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IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells

Arul Murugan Mani[1,2], Mark A Fenwick[1], Zhangrui Cheng[1], Mohan K Sharma[2], Dheer Singh[2] and D Claire Wathes[1]

[1]Reproduction Group, Department of Veterinary Basic Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL9 7TA, UK and [2]Animal Biochemistry Division, National Dairy Research Institute, Karnal, Haryana 132 001, India

Correspondence should be addressed to D C Wathes; Email: dcwathes@rvc.ac.uk

M A Fenwick is now at Division of Surgery, Oncology, Reproductive Biology and Anaesthetics (SORA), Faculty of Medicine, Imperial College London, Institute of Reproductive and Developmental Biology, Hammersmith Hospital, London W12 0NN, UK

Abstract

IGF1, a potent stimulator of cellular proliferation, differentiation and development, regulates granulosa cell steroidogenesis and apoptosis during follicular development. Depending upon species and stage of follicular growth, IGF1 acts on granulosa cell steroidogenesis either alone or together with FSH. We examined the mechanism of action of IGF1 in bovine granulosa cells in serum-free culture without insulin to determine its potential role in the regulation of steroidogenic and apoptotic regulatory gene expression and to investigate the interaction of FSH with IGF1 on this mechanism. Bovine granulosa cells treated with IGF1 demonstrated a significant increase in 17β-oestradiol (OE_2) production, cell number and in mRNA expression of *CYP11A1*, *HSD3B1*, *CYP19A1*, *BAX*, type 1 IGF receptor (*IGF1R*) and *FSHR*, while FSH alone had no significant effects. IGF1 or FSH alone or both together had no effect on *BCL2* expression. IGF1 with FSH resulted in a synergistic increase in granulosa cell number and in mRNA expression of *CYP19A1* and *IGF1R* without altering OE_2 production. IGF1 stimulated the phosphoinositide 3′-OH kinase (PI3K) but not the MAPK pathway in granulosa cells, as evidenced by increased phosphorylation of AKT but not extracellular-regulated kinase 1/2. Addition of the PI3K pathway inhibitor LY294002 (but not the MAPK pathway inhibitor PD98059) abrogated the increased expression of genes induced by IGF1. IGF1 therefore up-regulates the steroidogenic and apoptotic regulatory genes via activation of PI3K/AKT in bovine granulosa cells. The synergistic action of IGF1 with FSH is of likely key importance for the development of small antral follicles before selection; subsequently, other factors such as LH may also become necessary for continued cell survival.

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Introduction

FSH is an important pituitary hormone which controls granulosa cell steroidogenesis in the mammalian ovary by interacting with specific receptors located on granulosa cells (Richards 1994). The steroidogenic potential of granulosa cells can be modulated by locally produced growth factors acting through endocrine, paracrine and autocrine mechanisms, such that the modulation of granulosa cell steroidogenesis involves a complex interaction of both extra- and intra-ovarian signals. Insulin-like growth factor 1 (IGF1) plays a central role in these interactions with respect to both steroidogenesis and survival responses (Adashi & Roban 1992, Giudice 1992, Armstrong & Webb 1997).

The most important role of IGF1 appears to be reliant on its ability to synergize with gonadotrophins and to amplify their steroidogenic output (Adashi *et al*. 1985, 1988, Veldhuis *et al*. 1986, Urban *et al*. 1990, Balasubramanian *et al*. 1997). There are, however, species differences in these responses (deMoura *et al*. 1997, Chung *et al*. 1998, Devoto *et al*. 1999, Mamluk *et al*. 1999, Silverman *et al*. 1999), which may in part relate to the differing types of oestrous cycle. Cows are monovulatory species. At regular intervals during the bovine oestrous cycle, a group of small antral follicles grow rapidly from about 1 to 5 mm in size. Growth during this phase is dependent on gonadotrophin secretion and follicular production of both oestradiol (OE_2) and inhibin A increases, so circulating

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FSH concentrations fall. One follicle then achieves dominance, and is able to continue to grow in the face of declining and low FSH, whereas the remaining follicles within the wave undergo atresia (Webb *et al*. 2004, Mihm & Evans 2008). This process is influenced by a variety of growth factors. Among these, IGF1 and insulin are of key importance in the cow as they can link follicular growth and steroid production with the metabolic status of the animal (Spicer & Echternkamp 1995, Wathes *et al*. 2003). In ruminants, the major source of IGF1 in follicular fluid is the circulation (Funston *et al*. 1996, Perks *et al*. 1999) and there is substantial evidence that follicular maturation is compromised when cows are in negative energy balance and circulating concentrations of IGF1 and/or insulin are reduced (Wathes *et al*. 2003, Webb *et al*. 2004).

The effects of IGF1 are mediated through the type 1 IGF receptor (IGF1R), a transmembrane tyrosine kinase receptor that is structurally related to the insulin receptor. Depending upon the cell type, IGF1 activates the phosphoinositide 3′-OH kinase (PI3K) pathway and/or the MAPK pathway (Le Roith *et al*. 1995, Butt *et al*. 1999, Hancock 1999, Poretsky *et al*. 1999). PI3K signalling activates AKT (protein kinase B, PKB), an important mediator of proliferation and cell survival. Within the MAPK group, the extracellular-regulated kinase (ERK) can also regulate proliferation, differentiation and cell survival. Previous studies of both ovine and bovine follicles have suggested that both AKT and ERK signal transduction pathways are up-regulated during selection of the dominant follicle (Evans & Martin 2000, Ryan *et al*. 2007). Furthermore, administration of specific inhibitors for ERK or AKT to ovine follicles during the first follicular wave of the cycle inhibited their further growth and OE_2 production (Ryan *et al*. 2008). Despite their clear importance, the respective roles of these signalling pathways in the regulation of steroidogenesis and apoptosis by IGF1 alone or together with FSH in bovine granulosa cells still remain poorly understood.

While previous studies have shown that FSH and IGF1 can act synergistically to enhance follicular development, the mechanisms underlying this interaction remain uncertain (Richards *et al*. 2002). Furthermore, most previous studies involving cultured cattle granulosa cells have either provided serum during the first hours of culture or have pretreated the culture plates with serum to facilitate cell adhesion and increase cell viability (Langhout *et al*. 1991, Kawate *et al*. 1993, Wrathall & Knight 1993, Gong *et al*. 1994). Under these conditions, the cells luteinize spontaneously, independently of gonadotrophins (Luck *et al*. 1990, Wathes *et al*. 1995) and rapidly loose their granulosa cell phenotype including CYP19A1 activity (Roberts & Echternkamp 1994). Therefore, we used a serum-free system previously developed for bovine granulosa cells in which they maintain expression and activity of CYP19A1 and remain responsive to physiological concentrations of FSH and growth factors (Gutierrez *et al*. 1997).

It is also common practice in granulosa cell culture to add insulin to the medium. While insulin and IGF1 have distinct receptors (IGF1R and IR respectively), many of the downstream intracellular events resulting from ligand-induced receptor activation are very similar. Furthermore, at high concentrations insulin can cross react with the IGF1R, and when both receptors are present in the same cells IGF1R–IR hybrid receptors can form, which bind both IGF1 and insulin (Siddle *et al*. 2001, Le Roith 2003). In order to identify the specific effects of IGF1, we therefore tested IGF1 in the absence of any insulin.

