Dominance of type 1 angiotensin II receptor in the nonpregnant and pregnant bovine uterus

K. H. Schauser, A. H. Nielsen, H. Winther, V. Dantzer and K. Poulsen

Department of Anatomy and Physiology, The Royal Veterinary and Agricultural University, 7 Grønnegaardsvej, DK-1870 Frederiksberg C, Denmark

The present study was undertaken to characterize, determine and localize angiotensin II receptors in the nonpregnant and pregnant bovine uterus. In addition, the concentration of active renin, which is responsible for the generation of angiotensin, was determined. Autoradiography and angiotensin II receptor binding studies showed that all compartments of the bovine uterus contained high concentrations of angiotensin II receptors. In general, the type 1 angiotensin II receptor (AT₁) predominated over the AT₂ receptor. In the endometrium, the highest density was found in the caruncles and the AT₁ receptor was always predominant. The density of angiotensin II receptors in the endometrium increased at the beginning of pregnancy, but decreased and reached values similar to those in nonpregnant animals near term. In the myometrium, the density of angiotensin II receptors was highest at or near the endometrial–myometrial junction. In this area, the predominant type of angiotensin II receptor in the uterus of cyclic cows varied, whereas the AT₁ receptor always predominated during pregnancy. Non-AT₁ and non-AT₂ binding sites were found in the same locations as the angiotensin II receptors, but at lower densities. With the exception of the pregnant endometrium, all compartments contained higher active renin concentrations than found in plasma, indicating local synthesis of renin. This study demonstrates a difference in the expression of types of angiotensin II receptor in the bovine uterus compared with other species. The high densities of angiotensin II receptors localized in several important areas imply that the renin–angiotensin system participates in regulation of growth and tissue function in the bovine uterus.

Introduction

A number of studies have indicated the presence of a local tissue renin–angiotensin system (RAS) in the female reproductive tract (Hagemann *et al.*, 1994; Nielsen *et al.*, 1995; Vinson *et al.*, 1997). Angiotensin II synthesized locally in the tissues may be involved in the regulation of several aspects of tissue function through autocrine or paracrine actions, such as modulation of cell growth and neovascularization (Fernandez *et al.*, 1985; Le Noble *et al.*, 1993; Molloy *et al.*, 1993; Stoll *et al.*, 1995a,b; Meffert *et al.*, 1996; Steckelings *et al.*, 1996). The effect of angiotensin II is mediated by angiotensin II receptors, which comprise two main types, termed type 1 (AT₁) and type 2 (AT₂) receptors (Timmermans *et al.*, 1993), which can be distinguished by their different affinities for non-peptide antagonists (Chiu *et al.*, 1990; Dudley *et al.*, 1990). Both types of receptor, as well as non-AT₁ and non-AT₂ binding sites, have been demonstrated within the placenta of humans (Kalenga *et al.*, 1996; Li *et al.*, 1998) and cattle (Schauser *et al.*, 1998). Within the reproductive tract, the expression of the AT₁ and AT₂ receptors varies significantly

among species and there is evidence that it changes during the oestrous cycle and pregnancy (Nielsen *et al.*, 1995; Vinson *et al.*, 1997). In the uterus of women with normal cyclic menstruation, a cyclic change in the angiotensin II receptor densities is observed, indicating regulation by oestrogen and progesterones (Ahmed *et al.*, 1995; Mancina *et al.*, 1996). Uterine angiotensin II receptor densities decrease during pregnancy and in women taking oral contraceptives (de Gasparo *et al.*, 1994; Bing *et al.*, 1996; Mancina *et al.*, 1996; Matsumoto *et al.*, 1996). Furthermore, several studies have reported downregulation of angiotensin II receptors in the pig and ovine uterus during pregnancy (Cox *et al.*, 1993; Moeller *et al.*, 1996; Nielsen *et al.*, 1997).

Implantation fails in about 25% of bovine embryos (Smith *et al.*, 1982) and about 10% of this failure is due to maternal factors (Newman *et al.*, 1985). The uterine RAS may play a role in implantation and maintenance of pregnancy (Hagemann *et al.*, 1994; Vinson *et al.*, 1997). Therefore, as an extension of a recent study from this group on the expression of angiotensin II receptors in the bovine placenta (Schauser *et al.*, 1998), an investigation was undertaken into the RAS in the nonpregnant and pregnant bovine uterus. Since renin and the ubiquitous angiotensin-converting enzyme are the

Received 1 October 1998.

two enzymes that are important for the synthesis of the octapeptide angiotensin II, active renin concentrations were measured in all compartments. Furthermore, expression of the angiotensin II receptor was investigated using angiotensin II receptor binding studies and autoradiography.

Materials and Methods

Collection of tissues

Uteri from nonpregnant and pregnant cows were obtained at an abattoir 20–30 min after death. A total of 27 nonpregnant cows, which had apparently normal oestrous cycles, and 38 pregnant cows were studied. The stage of oestrous cycle of the nonpregnant animals was estimated by the gross appearance of the ovary (Ireland *et al.*, 1980; Grimes and Ireland, 1986) and the viscosity and amount of cervical and vaginal mucus. The stage of gestation was estimated by the fetal crown–rump length (Evans and Sack, 1973). A total of 10 nonpregnant animals and 25 animals at days 40–275 of pregnancy were used for the angiotensin II receptor binding studies. Tissue samples for angiotensin II receptor binding studies were transported to the laboratory on ice (1–2 h) and stored at -20°C . Tissue samples for autoradiography were collected from nonpregnant ($n = 17$) and pregnant animals ($n = 13$) at different days of gestation (range 50–252 days). The nonpregnant animals were grouped in oestrous period (day 1; five animals), post-oestrous period (days 2–4; four animals), dioestrous period (days 5–17; four animals) and pro-oestrous period (days 18–20; four animals). The tissue samples were snap-frozen in liquid nitrogen, wrapped in parafilm (American Can Company, Greenwich, CT) to prevent dehydration, transported on dry ice and stored at -80°C .

