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Kinase pathways in dominant and subordinate ovarian follicles during the first wave of follicular development in sheep

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Abstract

The mechanism by which one or more dominant ovarian follicles continue development while other subordinate follicles regress is not known. The mitogen activated protein kinases (MAPKs) are a group of kinases that are activated by hormonal factors and form a cascade of processes that regulate cell growth, division and differentiation. The aim of the present experiment was to characterise the presence of the MAPKs, Erk 1/Erk 2 and Akt in healthy dominant follicles and regressing subordinate follicles. Following in vivo monitoring of ovarian follicle development, three ewes were ovariectomised and the follicular fluid and follicle wall (theca and granulosa cells) saved from the dominant and largest subordinate follicle. The dissected diameter and follicular fluid oestradiol concentration of the dominant follicle was larger ($P < 0.01$) than the largest subordinate follicle (6.5 ± 0.0 mm and 41.3 ± 4.9 ng/ml versus 4.7 ± 0.3 mm and 0.6 ± 0.4 ng/ml). Western blot analyses showed that there was more Akt (202.7 ± 6.4 versus 59.6 ± 32.7 units; $P < 0.05$) and Erk 1/Erk 2 (104.5 ± 10.6 versus 0.3 ± 0.2 units; $P < 0.01$) present in follicle wall samples from the dominant compared to the largest subordinate follicles. Phosphorylated forms of Akt and Erk 1/Erk 2 were detected in samples from dominant but not subordinate follicles. We suggest that signal transduction pathways involving Akt and Erk 1/Erk 2 may play an important role in determining the outcome of ovarian follicle growth and development in sheep.

Keywords: Sheep-ovary; Follicle; Intracellular pathways

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1. Introduction

In cattle, sheep and humans, the dominant ovulatory follicle(s) is selected from a cohort of growing follicles 5–7 days prior to ovulation (Leyendecker et al., 1990; Roche et al., 1998; Evans et al., 2000). This follicle grows to a larger diameter than other follicles and appears to suppress the development of smaller (subordinate) follicles. Unlike the menstrual cycle of humans, cattle and sheep have two or more successive waves of follicular development during oestrous cycles (Webb et al., 1999; Evans et al., 2000). Healthy dominant follicles produce large quantities of oestradiol compared to atretic subordinate follicles that produce relatively more progesterone (Evans and Fortune, 1997; Evans et al., 2000).

The demise of cells within follicles occurs by apoptosis (Jolly et al., 1997; Van Wezel et al., 1999). All the follicles of a cohort develop in the same endocrine environment, yet how the dominant follicle survives while the neighbouring subordinate follicles die by apoptosis is not known. It seems plausible that individual follicles respond to their endocrine environment by regulating their own intracellular environments thereby controlling degradation or continued growth. Follicles produce intrafollicular growth factors that contribute to the success of folliculogenesis (Webb et al., 1999) and the later stages of follicle development are reliant upon the gonadotrophic hormones LH and FSH (Webb et al., 1992; Campbell et al., 1995). The actions of both the gonadotrophins and growth factors are mediated via cell surface receptors that rely on signal transduction mechanisms to relay information to the cell nucleus (Hill and Treisman, 1995). The nature of these pathways is complex and their interaction with the events that control apoptosis in ovarian cells is not clear.

The mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinases that are activated by a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus (Blumer and Johnson, 1994). Upon activation, MAPKs translocate to the nucleus and phosphorylate transcription factors (Cobb and Goldsmith, 1995). The MAPK cascade is involved in processes regulating cell growth, division and differentiation. The most widely studied cascades are the extracellular signal-regulated kinases-1 (Erk 1; p44 MAPK) and Erk 2 (p42 MAPK) one of whose functions when activated is to phosphorylate a range of transcription factors including signal transducers and activators of transcription (Stat) in the nucleus (Pircher et al., 1999). Most recently Erk has been shown to control the synthesis of nucleotides, the first step in the production of DNA and RNA (Graves et al., 2000). Another common signal transduction pathway involves the activation of protein kinase B (also known as Akt) by PI3 kinase (Coffer et al., 1998). Akt is a general mediator of cell survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli (Downward, 1998). Akt regulates the activity of transcription factors and modulates the activity of members of the Bcl2 family of proteins (Brunet et al., 1999; Kops et al., 1999) thus preventing the proapoptotic actions of a family of proteins referred to as caspases (Datta et al., 1997; Cardone et al., 1998; Kulik and Weber, 1998) that serve as regulators and effectors of apoptosis (see Mehmet, 2000). The activation of both Erks and Akt involve phosphorylation of an inactive form of the protein by specific upstream kinases (Khokhlatchev et al., 1998).