The primary aim of the experiments was thus to investigate the dose-dependent and synergistic effects of IGF1 and FSH in the absence of insulin on granulosa cells obtained from small to medium-sized follicles, the stage when follicle selection is occurring and many follicles become atretic. Cell number, OE_2 production and mRNA expression of steroidogenic (*CYP11A1*, *HSD3B1* and *CYP19A1*) and apoptotic regulatory (*BCL2* and *BAX*) genes, and genes encoding the IGF and FSH receptors (*IGF1R* and *FSHR*) were all measured under the same experimental conditions. The second aim was to determine the pathway(s) by which IGF1 exerts its effects on mRNA expression of selected genes and to investigate whether FSH influences the effects of IGF1 on these signalling pathways.

Results

Isolated granulosa cells were cultured for an initial 48 h establishment period in medium supplemented with androstenedione (10^{-7} M), low dose insulin (10 ng/ml), FSH (1 ng/ml) and low dose IGF1 (1 ng/ml) as described in more detail in the Materials and Methods section. Following this, the medium was replaced and that used in the experimental procedures continued to include androstenedione, but not insulin. Different treatment doses of IGF1, FSH or a combination were added as outlined below for the individual experiments.

Cell morphology and number

Shortly after seeding, granulosa cells were dispersed throughout the well with occasional clumps. After the initial 48 h of culture, cells were grouped into aggregates, similar to the spherical appearance of granulosa cells *in vivo*, which were attached to the culture plate by enlarged, flattened fibroblast-like cells present at the bottom of the clumps. There were significantly more granulosa cells present after culture for a further 48 h in the presence of 50 and 100 ng/ml of IGF1 in comparison

Table 1 Effects of insulin-like growth factor 1 (IGF1) and FSH on bovine granulosa cell number.

Expt. 1	IGF1 (ng/ml)	0	1	50	100
	Absorbance	0.65 ± 0.03^{a}	0.79 ± 0.05^{a}	1.85 ± 0.22^{b}	2.07 ± 0.17^{b}
	FSH (ng/ml)	0	1	25	50
	Absorbance	0.7 ± 0.05	0.68 ± 0.03	0.73 ± 0.04	0.74 ± 0.01
Expt. 2	(ng/ml)	0	FSH (25)	IGF1 (50)	FSH (25)+IGF1 (50)
	Absorbance	0.7 ± 0.03^{c}	0.96 ± 0.1^{c}	1.33 ± 0.21^{d}	1.98 ± 0.11^{e}

Cells were treated with different doses of IGF1, FSH and its combination as indicated in the table for 48 h in serum-free culture. Cell proliferation was assessed by CellTiter 96 Aqueous One Solution (Promega), and the values are given as absorbance values. Data from three separate batches of cells were analysed by mixed model analysis, and results are presented as the mean ± S.E.M.: a<b, $P<0.001$; c<d<e, $P<0.001$.

with the untreated cells, whereas the 1 ng/ml dose of IGF1 had no effect (Table 1). More fibroblast-like cells were also noted in the presence of the higher doses of IGF1 (data not shown). In contrast, FSH alone (1, 25 and 50 ng/ml) did not produce any significant effects on granulosa cell number. There was, however, a synergistic increase in cell number when FSH (25 ng/ml) was added with IGF1 (50 ng/ml; Table 1).

Effects of IGF1 and FSH on steroidogenesis

Granulosa cells were treated for 48 h with different doses of IGF1 and FSH alone or in combination. There was a significant increase in mRNA expression of *CYP11A1*, *HSD3B1* and *CYP19A1*, and in OE_2 production with 50 and 100 ng/ml IGF1 but no effect of the lowest 1 ng/ml dose. Although the difference between 50 and 100 ng/ml IGF1 was not significant, the maximum values were seen with the 50 ng/ml IGF1 treatment. In contrast, the different doses of FSH (1, 25 and 50 ng/ml) did not produce any significant effect on the above (Fig. 1). In a separate set of experiments, FSH (25 ng/ml) in combination with IGF1 (50 ng/ml) stimulated a further 2.2-fold increase in *CYP19A1* mRNA over IGF1 alone, but without altering OE_2 production. The combined treatment did not affect mRNA expression of *CYP11A1* or *HSD3B1* compared with IGF1 alone (Fig. 2).

Effects of IGF1 and FSH on the apoptotic regulatory genes BCL2 and BAX

The effects of IGF1 and/or FSH on mRNA expression of the apoptotic regulatory genes *BCL2* (anti-apoptotic) and *BAX* (pro-apoptotic) were also measured. *BCL2* mRNA was not affected by treatment with IGF1 or FSH either alone (Fig. 3A) or both together (Fig. 2D). Interestingly, IGF1 alone at 50 and 100 ng/ml increased the levels of *BAX* mRNA transcript. On the other hand, the highest dose of FSH (50 ng/ml) significantly reduced *BAX* expression (Fig. 3B). The stimulatory effect of IGF1 (50 ng/ml) on *BAX* mRNA expression was not, however, prevented by FSH (25 ng/ml; Fig. 2E). These results suggest that IGF1 might also participate in the apoptotic pathway in granulosa cells.

Type 1 IGF receptors and FSH receptors

The effect of IGF1 and FSH on *IGF1R* and *FSHR* mRNA expression was tested using selected doses, which had been shown to alter cell number and *CYP19A1* mRNA expression. Treatment with IGF1 (50 ng/ml) significantly ($P<0.05$) enhanced mRNA expression of both *IGF1R* and *FSHR*, but FSH (25 ng/ml) had no effect (Table 2).

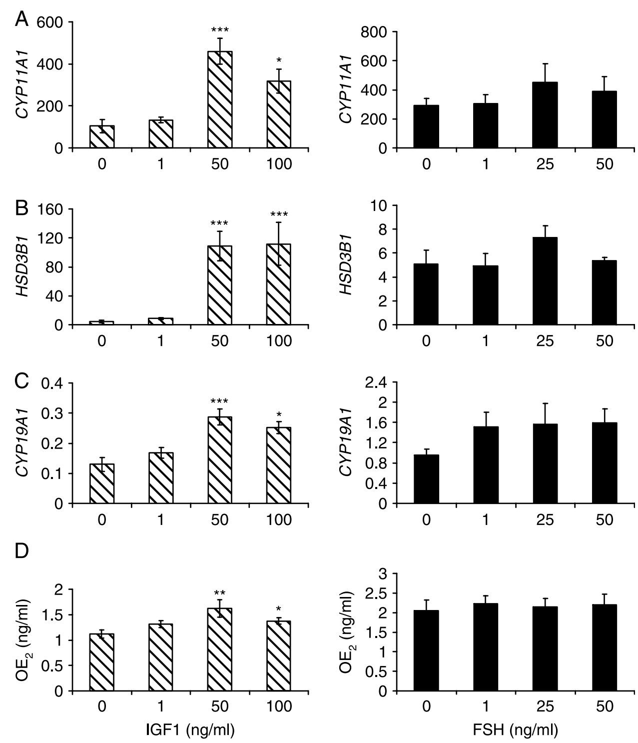

Figure 1 Dose-dependent effect of IGF1 and FSH on mRNA expression of the steroidogenic genes: (A) *CYP11A1*; (B) *HSD3B1*; (C) *CYP19A1*; and (D) on production of OE_2 in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently treated with IGF1 (0, 1, 50 and 100 ng/ml) or FSH (0, 1, 25 and 50 ng/ml) for 48 h. The reverse-transcribed RNA from cellular extracts was amplified by SYBR Green real-time PCR, and results are expressed as fg/µg reverse-transcribed RNA. Data from four separate batches of cells were analysed by mixed model analysis, and results are presented as the mean ± S.E.M. For IGF1, $*P<0.05$, $**P<0.01$ and $***P<0.001$ versus 0 control. There were no significant treatment effects for FSH.