Angiotensin II receptor binding studies

Cell membrane fractions for angiotensin II receptor binding studies were prepared by homogenization of 1–2 g tissue in 10 ml of 50 mmol Tris-HCl l^{-1} (pH 7.4) containing 10 mmol EDTA l^{-1} (Merck, Darmstadt) and 1 mmol phenylmethylsulfonyl fluoride (PMSF) l^{-1} (Sigma, St Louis, MO). The homogenization was performed at 0°C by using the dispersing instrument, Ultra Turrax T8 (IKA Labortechnik, Staufen) at setting 3 for 30 s, and a Potter-Elvehjem homogenizer (B. Braun, Melsungen) for 3 min at 250 r.p.m. The supernatant, obtained by centrifugation of the homogenate at 600 g for 10 min at 4°C , was centrifuged at 45 000 g for 25 min at 4°C . The pellet was suspended in 10 mmol sodium phosphate l^{-1} (pH 7.4) containing 120 mmol sodium chloride l^{-1} , 1 mmol EDTA l^{-1} and 1 mmol PMSF l^{-1} , centrifuged at 45 000 g for 25 min at 4°C and suspended in the same buffer with 5 mmol MgCl_2 l^{-1} . These cell membrane fractions were frozen at -20°C for later analysis.

The angiotensin II receptor assay was performed in duplicate, and incubation was conducted for 1 h at 37°C in a final volume of 150 μl . Twenty-five microlitres of [^{125}I][Sar 1 -Ile 5 -Ile 8]angiotensin II (specific activity 2200 Ci mmol^{-1} ; Du Pont, NEN Research Products, Wilmington, DE) in 10 mmol sodium phosphate l^{-1} (pH 7.4) containing 120 mmol sodium

chloride l^{-1} , 1 mmol EDTA l^{-1} , 1 mmol PMSF l^{-1} , 5 mmol MgCl_2 l^{-1} , 0.5 g soybean trypsin inhibitor type I-S l^{-1} (Sigma), 0.6 g bacitracin l^{-1} (Sigma) and 2 g human serum albumin l^{-1} (Statens Seruminstitut, Copenhagen) was added to 100 μl of the suspended cell membrane fraction. The final concentrations of [^{125}I][Sar 1 -Ile 5 -Ile 8]angiotensin II was 18–52 pmol l^{-1} . For characterization of the type of receptor, 25 μl of various concentrations of losartan (DuP 753; DuPont Merck Pharmaceutical Company, Wilmington, DE) or PD 123319 ((S)-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid, ditrifluoroacetate, monohydrate; Parke-Davis, Ann Arbor, MI) in 0.2 mol Tris-HCl l^{-1} (pH 7.5) containing 2 g human serum albumin l^{-1} were added. The resulting displacement curves were used to determine the relative amounts of AT $_1$ or AT $_2$ receptors. For determination of the density of angiotensin II binding sites and their binding characteristics for [Sar 1 -Ile 5 -Ile 8]angiotensin II, 25 μl of various concentrations (0.1–10 nmol l^{-1}) of unlabelled [Sar 1 -Ile 5 -Ile 8]angiotensin II (Sigma) were added to the mixture of [^{125}I][Sar 1 -Ile 5 -Ile 8]angiotensin II and cell membranes. After incubation, 3 ml ice-cold 10 mmol sodium phosphate l^{-1} (pH 7.4) containing 120 μmol sodium chloride l^{-1} was added and the mixture was immediately filtered through Whatman GF/F glass fibre filters (Whatman, Clifton, NJ) that were presoaked with 2 g human serum albumin l^{-1} . The filters were washed twice with 3 ml of the same buffer and counted in a gamma counter. The specific binding was calculated by subtracting the nonspecific binding, measured in the presence of 1 μmol unlabelled [Sar 1 -Ile 5 -Ile 8]angiotensin II l^{-1} , from the total binding. The dissociation constant (K_d) and the maximal binding capacity of ligand (B_{max}) were determined by Scatchard analysis of data obtained with unlabelled [Sar 1 -Ile 5 -Ile 8]angiotensin II, using the RADLIG program for the analysis of radio ligand binding experiments (Version 4, Biosoft, Cambridge). The binding data were best fitted with a one-site fit. The density of angiotensin II receptors was calculated using the B_{max} value and the protein concentration, assuming the binding of one molecule of ligand to each receptor.

Measurement of protein concentration

The protein concentration in cell membrane fractions was determined by the method of Lowry *et al.* (1951).

Measurement and identification of renin

For measurement of renin concentrations, 100 mg tissue was homogenized in 0.5 ml of 10 mmol sodium phosphate l^{-1} (pH 7.5) containing 140 mmol sodium chloride l^{-1} , 10 mmol EDTA l^{-1} , 10 mmol *N*-ethylmaleimide l^{-1} (Sigma), 2 mmol 8-hydroxyquinoline l^{-1} (Merck) and 0.3 mmol sodium azide l^{-1} . Homogenization was performed as described above. The homogenate was centrifuged at 1850 g for 15 min at 4°C . The supernatant was stored at -20°C before analysis.

