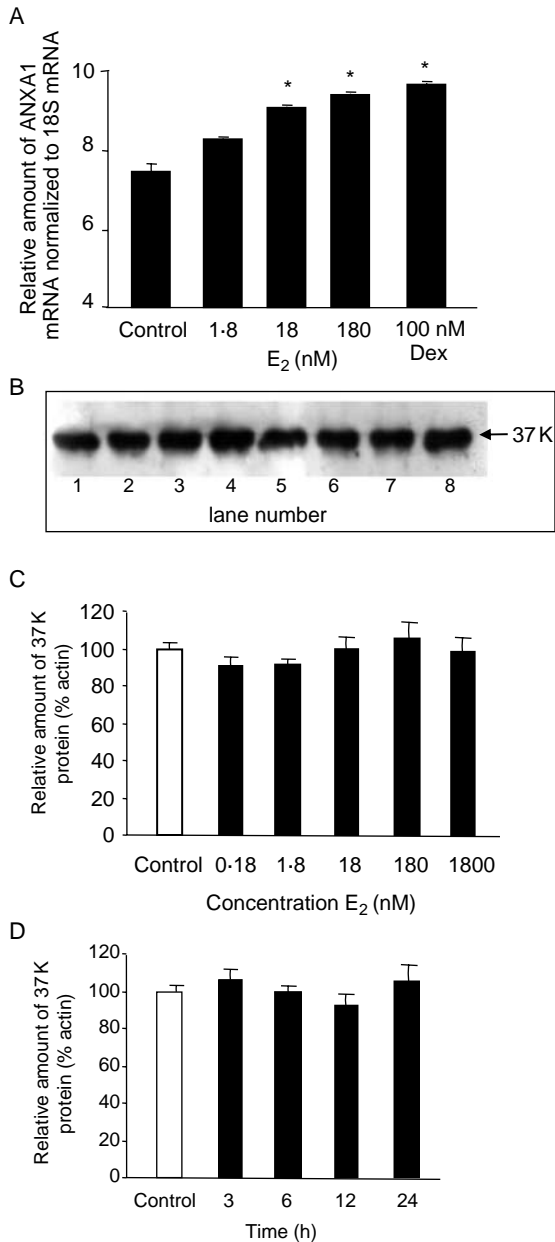
Figure 5 (A) Effect of 17β-estradiol (E$_2$; 1·8–180 nM, 24 h) and dexamethasone (Dex; 0·1 μM, 24 h) on the amount of ANXA1 mRNA in TtT/GF cells measured by quantitative PCR. Values expressed as means±S.E.M., $n=3$ experiments *$P<0·01$ versus negative control, ANOVA. (B) Effect of E$_2$ (1·8–180 nM, 24 h) on the amount of ANXA1 protein in TtT/GF cells. Western blot: lanes 1 and 2, control; lanes 3 and 4, 1·8 nM E$_2$-treated; lanes 5 and 6, 18 nM E$_2$-treated; lanes 7 and 8, 180 nM E$_2$-treated. (C) and (D) Integrated densitometry data of the effects of (C) increasing concentrations of E$_2$ (0·18–1800 nM, 24 h) and (D) duration of treatment with 18 nM E$_2$ (3–24 h) on the amount of ANXA1 in TtT/GF cells. Open columns, vehicle control; filled columns, 17β-estradiol-treated. Values expressed as means±S.E.M., $n=3$ experiments; no significant differences measured versus control, ANOVA.

with 17β-estradiol (100 nM, 24 h) significantly ($P<0·05$) increased the amount of surface ANXA1 detected in response to dexamethasone-stimulated (0·1 μM, 3 h) externalization (Fig. 6A, lanes 7 and 8 17β-estradiol and dexamethasone-treated versus lanes 3 and 4 control- and dexamethasone-treated; Fig. 6D), whereas the amount of intracellular ANXA1 measured was unaffected (Fig. 6C and E). No actin ir bands were detected in cell surface EDTA washes (Fig. 6B) confirming cell integrity. In subsequent experiments, the effect of co-treatment of TtT/GF cells with 17β-estradiol (18 nM) and dexamethasone (0·1 μM) for 24 h on ANXA1 content was measured (Fig. 7). Western blot analysis confirmed that dexamethasone alone significantly ($P<0·05$) increased ANXA1 content, whereas 17β-estradiol treatment (18 nM) alone had no significant effect (Fig. 7A, lanes 1 and 2 control versus lanes 3 and 4 dexamethasone-treated versus lanes 5 and 6 17β-estradiol-treated). However, co-treatment of TtT/GF cells with 17β-estradiol (18 nM) and dexamethasone (0·1 μM) for 24 h significantly ($P<0·05$) enhanced ANXA1 content compared with dexamethasone treatment alone (Fig. 7A, lanes 7 and 8 dexamethasone and 17β-estradiol-treated versus lanes 3 and 4 dexamethasone alone). However, no change was observed in the amount of β-actin detected (Fig. 7B).