The hormones thought to be central to follicle survival are FSH and insulin-like growth factors (IGFs). FSH acts via cAMP and PKA (Richards, 1995; Hansson et al., 1999) and

has been shown to induce the activation of Erks in porcine (Cameron et al., 1996) and rat (Das et al., 1996) granulosa cells in vitro. Insulin and/or IGF-I have been shown to activate Akt in fibroblasts, neuronal cells (Campana et al., 1999), epithelial cells (Alessi and Downes, 1998), Chinese hamster ovary cells (Takata et al., 1999) and porcine granulosa cells (Westfall et al., 1999) in vitro. However, the presence of Erk 1/Erk 2 and Akt pathways in ovarian follicles in vivo and their relative roles in dominant and subordinate follicles is not known.

The aim of the present experiment was to characterise the presence of Akt and Erk 1/Erk 2 in healthy dominant follicles and regressing subordinate follicles. This was achieved by Western blot analyses of proteins from follicles collected during the first follicular wave of follicular development on day 5 of the oestrous cycle in sheep.

2. Materials and methods

2.1. Animals, ultrasonography and tissue collection

During November and December, three Suffolk-cross cyclic maiden ewe lambs (10 months old) were kept at pasture (53°18′N) with free access to water. A raddled vasectomised ram was kept with the ewes to detect oestrus. They were fed 0.5 kg of a 19% protein ration each per day when they were brought in for ultrasonographic examinations. All procedures were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act, 1876 and European Community Directive 86/609/EC.

Prior to the start of the experiment oestrous cycles were synchronised using a progestogen releasing intravaginal device (Veramix; Upjohn Ltd., Cranley, UK) left in place for 13 days. Starting on day 0 (day of heat) and continuing for 20–23 days (until day 5 of the subsequent oestrous cycle), the ovaries of each animal were examined daily by transrectal ultrasonography using a rigid 7.5 mHz linear-array transducer (Concept 500; Dynamic imaging Ltd., Livingston, Scotland). Each day the position and diameter of individual follicles $\geq$2 mm in diameter and corpora lutea were recorded on a diagram as previously described (Evans et al., 2000). From the diagrams, the growth profiles of the dominant (largest) and largest subordinate (second largest) follicles of the first wave of follicle development in each cycle were determined. The day of emergence of identified follicles was retrospectively identified as the day on which the follicle was 2 or 3 mm in diameter. The days of detection was the number of days between which the follicle was first and last identified at 2 or 3 mm in diameter.

Animals were ovariectomised on day 5 of the second oestrous cycle. This day was chosen as it was the first day during the first follicular wave on which the dominant and subordinate follicles could be clearly identified using ultrasonongraphy. The dominant and largest subordinate follicles were dissected from the ovaries and measured to the nearest 0.5 mm by transillumination over a 1 mm grid (Evans and Fortune, 1997). The follicular fluid was then aspirated and stored at −20°C until radiommunoassay for oestradiol and progesterone concentrations. The follicles were cut open and the theca interna and adherent granulosa cells (i.e. follicle-wall) were peeled away from the theca externa/stroma and snap frozen in liquid nitrogen and stored at −75°C until immunoblotting analyses.

2.2. Radioimmunoassay

Estradiol concentrations were measured in follicular fluid samples as previously described (Prendiville et al., 1995) using a Biodata Estradiol MAIA kit (S.P.A radioimmunoassay kit, Code 12264, Biochem Immunosystems, Italy). The follicular fluid was diluted in assay buffer and no extraction of the sample was needed. The sensitivity was 0.031 pg per tube and the intra-assay coefficients of variation were 11.1 and 8.4% for samples containing 0.14 and 0.68 pg per tube, respectively. Progesterone concentrations were measured in follicular fluid samples in a single assay (Evans et al., 2000). The sensitivity of the assay was 3 pg per tube and intra-assay coefficients of variation were 7.4 and 7.0% for reference samples containing 7.0 and 26.0 pg per tube, respectively.

2.3. Immunoblotting analyses

Protein was extracted from the frozen follicle-wall samples by homogenisation in 200 μl of cold extraction buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 400 mM KCl, 5 mM benzamidine, 5 μg/ml each of pepstatin and aprotinin). The supernatant was retained after centrifugation at 14 000 g for 5 min at 4°C. Protein concentrations were determined using a spectrophotometric assay (BIORAD protein assay).

