Autoradiographic determination of angiotensin II receptors in prepubertal and postpubertal pig ovarian tissue

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The aim of this study was to determine whether specific receptors for angiotensin II are present in prepubertal and postpubertal pig ovaries using an autoradiographic technique and computerized densitometry. Frozen sections were cut from prepared samples, and ¹²⁵I-labelled angiotensin II and the angiotensin II receptor subtype-specific nonpeptide antagonists for receptor subtype 1, AT₁ (GR117289) and subtype 2, AT₂ (PD123319) were used. In both pre- and postpubertal pig ovarian tissue, specific receptors for angiotensin II were demonstrated. These receptors had a density of 2487.6 (range: 267.5–5177.6, *n* = 4) and 3703.8 (range: 1819.9–5207.8, *n* = 4) fmol per mm², respectively, and dissociation constants of 130.0 and 26.3 nmol l⁻¹, respectively (prepubertal ovarian range: 106.0–165.4 nmol l⁻¹; postpubertal ovarian range: 26.1–100.3 nmol l⁻¹; *P* < 0.05, Mann–Whitney U test). AT₁ receptors with a *K_i* for ¹²⁵I-labelled angiotensin II

of 346.9 nmol l⁻¹ in the prepubertal and 268.1 nmol l⁻¹ in the postpubertal ovary were located predominantly in follicle wall tissue. Competitive inhibition studies using both angiotensin II antagonists resulted in a decrease in *K_i* with prepubertal tissue (283.7 nmol l⁻¹) and an increase in postpubertal tissue (293.9 nmol l⁻¹). Immunocytochemistry using sections from paraffin wax-embedded prepubertal (*n* = 4) and postpubertal (*n* = 4) pig ovaries confirmed the presence of AT₁ receptors on the granulosa cell layer, but not the thecal cell layer, of antral follicles in both pre- and postpubertal pig ovarian tissue, and AT₂ receptors within the granulosa cell layer of prepubertal pig ovarian antral follicles. In summary, these results indicate that angiotensin II receptors are of higher affinity in postpubertal tissue than they are in prepubertal tissue, and indicate an active renin–angiotensin system within the pig ovary.

Introduction

There is considerable evidence for a local renin–angiotensin system in the mammalian ovary (Bumpus *et al.*, 1988; Lightman *et al.*, 1989; August and Sealey, 1990). The principal biologically active product of this cascade reaction is the octapeptide, angiotensin II (AngII), which acts through specific receptors, potentially to influence ovulation and steroidogenesis (Pellicer *et al.*, 1988; Yoshimura *et al.*, 1992; Peterson *et al.*, 1993). The two main types of AngII receptor, type 1 (AT₁) and type 2 (AT₂), have been classified using antagonists (Chiu *et al.*, 1989; Whitebread *et al.*, 1989; Timmermans *et al.*, 1993), primarily Losartan (a well characterized AT₁ antagonist) and PD 123319 (an AT₂ receptor antagonist). Glaxo compound GR117289 can also be used as an AT₁ antagonist (Robertson *et al.*, 1992). AT₁ receptors are involved in the regulation of vasoconstriction and salt and fluid balance

(Chiu *et al.*, 1989; Whitebread *et al.*, 1989), whereas AT₂ receptors are thought to play a role in growth regulation and apoptosis (Timmermans *et al.*, 1993). However, there is a general paucity of information regarding the presence or absence of AT₁ and AT₂ receptors within the mammalian ovary.

AT₂ receptors have been identified in cultured bovine thecal cells by preparing a cell membrane fraction and conducting an ¹²⁵I-binding study (Brunswick-Spickenheier and Mukhopadhyay, 1992a,b). Receptor autoradiography may be used to determine the presence of the receptor for a particular ligand within a type of tissue. Autoradiographic studies have demonstrated AngII receptor binding sites on rat granulosa cells (Daud *et al.*, 1989; Naftolin *et al.*, 1989), and on rat granulosa and theca interna cells (Husain *et al.*, 1987). Another autoradiographic study localized AngII receptors to granulosa cells within atretic follicles of the rat ovary (Daud *et al.*, 1988). However, these studies did not differentiate between the two characterized AngII receptor subtypes.

The present study used radiolabelled AngII to identify and locate receptors. This method has been used widely to process large numbers of ovarian samples, for example in

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localizing the type 1 IGF (insulin-like growth factor) receptor in the ovine ovary (Perks *et al.*, 1995), and ovarian gonadotrophin receptors in the rhesus monkey (Hutz *et al.*, 1993), human (Yamoto *et al.*, 1992) and rat ovary (Otani *et al.*, 1996). Quantitative receptor autoradiography *in vitro* is well established as a method to measure receptor binding to different regions of tissue (Palacios *et al.*, 1981; Som *et al.*, 1983; Yonekura *et al.*, 1983; Israel *et al.*, 1985; Zilles *et al.*, 1988, 1990).

There is some evidence of components of the renin-angiotensin system within the pig ovary. Prorenin and renin are present in pig follicular fluid (Hagemann *et al.*, 1992; Nielsen *et al.*, 1995). Previous studies in our laboratory have demonstrated renin activity in follicular fluid collected from small (1–3 mm in diameter) prepubertal follicles and at a lower level in larger (> 6 mm in diameter) postpubertal follicles (Shuttleworth, 2000). AngII inhibits the transcription of steroidogenic enzymes in pig granulosa cells, indicating an active role for this peptide in pig ovarian function (Li *et al.*, 1995). Nielsen *et al.* (1995) used a membrane fraction AngII receptor assay to indicate that there are receptors present in postpubertal pig follicle wall tissue, but did not characterize them.

