Characterization of the epidermal growth factor receptor in pig oviduct and endometrium

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The aim of this study was to determine whether the stage of the oestrous cycle (day 1, $n = 5$; day 6, $n = 5$; day 12, $n = 3$ gilts) has an influence on the expression and activity of epidermal growth factor receptor (EGF-R) in the pig oviduct and uterus. Histochemistry, cross-linking of ¹²⁵I-labelled EGF to isolated oviductal and endometrial membranes or on cryostat sections, and EGF-R binding assay were used to demonstrate the presence of the EGF-R in a qualitative and quantitative manner. The bioactivity of the EGF-R in the oviduct was estimated by means of a protein tyrosine kinase activity assay. This study suggests that EGF-R is widely distributed both in glandular and stromal cells of the endometrium and in epithelial cells of the oviduct in pigs. The concentrations of EGF-R were higher in oviductal membranes on day 1 (22.4 ± 8.7 fmol mg^{-1} protein) in comparison with day 6 (11.0 ± 0.42 fmol mg^{-1} protein; $P < 0.05$), but not on day 12 (16.0 ± 2.9 fmol mg^{-1} protein). The concentrations dropped similarly in the endometrium (day 1: 66.8 ± 16.4 fmol mg^{-1} protein; day 6: 39.1 ± 3.4 fmol mg^{-1} protein ($P < 0.05$); day 12: 38.0 ± 14.6 fmol mg^{-1} protein). The dissociation constant (K_d) showed the same pattern. These data were supported by cross-linking of ¹²⁵I-labelled EGF to a 170 kDa membrane protein representing the EGF-R. In contrast to day 6 and 12 of the cycle, a significantly ($P < 0.05$) higher endogenous protein tyrosine kinase activity was observed on day 1. In summary, changes in concentrations and functional status of the EGF-R may play a significant role in the cascade of cellular events in oviductal and endometrial tissues.

Introduction

The oviduct and uterus play an important role in sustaining the development of embryos. Their major function is to provide an essential or facilitative environment for the establishment of pregnancy, including the transport of ovum and spermatozoa, capacitation of spermatozoa, fertilization, and early cleavage stage embryonic development.

There is a complex series of interactions between the maternal oviduct, the uterus and the developing preimplantation conceptus. Studies have found that the oviduct or oviductal secretion contribute materials that are essential for the development of the zygotes to the blastocyst stage (Archibong *et al.*, 1989; White *et al.*, 1989). Many of these materials appear to be regulated by ovarian steroids, most importantly, oestrogens.

The expression of several growth factors and their receptors in the preimplantation embryo (Paria *et al.*, 1991, 1994; Vaughan *et al.*, 1992a; Zhang *et al.*, 1992; Fischer, 1996; Watson *et al.*, 1996a) and maternal reproductive tract (Brigstock *et al.*, 1989; Simmen *et al.*, 1989; Buhi, 1996) suggest

that growth factors are available to influence embryonic development in an autocrine–paracrine manner.

Epidermal growth factor (EGF) and its receptor (EGF-R) have been found in mouse (Huet-Hudson *et al.*, 1990) and rat uterine tissue (Lin *et al.*, 1988), in pig ovaries and follicular fluid (Vaughan *et al.*, 1992b), in pig oviducts and endometria (Kennedy *et al.*, 1994; Swanchara *et al.*, 1995) and in oviductal epithelial cell cultures (Lee, 1993).

The specific biological function of the EGF receptor and its ligands in the female reproductive tract is less certain. There is increasing evidence that EGF or transforming growth factor α (TGF α), or both factors, mediate the effects of steroid hormones on the tract (Watson *et al.*, 1996b). In rats (Mukku and Stancel, 1985) and mice, oestrogen upregulates the EGF receptor expression rapidly, resulting in the initiation of blastocyst activation (Paria *et al.*, 1993). Test results show that EGF or TGF α stimulate the growth of mouse preimplantation embryos and zona hatching *in vitro* (Paria and Dey, 1990). EGF increases the proliferation of human endometrium (Haining *et al.*, 1991) and stimulates the growth and DNA synthesis of oviductal cells (Tiemann and Hansen, 1995) and of uterine cells (Tiemann *et al.*, 1994).