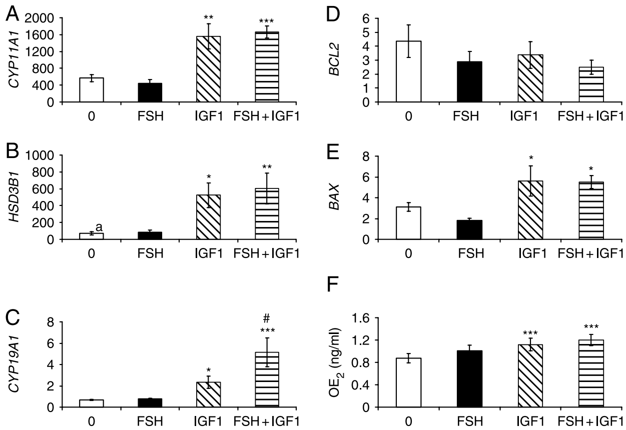

Figure 2 Effects of no treatment (0, control), FSH (25 ng/ml), IGF1 (50 ng/ml) or IGF1+FSH on mRNA expression of: (A) *CYP11A1*; (B) *HSD3B1*; (C) *CYP19A1*; (D) *BCL2*; (E) *BAX*; and (F) production of OE_2 in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently treated as described for 48 h. The reverse-transcribed RNA from cellular extracts was amplified by SYBR Green real-time PCR, and results are expressed as fg/μg reverse-transcribed RNA. Data from four separate batches of cells were analysed by mixed model analysis, and results are presented as the mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus 0 control. $^{\#}P<0.05$ versus IGF1 treatment.

When the two hormones were added in combination, *IGF1R* was higher by 1.3-fold than with IGF1 alone, whereas FSH completely prevented the IGF1 stimulated increase in the *FSHR*.

Effects of IGF1 on the PI3K and MAPK pathways in bovine granulosa cells

We next investigated the ability of IGF1 to induce phosphorylation of AKT and ERK in bovine granulosa cells. Cell lysates collected at different time periods from 0 to 48 h after treatment with 50 ng/ml IGF1 were subjected to immunoblotting with respective antibodies. The ratio between phosphorylated and total protein was then calculated. A very low level of phosphorylated AKT (p-AKT) was detected at $t=0$ h. This was immediately following the initial 48 h establishment period during which low dose insulin (10 ng/ml) and IGF1 (1 ng/ml) were both present. Overall time effects showed that values of p-AKT were increased at 5 min ($P<0.09$) after addition of test medium containing 50 ng/ml IGF1, peaked at 15–60 min ($P<0.001$) and were still raised at 24 h ($P<0.01$), but had returned to baseline within 48 h (Fig. 4A and B). The greatest treatment response was seen in control cells (IGF1 alone), and this was significantly reduced by the MEK inhibitor PD98059 ($P<0.01$) and to a greater extent by the PI3K inhibitor LY294002 either alone or together with PD98059 (both $P<0.001$ compared with control). The two forms of p-ERK, p-ERK1 (p44) and p-ERK2 (p42) were both detected at $t=0$ h. The overall treatment effect of inhibitors was highly significant ($P<0.001$) with p-ERK1/2 progressively decreased by LY294002 alone ($P<0.05$), PD98059 ($P<0.001$) and the combined inhibitor treatment ($P<0.001$). There was, however, no significant effect of time after IGF1 treatment and no treatment × time interaction. This implies that the MAPK pathway was not affected by IGF1 in the granulosa cells (Fig. 4C and D). The inhibition of p-ERK1/2 by LY294002 alone suggests that there is some degree of cross talk between the AKT and MAPK signalling pathways.

Effects of IGF1 and FSH on AKT and ERK signalling

We next examined the influence of FSH on IGF1-activated AKT and ERK signalling in bovine granulosa cells. Cell lysates were collected at 30 min after IGF1 treatment with or without FSH in the presence or absence of inhibitors and subjected to immunoblotting with respective antibodies. The ratios between phosphorylated and total protein were then calculated. In unstimulated cells, the expression of p-AKT was not detected, but IGF1 significantly ($P<0.01$) raised the levels of p-AKT. FSH alone did not produce any detectable level of AKT phosphorylation (Fig. 5A). Although the combined IGF1/FSH treatment resulted in a numerically higher level of phosphorylation than with IGF1 alone, this difference did not achieve statistical significance. The relationship between FSH/IGF1 stimulation and AKT phosphorylation was further determined by pretreatment with specific inhibitors. The PI3K inhibitor LY294002 alone, or together with the MEK inhibitor PD98059, tended to ($P<0.08$) reduce the

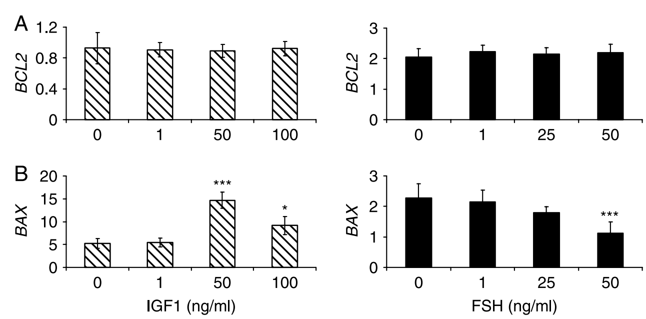

Figure 3 Dose-dependent effect of IGF1 and FSH on mRNA expression of (A) *BCL2* and (B) *BAX* in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently treated with IGF1 (0, 1, 50 and 100 ng/ml) or FSH (0, 1, 25 and 50 ng/ml) for a further 48 h in serum-free culture. The reverse-transcribed RNA from cellular extracts was amplified by SYBR Green real-time PCR, and results are expressed as fg/μg reverse-transcribed RNA. Data from four separate batches of cells were analysed by mixed model analysis, and results are presented as the mean ± S.E.M. With addition of IGF1, there were significant increases in *BAX*, whereas FSH caused a significant decrease: $^{*}P<0.05$, $^{***}P<0.001$ versus 0 control.

Table 2 Effects of insulin-like growth factor 1 (IGF1) and FSH on mRNA expression of *IGF1R* and *FSHR* in bovine granulosa cells.

	0	FSH	IGF1	FSH+IGF1
IGF1R	8.0 ± 0.80^a	8.5 ± 2.04^a	14.2 ± 1.14^b	18.5 ± 1.09^c
FSHR	0.9 ± 0.12^d	0.8 ± 0.20^d	2.0 ± 0.21^e	0.9 ± 0.06^d

Cells were treated with IGF1 (50 ng/ml) and/or FSH (25 ng/ml) for 48 h in serum-free culture. The reverse-transcribed RNA from cellular extracts was amplified by SYBR Green real-time PCR, and results are expressed as fg/μg reverse-transcribed RNA. Data from three separate batches of cells were analysed by mixed model analysis, and results are presented as the mean±S.E.M.: a<b, $P<0.05$; a<c, $P<0.001$; d<e, $P<0.01$.

p-AKT by 1.7- and 1.5-fold over the IGF1+FSH-treated group, whereas PD98059 alone had no effect (Fig. 5A). Phosphorylated ERK was observed at 30 min in all the treatment groups, and its concentration at this time did not differ significantly between cells treated with IGF1 and/or FSH. Pretreatment with PD98059 together with LY294002 significantly ($P<0.05$) inhibited phosphorylation of ERK1/2 when compared with the IGF1 alone, FSH alone or IGF1+FSH treatments, whereas LY294002 alone had no significant effect (Fig. 5B).