Therefore, the aim of the present study was to use autoradiography to determine whether there are receptors for AngII within the prepubertal and postpubertal pig ovary, and if so, to identify whether they are AT₁ or AT₂ subtypes. Such findings would give an indication of the role of AngII in the pig ovary. Computerized densitometry was applied to analyse ligand binding and hence determine the binding affinity (K_d or K_i) and binding capacity (B_{max}) of the receptor. Immunocytochemistry was used to localize the receptor subtypes to types of follicular cell within the pre- and postpubertal pig ovary.

Materials and Methods

Tissue and slide preparation

Prepubertal (12–15 weeks of age) and postpubertal ovarian tissue from Large White hybrid pigs was obtained from a local abattoir and transported to the laboratory on ice. Follicles within prepubertal ovaries were < 5 mm in diameter and there was no evidence of previous ovulation. Postpubertal ovarian samples were selected as being those in the follicular phase with several large follicles > 6 mm in diameter (Grant *et al.*, 1989), and therefore presenting no active corpora lutea, although corpora albicantia from previous cycles were present. These selection criteria ensured that similar structures (that is, follicles) in pre- and postpubertal ovaries were being compared. Both ovaries from five prepubertal and eight postpubertal animals were selected, dissected free of surrounding tissue and frozen in liquid nitrogen within 1 h of slaughter. Serial frozen sections (20 µm) were cut on a cryostat at –30°C, and duplicate consecutive sections were thaw-mounted on gelatin-coated slides. Slides were stored with desiccant at

–80°C and brought to room temperature immediately before use. Ovaries from a further four prepubertal and four postpubertal animals at a similar age or stage of the oestrous cycle as those used in the autoradiographic study were prepared and processed for embedding in paraffin wax. Four postpubertal adrenal glands were also prepared as per the ovarian samples and embedded in paraffin wax as positive controls. Serial sections (4 µm) were cut and mounted on glass slides pre-coated with 2.5% (v/v) 3-aminopropyltriethoxysilane (APES; Sigma Chemical Co., Poole) in acetone. Slides were stored at room temperature before immunocytochemical staining.

In vitro autoradiography

Prepared slides of frozen sections were preincubated at room temperature for at least 30 min in 50 mmol sodium phosphate buffer l⁻¹ (pH 7.2) containing 150 mmol NaCl l⁻¹, 10 mmol MgCl₂ l⁻¹, 5 mmol EDTA l⁻¹ (disodium salt), 0.4% (w/v) BSA and 0.005% (w/v) bacitracin (all Sigma Chemical Co.). The slides were incubated for 3 h at room temperature in a humidified chamber in fresh buffer containing increasing concentrations (10⁻¹²–10⁻⁴ mol l⁻¹) of the appropriate unlabelled ligand and 0.3 nmol ¹²⁵I-labelled AngII l⁻¹ (Amersham Pharmacia Biotech UK Ltd, Little Chalfont). Serial consecutive sections were incubated with increasing concentrations of unlabelled AngII to determine the specificity of AngII binding ($n = 4$ prepubertal ovaries, $n = 4$ postpubertal ovaries). Serial sections were incubated with increasing concentrations (10⁻¹²–10⁻⁴ mol l⁻¹) of the AT₁ receptor antagonist GR117289 (GlaxoSmithKline plc, Uxbridge), the AT₂ receptor antagonist PD123319 (Parke-Davies and Co. Ltd, Eastleigh) or a combination of GR117289 and PD123319 to characterize AngII receptor subtypes. After incubation, slides were washed twice for 5 min in sodium phosphate buffer at 4°C, and rinsed briefly in distilled water. The sections were dried in a stream of cool air. Slides were placed in a light tight X-ray cassette (Genetic Research Instruments Ltd, Braintree) with one set of ¹²⁵I microscale standards (mean values from 3552 to 1360 860 d.p.m. mg⁻¹; Amersham Pharmacia Biotech UK Ltd) and apposed to Hyperfilm ³H X-ray film (Amersham Pharmacia Biotech UK Ltd). The cassette was stored at room temperature for 5 days. The film was developed for 2.5 min in Kodak D19 developer (Sigma Chemical Co.) and rinsed briefly in tap water containing 1% (v/v) acetic acid and fixed for 5 min in Ilford Hypam Fixer (Ilford Ltd, Knutsford). The film was washed in running tap water for 20 min and air-dried. Slides were fixed in 10% (v/v) formol saline, and stained with haematoxylin and eosin to aid in identifying structures and type of cell to which ¹²⁵I-labelled AngII had bound.

Image analysis and computerized densitometry

Areas of the ovarian section that bound ¹²⁵I-labelled AngII produced a dark image when apposed to X-ray film. The film was analysed by computer densitometry to assess the binding of ¹²⁵I-labelled AngII to ovarian sections and to

determine the binding kinetics (Yonekura *et al.*, 1983; Israel *et al.*, 1984, 1985; Saavedra *et al.*, 1986). The developed autoradiograph was placed on a lightbox (DW Viewboxes Ltd, Milton Keynes) and viewed through a solid state video camera (COHU, San Diego, CA) linked to a computer running the Image Manager Package (Rev 5.21, Sight Systems, Foster Findlay Assoc. Ltd., Newcastle-Upon-Tyne), which gave a reading on a greyscale from 0 to 255 corresponding to the intensity of the developed image (background automatically subtracted). Each sample was analysed to give a greyscale value from 0 to 255, where 0 corresponds to 'black' and 255 corresponds to 'white'. Two areas were measured per ovarian section using computerized image analysis; the total area of the section including follicular antra, and the area corresponding to tissue only (not including the area occupied by follicular antra, deducted by altering the threshold setting to exclude these areas). Each standard was measured in triplicate, and a mean value determined. Grey scale values were converted to relative optical density (ROD) values.