Dexamethasone inhibition of basal- and CRH-stimulated ACTH release from pituitary segments *in* vitro

ANXA1 mediates certain acute inhibitory actions of the glucocorticoids on ACTH release (Taylor *et al*. 1997). As significant differences in ANXA1 anterior pituitary content were measured after 17β-estradiol treatment compared with OVX, and in proestrus compared with estrus, we compared the inhibitory effects of dexamethasone *in vitro* on basal and stimulated ACTH release in these groups. The inhibitory effects of dexamethasone (0·1 μM) on the release of ir-ACTH induced by CRH (10 nM) from OVX- and 17β-estradiol-treated OVX female pituitary segments *in vitro* is demonstrated in Fig. 8A. No significant difference in the basal release of ir-ACTH or the significant ($P<0·01$) increases in peptide release induced by CRH were detected between OVX- and 17β-estradiol-treated OVX groups. However, a significantly greater inhibition ($P<0·05$) by dexamethasone of CRH-stimulated ir-ACTH release was measured in OVX pituitary segments compared with 17β-estradiol-treated OVX segments (Fig. 8A). Figure 8B similarly compares the inhibitory effects of dexamethasone (0·1 μM) on the release of ir-ACTH induced by CRH (10 nM) from anterior pituitary segments from proestrus and estrus female rats. No significant difference in the basal