Renin concentrations were measured using the antibody trapping method (Poulsen and Jørgensen, 1974). This

Fig. 1. (a) Representative experiments showing the specific binding of ligand with increasing concentrations of [Sar¹-Ile⁵-Ile⁸]angiotensin II in cell membrane fractions prepared from the bovine endometrium (●) and myometrium (▲). (b) Scatchard analysis of the same data. In these experiments, the K_d and B_{max} values were 1.20 nmol l⁻¹ and 278 pmol l⁻¹, respectively, in the endometrium and 1.20 nmol l⁻¹ and 546 pmol l⁻¹, respectively, in the myometrium.

method is described in more detail for the measurement of renin in bovine plasma and tissues (Nielsen *et al.*, 1991, 1994; Hagemann *et al.*, 1993). The assay was standardized against a hog renin standard (MRC, Holly Hill, London) and the renin concentrations are given as Goldblatt units (GU) per litre. Renin was identified by inhibition of its enzymic activity with 1 µmol l⁻¹ of the specific renin inhibitor, remikiren (RO 42-5892, Hoffman-LaRoche, Basel).

Autoradiography

For *in situ* autoradiographic studies, a modified technique of Mendelsohn *et al.* (1987) was used. The samples were serial sectioned (10 µm) in a cryostat at -16°C and thaw-mounted on Polysine microscope slides (Menzel-Gläser, Braunschweig). The tissue sections were dried overnight at -2°C and used immediately or stored at -20°C for later use. Before the assay, the sections were brought to room temperature and preincubated for 15 min at room temperature in a buffer containing 10 mmol sodium phosphate l⁻¹ (pH 7.4), 120 mmol sodium chloride l⁻¹, 5 mmol MgCl₂ l⁻¹, 1 mmol EGTA l⁻¹ (Sigma), 0.3 mmol bacitracin l⁻¹ (Sigma) and 2 g proteinase-free bovine serum albumin l⁻¹ (Biofac A/S, Copenhagen). The sections were then incubated in a humidified chamber for 180 min in a fresh identical buffer containing 0.5 nmol [¹²⁵I]-[Sar¹-Ile⁵-Ile⁸]angiotensin II l⁻¹, a concentration close to the K_d value. Nonspecific binding was determined in serial sections using identical incubation conditions except for the addition of 1 µmol l⁻¹ of unlabelled

[Sar¹-Ile⁵-Ile⁸]angiotensin II. For localization and characterization of the angiotensin II receptor types, serial sections were incubated in the same buffer containing 0.5 nmol [¹²⁵I]-[Sar¹-Ile⁵-Ile⁸]angiotensin II l⁻¹ in the presence of 5 µmol losartan l⁻¹, in the presence of 5 µmol PD 123319 l⁻¹, or in the presence of both. After incubation, the sections were rinsed 4 × 3 min in ice-cold 50 mmol Tris-HCl l⁻¹ (pH 7.4) and then dipped in ice-cold distilled water. A stream of warm air (60°C) was used to dry the sections quickly. The sections were fixed in paraformaldehyde vapours for 2 h at 80°C and allowed to evaporate over night. Thereafter, they were dipped in photographic emulsion Ilford K2 (Ilford, Brønshøj) diluted 1:2 in distilled water and exposed for 3 days at 4°C. The developing process with Kodak D-19 developer (Kodak, Farum, Denmark) was stopped with Liquid Acid Hardener (Ilford). The slides were fixed in Rapid Fixer (Ilford) and washed for 1 h in rinsing water. After drying, the sections were weakly counterstained with haematoxylin and eosin to assist the localization of the silver grains. The angiotensin II receptor density was estimated by a visual subjective scoring of the silver grain deposition in the autoradiograms. The scoring was 0 (background), +, ++ and +++. Photographs were taken by using a Zeiss stereomicroscope with dark-field.

Statistical analysis

The data were analysed by non-parametric statistics (Conover, 1980), using the Stat-100 statistical analysis

Fig. 2. Representative curves showing the displacement of the specific binding of [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II by unlabeled [Sar¹-Ile⁵-Ile⁸]angiotensin II (■), PD 123319 (▲) and losartan (●) in a cell membrane fraction prepared from the myometrium of a cow at day 55 of pregnancy.

package (Version 1.24, Biosoft, Cambridge). Values are given as mean with the range in parentheses. $P < 0.05$ was considered significant.

Results

Characterization of angiotensin II receptors

Specific binding of [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II was found in all cell membrane fractions prepared from the bovine endometrium and myometrium. The nonspecific binding was low and unchanged during incubation at 37°C for 5 h ($n = 3$). Equilibrium at 37°C was reached after 30–60 min ($n = 3$). The specific binding of ligand approached saturation in experiments with [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II and increasing concentrations of unlabelled [Sar¹-Ile⁵-Ile⁸]angiotensin II (Fig. 1a). The dissociation constant (K_d) determined by Scatchard plots (Fig. 1b) was 1.08 nmol l⁻¹ (0.35–1.81 nmol l⁻¹; $n = 35$) in the endometrium and 0.69 nmol l⁻¹ (0.27–1.38 nmol l⁻¹; $n = 35$) in the myometrium.