Standards were corrected for radioactive decay and a standard curve was plotted (linear regression) of ROD versus the logarithm of this value; a good fit was considered to give an *r* value approaching 1 (range 0.9613–0.9857, to four decimal places) and *P* < 0.05. All standard curves in this study fitted this criterion. The logarithm of sample d.p.m. per mm² was determined from the calculated ROD using the standard curve. This value was raised to the power 10, corrected for radioactive decay, and the logarithm of this corrected value was obtained. The equation generated for the standard curve was used to determine the corrected ROD. A displacement curve was plotted of the corrected ROD versus the logarithm of the concentration of the unlabelled compound studied to give a sigmoidal curve symmetrical about the dissociation constant *K_d* (when the displacing ligand is other than AngII, for example the receptor antagonists, this value is termed *K_i*). A Scatchard plot was constructed using GraphPad Prism v1.03 (Graphpad, San Diego, CA) and a line generated (for example, see Fig. 1). The mean area of the tissue (less antral spaces) was calculated for each series of displacement and was used in the calculation of *B_{max}* (fmol per mm²) to allow comparison between tissues. The calculated *K_d* values for pre- and postpubertal ovarian tissue were analysed statistically using the Mann–Whitney U test.

Immunocytochemistry

Immunocytochemistry was completed on the paraffin wax-embedded sections using the Dako EnVision+® System (Dako, Ely). The protocol was as per the manufacturer's instructions except that blocking for endogenous peroxidase was undertaken using a standard methanol–hydrogen peroxide step for 15 min (Jackson and Blythe, 1993). The monoclonal antibody to the AT₁ receptor (6313/G2, a kind gift from G. P. Vinson (Barker *et al.*, 1993)) was used at a 1 in 10 dilution in Tris buffer containing 0.25% (w/v) BSA. A polyclonal anti-AT₂

Fig. 1. Scatchard analysis of representative data obtained by the displacement of ¹²⁵I-labelled angiotensin II from pig postpubertal ovarian tissue and various concentrations of unlabelled angiotensin II. *K_d* = 26.6 nmol l⁻¹ and *B_{max}* = 5207.8 fmol per mm² in this experiment.

receptor antibody (a kind gift from W. T. Cheung (Yiu *et al.*, 1997)) was used at a dilution of 1 in 4000 in Tris buffer containing 0.25% (w/v) BSA. Sections were counterstained with Harris' haematoxylin. Positive controls for both receptor subtypes comprised paraffin wax-embedded postpubertal pig adrenal gland. Negative controls initially comprised sections of all types of tissue (including adrenal gland) processed as above, with mouse non-immune serum (anti-AT₁ negative control) and rabbit non-immune serum (anti-AT₂ negative control). As neither control produced false positive immunoreactivity, all subsequent negative controls comprised Tris buffer containing 0.25% (w/v) BSA replacing receptor subtype antibody in the protocol.

Results

Specificity of AngII binding

An example displacement of 0.3 nmol ¹²⁵I-labelled AngII l⁻¹ using increasing concentrations of unlabelled AngII is shown (Fig. 2). With an increasing amount of unlabelled ligand, there was an increase in displacement first observed visually between 10⁻¹⁰ and 10⁻⁸ mol l⁻¹, indicating the specific binding of AngII to receptors in both types of tissue. Some sites were identified histologically by comparison with haematoxylin and eosin stained slides as being blood vessels in which ¹²⁵I-labelled AngII was not displaced with high concentrations of unlabelled ligand. Resolution of autoradiographs was not sufficient to identify conclusively other sites within ovarian stroma to which ¹²⁵I-labelled AngII was bound even at × 200 magnification. *K_d* and *B_{max}* values were calculated for four ovarian samples studied autoradiographically. As the postpubertal tissue data were skewed (Fig. 3), median values are given. The median *K_d* values for pre- and postpubertal ovarian tissue were 130.0 and 26.3 nmol l⁻¹, respectively (prepubertal ovarian range: 106.0–165.4 nmol l⁻¹; postpubertal ovarian range:

Fig. 2. Displacement of 0.3 nmol ^{125}I -labelled angiotensin II (AngII) I^{-1} from pig (a) prepubertal and (b) postpubertal ovarian tissue, with increasing concentrations of unlabelled AngII. Examples of blood vessels are indicated by arrowheads; examples of the antral space of an antral follicle are denoted by 'A'. Scale bar for each series represents 5 mm (note different magnifications).

26.1–100.3 nmol I^{-1} ; $P < 0.05$, Mann–Whitney U test). The median B_{max} was 2487.6 fmol per mm^2 (range: 267.5–5177.6 fmol per mm^2) for prepubertal tissue, and 3703.8 fmol per mm^2 (range: 1819.9–5207.8 fmol per mm^2) for postpubertal tissue. There was no significant difference between these B_{max} values.