Role of AKT and ERK pathways in IGF1-induced up-regulation of mRNA expression of steroidogenic and apoptotic genes

Finally, we determined whether the up-regulation of steroidogenesis and *BAX* expression by IGF1 in bovine granulosa cells was mediated via AKT or ERK phosphorylation. Cells were treated with IGF1 for 48 h in the presence or absence of specific inhibitors. Pretreatment with the PI3K inhibitor LY294002 alone, or together with the MEK inhibitor PD98059, significantly ($P<0.05$) inhibited the IGF1-induced increase in expression of mRNA for *CYP11A1*, *HSD3B1* and *CYP19A1*. LY294002 pretreatment also tended ($P<0.1$) to prevent any increase in *BAX* and OE_2 production (Fig. 6). These results suggested that IGF1 induced up-regulation of mRNA expression of steroidogenesis and *BAX* requires activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells.

Discussion

Cows undergo two or three waves of follicular development during each oestrous cycle. At the start of each wave, several small 1–5 mm follicles begin to grow and at this point the future dominant follicle and largest

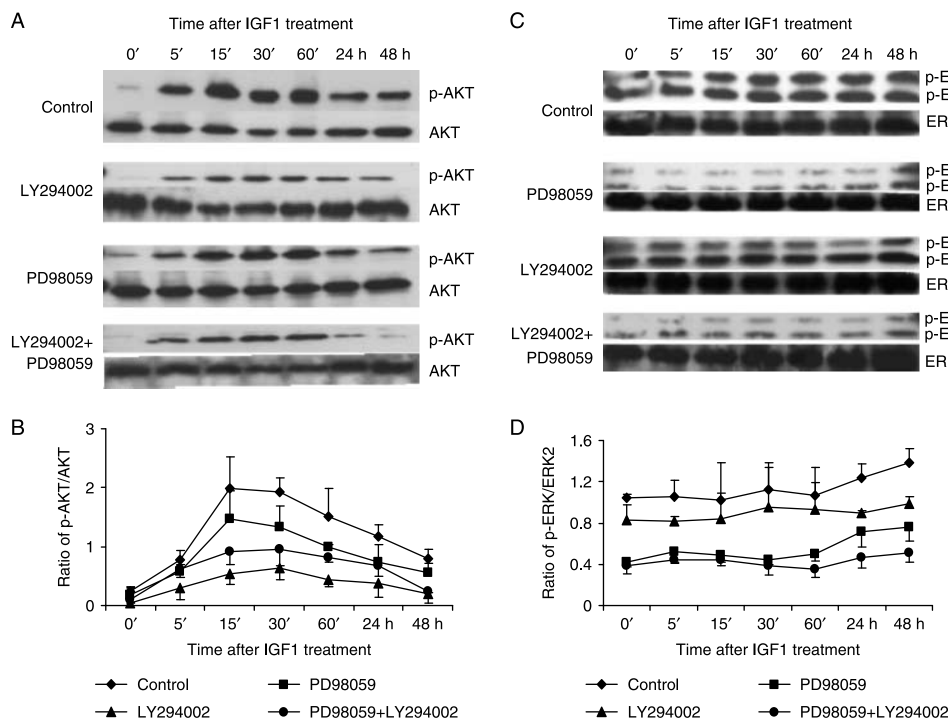

Figure 4 Activation of AKT and ERK by IGF1 in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently pretreated for 30 min with no inhibitor (control), 10 μM LY294002 (a specific PI3 kinase inhibitor), 15 μM PD98059 (a specific MEK inhibitor) or both inhibitors in combination. IGF1 (50 ng/ml) was added and cells were collected for preparation of lysates at 0, 5, 15, 30 and 60 min, and 24 and 48 h. (A) Representative immunoblots showing phosphorylated (p-AKT) and total AKT. (B) Ratio of the signal intensities of p-AKT/total AKT. (C) Representative immunoblots showing p-ERK1/2 and total ERK2. (D) Ratio of the signal intensities of p-ERK1/2/total ERK2. Data from three separate batches of cells were analysed by mixed model analysis, and results are presented as the mean±S.E.M. In relation to p-AKT, there were highly significant ($P<0.001$) effects of both time after IGF1 addition and inhibitor treatment, but no interaction (see text for further details). With respect to p-ERK1/2, inhibitor treatment was highly significant ($P<0.001$), but there was no significant effect of time after IGF1 and no treatment×time interaction as differences between treatments were already apparent before the addition of IGF1.

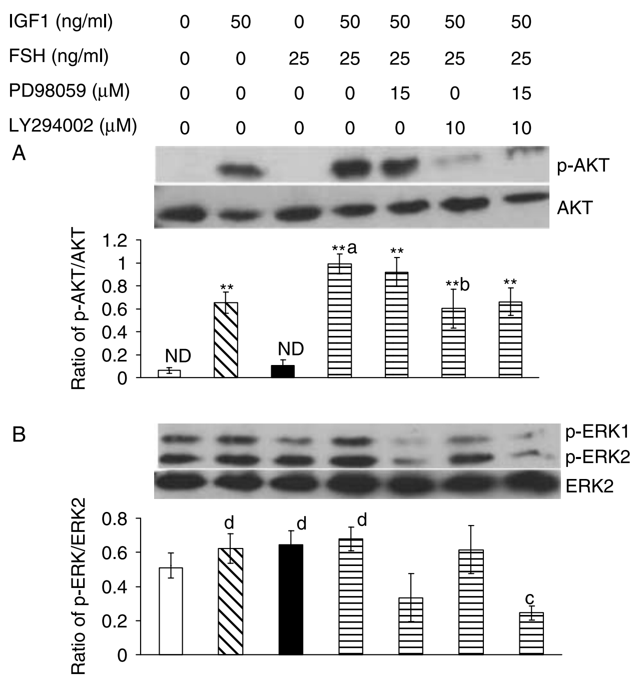

Figure 5 Effects of IGF1 and FSH on the activation of AKT and ERK in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently pretreated with no inhibitor (control), 15 μM PD98059 (a specific MEK inhibitor) and/or 10 μM LY294002 (a specific PI3 kinase inhibitor) for 30 min. Cells were then treated with IGF1 (50 ng/ml) or FSH (25 ng/ml) alone or in combination for 30 min in the presence or absence of the inhibitors. After incubation, cells were collected for preparation of lysates. (A) Representative immunoblots showing phosphorylated (p-AKT) and total AKT with histogram giving the ratio of the signal intensities of p-AKT/total AKT. (B) Representative immunoblots showing p-ERK1/2 and total ERK2 with histogram giving the ratio of the signal intensities of p-ERK1/2/total ERK2. Data from three separate batches of cells were analysed by mixed model analysis, and results are presented as the mean±s.e.m. For p-AKT, there was a highly significant overall treatment effect ($P<0.001$). All combinations except FSH alone increased p-AKT above the untreated control value (**$P<0.01$), which was not detectable (ND). There was a tendency for the addition of LY294002 to reduce the concentration of p-AKT below that found in the combined IGF1/FSH treatment (b<a, $P<0.08$). Neither IGF1 nor FSH alone or in combination increased p-ERK1/2 above control values. However, the combined PD98059+LY294002 treatment reduced p-ERK1/2 concentrations (c<d, $P<0.05$).