EGF and TGF α are structurally related, and their cellular effects are apparently mediated by binding to the EGF-R (Todaro *et al.*, 1980). An early molecular event mediated by EGF-TGF α after binding to the EGF receptor is the stimulation of subcellular protein tyrosine phosphorylation, including rapid receptor autophosphorylation (Carpenter *et al.*, 1979; Hunter and Cooper, 1981).

These findings suggest that EGF-related growth factors and changes in the functional status of EGF-R play an important role in the regulation of oviductal and uterine functions and in the stimulation of embryonic development.

In rodents and humans, there is evidence for the regulation of expression both of the EGF-R and of EGF itself in the female reproductive tract by oestrogens (Mukku and Stancel, 1985; DiAugustini *et al.*, 1988; Huet-Hudson *et al.*, 1990; Troche *et al.*, 1991). In pigs, the expression of mRNA encoding EGF-R and EGF has been shown on all days of the cycle examined using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Kennedy *et al.*, 1994). Thus, if oestrogen regulates this expression, it is in a quantitative rather than a qualitative manner; consequently, procedures yielding more elaborate quantitative analyses than the RT-PCR analysis will be required.

The aims of the present study were to determine whether the stage of the oestrous cycle has an influence on the EGF-R concentration in pig oviduct and endometrium, and to determine whether there are changes in the bioactivity of the receptor protein depending on the day of the cycle.

Materials and Methods

Materials

Biotinylated mouse EGF, mouse EGF, human transforming growth factors α and β (TGF α and TGF β), human insulin-like growth factors I and II (IGF-I and IGF-II), basic fibroblast growth factor (bFGF), and Streptavidin-horseradish peroxidase were purchased from Boehringer (Mannheim). 125 I-labelled EGF (specific activity 100 μ Ci μ g $^{-1}$; mouse) and [γ - 32 P]ATP (specific activity 6000 Ci mmol $^{-1}$) were obtained from Amersham (Braunschweig). 3-3'-Diaminobenzidine Tablet sets, Sigma Fast[®] and all other reagents were acquired from Sigma GmbH (Deisenhofen).

Regumate[®] was obtained from Serum-Werk (Bernburg), Pregmagon[®] from Impfstoffwerk (Dessau) and Choriolutin[®] from Albrecht (Aulendorf).

Animals and preparation of oviductal and endometrial tissue

A total of 13 pubertal crossbred Landrace gilts, aged approximately 8.5 months, with a body mass of 120–125 kg were used. The oestrous cycle was synchronized by feeding altrenogest (20 mg Regumate[®] per animal per day) for 15 days. Gilts were injected with 1,000 i.u. PMSG (Pregmagon[®]) 24 h after the last Regumate[®] feeding to stimulate follicular growth. Ovulation was induced by the application of 500 i.u. hCG (Choriolutin[®]) 80 h after PMSG injection. The animals were killed on day 1 (day 1 = the second day after hCG application, $n = 5$), day 6 ($n = 5$) and day 12 ($n = 3$) of the cycle.

The reproductive tracts of the gilts were removed at the local abattoir and brought to the laboratory within 10 min after slaughter. The reproductive tracts were trimmed free of mesosalpinx. The uterus was cut open longitudinally and the endometrium was isolated. Oviduct and endometrium samples were stored at -80°C .

Histochemical determination of EGF receptor binding

Frozen tissue sections were obtained from shock-frozen tissue samples. EGF receptor binding using biotinylated EGF was performed on 5–8 μ m oviductal and on endometrial cryostat sections from gilts on day 1 ($n = 2$) and day 6 ($n = 2$) of the cycle. The cryostat slices were taken from the region of the ampullary-isthmus junction of the oviduct and from the middle region of the endometrium. The slices were fixed in acetone for 2 min at 4°C ; the tissue sections were washed three times with PBS and incubated with 30 μ l (10 μ g ml $^{-1}$) biotinylated EGF at 4°C under humidified conditions for 1 h. After being washed in PBS three times, the sections were incubated with 1.5% (v/v) hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity, and then washed again. The sections were subsequently incubated with 10 μ l (500 mU ml $^{-1}$) Streptavidin-horseradish peroxidase at room temperature for 30 min. The chromogenic reactions were carried out with 3-3'-diaminobenzidine. Control experiments included the incubation of sections in the absence of biotinylated EGF, and the addition of a 1000-fold excess of unlabelled EGF to displace the biotinylated EGF from the receptor.