Characterization and localization of receptors

Displacement of ^{125}I -labelled AngII by the AT_1 antagonist (GR117289) and the AT_2 antagonist (PD123319) is presented (Figs 4 and 5). Displacement was seen with lower concentrations of the AT_1 antagonist than the AT_2 antagonist, and quantified data are presented (Table 1). It was not possible to calculate K_i for PD123319 owing to the minimal displacement observed. The K_i and B_{max} values for the AT_1 antagonist calculated for prepubertal ovarian tissue were 346.9 nmol I^{-1} and 241.8 fmol per mm^2 , respectively, and for postpubertal ovarian tissue were 268.1 nmol I^{-1} and 6868.8 fmol per mm^2 , respectively. The use of both antagonists gave greater displacement than use of GR117289 alone (in prepubertal tissue, ROD using GR117289 alone was 0.047, and with both antagonists it was 0.042; in postpubertal tissue, ROD using GR117289 alone was 0.045 and with both antagonists it was 0.039) (Fig. 5), indicating some additional displacement due to

PD123319. Sites of binding corresponding to the theca interna–granulosa cell layers within the follicle were identified (Fig. 5). Immunocytochemical staining for the two receptor subtypes demonstrated that there were AT_1 receptors clearly localized to the adrenal positive control and also to the granulosa cell layer in both pre- and postpubertal ovaries (see Fig. 6) despite predominant edge-effect staining observed at the edge of antral follicles. There was faint immunostaining for AT_2 receptors in the granulosa cell layer of prepubertal but not postpubertal antral follicles. There did not appear to be AT_1 or AT_2 receptors localized to the theca cell layer.

Discussion

This study shows that AngII binds specifically to receptors in the pre- and postpubertal pig ovary, and that the receptors were of higher affinity in postpubertal than in prepubertal tissue. Furthermore, most of these receptors were of the AT_1 receptor subtype and were localized using immunocytochemistry to the granulosa cell layers of antral follicles. The calculated K_i values were high, indicating a low affinity receptor (high affinity receptors will have low K_d and K_i values, typically in the < 10 nmol I^{-1} range: Bumpus *et al.*, 1988; Daud *et al.*, 1988; Aguilera *et al.*, 1989; Nielsen *et al.*, 1994a, 1995). However, studies in pregnant rat uteri

Table 1. Binding kinetics of ^{125}I -labelled angiotensin II displacement with angiotensin II (AngII), GR117289 or PD123319 in pre- and postpubertal pig ovarian tissue

Pig ovarian tissue	Antagonist	Binding affinity (nmol l^{-1}) [†]	B_{max} (fmol per mm^2)
Prepubertal	AngII	165.4	267.5
Prepubertal	GR117289	346.9	241.8
Prepubertal	PD123319	*	*
Prepubertal	GR117289/PD123319	283.7	955.5
Postpubertal	AngII	26.6	5207.8
Postpubertal	GR117289	268.1	6868.8
Postpubertal	PD123319	*	*
Postpubertal	GR117289/PD123319	293.9	937.2

Each value is based on one experiment.

[†]Binding affinity dissociation constant is K_d when AngII is the antagonist and K_i for other antagonists.

*Values could not be calculated owing to the minimal displacement observed with PD123319.

Fig. 3. Calculated K_d values for four samples of prepubertal and postpubertal pig ovarian tissue when adding ^{125}I -labelled angiotensin II (AngII) with unlabelled AngII. The median K_d value is $130.0 \text{ nmol l}^{-1}$ ($n = 4$) for prepubertal tissue and 26.3 nmol l^{-1} ($n = 4$) for postpubertal tissue. Note that there is no overlap in data from the two tissue stages.

indicate that there is a single receptor with a high K_d value ($18.3\text{--}24.2 \text{ nmol l}^{-1}$; Yang *et al.*, 1994). Thus, K_d values differ among species and types of tissue and therefore extrapolation to pigs, for which there is a paucity of information regarding ovarian AngII receptor kinetics, is problematic.

In the present study, some sites in both tissues examined were not associated with any specific area of the ovarian stroma where the ligand bound non-specifically (that is, did not show displacement even at high concentrations of unlabelled ligand). These sites may act in a regulatory fashion to remove excess AngII from the ovary. However, the calculated median K_d values of 26.3 nmol l^{-1} for postpubertal tissue and $130.0 \text{ nmol l}^{-1}$ for prepubertal tissue indicate that AngII has a higher affinity for postpubertal tissue, and that there was no overlap in K_d value between the two types of tissue. The difference in affinity between pre- and postpubertal tissue may be due to the age of the