release of ir-ACTH or the significant ($P<0·01$) increases in peptide release induced by CRH were detected between proestrus and estrus pituitary segments. However, a significantly greater inhibition ($P<0·05$) by dexamethasone of CRH-stimulated ir-ACTH release was measured in proestrus compared with estrus segments (Fig. 8B).
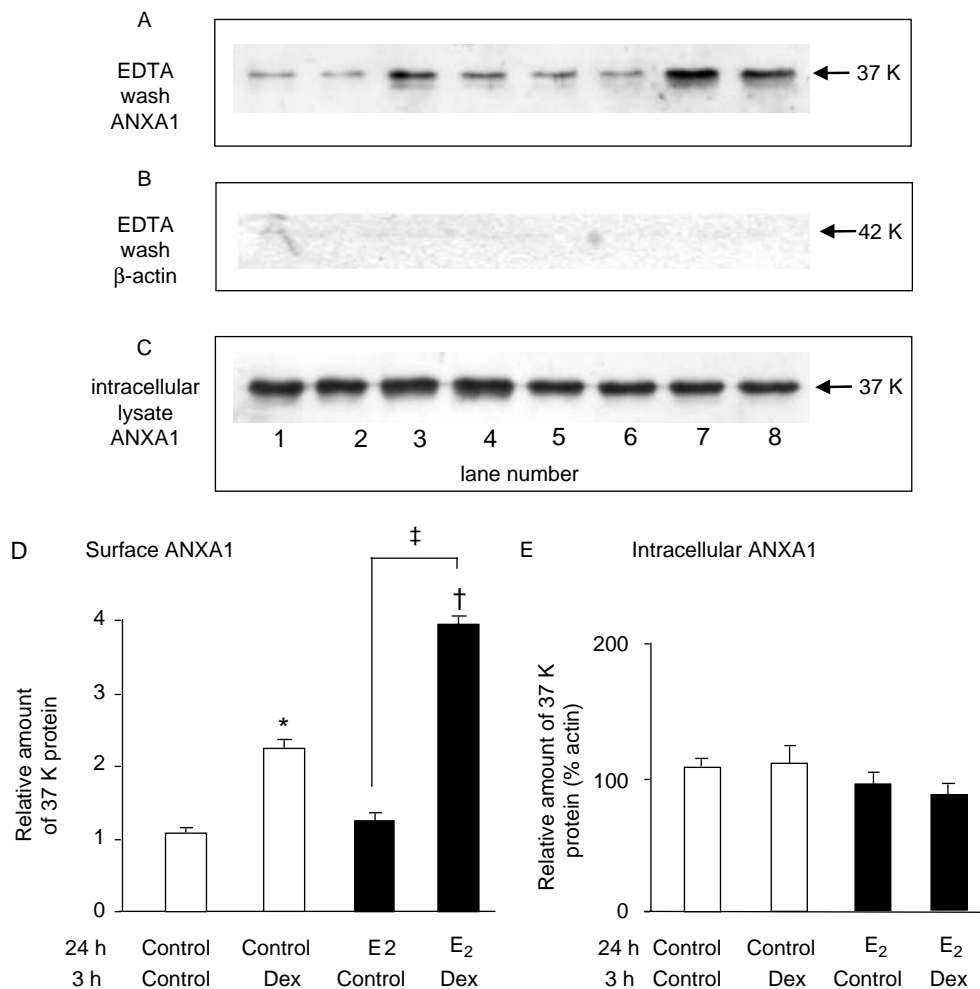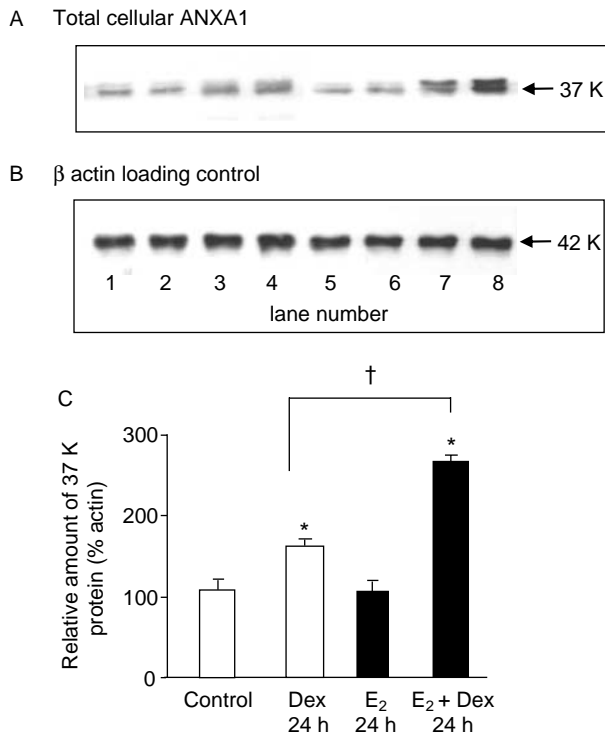
Figure 6 Effect of 17β-estradiol (E_2) on (A) the amount of ANXA1 in the EDTA wash, i.e. cell surface ANXA1, (B) β-actin in the EDTA wash (negative control), and (C) intracellular ANXA1 in control and dexamethasone (Dex)-stimulated (0·1 μM, 3 h) TtT/GF cells. ANXA1 and β-actin were measured by western blot analysis. Western blots: control, lanes 1 and 2; vehicle (24 h) plus Dex-treated (0·1 μM, 3 h), lanes 3 and 4; E_2-treated (180 nM, 24 h) plus vehicle (3 h), lanes 5 and 6; E_2 plus Dex-treated, lanes 7 and 8. (D) Integrated densitometry data for surface ANXA1 measured. (E) Integrated densitometry data for intracellular ANXA1 measured. Open columns, vehicle control; filled columns, 17β-estradiol-treated; treatment group is shown below the columns. Values expressed as means ± S.E.M., $n = 3$ experiments; *$P < 0.05$, †$P < 0.01$ versus negative control; ‡$P < 0.05$ versus corresponding E_2-free control, ANOVA.