Membrane preparation

The whole oviduct (including ampulla and isthmus, without infundibulum) and endometrium (1.5 g) were homogenized in 4 ml of homogenization buffer (20 mmol HEPES l $^{-1}$, 0.25 mol sucrose l $^{-1}$, 1 mmol EGTA l $^{-1}$, 1 mmol EDTA l $^{-1}$, 0.5 mmol PMSF l $^{-1}$, 1 μ g leupeptin ml $^{-1}$, 1 μ g pepstatin ml $^{-1}$; pH 7.4) using three bursts of 15 s duration in an Ultra-Turrax (IKA, Staufen). The homogenates were centrifuged at 800 g and 4°C for 10 min and the supernatants were collected. Supernatants were centrifuged at 100 000 g for 1 h. For cross-linking, the pellet was suspended in 1.5 ml 20 mmol HEPES l $^{-1}$, 0.14 mol NaCl l $^{-1}$ (pH 7.4). For the binding assay, the pellet was resuspended in 2 ml of binding assay buffer containing 25 mmol Tris-HCl l $^{-1}$, 5 mmol MgCl $_2$ l $^{-1}$, 0.1% (w/v) bovine serum albumin (BSA) and stored at -80°C until use. Protein concentration was measured as described by Lowry *et al.* (1951) using BSA as the standard.

125 I-labelled EGF binding assay

Oviductal and endometrial membranes (400 μ g protein) from cyclic gilts were incubated overnight with gentle shaking at 25°C in 500 μ l of binding reaction buffer (25 mmol Tris l $^{-1}$ (pH 7.2), 5 mmol MgCl $_2$ l $^{-1}$, 0.1% (w/v) BSA) containing 0.065–2.08 nmol 125 I-labelled EGF l $^{-1}$ (19 000–162 000 c.p.m.). Nonspecific binding was determined by

performing parallel binding reactions in the presence of a 100-fold molar excess of unlabelled EGF for 15–18 h.

After incubation, the reaction was stopped by adding 1000 μ l ice-cold binding reaction buffer, and the membranes were collected by centrifugation at 6000 *g* and 4°C for 30 min, and then washed by resuspending the pellet in 1000 μ l of binding buffer, followed by centrifugation. The pellets were collected, and the bound radioactivity was quantified by γ counting (Berthold LB 2104, Wildbad). The nonspecific binding was subtracted from the total binding to yield the specific binding. The number of binding sites ($B_{\max}$) and the dissociation constant (K_d) were calculated by Scatchard analysis (1949).

A specificity of 125 I-labelled EGF binding was determined by exposing the membrane solution to 0.13 nmol 125 I-labelled EGF l^{-1} in the presence and in the absence of a 10-, 100-, and 1000-fold molar excess of unlabelled mouse EGF, human TGF α , TGF β , human IGF-I, IGF-II and bFGF.

Cross-linking of 125 I-labelled EGF to EGF-R

Oviductal and endometrial membranes (80 μ g protein) were incubated with shaking at room temperature with 1 mmol 125 I-labelled EGF l^{-1} (100 000 c.p.m.) in 100 μ l 50 mmol Hepes l^{-1} (pH 7.4) containing 0.5% (w/v) BSA, 128 mmol NaCl l^{-1} , 5 mmol KCl l^{-1} , and 1.2 mmol MgSO $_4$ l^{-1} for 2 h in the presence and in the absence of a 500-fold molar excess of unlabelled EGF (Paria *et al.*, 1994). The reaction products were diluted with 10 volumes of ice-cold buffer and centrifuged at 100 000 *g* for 30 min. The pellet of membranes was washed twice in 20 mmol Hepes l^{-1} buffer without BSA and resuspended in 30 μ l of the same buffer containing 1 mmol disuccinimidyl suberate (DSS) l^{-1} . The reaction was allowed to continue for 15 min at room temperature. The reaction was terminated by adding 30 μ l 10 mmol Tris l^{-1} (pH 7.4) containing 1 mmol EDTA l^{-1} and 0.15 mol NaCl l^{-1} for 15 min at 4°C. Double-strength SDS sample buffer (125 mmol Tris l^{-1} (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) β -mercaptoethanol; 60 μ l) was added to the reaction mixtures. The cross-linked product was analysed by 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970). The gels were dried and exposed to Hyperfilm MP with intensifying screens (Amersham, Braunschweig) at –80°C for 3 weeks.