pig studied and the influence of the change in hormonal milieu between pre- and postpubertal animals. This finding is not unexpected, as a change in receptor affinity within the myometrium from the non-pregnant to pregnant state has been reported in both women (fourfold: Cox *et al.*, 1996) and rats (9–12-fold: Yang *et al.*, 1994). Analysis of four postpubertal ovarian samples showed one high outlier K_d value. The mean K_d and B_{max} values of the other three samples were 25.6 nmol l^{-1} and $4335.1 \text{ fmol per mm}^2$, respectively. The 'high' outlier gave a K_d value of $100.3 \text{ nmol l}^{-1}$ and B_{max} value of $1818.9 \text{ fmol per mm}^2$, indicating a lower number of receptors at a lower affinity. Analysis of slides stained with haematoxylin and eosin demonstrated that this sample was at a later stage of the follicular phase of the oestrous cycle, as it contained larger antral follicles than the other three samples. Differences in AngII receptor expression during the oestrous cycle have been observed in immunocytochemical studies in rats, in which specific ^{125}I -labelled AngII binding in stromal and thecal cells surrounding large antral follicles in normally cyclic ovaries was as follows: dioestrus > pro-oestrus > oestrus (Lightman *et al.*, 1988). These differences indicate that as the animal proceeds through the oestrous cycle, the K_d value increases, as supported by the present study. In contrast to the present study, there was no evidence in rats of binding in the granulosa cells of preantral or antral follicles, or immature ovaries. However, specific high affinity AngII receptors have been demonstrated predominantly on the rat granulosa cell and theca interna layers of a subpopulation of mature and immature follicles (Speth *et al.*, 1986; Husain *et al.*, 1987). Daud *et al.* (1988) reported that, of the follicles containing AngII receptor in the cyclic rat ovary, approximately 95% showed signs of atresia and, using a membrane binding assay, showed that specific binding was lowest at oestrus and highest at dioestrus. Strong AT_2 mRNA expression has been demonstrated in granulosa cells from rat follicles in tertiary stages of development exhibiting signs of atresia, and in individual collapsed atretic follicles, indicating that the AT_2 receptor mediates effects leading to

Fig. 4. ^{125}I -Labelled angiotensin II (AngII) displacement observed with unlabelled AngII (■), GR117289 (Δ), PD123319 (∇) and GR117289 plus PD123319 (●), using prepubertal (a) and postpubertal (b,c) pig ovarian tissue ($n = 1$).

antiproliferative actions and apoptosis (Obermuller *et al.*, 1998). This contention was supported by a further study demonstrating that angiotensin-converting enzyme was linked to the early stages of follicular maturation and atresia (Daud *et al.*, 1990).

The present study demonstrates for the first time that most receptors within the pig pre- and postpubertal ovary are of the AT_1 subtype. The use of the pig adrenal gland as a positive control confirms that the results observed were due to the specificity of the antibodies used. This finding is in contrast to studies in other mammals (rats: Daud *et al.*, 1988; Obermuller *et al.*, 1998; Kotani *et al.*, 1999; cows:

Brunswick-Spickenheier and Mukhopadhyay, 1992b; Aiyar *et al.*, 1993; Nielsen *et al.*, 1995) indicating the AT_2 receptor subtype is associated with ovarian tissue, but is in agreement with studies on rabbit ovaries (Feral *et al.*, 1996). In rats, workers using membrane binding assays have argued for the presence of a single receptor class, with higher concentrations found in corpora lutea and stromal tissue than in granulosa or luteal cells (Aguilera *et al.*, 1989). However, Pucell *et al.* (1991) also used a membrane binding assay to demonstrate that high affinity AT_2 receptors were present in rat granulosa cells and that AT_1 receptors were present on other ovarian structures (that is, the thecal cell layer, interstitium, corpus luteum, blood vessels and surface epithelium). There may be confusion in the detection of the AT_1 receptor in the rat, as studies have shown the presence of two forms, AT_{1A} and AT_{1B} (Kakar *et al.*, 1992), and report that AT_{1A} receptors have a lower affinity for Losartan (Tian *et al.*, 1996). There are no reported differences in AT_1 receptors in pigs and it appears from the results of the present study that the pig ovary possesses both characterized subtypes within its ovarian tissue.

In the bovine ovary, many studies have shown that the predominant receptor is subtype 2 (Brunswick-Spickenheier and Mukhopadhyay, 1992a,b; Aiyar *et al.*, 1993; Nielsen *et al.*, 1994a,b, 1995). Studies by Aiyar *et al.* (1993) indicate that approximately 80% of AngII receptors are subtype 2. It has been suggested that high receptor density is a feature of large bovine ovarian follicles, and reflects an increase in expression with follicular growth and that therefore, unlike in rats, AngII receptor expression is not confined to atretic follicles (Nielsen *et al.*, 1994a,b). In the present study, it was not possible to determine the binding kinetics per follicle, and therefore the receptor density per follicle, although there did appear to be an increase in binding with increasing follicle size.

Nielsen *et al.* (1995) compared components of the ovarian renin-angiotensin system in bovine and pig ovaries and demonstrated that AT_2 receptors in bovine corpora lutea had a K_i value of 1.01 (range 0.64–1.79) nmol l^{-1} ($n = 8$) and a B_{max} value of 22.7 (range 1.9–93) fmol mg^{-1} membrane protein. The authors suggested that this value is ten times higher than it is in pig corpora lutea and reported that the receptor density in postpubertal pig follicular wall tissue was 35-fold lower than it was in bovine follicles (47 (range 19–97) fmol mg^{-1} protein; $n = 13$). It is difficult to compare the results of Nielson *et al.* (1995) with the results of the present study, which are expressed in terms of the area studied in the autoradiogram. However, Nielson *et al.* (1995) comment that it was not possible to determine the subtype or K_i value of the receptor in the pig ovary owing to varying but low specific binding, and assume the presence of AT_2 receptors, as in the bovine ovary, with binding characteristics similar to AT_2 receptors in the pig placental amniochorion-endometrium, without testing the hypothesis. The present autoradiographic study demonstrates clearly the presence of AT_1 receptors in the pre- and postpubertal