Curves, obtained by displacement of the specific [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II binding with the type-specific non-peptide angiotensin II receptor antagonists, losartan (DuP 753) and PD 123319, are shown (Fig. 2). At concentrations below 10^{-6} – 10^{-5} mol l⁻¹, the antagonists displace the binding type-specific (Chiu *et al.*, 1990; Dudley *et al.*, 1990), which allows for an estimation of the relative distribution of the two receptor types. Representative curves for the myometrium of a pregnant cow are shown (Fig. 2), together with the improving displacement curve with unlabelled [Sar¹-Ile⁵-Ile⁸]angiotensin II. It was estimated that about 90% of the total angiotensin II receptors were AT₁ receptors in this

Table 1. Percentage of type 1 angiotensin II receptors of the total angiotensin II receptors in the bovine uterus

	Nonpregnant cows	Pregnant cows
Endometrium	85% (70–95%, $n = 7$)	95% (80–100%, $n = 9$)
Myometrium	65% (20–95%, $n = 8$)	90% (70–95%, $n = 9$)

preparation. The percentage of AT₁ receptors in the bovine uterus is shown (Table 1). There was no significant difference in the relative distribution of the angiotensin II receptor types between the endometrium and myometrium and between nonpregnant and pregnant animals (Table 1). No non-AT₁-non-AT₂ binding sites were detected in any of the angiotensin II receptor displacement experiments.

Angiotensin II receptor densities

In the endometrium (Fig. 3), the angiotensin II receptor density was higher ($P < 0.01$) in the pregnant animals (955 fmol mg⁻¹, range 115–2440 fmol mg⁻¹, $n = 25$) than in the nonpregnant animals (375 fmol mg⁻¹, range 185–640 fmol mg⁻¹, $n = 10$). There was a negative correlation between the duration of pregnancy and the receptor density (Spearman's $\rho = -0.625$, $P < 0.001$). In the myometrium (Fig. 4), the angiotensin II receptor density did not differ between nonpregnant (445 fmol mg⁻¹, range 140–805 fmol mg⁻¹, $n = 10$) and pregnant animals (495 fmol mg⁻¹, range 125–815 fmol mg⁻¹, $n = 25$). There was a negative correlation between the duration of pregnancy and the receptor density (Spearman's $\rho = -0.426$, $P < 0.05$). The angiotensin II receptor densities in the endometrium and myometrium (Figs 3 and 4) did not differ in the nonpregnant animals, whereas the densities were higher in the endometrium during pregnancy ($P < 0.002$).

Renin concentrations

The renin concentrations (Table 2) were higher in the myometrium than in the endometrium in both nonpregnant ($P < 0.05$) and pregnant animals ($P < 0.001$). In the myometrium, the renin concentration did not differ between nonpregnant and pregnant animals, whereas it was lower ($P < 0.025$) in the endometrium during pregnancy. No relationship was found between the duration of pregnancy and the renin concentrations in either the endometrium or the myometrium.

Autoradiography in nonpregnant cows

The labelling in the functional zone of the endometrial lamina propria ($n = 17$) was most dense in the caruncles and extended into the intercaruncular region, where it was concentrated in the subepithelial part (Figs 5 and 6). The AT₁ receptor was predominant. There were fewer AT₂ receptors and non-AT₁-non-AT₂ binding sites. The uterine epithelium showed no labelling. The basal zone revealed a moderate number of AT₁ receptors and AT₂ receptors, and few non-

Fig. 3. Angiotensin II receptor densities in the bovine endometrium (fmol per mg cell membrane protein).

Fig. 4. Angiotensin II receptor densities in the bovine myometrium (fmol per mg cell membrane protein).

AT₁-non-AT₂ binding sites. In the glands and their surrounding stroma, there was a large variability with respect to binding density and receptor type expression. However, binding was always present in this area and, in general, the AT₁ receptor predominated. The binding density and receptor type expression did not correlate with the stage of the oestrous cycle.

In the myometrium ($n = 17$), a very characteristic finding was the high expression of angiotensin II receptors towards the border of the endometrium (Figs 5 and 6). Here, the main receptor type could differ from that in the rest of the myometrium. Eight animals (four post-oestrous, one pro-oestrous, three oestrous) had mainly AT₂ receptors (Figs 5b and 6b), while five animals (two dioestrous, two pro-oestrous, one oestrous) showed mainly AT₁ receptor binding in this area (Fig. 6d). In the external part of the myometrium, the predominant receptor type also varied. In four animals (one post-oestrous, one dioestrous, one pro-oestrous, one oestrous), the AT₁ receptor was the main type (Fig. 6 d-f). The AT₂ receptor predominated in three animals (two pro-oestrous, one oestrous). Most non-AT₁-non-AT₂ binding sites were observed in the endometrial-myometrial junction (Figs 5c and 6c,f).

Binding was always present in the arteries of the

Table 2. Renin concentrations in the bovine uterus given in Goldblatt units (GU) kg⁻¹ wet tissue mass

	Nonpregnant cows	Pregnant cows
Endometrium	0.28 (0.020–0.82, $n = 9$) bd, $n = 1$	0.043 (0.014–0.146, $n = 22$) bd, $n = 3$
Myometrium	0.67 (0.040–2.69, $n = 10$)	0.53 (0.063–2.14, $n = 25$)

n : number of animals; bd, below the detection limit of the renin assay (0.014 GU kg⁻¹)

endometrium and the myometrium, but the angiotensin II receptor expression varied extensively, and no cyclic scheme was apparent. In general, the AT₁ receptor predominated (Fig. 6) but, in two cows, the AT₂ receptor predominated and was located in the periphery of the tunica media (data not shown). In the stroma surrounding arteries, the labelling density varied greatly, with the AT₁ receptor most prominently expressed.