EGF-dependent protein tyrosine kinase activity

EGF-specific protein tyrosine kinase (PTK) activity in oviductal membranes was determined as described by McCune *et al.* (1990) and Paria *et al.* (1991) using the peptide substrate Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly. The whole oviducts (1.5 g) were homogenized in 4 ml ice-cold homogenization buffer (10 mmol Tris l^{-1} (pH 7.4), 0.25 mol sucrose l^{-1} , 10 μ g leupeptin ml^{-1} , and 20 μ g PMSF ml^{-1}) by three bursts of 15 s duration in an Ultra-Turrax. The homogenates were centrifuged at 3000 *g* and 4°C for 15 min. Aliquots (40 μ g protein) of supernatants were mixed with the reaction buffer (20 mmol Pipes l^{-1} (pH 7.0), 1 mmol MnCl $_2$ l^{-1} , 0.1 mmol sodium vanadate l^{-1} , 0.1% (v/v) Triton X-100) to a final volume of 50 μ l in the presence and in the absence of the

peptide substrate (1 mmol l^{-1}). The reaction mixture was first incubated on ice with and without EGF (1 μ g ml^{-1}) for 5 min, and then 20 μ mol ATP l^{-1} containing 10 μ Ci [γ - 32 P]ATP was added. The incubation was continued at 30°C for 8 min. The reactions were terminated by the addition of 15 μ l 2 mmol ATP l^{-1} in 0.1% (w/v) BSA and 15 μ l 5% (w/v) TCA and cooling on ice for 20 min. The samples were centrifuged at 16 000 *g* and 4°C for 5 min. Spots (30 μ l) of supernatant were placed onto a phosphocellulose filter. The filters were washed in 30% (v/v) acetic acid and acetone, dried, and the amount of γ - 32 P-labelled peptide was quantified by liquid scintillation counting (LKB–Wallac 1219, Rack Beta, Turkow). Basal activity (without a peptide substrate) was subtracted from the experimental value (with a peptide substrate) to obtain the PTK activity. Two (day 1) or three (day 6, day 12) animals in each stage of the oestrous cycle were used and, by means of two separate experiments (two tissue pools per animal, aliquots from each pool were assayed in duplicate), the PTK activity was analysed. The PTK activity was expressed as a percentage increase over the basal activity.

Statistical analysis

The data are expressed as the mean $\pm$ SD. All data were first examined by ANOVA. Differences between means were determined by Student–Newman–Keuls test; *P* values < 0.05 were considered statistically significant.

Results

Histochemical determination of EGF receptor binding

Haematoxylin and eosin staining of sections throughout the pig oviduct and endometrium illustrating the tubal epithelium and luminal and glandular epithelia of the endometrium layers are shown (Fig. 1). The EGF receptor was detected by histochemical ligand binding assay (Fig. 2). The accumulation of biotinylated EGF was primarily noted in the tubal epithelial cells in gilts. Staining was most intense at the apical border of some epithelial cells. It is supposed that the ciliated cells of oviduct epithelium possess more EGF-R in comparison with the secretory cells (Lei and Rao, 1992). EGF-R was seen throughout the endometrium in both luminal and glandular epithelia. Stromal cells were essentially weaker for immunostaining. Between the stages of the cycle investigated, no consistent changes in the intensity of biotinylated EGF binding in the region of the oviduct or uterus examined were observed. Therefore, results shown are representative pictures for one stage of the cycle. In control sections in which either biotinylated EGF had been omitted or had been displaced by a 1000-fold excess of unlabelled EGF (data not shown), a considerable reduction in immunostaining in tubal epithelium, and luminal and glandular epithelia of the endometrium was observed.

125 I-labelled EGF binding in oviduct and endometrium

The binding of 125 I-labelled EGF on membranes of the oviduct and endometrium of gilts resulted in approximately

Fig. 1. Photomicrograph of the haematoxylin and eosin staining of a cryostat section (5–8 μm) of (a) oviduct and (b) endometrium from gilt. TE, tubal epithelium; LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 100 μm .