subordinate follicle are similar in size and growth rates. However, divergence soon occurs as OE_2 production increases and the future dominant follicle continues to grow in the face of declining plasma FSH, whereas the subordinate follicles do not (Mihm *et al*. 2000, Ginther *et al*. 2001). This difference is probably achieved through enhanced responsiveness of the dominant follicle to gonadotrophin, an effect which is possibly mediated through higher concentrations of OE_2 and free IGF1 present in the dominant follicle (Fortune *et al*. 2001, Quirk *et al*. 2004). In addition, granulosa cells from the selected dominant follicle increase their LH-binding capacity (Mihm & Evans 2008). Little is currently known about the mechanisms involved in the synergistic effects of FSH with IGF1 on ovarian cell function in cattle.

In agreement with previous studies on bovine granulosa cells, IGF1 significantly enhanced granulosa cell number (Spicer *et al*. 1993, Gutierrez *et al*. 1997), whereas FSH alone did not (Langhout *et al*. 1991, Gong *et al*. 1993). Although Ryan *et al*. (2008) did report a small increase in cell number following FSH treatment, this may reflect differences in experimental design, in particular their inclusion of insulin in the medium during the FSH treatment. Previous studies have reported that FSH in the presence of 10 ng/ml of insulin stimulated granulosa cell proliferation in both cattle and sheep (Campbell *et al*. 1996, Gutierrez *et al*. 1997). Similarly in our experiments, FSH in the presence of 50 ng/ml of IGF1 did synergistically stimulate cell number when compared with IGF1 alone. This effect is more likely due to cell proliferation than enhanced cell survival, but it is possible that both mechanisms were operational as we could not differentiate between them with our experimental design. It is thus clear that FSH alone had no direct effect on granulosa cell proliferation, but did enhance the sensitivity of granulosa cells to the mitotic effect of IGF1. It is possible that this effect could be mediated through the reported increase in IGF1R following FSH treatment.

The most effective dose of IGF1 tested was 50 ng/ml. This is slightly below the normal physiological range found in the follicular fluid of adult cows (e.g. 90–100 ng/ml, Funston *et al*. 1996). In the normal circulation, over 90% of IGF are complexed to IGF-binding proteins (IGFBPs; Jones & Clemmons 1995) and the same is true within follicular fluid (Monget *et al*. 1993, Funston *et al*. 1996, Webb *et al*. 2004). Although we did not add any IGFBPs to the culture medium, the granulosa cells expressed mRNA for both IGFBP2 and IGFBP5 (AM Mani 2008, unpublished observations), so it is likely that these proteins were present in the medium and would, therefore, have influenced the bioavailability of the added IGF1. The dose–response studies found no effects of FSH alone at concentrations between 1 and 25 ng/ml; only the higher dose of 25 ng/ml was subsequently tested in the synergistic experiments with IGF1. Basal concentrations of FSH in cows are around 20 ng/ml, peaking at 30–60 ng/ml during the pre-ovulatory surge (Taya *et al*. 1991, Mihm *et al*. 1997), so this effective concentration was also within the normal physiological range.

Our current data demonstrated the effects of IGF1 on three genes controlling the rate of steroid hormone synthesis, namely *CYP11A1*, *HSD3B1* and *CYP19A1* and related this to steroidogenic output by measuring OE_2 production. Treatment with IGF1 (50 and 100 ng/ml) significantly enhanced the mRNA expression of *CYP11A1*, an inner mitochondrial membrane protein that catalyses the conversion of cholesterol to

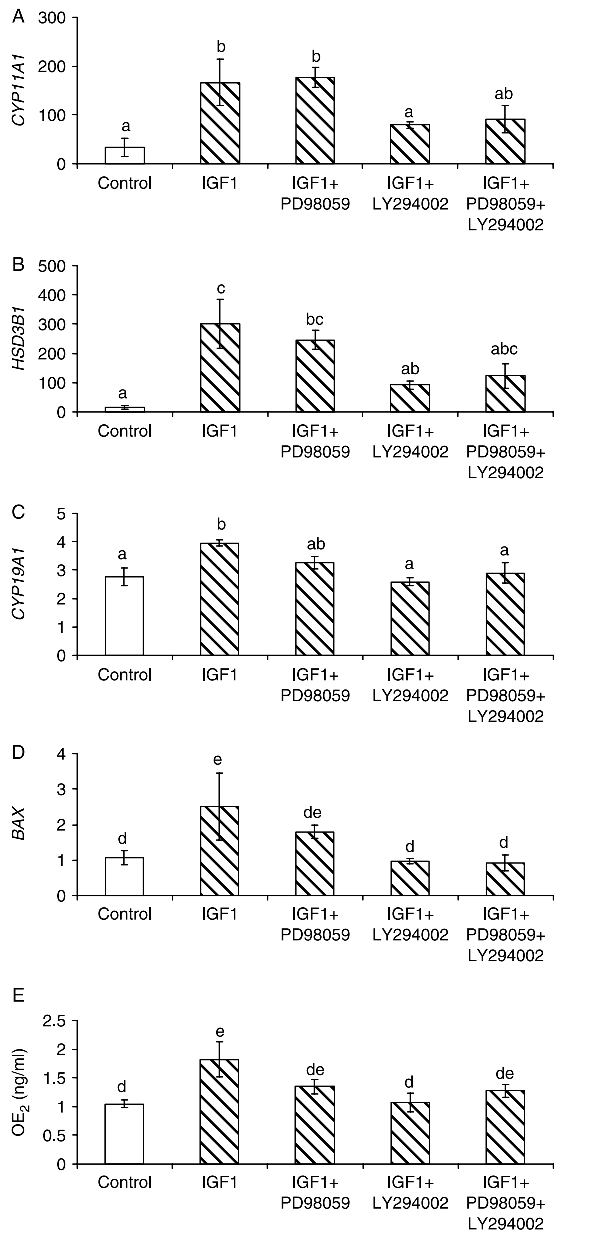

Figure 6 Effect of PD98059 and/or LY294002 on mRNA expression of the steroidogenic genes (A) *CYP11A1*; (B) *HSD3B1*; (C) *CYP19A1*; (D) *BAX*; and (E) OE_2 production induced by IGF1 in bovine granulosa cells. Cells were cultured for an initial 48 h establishment period in supplemented serum-free medium. Cells were subsequently pretreated with no inhibitor (control), 15 μM PD98059 and/or 10 μM LY294002 for 30 min. IGF1 (50 ng/ml) was then added and cells were cultured for a further 48 h. After the treatment period, the spent media were used for measuring OE_2 by RIA, and the cells were used for RNA isolation. The reverse-transcribed RNA was amplified by SYBR Green real-time PCR, and results are expressed as fg/μg reverse-transcribed RNA. Data from two separate batches of cells were analysed by mixed model analysis, and results are presented as the mean ± s.e.m. In all cases, the overall treatment effect was significant and IGF1 stimulated increases in comparison with the controls. This increase was consistently prevented by the presence of LY294002 either alone or together with PD98059. However, the addition of PD98059 alone did not significantly alter the response to IGF1: a<b<c, $P<0.05$; d<e, $P<0.1$.

pregnenolone. Similar results were previously observed in pig granulosa cells (Urban *et al*. 1990). In contrast, IGF1 alone did not produce any significant effect on *CYP11A1* message in rat granulosa cells, requiring the presence of FSH to produce a synergistic stimulation (deMoura *et al*. 1997, Eimerl & Orly 2002). FSH alone, or in synergy with IGF1, also enhanced mRNA expression of *CYP11A1* in pig granulosa cells (Urban *et al*. 1994). Silva & Price (2002) found that FSH but not insulin was important in maintaining *CYP11A1* expression in bovine follicles. Surprisingly, in the present study, addition of FSH was without significant effect on *CYP11A1* under either basal or IGF1-stimulated circumstances. These differences in results are currently hard to reconcile and indicate that the species, precise stage of follicular development and/or experimental treatment protocols can influence follicular *CYP11A1* expression through mechanisms which remain to be fully elucidated.