Autoradiography in pregnant cows

The main receptor type in the uterus of the pregnant animals ($n = 13$) was the AT₁ receptor. In the endometrium, the angiotensin II receptor binding was densest in the functional zone of the caruncular stalk (Figs 7 and 8). In the caruncles, the binding followed the walls of the main crypts (Fig. 8). The binding extended into the functional zone in the intercaruncular region (Fig. 7). A moderate number of AT₂ receptors and few non-AT₁-non-AT₂ binding sites were observed in the same structures (Fig. 7). The angiotensin II receptor expression in the myometrium was similar to that in the nonpregnant cow, although the binding appeared less dense (Figs 7 and 8). In the myometrium of pregnant animals, the AT₁ receptor always dominated in the endometrial-myometrial junction. In general, the arteries and the surrounding stroma showed little labelling, with the AT₁ receptor as the main type (Fig. 7) but, in one cow, the AT₂ receptors predominated (Fig. 8).

Discussion

In the bovine endometrium, the AT₁ receptor predominated in nonpregnant animals. This is in agreement with findings in women, sows and the ewes (Ahmed *et al.*, 1995; Moeller *et al.*, 1996; Nielsen *et al.*, 1997). The angiotensin II receptor density was increased at the beginning of the pregnancy, and reached the same density at term as in nonpregnancy. This finding differs from the findings in the pig endometrium, where the predominant AT₁ receptor was downregulated throughout pregnancy (Nielsen *et al.*, 1997). Most of the angiotensin II receptors were localized in the caruncles. In pregnancy, the caruncles are the maternal site of attachment of the placenta. The caruncles grow fast with intense angiogenesis and develop an intimate contact with the

Fig. 5. Autoradiograms showing the distribution of [125 I][Sar¹-Ile³-Ile⁶]angiotensin II binding in consecutive uterine sections of a cow in dioestrus. (a) Dark-field micrograph of a section incubated in the presence of PD 123319, showing AT₁ receptor binding. (b) Dark-field micrograph of section incubated in the presence of losartan, showing AT₂ receptor binding. (c) Dark-field micrograph of section incubated in the presence of both PD 123319 and losartan, showing non-AT₁-non-AT₂ binding sites. (d) Dark-field micrograph of section showing total binding. (e) Dark-field micrograph of section incubated in the presence of 1 μ mol unlabelled [Sar¹-Ile³-Ile⁶]angiotensin II [125 I]. (f) Bright field micrograph of a section stained with haematoxylin and eosin. Arrow, luminal epithelium lining the lamina propria of the endometrium; open arrowhead, pointing from the intercaruncular region towards the caruncle; filled arrowhead, border between endometrium and myometrium; asterisks, arteries; E, endometrium; M, myometrium; Scale bar represents 1.6 mm.

cotyledons of the fetal membranes. In the intercaruncular region, the most intense receptor binding was found in the functional zone of the lamina propria just beneath the luminal epithelium. No angiotensin II receptors were observed in the luminal epithelium. In contrast, the lining epithelium of the uterine glands expressed all receptor types in varying proportions, although, in general, the AT₁ receptor

predominated. In the adjacent stroma, the AT₁ receptor was a constant finding. No cyclic variation was detected.

In the bovine myometrium, the angiotensin II receptor density was highest at or near the endometrial–myometrial junction. Autoradiography in nonpregnant animals showed a large variation of angiotensin II receptor type expression in this area. The percentage of the AT₁ receptor varied from 20

Fig. 6. Autoradiograms showing the distribution of [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II binding in consecutive uterine sections of two cows in oestrus, showing variability in angiotensin II receptor types. (a–c) Cow 1: (a) Dark-field micrograph of section incubated in the presence of PD 123319 showing AT₁ receptor binding. (b) Dark-field micrograph of section incubated in the presence of losartan, showing AT₂ receptor binding. (c) Dark-field micrograph of section incubated in the presence of both PD 123319 and losartan showing non-AT₁-non-AT₂ binding sites. (d–f) Cow 2: (d) Dark-field micrograph of section incubated in the presence of PD 123319 showing AT₁ receptor binding. (e) Dark-field micrograph of section incubated in the presence of losartan showing AT₂ receptor binding. (f) Dark-field micrograph of section incubated in the presence of PD 123319 and of losartan showing non-AT₁-non-AT₂ binding sites. Arrow, uterine glands; open arrowhead, caruncle; filled arrowhead, border between endometrium and myometrium; asterisk, arteries in myometrium; E, endometrium; M, myometrium. Scale bars represent 1.2 mm.

Fig. 7. Autoradiograms showing the distribution of [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II binding in consecutive sections from a bovine uterus with fetal membranes (day 86) incubated with different antagonists. (a) Dark-field micrograph of section incubated in the presence of PD 123319 showing AT₁ receptor binding. (b) Dark-field micrograph of section incubated in the presence of losartan, showing AT₂ receptor binding. (c) Dark-field micrograph of section incubated in the presence of both PD 123319 and losartan, showing non-AT₁-non-AT₂ binding sites. (d) Dark-field micrograph of section showing total binding. (e) Dark-field micrograph of section incubated in the presence of 1 μ mol unlabelled [Sar¹-Ile⁵-Ile⁸]angiotensin II. (f) Bright field micrograph of a section stained with haematoxylin and eosin. Arrow, uterine glands; open arrowhead, pointing at caruncular stalk (placentome) to the right; filled arrowhead, border between endometrium and myometrium; asterisk, myometrial arteries; F, fetal membrane; E, endometrium; M, myometrium. Scale bar represents 960 μ m.