Fig. 5. Displacement of ^{125}I -labelled angiotensin II (AngII) by $10^{-6} \text{ mol l}^{-1}$ unlabelled AngII, GR117289 and PD123319 in pig (a) prepubertal and (b) postpubertal ovarian tissue. Note the presence of binding to blood vessels, particularly in those sections corresponding to addition of unlabelled AngII or GR117289 (examples indicated by open arrowheads), and binding in the theca interna-granulosa cell layer of antral follicles (examples indicated by closed arrowheads). Scale bar for each series represents 5 mm (note different magnifications).

pig ovary, yet does not establish the presence of AT_2 receptors conclusively. There was displacement in both tissues at high concentrations of PD123319 that was more clearly observed in postpubertal ovaries. The use of both AT_1 and AT_2 antagonists on the same section resulted in a shift in the displacement curve from that using just GR117289, implying the presence of a small number of AT_2 receptors in these tissues. Thus, it appears AT_2 receptors are present in postpubertal tissues, a finding that is in agreement with the contention that this receptor is linked strongly to apoptosis and growth regulation, although the results of the immunocytochemical study indicate that these receptors are not associated with antral follicles. However, AT_2 receptors were also associated with the granulosa cell layer of follicles in the prepubertal ovary and their putative role in this tissue needs to be clarified.

Treatment of isolated rabbit thecal and granulosa cell cultures with AngII leads to a decrease in oestradiol secretion and an increase in thecal cell testosterone production, but does not affect the release of progesterone from granulosa cells (Feral *et al.*, 1996). The addition of AngII to cultured rat granulosa cells also elicits no change in progesterone production (Cannon *et al.*, 1997). The results from Feral *et al.* (1996) indicate that AngII plays a role in steroid synthesis by inhibiting aromatase activity. However, two studies on luteinized human granulosa cells *in vitro*

demonstrated that the addition of AngII leads to an increase in progesterone synthesis (Morris *et al.*, 1994; Johnson *et al.*, 1997), indicating a modulatory action on 3β -hydroxysteroid dehydrogenase (3β -HSD; Johnson *et al.*, 1997). Treatment with the non-specific receptor blocker saralasin resulted in enhanced oestradiol production, indicating that oestradiol secretion is inhibited by endogenous AngII (Morris *et al.*, 1994). It has been suggested that AngII reduces the ability of cultured prepubertal pig granulosa cells to synthesize steroids by inhibiting LH-induced transcription of the 3β -HSD gene or by altering the stability of 3β -HSD mRNA, and that AngII stimulates apoptosis in a subpopulation of cells (Li *et al.*, 1995). The ability to produce steroids differentiates between healthy and atretic follicles (Daud *et al.*, 1988). In rats, AT_2 receptors inhibit FSH actions and mediate follicle atresia through granulosa cell apoptosis (Kotani *et al.*, 1999).

In conclusion, the present study has demonstrated the presence of specific binding sites for AngII within the pre- and postpubertal pig ovary. The receptors were predominantly AT_1 , a subtype associated with the classical effects of AngII, such as on water and salt balance, and which may therefore be associated with fluid transfer into the follicle. High concentrations of AT_1 receptors were present in the granulosa cell layer of antral follicles of both pre- and postpubertal ovaries, in contrast to reports in other species

Fig. 6. Immunohistochemical staining for angiotensin II receptor subtype-specific non-peptide antagonists for receptor subtype 1 (AT_1) (i) or AT_2 (ii) receptors in pig (a) prepubertal ovarian tissue (b) postpubertal ovarian tissue, and (c) adrenal medulla. The negative control is presented in (iii). A: antral space; G: granulosa cells; T: thecal cells. Scale bars represent 100 μ m.

in which AT_2 receptors dominate and have been suggested to be associated with apoptosis and angiogenesis. Low concentrations of AT_2 receptors were present within the prepubertal ovary, although localization of this receptor remains to be clarified for postpubertal ovaries. These data indicate that there are differences in the expression of AngII receptor subtypes and therefore variations in the role of AngII in the ovaries of different species.

The authors would like to thank C. Pearson (University of Sheffield) for assistance and advice with densitometry, G. P. Vinson (University of London) and W. T. Cheung (University of Hong Kong) for generous gifts of antibodies to AT_1 and AT_2 receptors, respectively.

References

- Aguilera G, Millan MA and Harwood JP (1989) Angiotensin II receptors in the gonads *American Journal of Hypertension* **2** 395–402
- Aiyar N, Griffin E, Edwards R, Weinstock J, Samanen J and Nambi P (1993) Characterization of bovine ovary angiotensin II receptors using subtype-selective antagonists *Pharmacology* **46** 1–8
- August P and Sealey JE (1990) The renin–angiotensin system in normal and hypertensive pregnancy and in ovarian function. In *Hypertension: Pathophysiology, Diagnosis and Management* pp 1761–1778 Eds JH Laragh and BM Brenner. Raven Press Ltd, New York
- Barker S, Marchant W, Ho MM, Puddefoot JR, Hinson JP, Clark AJL and Vinson GP (1993) A monoclonal antibody to a conserved sequence in the extracellular domain recognizes the angiotensin II AT_1 receptor in mammalian target tissues *Journal of Molecular Endocrinology* **11** 241–245
- Brunswick-Spickenheier B and Mukhopadhyay AK (1992a) Angiotensin II (AT_1) receptors in the bovine ovary are regulated by LH *FASEB Journal* **6** A1861 (Abstract)
- Brunswick-Spickenheier B and Mukhopadhyay AK (1992b) Characterization of angiotensin II receptor subtype on bovine thecal cells and its regulation by luteinizing hormone *Endocrinology* **131** 1445–1452
- Bumpus FM, Pucell AC, Daud AI and Husain A (1988) Angiotensin II: an intraovarian regulatory peptide *American Journal of the Medical Sciences* **295** 406–408
- Cannon CM, Keeble SC and Curry TE (1997) Effect of A23187 on angiotensin II on ovarian metalloproteinase inhibitors and steroidogenesis in rats *Journal of Reproduction and Fertility* **111** 71–79
- Chiu AT, Herblin WF, McCall DE et al. (1989) Identification of angiotensin II receptor subtypes *Biochemical and Biophysical Research Communications* **165** 196–203
- Cox BE, Word RA and Rosenfeld CR (1996) Angiotensin II receptor