Follicle-wall proteins were separated using SDS–polyacrylamide gel electrophoresis (20 μg total protein per lane) and were then electrophoretically transferred onto a nitrocellulose membrane. Equal loading was confirmed visually after staining with Ponceau S solution (Sigma–Aldrich, Ireland). Membranes were blocked for 1–3 h in TBS-T (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dried milk and then overnight (14–16 h) with the appropriate primary antibody diluted in 5% BSA in TBS-T at 4°C. The antibodies (all rabbit anti-mouse and supplied by New England BioLabs) for Akt and Erk 1/Erk 2 were used at a dilution of 1:2000, the antibodies for phospho-Akt and phospho-Erk 1/Erk 2 were used at a dilution of 1:1000, the antibody against Stat-1 was used at a dilution of 1:4000. Membranes were then rinsed twice in TBS-T for 10 min and incubated with the secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (Dako, Cambridge, UK) at room temperature for 60 min. After rinsing five times for 7 min in TBS-T the bands were visualised using enhanced chemiluminescence (Supersignal® West Dura, Pierce) and autoradiography.

The intensity of protein bands were quantified using Scion Image software (http://www.scioncorp.com). Autoradiograms were digitised, the area of each band of interest was selected, the mean pixel intensity was determined (0 = white, no intensity; 256 = black, maximum intensity) and background intensities (measures on adjacent areas of each autoradiogram) were subtracted from individual values.

2.4. Statistical analyses

Mean values were compared using *t*-test and all values are given as the mean ± S.E.M.

3. Results

As expected, the dominant follicle of the first follicle wave of the first complete oestrous cycle was detected for a longer period (11.3 ± 2.2 days; $P < 0.05$) than the largest subordinate follicle (5.3 ± 0.3 days; Fig. 1a). After ovariectomy on day 5 of the second oestrous cycle, the dominant follicle had a larger ($P < 0.01$) dissected diameter than the largest

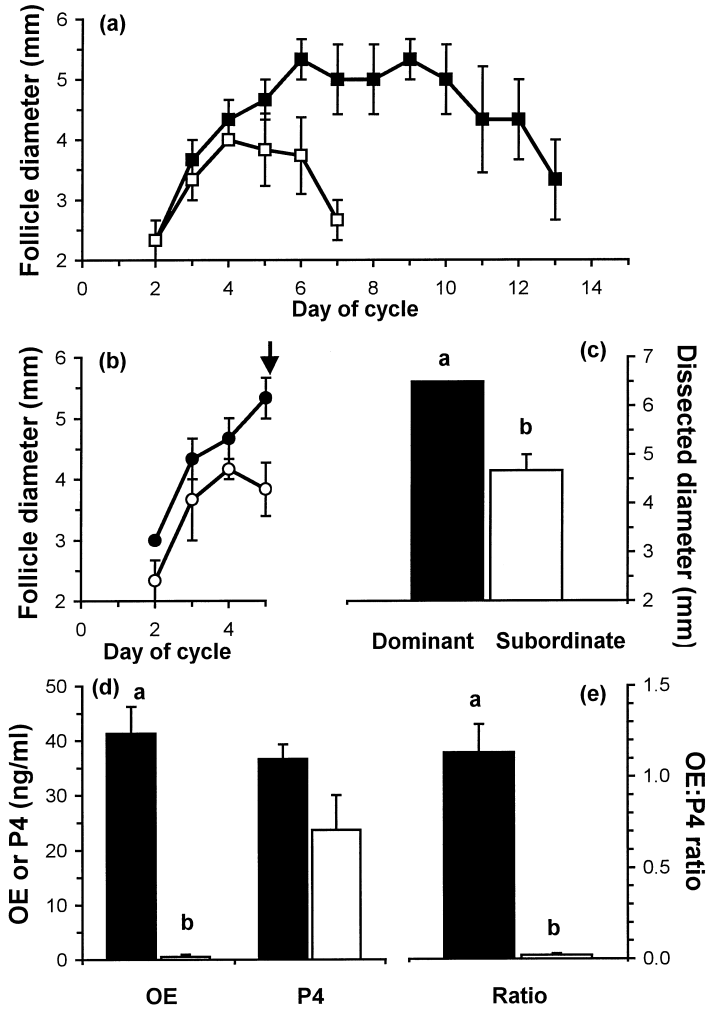

Fig. 1. Mean (±S.E.M) diameters (measured using ultrasonography) of the dominant and largest subordinate follicles of the first wave of follicular development during the complete first oestrous cycle (a) and leading up to ovariectomy on day 5 of the second oestrous cycle (b); dissected follicle diameters (c); oestradiol (OE) and progesterone (P4) concentrations in follicular fluid (d) and the follicular fluid OE:P4 ratio (e) in the dominant (solid symbols and bars) and largest subordinate (open symbols and bars) follicles in three cyclic ewe lambs. Arrow indicates time of ovariectomy. Within variable, columns with no common superscript are different (ab, $P < 0.01$).