Fig. 8. Autoradiograms showing the distribution of [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II binding in consecutive sections of a bovine uterus wall, with the maternal side of the placental at the top (day 204). (a) Dark-field micrograph of section incubated in the presence of PD 123319, showing AT₁ receptor binding. (b) Dark-field micrograph of section incubated in the presence of losartan, showing AT₂ receptor binding. Arrow, wall of main crypt; filled arrowhead, border of myometrium towards endometrium; asterisk, arteries; E, endometrium at the base of a placental; M, myometrium. Scale bar represents 1.2 mm.

to 95% in the myometrium of these animals in the angiotensin II receptor binding studies. These findings indicate changes in the angiotensin II receptor expression during the oestrous cycle. However, the expression of the angiotensin II receptors observed during autoradiography also varied markedly among animals within the same stage of the cycle.

Throughout bovine pregnancy, the AT₁ receptor type predominated. There were no significant differences in the receptor density between the nonpregnant and pregnant bovine myometrium. This finding differs markedly from the findings in other species, where the angiotensin II receptor density is downregulated during pregnancy. In sheep and pigs, a downregulation of the predominant AT₂ receptor was observed in the myometrium during pregnancy (Moeller *et al.*, 1996; Nielsen *et al.*, 1997). In the human myometrium, Matsumoto *et al.* (1996) found a decrease in the predominant AT₂ receptor mRNA expression during pregnancy but no changes in AT₁ receptor mRNA expression. In human myometrium and its blood vessels, the predominant AT₂ receptor was downregulated already during the secretory phase (Ahmed *et al.*, 1995; Bing *et al.*, 1996), indicating a preparation for pregnancy.

All autoradiograms, in which uterine sections were incubated with high concentrations of both PD 123319 and losartan, showed [125 I][Sar¹-Ile⁵-Ile⁸]angiotensin II binding, indicating the presence of non-AT₁-non-AT₂ binding sites. These sites were located in the same compartments as the AT₁ and AT₂ receptors but at much lower densities. Such

novel non-AT₁-non-AT₂ binding sites have been observed in the human endometrium (Ahmed *et al.*, 1995) and placenta (Li *et al.*, 1998), and in the bovine placenta and fetal membranes (Schauser *et al.*, 1998). The nature of these binding sites is still unknown.

The active renin concentrations in the nonpregnant and pregnant myometrium did not differ. In the nonpregnant endometrium, the active renin concentrations were lower than in the myometrium and decreased further during pregnancy. This finding is in contrast to findings in pigs, in which endometrial renin concentrations remained unchanged (Hagemann *et al.*, 1993). In humans and all other animals investigated, renin increased in the uterus during pregnancy (Hagemann *et al.*, 1994). Except for the pregnant bovine endometrium, the active renin concentrations were higher than those found earlier in bovine plasma (Nielsen *et al.*, 1991). This finding indicates local synthesis of active renin, in agreement with a previous study in the human endometrium (Johnson, 1980).

In contrast to the abundant expression of the AT₂ receptor type during fetal development, AT₂ receptor is expressed in few tissues in adult life. The ovary and the uterus express the AT₂ receptor extensively in adults (Ahmed *et al.*, 1995; Nielsen *et al.*, 1995, 1997) indicating an important role in reproduction. In the bovine placenta, the angiotensin II receptors were predominantly AT₂ in the fetal and AT₁ in the maternal compartment (Schauser *et al.*, 1998). The distribution of the angiotensin II receptors did not change throughout gestation. The high expression of angiotensin II receptors in the bovine placenta and fetal membranes indicates an effect on regulatory as well as growth processes in these tissues (Schauser *et al.*, 1998). In the bovine uterus, the two angiotensin II receptor types were located in the same compartments but in variable proportions. A growth-promoting effect of angiotensin II has been shown to be mediated by the AT₁ receptor (Stoll *et al.*, 1995a). In endothelial cells, this effect was counteracted by the antiproliferative actions of the AT₂ receptor (Stoll *et al.*, 1995a,b; Meffert *et al.*, 1996). In a culture of human keratinocytes, angiotensin II stimulated proliferation via non-AT₁-non-AT₂ binding sites (Steckelings *et al.*, 1996). These findings indicate that angiotensin II plays a role as a growth factor. Cross-talk between the AT₁ and AT₂ receptors, and perhaps even between the non-AT₁-non-AT₂ angiotensin II binding sites, may act as a modulator of the effect of angiotensin II (Gelband *et al.*, 1997; Li *et al.*, 1998).

Angiotensin II is a potent systemic vasoconstrictor, and its effect is exerted via the AT₁ receptor but is less pronounced in human and ovine uterine arteries (Maigaard *et al.*, 1986). It is likely that angiotensin II also modulates and regulates vascular resistance and blood flow in the bovine uterus, since AT₁ receptors were found in the tunica media of the arteries. Although the expression often appeared very weak, a marked effect is possible. The AT₂ receptor dominated in the human uterine arteries and was downregulated during pregnancy (Cox *et al.*, 1996). The AT₂ receptor was predominant in the arteries of only 3 of 30 bovine uteri.