- characteristics and subtype expression in uterine arteries and myometrium during pregnancy *Journal of Clinical Endocrinology and Metabolism* **81** 49–58
- Daud AI, Bumpus FM and Husain A** (1988) Evidence for selective expression of angiotensin II receptors on atretic follicles in the rat ovary: an autoradiographic study *Endocrinology* **122** 2727–2734
- Daud AI, Bumpus FM and Husain A** (1989) Angiotensin II – does it have a direct obligate role in ovulation? *Science* **245** 870–871
- Daud AI, Bumpus FM and Husain A** (1990) Characterization of angiotensin I-converting enzyme (ACE)-containing follicles in the rat ovary during the estrous cycle and effects of ACE inhibitor on ovulation *Endocrinology* **126** 2927–2935
- Feral C, Benhaim A and Leymarie P** (1996) Angiotensin receptor type 1 on granulosa and thecal cells of rabbit preovulatory follicles *Biochimica et Biophysica Acta* **1284** 221–226
- Grant SA, Hunter MG and Foxcroft GR** (1989) Morphological and biochemical characteristics during ovarian follicular development in the pig *Journal of Reproduction and Fertility* **86** 171–183
- Hagemann A, Nielsen AH, Dantzer V, Avery B and Poulsen K** (1992) Measurement and identification of prorenin and renin in ovarian follicular fluid from cattle and pig *Clinical and Experimental Pharmacology and Physiology* **19** 267–273
- Husain A, Bumpus FM, De Silva P and Speth RC** (1987) Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries *Proceedings National Academy of Sciences of the USA* **84** 2489–2493
- Hutz RJ, Wagner N, Krause P, Fisher C, Syed N, Dierschke DJ, Monniaux D and Tomanek M** (1993) Localization of estrogen receptors in rhesus monkey ovary *American Journal of Primatology* **31** 299–309
- Israel A, Correa FMA, Niwa M and Saavedra JM** (1984) Quantitative determination of angiotensin II binding sites in rat brain and pituitary gland by autoradiography *Brain Research* **322** 341–345
- Israel A, Plunkett LM and Saavedra JM** (1985) Quantitative autoradiographic characterization of receptors for angiotensin II and other neuropeptides in individual brain nuclei and peripheral tissues from single rats *Cellular and Molecular Neurobiology* **5** 211–222
- Jackson P and Blythe D** (1993) Immunolabelling techniques for light microscopy. In *Immunocytochemistry: A Practical Approach* pp 15–42 Ed. JE Beesley. IRL Press, Oxford
- Johnson MC, Vega M, Vantman D, Troncoso JL and Devoto L** (1997) Regulatory role of angiotensin II on progesterone production by cultured human granulosa cells. Expression of angiotensin II type-2 receptor *Molecular Human Reproduction* **3** 663–668
- Kakar SS, Sellers JC, Devor DC, Musgrove LC and Neill JD** (1992) Angiotensin II type-I receptor subtype cDNAs: differential tissue expression and hormonal regulation *Biochemical and Biophysical Research Communications* **183** 1090–1096
- Kotani E, Sugimoto M, Kamata H et al.** (1999) Biological roles of angiotensin II via its type 2 receptor during rat follicle atresia *American Journal of Physiology, Endocrinology and Metabolism* **39** E25–E33
- Li XM, Juorio AV and Murphy BD** (1995) Angiotensin II interferes with steroidogenesis in porcine granulosa cells *Biology of Reproduction* **53** 791–799
- Lightman A, Jones CL, Macluskus NJ, Palumbo A, De Cherney AH and Naftolin F** (1988) Immunocytochemical localization of angiotensin II immunoreactivity and demonstration of angiotensin II binding in the rat ovary *American Journal of Obstetrics and Gynecology* **159** 526–530
- Lightman A, Palumbo A, De Cherney AH and Naftolin F** (1989) The ovarian renin–angiotensin system *Seminars in Reproductive Endocrinology* **7** 79–87
- Morris RS, Francis MM, Do YS, Hsueh WA, Lobo RA and Paulson RJ** (1994) Angiotensin II (All) modulation of steroidogenesis by luteinized granulosa cells *in vitro*. *Journal of Assisted Reproduction and Genetics* **11** 117–122
- Naftolin F, Andrade-Gordon P, Pellicer A, Palumbo A, Apa R, Zreik T, Yoon TK and De Cherney A** (1989) Angiotensin II – does it have a direct obligate role in ovulation? (response) *Science* **245** 871
- Nielsen AH, Hagemann A, Svenstrup B, Nielsen J and Poulsen K** (1994a) Angiotensin II receptor density in bovine ovarian follicles relates to tissue renin and follicular size *Clinical and Experimental Pharmacology and Physiology* **21** 463–469
- Nielsen AH, Hagemann A, Svenstrup B, Nielsen J and Poulsen K** (1994b) Relationship of tissue renin and follicular size with angiotensin II receptor density in bovine ovarian follicles *Regulatory Peptides* **53** 149