80% specific binding and 20% nonspecific binding. The receptor affinity and binding capacity of EGF in membranes were determined by the Scatchard (1949) method. The binding data from the oviductal and endometrial membranes fit straight lines, suggesting a single class of noninteracting binding sites (Fig. 3). The specificity of EGF binding was determined by examining the ability of various unlabelled growth factors to displace the binding of ^{125}I -labelled EGF to the endometrial

membranes of gilts (Fig. 4). The data show that the displacement of bound ^{125}I -labelled EGF by EGF and TGF α was dose dependent. In the presence of 10-, 100-, and 1000-fold molar excesses of TGF β , IGF-I, IGF-II and bFGF, these ligands did not compete for ^{125}I -labelled EGF binding.

Analysis of the binding parameters revealed that the binding capacity of EGF to membranes of the oviduct and endometrium was higher on day 1 of the cycle when compared with the

Fig. 2. Epidermal growth factor (EGF) binding sites (brown products) detected by a histochemical binding assay with biotinylated EGF on oviductal and endometrial cryostat sections (5–8 μ m) of the gilt. EGF receptor was localized primarily in (a) tubal epithelium and (b) luminal and glandular epithelia of endometrium. In the control experiment, in which biotinylated EGF had been omitted, the intensity of staining was substantially reduced in both oviduct (c) and endometrium (d). Scale bar represents 100 μ m.

Fig. 3. Scatchard analysis of binding of ^{125}I -labelled epidermal growth factor to membranes from (a) oviducts and (b) endometrium of gilts on day 1 ($\circ$; $n=3$), day 6 ($\square$; $n=3$) and day 12 ($\triangle$; $n=2$) of the oestrous cycle. The plot (a) results in a linear relationship, with correlation coefficients of $r=0.92$ for day 1, $r=0.98$ for day 6, $r=0.99$ for day 12 gilts, respectively. The plot (b) results in a linear relationship, with correlation coefficients of $r=0.98$ for day 1, $r=0.88$ for day 6, and $r=0.97$ for day 12 gilts, respectively.

values taken for membranes on day 6 ($P<0.05$), but not on day 12, of the cycle. The binding affinities showed no significant changes (Table 1).

Affinity cross-linking of ^{125}I -labelled EGF to oviductal and endometrial membranes

Membrane preparations of the oviduct and endometrium were cross-linked to ^{125}I -labelled EGF in the presence and in the absence of an excess of unlabelled EGF. Cross-linking experiments were performed to ascertain whether the ^{125}I -labelled EGF binding protein was similar to that reported in other species. A 170 kDa protein, as expected for the EGF-R, and two radiolabelled bands detected on the top of the stacking and separating gel were found in the oviductal and endometrial membranes (Fig. 5). The 170 kDa protein was the result of specific binding of EGF and was eliminated by excess unlabelled EGF. The cross-linked proteins were detected in membrane preparations from the oviduct and endometrium of all stages of the cycle. The radiolabelled bands detected on the top of the stacking and separating gel were presumably the result of aggregated material that did not enter the gel, or they are a heteromized form of the 170 kDa protein, as less

Fig. 4. Specificity of binding of ^{125}I -labelled epidermal growth factor (EGF) to membranes from endometrium of gilts that were incubated in the presence of $0.13 \text{ nmol } ^{125}\text{I}$ -labelled EGF l^{-1} with 10-, 100-, and 1000-fold molar excess of unlabelled competitors. Each value is the mean of two experiments. ∇ , mouse EGF; $\square$, human transforming growth factor α ; $\bullet$, human transforming growth factor β ; $\blacklozenge$, human insulin-like growth factor I; $\blacktriangle$, human insulin-like growth factor II; $\bullet$, basic fibroblast growth factor.

radioactivity was measured in the presence of an excess of unlabelled EGF.

EGF-dependent protein tyrosine kinase activity

The biological activity of oviductal EGF-R was tested by determining subcellular PTK activity. In this way, the supernatants of oviductal homogenates were assayed for their ability to phosphorylate the synthetic peptide from the region around a major autophosphorylation site. The results of these experiments are shown (Fig. 6). Endogenous PTK activity was detected in oviductal extracts on days 1, 6 and 12 of the cycle. In comparison with day 6 and 12 of the cycle, on day 1 of the cycle, a significant increase in peptide phosphorylation was observed in those preparations without added EGF. On day 6 of the cycle, the endogenous PTK activity was undetectable in homogenates derived from two of the gilts. After exogenous EGF application to oviductal homogenates collected on days 1, 6 and 12 of the cycle, the PTK activity was not stimulated.