The pregnenolone produced by the action of CYP11A1 is subsequently converted to progesterone by HSD3B. The enhancement of *HSD3B1* transcripts by IGF1 treatment in the present study was consistent with work on rat granulosa cells (deMoura *et al*. 1997, Eimerl & Orly 2002). FSH alone also induced *HSD3B1* transcripts significantly in rat granulosa cells (deMoura *et al*. 1997), but not in the present study or in the work of Zheng *et al*. (2008), also on bovine tissue. In accord with previous work involving rat granulosa cells (deMoura *et al*. 1997, Eimerl & Orly 2002), there was no significant synergistic effect of IGF1 and FSH on *HSD3B1* expression. The increase in CYP11A1 and HSD3B1 stimulated by IGF1 may increase progesterone production *per se*, as well as providing precursor for OE_2 production. In accord with this, both Schams *et al*. (1988) and Ryan *et al*. (2008) found that addition of IGF1 increased granulosa cells' progesterone production. A similar action of IGF1 may therefore be of particular importance *in vivo* after the LH surge when follicles are starting to luteinize.

Similarly, we found that IGF1 significantly enhanced expression of *CYP19A1* mRNA and caused increased OE_2 production. Unlike *CYP11A1* and *HSD3B1*, the presence of FSH significantly enhanced IGF1-induced *CYP19A1*, consistent with previous reports in cultured bovine (Spicer *et al*. 2002, Ryan *et al*. 2008) and rat granulosa cells (Adashi *et al*. 1985). Although IGF1 and FSH produced a significant synergism on *CYP19A1* expression, there was no accompanying alteration in OE_2 production, possibly due to the relative sensitivities of the PCR and RIA methodologies employed. Moreover, it has been reported that, in the presence of FSH, the maximal stimulatory effect achieved by IGF1 on OE_2 production was only a fraction (8–20%) of that observed for insulin (Spicer *et al*. 2002). FSH alone had no effect on OE_2 production, but it significantly enhanced OE_2 production by bovine granulosa cells obtained from heifers pretreated with pregnant mare serum gonadotrophin and cultured in the presence of 20–1000 ng/ml of insulin (Saumande 1991). Thus, insulin may be a more important stimulator of OE_2 production by follicles than IGF1 in an FSH-rich environment.

To find out the apoptotic status of granulosa cells during IGF1 and FSH treatment, we also demonstrated the mRNA expression of *BCL2*, an anti-apoptotic gene, and *BAX*, a pro-apoptotic gene. The ratio between these two is the critical determinant of cell fate, such that elevated *BCL2* favours extended survival of cells, whereas increasing levels of *BAX* expression accelerate cell death (Oltivai *et al*. 1993, Williams & Smith 1993). IGF1 or FSH alone or together did not affect *BCL2* mRNA expression. Higher doses of IGF1 (50 and 100 ng/ml) increased, whereas higher doses of FSH (50 ng/ml) decreased, *BAX* mRNA expression. However, the anti-apoptotic FSH did not affect the up-regulation of *BAX* mRNA following IGF1. There was thus a significant decrease in the *BCL2/BAX* ratio when cells were treated with IGF1 (data not shown). This could be detrimental to cells, whereas higher dose of FSH (50 ng/ml) given alone increased the ratio, indicating improved potential for survival. The rise in *BAX* mRNA expression was not caused by reduced *IGF1R*, as mRNA expression of both genes was raised with the 50 ng/ml IGF1 dose. These results on gene expression contrast those on cell number, which was highest following IGF1 treatment and not influenced by FSH. Previous studies similarly found that IGF1 significantly stimulated cell proliferation in granulosa cell culture, but that FSH alone had no effect (Campbell *et al*. 1995, 1996).

It is possible that any decreases in cell number due to high concentrations of IGF1-inducing apoptosis may take longer than the 48 h culture period we tested to become manifest. Further studies involving a longer time course and incorporating TUNEL staining in addition to measurements of cell number as reported here would help to clarify this issue. According to Yang & Rajamahendran (2000), a low dose of IGF1 (10 ng/ml) attenuated apoptosis, while a higher dose of 100 ng/ml increased apoptosis in cultured bovine granulosa cells. A combination of treatment with FSH (1 ng/ml) and IGF1 (100 ng/ml) inhibited apoptosis, and a similar finding was observed in pigs (Guthrie *et al*. 1997) in that both IGF1 and FSH suppressed apoptosis in cultured granulosa cells. The mechanism by which IGF1 at 50–100 ng/ml can increase *BAX* expression (present study) and apoptosis (Yang & Rajamahendran 2000) is not clear. However, a high concentration of IGF1 (evident at >30 ng/ml) inhibited oestrogen production by granulosa cells from small (1–5 mm) cattle follicles (Spicer *et al*. 1994*a*). The data are also consistent with the fact that rapidly dividing cells are the most susceptible to apoptosis (Quirk *et al*. 2004), so high IGF1 may increase the vulnerability of granulosa cells to apoptosis in the defined medium used here in which other essential survival factors present in serum (e.g. insulin and LH) were lacking. Another possibility is that the induction of apoptosis requires an intermediary role of the IGFBP2 and -5, which are present in granulosa cells and can inhibit IGF1 bioactivity in follicles (Ui *et al*. 1989, Monget *et al*. 1993). Increased IGF bioactivity as a result of decreased follicular IGFBP production may be part of the local control mechanism that drives follicle growth. Conversely, increased IGFBP production by atretic follicles would be expected to decrease IGF bioactivity and could be a key factor in the initiation of atresia (Ui *et al*. 1989, Monget *et al*. 1993).