Discussion

To test the hypothesis that 17β-estradiol regulates ANXA1 expression, we measured pituitary ANXA1 content following ovariectomy and 17β-estradiol replacement in rats. OVX- and sham-operated rats were treated with a concentration of 17β-estradiol previously shown to mimic physiological levels (Young *et al.* 2001). The uterine weight in 17β-estradiol-treated OVX rats was not significantly different to sham-operated rats suggesting that normal circulating concentrations of the hormone were restored. In OVX rats, the anterior pituitary content of ANXA1 was significantly reduced

compared with sham-operated controls and in both the sham-operated and OVX animals 17β-estradiol replacement caused a significant increase in anterior pituitary ANXA1 content. Pituitary ANXA1 is expressed strongly in FS cells (Traverso *et al.* 1999) and to date, glucocorticoids are the principal factors known to stimulate ANXA1 synthesis in FS cells (Ozawa *et al.* 2002, John *et al.* 2004). The potent effects of ovariectomy on HPA axis activity have been well documented (Burgess & Handa 1992, Carey *et al.* 1995, Seale *et al.* 2004) and consistent with these studies we found that ovariectomy produced a significant decrease in blood corticosterone that was reversed by administration of 17β-estradiol. 17β-Estradiol

A   Total cellular ANXA1

B   β actin loading control

C

Figure 7 Effect of 17β-estradiol (E$_2$; 18 nM, 24 h) and dexamethasone (Dex; 0·1 µM, 24 h) on the amount of total ANXA1 protein in TtT/GF cells. Western blots showing (A) ANXA1, (B) β-actin, loading control: lanes 1 and 2, control; lanes 3 and 4, dexamethasone-treated; lanes 5 and 6, E$_2$-treated; lanes 7 and 8 co-treated with Dex and E$_2$. (C) Integrated densitometry data. Open columns, vehicle control; filled columns, 17β-estradiol-treated; treatment group is shown below the columns. Values expressed as means±S.E.M., $n=3$ experiments; *$P<0.05$, versus corresponding Dex-free control; †$P<0.05$ versus corresponding E$_2$-free control, ANOVA.

has been shown to stimulate adrenal corticosterone release both directly and via modulation of glucocorticoid feedback mechanisms (Peiffer & Barden 1987, Peiffer *et al.* 1991, Burgess & Handa 1992, Redei *et al.* 1994, Lo *et al.* 2000). It was therefore possible that 17β–estradiol treatment increased the amount of anterior pituitary ANXA1 expression via changes in circulating glucocorticoid concentrations secondary to estradiol treatment and/or via a direct pituitary action on FS cells.

In order to test whether the 17β–estradiol-induced increase in anterior pituitary ANXA1 content *in vivo* may be via increased corticosterone release from the adrenal gland, 17β–estradiol was administered to ADX and combined OVX/ADX animals, and ANXA1 mRNA and protein measured. 17β-Estradiol significantly increased anterior pituitary ANXA1 mRNA and protein content in the OVX rats relative to vehicle OVX controls but no such increase in response to 17β-estradiol was observed in the ADX or double OVX/ADX groups. These findings show that in the absence of the adrenal gland and circulating corticosterone,

A

B
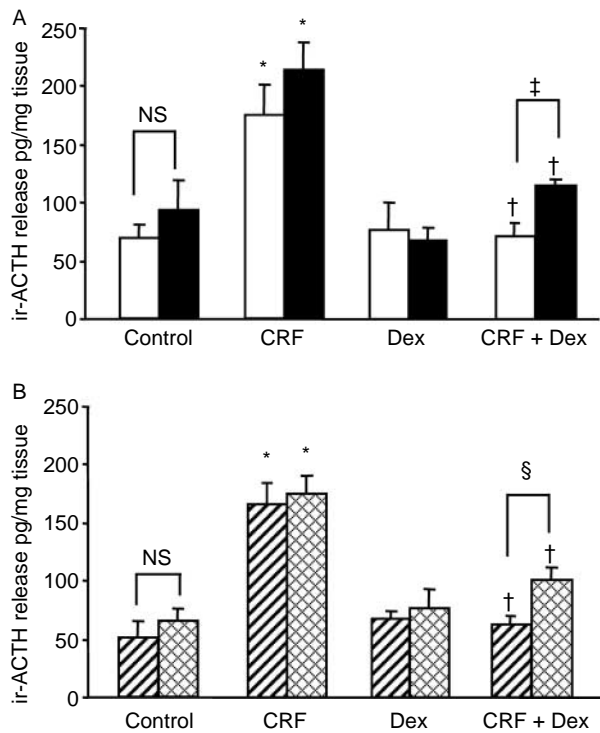
Figure 8 The inhibitory effects of dexamethasone (0·1 µM) on the release of ir-ACTH induced by CRH (10 nM) from (A) vehicle versus 17β-estradiol (E$_2$)-treated anterior pituitary segments from OVX rats *in vitro*; open columns, vehicle; filled columns, E$_2$-treated; and (B) anterior pituitary tissue from proestrus and estrus females; hashed columns, proestrus, crossed pattern columns, estrus. Values are expressed as means±S.E.M., $n=6$. *$P<0.05$ versus corresponding control. †$P<0.05$ versus corresponding dexamethasone-free control. ‡$P<0.05$ OVX versus OVX E$_2$ 24 h; §$P<0.05$ proestrus versus estrus, ANOVA plus Scheffe's test.