Discussion

Using histochemistry, cross-linking and ^{125}I -labelled EGF receptor binding assay, we have demonstrated the presence of the EGF-R in a qualitative and quantitative manner in pig oviduct and endometrium on different days of the oestrous cycle. By determining EGF-dependent PTK activity, we also tested the bioactivity of the EGF-R in the oviduct to gather information on the functional state of the receptor.

Table 1. Scatchard analysis of EGF binding to oviductal and endometrial membranes

Day of cycle	Oviduct		Endometrium	
	$B_{\max}$ (fmol mg ⁻¹ protein)	K_d (nmol)	$B_{\max}$ (fmol mg ⁻¹ protein)	K_d (nmol)
1	22.4 ± 8.7 ^a	0.67 ± 0.40 ^a	66.8 ± 16.4 ^a	0.75 ± 0.30 ^a
6	11.0 ± 0.42 ^b	0.40 ± 0.20 ^a	39.1 ± 3.4 ^b	0.38 ± 0.29 ^a
12	16.0 ± 2.97 ^{ab}	0.50 ± 0.20 ^a	38.0 ± 14.6 ^{ab}	0.43 ± 0.19 ^a

Each value represents mean ± SD. ^{ab}Values with the same letters within the same column are not significantly different ($P > 0.05$).

Fig. 5. Autoradiograph of a polyacrylamide gel (7.5%) showing cross-linking of ¹²⁵I-labelled epidermal growth factor (EGF) to its receptor in (a) oviductal and (b) endometrial membranes (30 µg protein per lane). Lane 1: day 1, ¹²⁵I-labelled EGF; lane 1': day 1, ¹²⁵I-labelled EGF in the presence of excess nonlabelled EGF; lane 2: day 6, ¹²⁵I-labelled EGF; lane 2': day 6, ¹²⁵I-labelled EGF in the presence of excess nonlabelled EGF; lane 3: day 12, ¹²⁵I-labelled EGF; lane 3': day 12, ¹²⁵I-labelled EGF in the presence of excess nonlabelled EGF. Membranes were incubated with ¹²⁵I-labelled EGF for 2 h. Lane 4: molecular weight markers.

Histochemical EGF-R binding using biotinylated EGF was used to determine the localization of the receptor in oviductal and endometrial regions. The results suggest that EGF-R is widely distributed both in glandular and stromal cells of the endometrial, and epithelial cells of the oviduct in pigs, and are

Fig. 6. Protein tyrosine kinase activity was measured by its ability to phosphorylate an exogenous substrate. Protein tyrosine kinase activity was determined in supernatants of oviductal homogenates of day 1 ($n = 2$), day 6 ($n = 3$) and day 12 ($n = 3$) animals without (□) and with (■) application of epidermal growth factor in the cell-free assay. ^{ab}Values with different superscripts are significantly different ($P < 0.05$).

in agreement with the data on immunocytochemical localization of EGF-R using EGF-R antibodies in the human oviduct and uterus (Chegini *et al.*, 1992, 1994). Staining was most intense at the apical border of the epithelial cells of the oviduct. In addition, Swanchara *et al.* (1995) showed that the EGF-R is dispersed primarily throughout the pig oviductal epithelial cells, most prominently in the apical periphery of these cells, suggesting that EGF-R is a membrane-bound receptor complex. No consistent changes in the intensity of biotinylated EGF binding to different regions of the oviduct or uterus were observed between the stages of the cycle investigated. This fact is not likely to be due to tissue handling, but may be related to the limits of quantification using histochemical techniques. Our results are in agreement with the data attained by Kennedy *et al.* (1994), who used RT-PCR and immunohistochemistry to detect mRNA transcripts for EGF, EGF-R and EGF protein in pig endometrial and oviductal tissue. These

authors found no obvious quantitative changes in expression of mRNA encoding EGF-R and EGF in relation to the stages of the oestrous cycle or pregnancy.

In the present study, EGF-R was quantitatively measured in membranes from oviductal and endometrial tissue by heterologous assay using mouse ^{125}I -labelled EGF. The specificity of EGF binding was clearly shown by the observation that only EGF and TGF α , recognized as EGF receptor ligands (Massague, 1983), decreased the ^{125}I -labelled EGF binding to the membranes. These results were supported by cross-linking of ^{125}I -labelled EGF to a 170 kDa membrane protein.