The AT₁ receptor is known to mediate contraction of the myometrium (Maigaard *et al.*, 1986; Odum and Broughton, 1988). Nonpregnant women did not respond to angiotensin

II, whereas there was a response in pregnant and labouring women (Cox *et al.*, 1996).

Angiotensin II was shown to increase vascular permeability (Robertson and Khairallah, 1972) and to possess angiogenic properties (Fernandez *et al.*, 1985, Le Noble *et al.*, 1993). The high densities of angiotensin II receptors in the caruncles, especially in the beginning of the pregnancy, indicate that angiotensin II plays a role in implantation, the subsequent vascular development and in the maintenance of pregnancy.

In conclusion, all parts of the bovine uterus contained high densities of angiotensin II receptors. In general, the AT₁ receptor predominated in all compartments. In the endometrium, the highest density was found in the caruncles. The endometrial angiotensin II receptor density was increased at the beginning of pregnancy but decreased and, near term, reached a density similar to that observed during nonpregnancy. In the myometrium, the angiotensin II receptor density was highest at or near the endometrial-myometrial junction. High active renin concentrations were found in all compartments but, in the endometrium, it was low during pregnancy. Further studies of the uterine compartments expressing angiotensin II receptors are needed to elucidate the role of angiotensin II in the uterus of the cyclic and pregnant cows.

The skilful technical assistance of S. Holm, C. T. Larsen and I. Thomsen is gratefully acknowledged. The renin inhibitor remikiren (RO 42-5892) was kindly provided by W. Fischli, Hoffman-LaRoche, Basel, Switzerland. The non-peptide type-specific angiotensin II receptor antagonists losartan (DuP 753) and PD 123319 were gifts from the Du Pont Merck Pharmaceutical Company, Wilmington, DE and Parke-Davis, Ann Arbor, MI, respectively.