- Nielsen AH, Hagemann A, Avery B and Poulsen K** (1995) Differences in expression of angiotensin II receptors and renin in porcine and bovine ovaries *Experimental and Clinical Endocrinology and Diabetes* **103** 332–338
- Obermuller N, Schlamp D, Hoffmann S, Gentili M, Inagami T, Gretz N and Weigel M** (1998) Localization of the mRNA for the angiotensin receptor subtype 2 (AT₂) in follicular granulosa cells of the rat ovary by nonradioactive *in situ* hybridization *Journal of Histochemistry and Cytochemistry* **46** 865–870
- Otani H, Yamoto M, Fujinaga H and Nakano R** (1996) Presence and localization of endothelin receptor in the rat ovary and its regulation by pituitary gonadotrophins *European Journal of Endocrinology* **135** 449–454
- Palacios JM, Niehoff DL and Kuhar MJ** (1981) Receptor autoradiography with tritium sensitive film: potential for computerized densitometry *Neuroscience Letters* **25** 101–105
- Pellicer A, Palumbo A, De Cherney AH and Naftolin F** (1988) Blocking of ovulation by an angiotensin antagonist *Science* **240** 1660–1661
- Perks CM, Denning-Kendall PA, Gilmour RS and Wathes DC** (1995) Localization of messenger ribonucleic acids for insulin-like growth factor I (IGF-I), IGF-II, and the type I IGF receptor in the ovine ovary throughout the estrous cycle *Endocrinology* **136** 5266–5273
- Peterson CM, Zhu C, Mukaida T, Butler TA, Woessner JF and LeMarie WJ** (1993) The angiotensin II antagonist saralasin inhibits ovulation in the perfused rat ovary *American Journal of Obstetrics and Gynecology* **168** 242–245
- Pucell AG, Hodges JC, Sen I, Bumpus FM and Husain A** (1991) Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor *Endocrinology* **128** 1947–1959
- Robertson JM, Barnes JC, Drew GM et al.** (1992) Pharmacological profile of GR117289 *in vitro*: a novel, potent and specific non-peptide angiotensin AT₁ receptor antagonist *Journal of Pharmacology* **107** 1173–1180
- Saavedra JM, Israel A, Plunkett LM, Kurihara M, Shigematsu K and Correa FMA** (1986) Quantitative distribution of angiotensin II binding sites in rat brain by autoradiography *Peptides* **7** 679–687
- Shuttleworth, G** (2000) *Porcine Ovarian Follicle Development and the Renin–Angiotensin System* PhD Thesis, University of Nottingham
- Som P, Yonekura Y, Oster ZH et al.** (1983) Quantitative autoradiography with radiopharmaceuticals, part 2: applications in radiopharmaceutical research: concise communication *Journal of Nuclear Medicine* **24** 238–244
- Speth RC, Bumpus FM and Husain A** (1986) Identification of angiotensin II receptors in the rat ovary *European Journal of Pharmacology* **130** 351–352
- Tian Y, Baukal AJ, Sandberg K, Bernstein KE, Balla T and Catt KJ** (1996) Properties of AT_{1a} and AT_{1b} angiotensin receptors expressed in adrenocortical Y-1 cells *American Journal of Physiology* **270** E831–E839
- Timmermans PWM, Wong PC, Chiu AT et al.** (1993) Angiotensin II receptors and angiotensin II receptor antagonists *Pharmacological Reviews* **45** 205–251
- Whitebread S, Meme M, Kamber B and de Gasparo M** (1989) Preliminary biochemical characterisation of two angiotensin II receptor subtypes *Biochemical and Biophysical Research Communications* **163** 284–291
- Yamoto M, Shima K and Nakono R** (1992) Gonadotrophin receptors in human ovarian follicles and corpora lutea throughout the menstrual cycle *Hormone Research* **37** 5–11
- Yang Y, Macdonald GJ and Duggan KA** (1994) Differential regulation of uterine and glomerular angiotensin II receptors in normal and hypertensive pregnancy in the rat *Clinical and Experimental Pharmacology and Physiology* **21** 253–256
- Yiu AKL, Wong PF, Yeung SY, Lam SM, Luk SKS and Cheung WT** (1997) Immunohistochemical localization of type-II (AT₂) angiotensin receptors

with a polyclonal antibody against a peptide from the C-terminal tail
Regulatory Peptides **70** 15–21

Yonekura Y, Brill AB, Som P, Bennett GW and Fand I (1983) Quantitative autoradiography with radiopharmaceuticals, part 1: digital film-analysis system by videodensitometry: concise communication *Journal of Nuclear Medicine* **24** 231–237

Yoshimura Y, Karube M, Koyama N, Shiokawa S, Nanno T and Nakamura Y (1992) Angiotensin II directly induces follicle rupture and oocyte maturation in the rabbit *FEBS Letters* **307** 305–308

Zilles K, Schleicher A, Rath M and Bauer A (1988) Quantitative receptor

autoradiography in the human brain: methodical aspects *Biochemistry* **90** 129–137

Zilles K, zur Nieden K, Schleicher A and Traber J (1990) A new method for quenching correction leads to revisions of data in receptor autoradiography *Histochemistry* **94** 569–578

Resubmitted manuscript received 26 February 2001.

First decision 3 May 2001.

Accepted 3 July 2001.