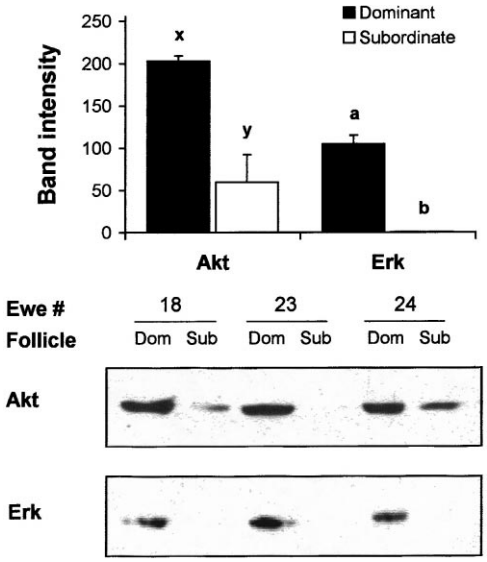

Fig. 2. Immunoblotting signal intensity (top panel) and Western blot analyses of Akt and Erk 1/Erk 2 in follicle wall samples from dominant (Dom, solid bars) and subordinate (Sub, open bars) follicles on day 5 of the oestrous cycle in three cyclic ewe lambs. Columns with no common superscript are different (xy, $P < 0.05$; ab, $P < 0.01$).

subordinate follicle (Fig. 1c). Oestradiol and the ratio of oestradiol to progesterone concentrations in follicular fluid were greater ($P < 0.01$) in the dominant compared to the largest subordinate follicles (Fig. 1d and e) indicating the good and poor health of the dominant and subordinate follicles, respectively.

There was more Akt ($P < 0.05$) and Erk 1/Erk 2 ($P < 0.01$) present in follicle wall samples from the dominant compared to the largest subordinate follicles (Fig. 2) and the phosphorylated forms of Akt and Erk 1/Erk 2 were detected in samples from dominant follicles (Fig. 3). Phosphorylated forms of Akt and Erk 1/Erk 2 were not detected in samples from subordinate follicles (data not shown). More Stat 1 was detected in follicle wall samples from the dominant compared to the largest subordinate follicles ($P < 0.01$; Fig. 4).

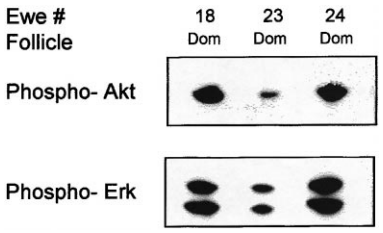

Fig. 3. Western blot analyses of phosphorylated Akt and phosophorylated Erk 1/Erk 2 in follicle wall samples from dominant (Dom) follicles on day 5 of the oestrous cycle in three cyclic ewe lambs.

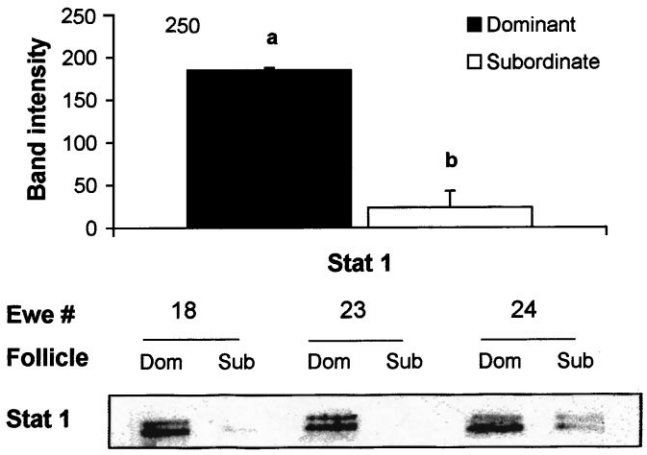

Fig. 4. Immunoblotting signal intensity (top panel) and Western blot analyses of Stat 1 in follicle wall samples of dominant (Dom) and subordinate (Sub) follicles on day 5 of the oestrous cycle in three cyclic ewe lambs. Columns with no common superscript are different (ab, $P < 0.01$).