Their respective receptors are obvious candidates by which IGF1 and FSH can induce up-regulation of steroidogenic and apoptotic regulatory genes. In cows, *IGF1R*, the physiological target of both IGF1 and IGF2, is expressed in granulosa cells throughout follicle development, with higher levels of expression in healthy than atretic follicles (Perks *et al*. 1999, Armstrong *et al*. 2000). In the present study, the levels of both *IGF1R* and *FSHR* were increased significantly when the cells were cultured with IGF1 (50 ng/ml). FSH alone had no effect on *IGF1R* mRNA, but FSH (25 ng/ml) in the presence of IGF1 (50 ng/ml) enhanced *IGF1R*. FSH alone did not alter *FSHR* mRNA, but it prevented the increase in *FSHR* mRNA found with IGF1 treatment alone. This effect of FSH was unexpected, but may be associated with enhanced differentiation of granulosa cells. These results clearly indicated that IGF1 can in part up-regulate steroidogenic and apoptotic regulatory pathways through increasing receptor numbers for both IGF1 and FSH. In contrast, Minegishi *et al*. (2000) reported a synergistic effect of IGF1 and FSH on *FSHR* expression in rat granulosa cells. Furthermore, they provided evidence that the addition of IGF1 increased the half-life of the *FSHR* transcript, thus providing a further mechanism by which IGF1 can affect FSH action. As the IGF1 treatment used here also increased OE_2 production, it remains to be determined whether the effect on *IGF1R* was direct or mediated via OE_2. Spicer *et al*. (1994*b*) previously reported that OE_2 treatment can increase the number of IGF1R in granulosa cells from small bovine follicles.

The downstream mechanisms by which activated IGF1R influences the steroidogenic and apoptotic regulatory genes are poorly defined. The potential roles of the AKT and MAPK pathways in mediating the protective effect of IGF1 were examined because each of these pathways has been shown to have effects on cell survival as well as proliferation in a number of cell types (Gallaher *et al*. 2001). We present evidence that IGF1 stimulation alone caused an activation of PKB/AKT in granulosa cells, as evidenced by increased phosphorylation of AKT. These results are consistent with reports that survival of bovine granulosa cells in serum-free culture medium in response to IGF1 in the presence of 100 ng/ml insulin is associated with increased phosphorylation of AKT (Hu *et al*. 2004). Moreover, we found that inhibition of the PI3K pathway by LY294002 reduced the IGF1-stimulated mRNA expression of *CYP11A1*, *HSD3B1*, *CYP19A1*, *BAX* and OE_2 production. Similarly, addition of PI3K inhibitor significantly decreased the insulin-stimulated *CYP19A1* mRNA levels and OE_2 accumulation in bovine granulosa cells (Silva *et al*. 2006). Although there was a tendency for ERK1/2 phosphorylation to rise 24–48 h after the addition of IGF1 (Fig. 4), this was not statistically significant. A further complication was the finding that LY294002, a PI3 kinase inhibitor, also produced a modest reduction in the level of p-ERK1/2. This supports previous suggestions that there is some degree of cross talk between the AKT and MAPK signalling pathways (Kumar *et al*. 2008). In previous work on other cell types, IGF1 stimulated both PI3K and MAPK pathways, but only the PI3K pathway was required for protection against apoptosis (Kulik *et al*. 1997, Miller *et al*. 1997, Campana *et al*. 1999). Therefore, we concluded that IGF1-enhanced mRNA expression of steroidogenic and apoptotic regulatory genes was via the PI3K pathway and not by the MAPK pathway. It is possible that the enhancement of IGF1 action by FSH found in relation to cell number and increased expression of *IGF1R* and *CYP19A1* was mediated via further increases in AKT signalling. Although the numerical increase in AKT following the combined IGF1 and FSH treatment failed to achieve statistical significance, such an effect was reported by Ryan *et al*. (2008).

In summary, our results show that FSH acts synergistically with IGF1 to increase cell number and expression of *CYP19A1* mRNA in bovine granulosa cells, and that the actions of IGF1 involved the PI3K signalling cascade. The model we have used is most relevant to the growth phase of antral follicles during the start of a follicular wave. Our studies support a key role for IGF1 in the regulation of both proliferation and steroidogenesis in the dairy cow, where follicular development may be compromised in early lactation, in association with reduced circulating IGF1 concentrations (Wathes *et al*. 2003). Once selection occurs, the dominant follicle continues to grow and increase OE_2 production, whereas the subordinate follicles undergo apoptosis. The increased *BAX* mRNA expression found with IGF1 after 48 h in our serum-free culture system implies that apoptotic pathways were also up-regulated at this time. It is likely that further development at this stage would be dependent on the provision of LH, insulin and possibly other additional factors, which may be necessary to promote continued survival of the dominant follicle.

Materials and Methods

Reagents

All culture media and additives as well as kinase inhibitors (LY294002 and PD98059) were purchased from Sigma Chemical Co. Ovine FSH (Ovagen; 15.6–19.6 mg potency per vial relative to NIADDK-oFSH-17) was from ICPbio (Auckland, New Zealand). Recombinant human IGF1 was from Bachem (St Helens, Merseyside, England). Antibody against OE_2 was from Biogenesis (Poole, Dorset, UK). RNeasy mini kits were from Qiagen. Superscript first-strand synthesis system for RT-PCR was from Invitrogen. Absolute qPCR SYBR Green mix was from ABgene (Epsom, Surrey, UK). Rabbit anti-mouse p-AKT (Ser473, no. 9271) and total AKT antibodies (no. 9272) were obtained from Cell Signaling Technology (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire, UK). Mouse anti-human p-ERK (Tyr204, sc-7383) and polyclonal goat anti-rat ERK2 (sc-154G) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HRP-conjugated rabbit anti-goat IgG and HRP-conjugated goat anti-rabbit IgG were from Pierce Biotechnology (Chester, Cheshire, UK). HRP-conjugated goat anti-mouse IgG was from BD Biosciences (Oxford, UK).

Isolation and culture of granulosa cells

Freshly excised bovine ovaries were obtained from the local abattoir and transported in M199 at 37 °C (∼2 h) and then processed immediately. Granulosa cells from follicles sized 2–8 mm were isolated and cultured in serum-free McCoy's 5A medium as previously described (Gutierrez *et al*. 1997). Briefly, follicles were isolated manually by dissection and selected for isolation of granulosa cells if they appeared healthy based on a transparent appearance, highly vascularized theca and clear follicular fluid without any visible debris. Follicles were hemisected and granulosa cells were obtained by flushing the hemisected shells with medium and collecting the cell-rich supernatant. Cells were plated at a density of 5×10^5 cells/ml in 24-well plates (Iwaki, Osaka, Japan) using serum-free McCoy's 5A medium supplemented with bicarbonate (2.2 mg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (1.25 μg/ml), L-glutamine (3 mM/ml), BSA (1 mg/ml), androstenedione (10^{-7} M), transferrin (2.5 μg/ml), sodium selenite (0.04 ng/ml), bovine insulin (10 ng/ml), ovine FSH (1 ng/ml) and human rIGF1 (1 ng/ml). They were then cultured for a 48 h establishment period at 37 °C in humidified air with 5% CO_2. The hormone treatments during this initial phase were selected based on the study of Gutierrez *et al*. (1997) who established that this culture system could maintain a granulosa cell phenotype.

After the initial 48 h establishment period, the spent media were carefully removed and replaced with fresh medium prepared as above and including androstenedione, but without the other hormonal additives (insulin, FSH and IGF1) as standard. This was to remove the possibility that insulin might produce responses in addition to the IGF1 being tested. At this stage, cells were left untreated or were treated with different treatment doses of IGF1, FSH or a combination of both with or without inhibitors as indicated in the figure legends. After a further 48 h treatment period, media were carefully removed and stored at −20 °C until assayed, and cells from triplicate wells were pooled and used for RNA isolation. In some experiments, cells were pretreated with vehicle (0.007% DMSO) or inhibitors for 30 min before the addition of IGF1 ±FSH. In a separate series of experiments, cells from triplicate wells were collected separately and pooled for immunoblotting at various times from 0 to 48 h during the treatment period. For cell proliferation assay, cells (50×10^3) were plated in 250 μl culture medium per well in 96-well plates (Iwaki), but other aspects of the experimental treatments remained the same.