References

- Ahmed A, Li XF, Shams M, Gregory J, Rollason T, Barnes NM and Newton JR (1995) Localization of the angiotensin II and its receptor subtype expression in human endometrium and identification of a novel high-affinity angiotensin II binding site *Journal of Clinical Investigation* **96** 848–857
- Bing C, Johnson IR and Pipkin FB (1996) Angiotensin receptors in myometrium and myometrial vessels from uteri of women during the follicular and luteal phases of the menstrual cycle and in late pregnancy *Clinical Science* **90** 499–505
- Chiu AT, McCall DE, Ardecky RJ, Duncia JV, Nguyen TT and Timmermans PB (1990) Angiotensin II receptor subtypes and their selective nonpeptide ligands. In *Receptor* pp 33–40 Ed. G Litwack. The Humana Press, Clifton
- Conover WJ (1980) *Practical Non-Parametric Statistics* 2nd edn. John Wiley, New York
- Cox BE, Ipson MA, Shaul PW, Kamm KE and Rosenfeld CR (1993) Myometrial angiotensin II receptor subtypes change during ovine pregnancy *Journal of Clinical Investigation* **92** 2240–2248
- 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
- de Gasparo M, Whitebread S, Kalenga MK, De Hertogh R, Crevoisier P and Thomas K (1994) Downregulation of the angiotensin II receptor subtype AT₂ in human myometrium during pregnancy *Regulatory Peptides* **53** 39–45
- Dudley DT, Panek RL, Major TC, Lu GH, Bruns RF, Klinkefus BA, Hodges JC and Weishaar RE (1990) Subclasses of angiotensin II binding sites and their functional significance *Molecular Pharmacology* **38** 370–377
- Evans HE and Sack WO (1973) Prenatal development of domestic and laboratory mammals: growth curves, external features and selected references *Zentralblatt für Veterinärmedizin, Reihe C* **2** 11–45
- Fernandez LA, Twickler J and Mead A (1985) Neovascularization produced by angiotensin II *Journal of Laboratory and Clinical Medicine* **105** 141–145
- Gelband CH, Zhu M, Lu D, Reagan LP, Fluharty SJ, Posner P, Raizada MK and Sumners C (1997) Functional interaction between neuronal AT₁ and AT₂ receptors *Endocrinology* **138** 2195–2198
- Grimes RW and Ireland JJ (1986) Relationship of macroscopic appearance of the surface of bovine ovarian follicles, concentrations of steroids in follicular fluid, and maturation of oocytes *in vitro*. *Biology of Reproduction* **35** 725–732
- Hagemann A, Dantzer V, Nielsen AH and Poulsen K (1993) Renin and prorenin in reproductive tissues during gestation in pigs and cattle *Clinical and Experimental Pharmacology and Physiology* **20** 41–50
- Hagemann A, Nielsen AH and Poulsen K (1994) The uteroplacental renin-angiotensin system: a review *Experimental and Clinical Endocrinology* **102** 252–261
- Ireland JJ, Murphee RL and Coulson PB (1980) Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum *Journal of Dairy Science* **63** 155–160
- Johnson IR (1980) Renin substrate, active and acid-activatable renin concentrations in human plasma and endometrium during the normal menstrual cycle *British Journal of Obstetrics and Gynaecology* **87** 875–882
- Kalenga MK, de Gasparo M, Thomas K and De Hertogh R (1996) Downregulation of angiotensin AT₁ receptor by progesterone in human placenta *Journal of Clinical Endocrinology and Metabolism* **81** 998–1002
- Le Noble FAC, Schreurs NHJS, Van Straaten HWM, Slaaf DW, Smits JFM, Rogg H and Struijker-Boudier HAJ (1993) Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane *American Journal of Physiology* **264** R460–R465
- Li X, Shams M, Zhu J, Khalig A, Wilkes M, Whittle M, Barnes N and Ahmed A (1998) Cellular localization of AT₁ receptor mRNA and protein in normal placenta and its reduced expression in intrauterine growth restriction *Journal of Clinical Investigation* **101** 442–454
- Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the folin phenol reagent *Journal of Biological Chemistry* **193** 265–275
- Maigaard S, Forman A, and Andersson KE (1986) Differential effects of angiotensin, vasopressin and oxytocin on various smooth muscle tissues within the human uteroplacental unit *Acta Physiologica Scandinavica* **128** 23–31
- Mancina R, Susini T, Renzetti A, Forti G, Razzoli E, Serio M and Maggi M (1996) Sex steroid modulation of AT₂ receptors in human myometrium *Journal of Clinical Endocrinology and Metabolism* **81** 1753–1757
- Matsumoto T, Sagawa N, Mukoyama M, Tanaka I, Itoh H, Goto M, Horiuchi M, Dzau VJ, Mori T and Nakao K (1996) Type 2 angiotensin II receptor is expressed in human myometrium and uterine leiomyoma and is downregulated during pregnancy *Journal of Clinical Endocrinology and Metabolism* **81** 4366–4372
- Meffert S, Stoll M, Steckelings UM, Bottari SP and Unger T (1996) The angiotensin II AT₂ receptor inhibits proliferation and promotes differentiation in PC12W cell *Molecular and Cellular Endocrinology* **122** 59–67
- Mendelsohn FA, Millan M, Quirion R, Aguilera G, Chou ST and Catt KJ (1987) Localization of angiotensin II receptors in rat and monkey kidney by *in vitro* autoradiography *Kidney International* **31** Supplement 20 S40–S44
- Moeller I, Chai SY, MacGregor DP, Aldred GP and Mendelsohn FA (1996) Localization and quantitation of angiotensin AT₁ and AT₂ receptors in the pregnant and non-pregnant sheep uterus *Regulatory Peptides* **61** 213–218
- Molloy CJ, Taylor DS and Weber H (1993) Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells *Journal of Biological Chemistry* **268** 7338–7345
- Newman S, Harris DL and Doolittle DP (1985) Lifetime parental productivity in twenty-seven crosses of mice II. Weaning traits reflecting reproduction and lactation *Journal of Animal Science* **61** 367–375
- Nielsen AH, Gotfredsen P, Nielsen PB, Hyttel P and Poulsen K (1991) Measurement of renin and prorenin in cattle, hog and horse *Comparative Biochemistry and Physiology A* **100** 127–131
- Nielsen AH, Hagemann A, Svenstrup B, Nielsen J and Poulsen K (1994) 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 and Poulsen K (1995) The tissue renin-angiotensin system in the female reproductive tissues: a review *Advances in Experimental Medicine and Biology* **377** 253–268
- Nielsen AH, Schauser K, Winther H, Dantzer V and Poulsen K (1997) Angiotensin II receptors and renin in the porcine uterus: myometrial AT₂ and endometrial AT₁ receptors are downregulated during gestation *Clinical and Experimental Pharmacology and Physiology* **24** 309–314
- Odum CU and Broughton PF (1988) Studies on the effects of nitrendipine on oxytocin-, angiotensin II- and ergometrine-induced contraction of pregnant

- human myometrium *in vitro*. *British Journal of Obstetrics and Gynaecology* **95** 765–770
- Poulsen K and Jørgensen J** (1974) An easy radioimmunological microassay of renin activity, concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody *Journal of Clinical Endocrinology and Metabolism* **39** 816–825
- Robertson AL and Khairallah PA** (1972) Effects of angiotensin II and some analogues on vascular permeability in the rabbit *Circulation Research* **31** 923–931
- Schauser KH, Nielsen AH, Winther H, Dantzer V and Poulsen K** (1998) Autoradiographic localization and characterization of angiotensin II receptors in the bovine placenta and fetal membranes *Biology of Reproduction* **59** 684–692
- Smith MF, Nix KJ, Kraemer DC, Amoss MS, Herron MA and Wiltbank JN** (1982) Fertilization rate and early embryonic loss in Brahman crossbred heifers *Journal of Animal Science* **54** 1005–1011
- Steckelings UM, Artuc M, Paul M, Stoll M and Henz BM** (1996) Angiotensin II stimulates proliferation of primary human keratinocytes via a non-AT₁, non-AT₂ angiotensin receptor *Biochemical and Biophysical Research Communications* **229** 329–333
- Stoll M, Meffert S, Stroth U and Unger T** (1995a) Growth or antigrowth: angiotensin and the endothelium *Journal of Hypertension* **13** 1529–1534
- Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R and Unger T** (1995b) The angiotensin AT₂-receptor mediates inhibition of cell proliferation in coronary endothelial cells *Journal of Clinical Investigation* **95** 651–657
- Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA and Smith RD** (1993) Angiotensin II receptors and angiotensin II receptor antagonists *Pharmacological Reviews* **45** 205–251
- Vinson GP, Saridogan E, Puddefoot JR and Djahanbakhch O** (1997) Tissue renin–angiotensin systems and reproduction: a review *Human Reproduction* **12** 651–662