4. Discussion

The presence of Akt and Erk 1/Erk 2 in dominant or subordinate follicles of a follicular wave has not been described previously. In dominant follicles, the signalling pathways that include Akt and Erk 1/Erk 2 are active as shown by the presence of the native protein (Fig. 2) and the activated phosphorylated forms (Fig. 3). In addition, the high concentration of oestradiol in follicular fluid (Fig. 1) and the abundant presence of Stat 1 in follicle wall samples (Fig. 4) indicates that on day 5 of the cycle the dominant follicles were healthy (Jolly et al., 1997) and transcriptionally active. In contrast, the smaller subordinate follicles had a very low ratio of oestradiol to progesterone in follicular fluid (Fig. 1), indicating an advanced atretic state, and much reduced levels of Akt, Erk 1/Erk 2 and Stat 1 compared to the dominant follicle (Figs. 2 and 4). We suggest that the mechanisms that regulate the continued development of dominant and regression of subordinate follicles are mediated by signal transduction pathways that utilise the Akt and Erk 1/Erk 2 pathways. It remains to be established if these pathways play a role in the process of dominant follicle selection earlier in the wave.

FSH is critical for the later stages of follicle development (Webb et al., 1999) and FSH has been shown to activate Erk 1/Erk 2 in porcine and rat granulosa cells in vitro (Cameron et al., 1996; Das et al., 1996). Dominant follicles in ewes contain more FSH receptors (Carson et al., 1979) and produce more cAMP in response to FSH stimulation in vitro (Henderson et al., 1985) than atretic subordinate follicles. Hence, differences in FSH receptor concentrations on granulosa cells in dominant and subordinate follicles may explain differences in the levels of Erk 1/Erk 2. However, exact relationships between receptor concentrations and intracellular pathways are not clear and it has been suggested that the coupling of adenylate cyclase to down-stream pathways may be a regulated event in controlling dominant follicle selection and development in cattle (Jolly et al., 1994).

Insulin and IGFs have been shown to activate Akt in a wide variety of cells in vitro, including porcine granulosa cells (Westfall et al., 1999). The regulation of IGF availability within follicles is regulated by IGF binding proteins (IGFBPs) and it has been shown that IGFBP concentrations are lower in healthy dominant follicles compared to regressing subordinate follicles in cattle (Echternkamp et al., 1994; Armstrong et al., 1998) and sheep (Besnard et al., 1996). Hence, differences between follicle types for levels of Akt (Fig. 2) may be an eventual consequence of differing levels of stimulation by growth factors.

While the weight of literature would suggest that FSH acts via Erk 1/Erk 2 and that insulin/IGFs act through Akt (Price and Silva, 1999), it is likely that there is crossover and that other pathways are involved. IGF-I activates Erk 1/Erk 2 in rat adipocytes (Porras et al., 1998) and Chinese hamster ovary cells (Sellers, 1999) in vitro and FSH stimulates the activation of p38 MAPK in rat granulosa cells in vitro (Maizels et al., 1998). Consequently, elucidation of the exact intracellular pathways by which FSH and IGFs control follicle growth and development remain to be established.

The characteristics of dominant compared to subordinate follicles are that they continue to grow and to produce large quantities of oestradiol (Roche et al., 1998). It is widely assumed that the insulin/IGF system controls growth and development and that FSH controls aromatase production. While this may largely be the case there is some crossover as FSH can stimulate the development of granulosa cells (Robker and Richards, 1998) and insulin and IGF-I can stimulate oestradiol production by ovine granulosa cells in vitro (Campbell et al., 1996). The interaction of these systems is further complicated as each system seems to augment the actions of the other by regulating binding protein production (Armstrong et al., 1998) and receptor expression (Zhou et al., 1997). To completely understand the role of these hormones in controlling development and oestradiol production we must fully understand the intracellular signalling pathways within cells. To date, research has concentrated on the relationships among follicle development and endocrine and local factors. However, how these factors regulate intracellular events within the ovary and the biochemical links between hormonal changes and apoptosis and oestradiol production need to be understood. The present data demonstrate the presence of AKT and Erk 1/Erk 2 pathways in dominant and subordinate follicles and provide a basis for further studies on the hormonal regulation of follicle development.

5. Conclusions

In mammals a species-specific number of dominant follicles are selected from a cohort of follicles to ovulate when the endocrine environment permits. The mechanism by which one or more follicles continue development while other follicles regress is not known. However, it is likely that this occurs as a result of the integration of external endocrine signals and locally produced paracrine signals via signal transduction pathways to affect the cell survival machinery within individual cells. The present experiment shows that dominant follicles contained more of the protein kinases Akt and Erk 1/Erk 2 and also activated phosphorylated forms of these proteins than subordinate follicles. We suggest that signal transduction pathways involving Akt and Erk 1/Erk 2 play an important role in the

continued development of dominant follicles in sheep and that these proteins may play a role during follicle selection; however, this has yet to be determined.

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