17β–estradiol was unable to induce an increase in anterior pituitary ANXA1 expression. An unexpected observation was that combined ADX and OVX produced an increase in ANXA1 expression, greater than in the adrenalectomy alone groups. The ability of OVX to partially reduce the effects of ADX on ANXA1 expression implies that gonadal steroids may have inhibitory as well as permissive actions. It is therefore possible that progesterone could also play a role in the regulation of ANXA1 expression opposing the actions of estrogen, which we are currently investigating.

Western blot analysis revealed that anterior pituitary ANXA1 content varied with the estrous cycle. ANXA1 content was robustly decreased during proestrus and increased in estrus. Estradiol concentrations vary with the rat estrous cycle, being low at estrus, beginning to increase at late metestrus, reaching a sharp peak at early afternoon of proestrus from which levels start to decline by early evening of proestrus. Corticosterone blood concentrations also vary with the estrous cycle such that levels are highest in the afternoon of proestrus (Carey *et al.* 1995), coinciding with the time of maximal 17β-Estradiol secretion. One possible

explanation is that the change in ANXA1 is due to the surge in estradiol during proestrus, and mediated via changes in plasma corticosterone. However, it could also be that the combination of estradiol and progesterone in proestrus drives ANXA1 synthesis down and that the presence of estradiol in the absence of progesterone during estrus increases ANXA1 synthesis. The ability of OVX to partially reduce the effects of ADX on ANXA1 expression supports the hypothesis that gonadal steroids also have inhibitory actions. Although estrogen treatment in male rats has been shown to either induce (48 h treatment; Feldman *et al.* 1979) or reduce (72 h treatment; Smith & Hammond 1992) corticosterone binding globulin (CBG) production and secretion, CBG concentrations remain unchanged in estrogen-treated intact or OVX female rats (up to 10 days treatment tested; Gala & Westphal 1965, 1966). Therefore, in the present study, it is unlikely that 24-h estrogen treatment induced changes in CBG that contributed to the effects observed.

Although the promoter region of the ANXA1 gene lacks a classical estrogen response element sequence, it does contain a cAMP-responsive element through which dexamethasone and estradiol have been shown to induce ANXA1 transcription (Wallner *et al.* 1986, Horlick *et al.* 1991). Studies in a human CCRF-CEM acute lymphoblastic leukemia cell line have demonstrated that 17β-estradiol (at 1 μM) increases ANXA1 mRNA synthesis and cell surface ANXA1 (Castro-Caldas *et al.* 2001) by a mechanism requiring AMP response element (CRE)-binding protein (Castro-Caldas *et al.* 2003). We therefore investigated the possibility of direct effects of 17β-estradiol on ANXA1 expression in a FS cell line. The TtT/GF cell line is a well-characterized pituitary FS cell line, which closely models pituitary FS cells (Chapman *et al.* 2003, Tierney *et al.* 2003, Omer *et al.* 2006). FS cells express intracellular estrogen receptors (Mitchner *et al.* 1998) and RT-PCR analysis has demonstrated the expression of ERα and ERβ mRNA in TtT/GF cells (Nagashima *et al.* 2003). By immunofluorescence methods, we localized the subcellular distribution of ERα and ERβ in TtT/GF cells and revealed that in control- and 17β-estradiol-treated conditions, ERα and ERβ were localized predominantly in the nucleus, consistent with previous receptor localization studies in gonadotropes (Sanchez-Criado *et al.* 2005). Treatment of TtT/GF cells with 17β-estradiol stimulated an increase in ANXA1 mRNA as measured by QPCR. No significant change in the amount of ANXA1 protein was measured in response to the range of concentrations of 17β-estradiol tested. However, the trend for estradiol regulation of ANXA1 protein expression was upward and it is not unusual for protein changes to be smaller than mRNA changes as mRNA and protein stoichiometry is not 1:1. It is well established that glucocorticoids induce the expression and externalization of ANXA1 in TtT/GF cells (Chapman *et al.* 2002, Omer *et al.* 2006). Pretreatment of TtT/GF cells with 17β-estradiol for 24 h increased the amount of ANXA1 synthesized and externalized in response to a subsequent dexamethasone stimulus compared with untreated controls. Furthermore,