In the present study, the K_d values for pig oviduct and endometrium are in the range reported for EGF binding in the organs of various species. Gardner *et al.* (1989) measured K_d values of 0.7–2.4 nmol l^{-1} for specific EGF binding to the uterine membranes of mature rats, whereas K_d values of 0.2–2.5 nmol l^{-1} have been observed in pregnant mouse uteri (Das *et al.*, 1994). Zhang *et al.* (1992) determined K_d values of 2.96 nmol l^{-1} and 2.48 nmol l^{-1} for stromal and glandular cells in endometria of pregnant (day 13) pigs.

To our knowledge, this is the first report of quantitative EGF-R concentration in the female genital tract of pigs during various days of the oestrous cycle. The data suggested that the receptor concentration of the oviduct and endometrium were dependent upon the stage of the oestrous cycle. Use of RT-PCR analysis and a direct fluorescence staining technique (Kennedy *et al.*, 1994; Swanchara *et al.*, 1995) did not show any obvious changes in relation to the stages of the oestrous cycle or pregnancy examined. In rodents and humans, there is evidence for the regulation of the expression of both the EGF-R and EGF itself in the uterus by oestrogens (Mukku and Stancel, 1985; Huet-Hudson *et al.*, 1990; Troche *et al.*, 1991).

During the oestrous cycle of pigs, the concentrations of oestrogens in the circulation are high at about day 20 of the cycle (Henricks *et al.*, 1972), and there have also been reports of increased concentrations of oestrogens on day 0 of the cycle (Blöndow *et al.*, 1977; Schneider *et al.*, 1979; Brüssow, 1992). We observed that the concentration of EGF-R increased significantly in both the oviduct and endometrium on day 1 of the oestrous cycle as compared with the concentrations on day 6 and day 12. It can be assumed that increased oestrogen concentrations during the time of ovulation are responsible for the increased EGF-R concentration on day 1. This assumption is supported by Mukku and Stancel (1985), who showed increased EGF-R concentrations 18–24 h after the treatment of ovariectomized rats with oestrogen. The changes in the concentrations of EGF-R were similar in the oviduct and endometrium and it can be assumed that the expression of the EGF-R in pig reproductive tracts is effected by maternal plasma hormone concentrations.

Ligand-dependent receptor autophosphorylation and PTK activity are important determinants of the EGF-R bioactivity (Carpenter and Cohen, 1990). Our results demonstrated an increased endogenous PTK activity in oviductal membranes collected on day 1 of the oestrous cycle in comparison with those collected on day 6 and day 12. However, it was not possible to increase PTK activity after the application of exogenous EGF in a cell-free system, which suggests that endogenous EGF could have already activated the receptor system. This could be due to the oestrogenic induction of EGF

during day 1 of the cycle. It has been shown that steroids regulate the EGF-induced EGF-R activity in the mouse uterus (Das *et al.*, 1994). The data obtained from membranes on day 12 suggest that EGF is unable to induce the peptide phosphorylation, possibly due to the reduced receptor activity at this time. However, it could be shown that the serine-threonine residues of EGF-R are phosphorylated by protein kinase C after ligand binding, resulting in a downregulation of its tyrosine phosphorylation (Hunter *et al.*, 1984; Carpenter and Cohen, 1990; Countaway *et al.*, 1992). In this respect, protein kinase C activity in the ovine uterus increased with increased progesterone concentrations and decreased with increasing oestrogen concentrations (Magness *et al.*, 1991). Thus, lower endogenous PTK activity and the enhanced progesterone concentrations in the circulation of pigs during metoestrus appear possible. The increased PTK activity (not significant) after the application of EGF on day 6 suggests that the concentration of progesterone could also be critical for the bioactivation of the EGF-R. A comparable stimulation of the EGF-R concentrations in human endometrial cultures has been observed (Taketani and Mizuno, 1991). The correct interpretation of these data must await further investigation. On the basis of the quantitative detection of transcripts for EGF and TGF α in the reproductive tissues, it seems possible to gather information on the regulation of these factors and their influence upon the expression and the bioactivity of the EGF-R. The results of the present study suggest that changes in the concentration and functional status of the EGF-R in the oviduct and the endometrium of pigs play a role in the cascade of cellular events by which oestrogens mediate oviductal and uterine growth and so may have an indirect effect on early embryonic development.

It can be concluded that the expression of the maternal oviductal and endometrial EGF-R of gilts depends on the stage of the oestrous cycle, suggesting that sex steroids play an important role in its regulatory processes.

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