co-treatment of cells with 17β-estradiol and dexamethasone for 24 h enhanced pituitary ANXA1 protein content compared with the response to dexamethasone alone. Together, these results in a FS cell line suggest that 17β-estradiol acts to enhance the production of ANXA1 mRNA and release of ANXA1 in response to glucocorticoid. However, *in vivo* 17β-estradiol did not influence ANXA1 mRNA or protein expression in ADX rats suggesting that either 17β-estradiol does not have a direct effect in the rat pituitary or the effect is inhibited by an unknown factor in ADX conditions.

In the male anterior pituitary, ANXA1 plays a key role in the manifestation of the early delayed feedback effects of glucocorticoids (reviewed in John *et al.* 2004). We compared the suppression of CRH-stimulated ACTH release by dexamethasone in proestrus pituitary compared with estrus and in OVX compared with estradiol-treated OVX tissue. Suppression of CRH-stimulated ACTH release by dexamethasone produced significantly greater inhibition in proestrus pituitary compared with estrus and in OVX compared with estradiol-treated OVX tissue. Therefore, in conditions of greater pituitary ANXA1 expression and greater estradiol exposure, the degree of glucocorticoid suppression of ACTH release *in vitro* was less. These data suggest that there is not a direct correlation between the concentration of ANXA1 in the pituitary and the glucocorticoid feedback inhibition. However, the ANXA1 antibody used in these studies does not discriminate between the different phosphorylation states of ANXA1 and it may be that the phosphorylation (or other modification) of ANXA1 is a more important indicator of the functional action of ANXA1 than the total quantity (Solito *et al.* 2006). Conversely, in the female ANXA1 null mouse, basal HPA parameters were not altered compared with wild type, whereas in male ANXA1 null mice anterior pituitary corticotrope number and ACTH content were increased suggesting there may be a less critical role for ANXA1 in females (Morris *et al.* 2006).

Sexual dimorphism in the immune and inflammatory response in humans is well known. Females produce more vigorous cellular and humoral reactions, are more resistant to certain infections, and suffer a higher incidence of auto-immune diseases (e.g. rheumatoid arthritis) than males (Bouman *et al.* 2005). Disease expression is also influenced by the reproductive status of the female. In ANXA1 null mice, the male ANXA1 null leukocyte response to experimental inflammation was enhanced to a greater extent than the female response (Hannon *et al.* 2003). Our findings demonstrating gender differences in ANXA1 expression may therefore contribute to the gender differences underlying susceptibility to autoimmune and inflammatory diseases.

In summary, 17β-estradiol treatment *in vivo* increased ANXA1 expression in the anterior pituitary by a mechanism that involves the adrenal gland. The parallel changes in ANXA1 expression and in circulating corticosterone in response to 17β-estradiol, suggest it is possible that

corticosterone has a role in the mechanism of action of 17β-estradiol. Further studies investigating the effects of a glucocorticoid receptor antagonist, such as RU486, are required to verify this possibility. The exact cell targets of 17β-estradiol action *in vivo* that lead to upregulation of ANXA1 are unknown but could include indirect regulation of brain, pituitary, and/or adrenal receptors or secretagogs that influence corticosterone release or via a direct adrenal action (Peiffer & Barden 1987, Peiffer *et al.* 1991, Burgess & Handa 1992, Lo *et al.* 2000). Experiments in TtT/GF cells indicate a direct action of 17β-estradiol to enhance ANXA1 mRNA content and externalization in response to glucocorticoid. Together, these data are consistent with the hypothesis that estrogens contribute to the regulation of pituitary ANXA1 expression and suggest that changes in ANXA1 may relate to known gender differences in stress responsivity and susceptibility to autoimmune and inflammatory diseases.

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