Monitoring cell growth and proliferation

Growing cells were photographed using a Canon PowerShot A540 digital camera (6MP 4× Optical) attached to an inverted microscope after the initial 48 h establishment period and again after the 48 h treatment. Viable cell numbers were measured after the 48 h treatment period using the Cell Titer96 Aqueous One Solution Cell Proliferation Assay (Promega). For this, 20 μl cell proliferation assay solution was added into each well of a 96-well culture plate containing cells in 100 μl culture medium and incubated for 4 h at 37 °C with 5% CO_2. At the end of the incubation period, the quantity of formazan product was measured as absorbance at 490 nm using a 96 well ELISA plate reader. This was directly proportional to the number of living cells in culture. Each treatment was performed in triplicate upon each batch of cells and cells from each well were assayed individually.

Hormone assay

OE_2 was measured in culture media by RIA as previously described (Lane & Wathes 1998). The sensitivity of the assay was 40 pg/ml. The intra- and inter-assay coefficients of variation were 7 and 9% respectively. Each treatment was performed in triplicate upon each batch of cells and media from each well were assayed individually.

RNA isolation and RT

Total RNA was isolated from cell cultures using an RNeasy mini kit (Qiagen) and quantitated using a NanoDrop spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE, USA). All samples had an A260/280 ratio of absorbance between 1.8 and 2.0. The integrity of isolated RNA was assessed by ethidium bromide stained as 28S and 18S ribosomal bands on agarose gel. Following DNase treatment (Promega Corporation), RNA was reverse-transcribed into first-strand cDNA using random primers and SuperScript II Rnase H^- Reverse Transcriptase (Invitrogen Ltd, Life Technologies) according to the manufacturer's protocols.

Primer design and optimization of RT-PCR

Assays were designed for steroidogenic genes (*CYP11A1*, *HSD3B1* and *CYP19A1*), apoptotic regulatory genes (*BCL2* and *BAX*) and receptors (*IGF1R* and *FSHR*). Primer sequences were taken from a previous reference (Fenwick *et al*. 2008) or were designed online using the Primer3 web software (http://frodo.wi.mit.edu/primer3/input.htm) based on coding regions of core bovine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/) or Ensembl (http://www.ensembl.org). Wherever possible, primers were designed to amplify products spanning the boundary of at least two adjacent exons. All oligonucleotides were synthesized commercially as highly purified salt-free products (MWG-Biotech AG, London, UK). The primer sequences, accession number and expected product lengths are listed in Table 3.

For each gene, PCR conditions were optimized by conventional PCR amplification using Platinum PCR SuperMix containing Taq polymerase (Invitrogen Ltd, Life Technologies) and the addition of 50 ng DNase-treated reverse-transcribed RNA and primers (20 μM). Once optimized, external standards were prepared from cDNAs identical to real-time PCR products and purified using QIAquick PCR purification columns (Qiagen). The precise concentrations of purified cDNA product were determined using the NanoDrop ND-100 spectrophotometer, and products were then diluted in nuclease-free water and used as qPCR standards. The identity of the cDNA products was confirmed by DNA sequences analysis (Geneservice Ltd, Cambridge, UK).

SYBR Green real-time PCR

Gene transcripts were quantified by real-time PCR using the DNA engine Option 2 (MJ Research Inc., Waltham, MA, USA). For each assay, a mastermix was prepared that contained a final concentration of 2× Absolute qPCR SYBR Green mix (ABgene), 500 nM forward and reverse primers and nuclease-free water. For all unknown samples measured, 20 μl reactions were prepared in white tubes (TLS-0851, Bio-Rad) and sealed with optical clarity flat caps (TCS-0803, Bio-Rad). Each reaction containing the above mastermix was added with 50 ng reverse-transcribed RNA and was analysed as duplicates. External standards were run on the same plate in triplicate 20 μl reaction volumes. Thermal cycling conditions applied to each assay consisted of an initial *Taq* activation step at 95 °C for 15 min followed by 38 cycles of denaturation, annealing, extension and an amplicon-specific fluorescence reading (Table 4). A melting curve analysis was performed for each amplicon between 50 and 95 to ensure any smaller non-specific products such as dimers (if present) were melted prior to fluorescence acquisition.

All quantitative PCR results were recorded with the Opticon Monitor analysis software. Background fluorescence was subtracted using the global minimum function, and the threshold was manually placed in the linear amplification phase. For comparison of expression data, absolute values expressed as fg/μg reverse-transcribed RNA were derived from the mean C_t value of all unknown samples. The stable expression levels of several genes were tested (data not shown) for normalization of qPCR data; however, no such transcripts were found to be satisfactory and therefore a set of highly pure and precise cDNA standards were generated to ascertain the absolute expression levels of unknown samples. Serial dilutions of each standard exhibited high amplification

Table 3 Oligonucleotide primer sequences and expected amplicon size used for real-time PCR assays.

Gene	Sequence (5′→3′)	Size (bp)	Accession no.
CYP11A1	For: AGACTTGGAGGGACCATGTAGC Rev: TGCCTGGGTAATTCCTAAATTC	117	ENSBTAT 00000009106
HSD3B1	For: AATCCGGGTGCTAGACAAAGT Rev: CACTGCTCATCCAGAATGTCTC	111	ENSBTAT 00000010992
CYP19A1	For: TGGCTGTGCAGAAAGTATGAA Rev: CAGTGGCGAAATCTATGCTGT	127	ENSBTAT 00000019823
BCL2	For: GTGGATGACCGAGTACCTGAAC Rev: AGACAGCCAGGAGAAATCAAAC	124	ENSBTAT 00000025701
BAX	For: GACATTGGACTTCCTTCGAGA Rev: AGCACTCCAGCCACAAAGAT	126	ENSBTAT 00000017739
IGF1R	For: GATCCCGTGTTCTTCTACGTTC Rev: AAGCCTCCCACTATCAACAGAA	101	X54980
FSHR	For: GCCAAGTCAACTTACCGCTT Rev: TGACCCCTAGCCTGAGTCAT	193	NM174061

CYP11A1, cytochrome P450 side chain cleavage; *HSD3B1*, 3β-hydroxysteroid dehydrogenase type 1; *CYP19A1*, cytochrome P450 aromatase; *BCL2*, B-cell lymphoma-2; *BAX*, Bcl-2 associated X protein; *IGF1R*, insulin-like growth factor receptor type 1; *FSHR*, FSH receptor.

efficiencies (all >0.91) and linearity (all >0.99). For each batch of cells, cell isolation and RNA measurement were performed using cells pooled from triplicate culture wells. The RNA was then converted to cDNA and this was used to generate duplicate measurements by qPCR, ensuring that equal amounts of cDNA were loaded into each well.

Immunoblotting

Cells were washed with ice-cold PBS (pH 7.5) and the culture wells were aspirated to dryness. Then, lysis buffer (63.5 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 10 μl of 1× protease inhibitor cocktail (Calbiochem, Sandiego, CA, USA) and 200 mM sodium orthovanadate) was added to the cells, which were then incubated on ice for 10 min. The cells were scraped from the culture wells into 1.5 ml microfuge tubes and were then boiled at 100 °C for 5 min and briefly centrifuged to pellet the cell debris, if present. After determination of approximate protein content using absorbance at 280 nm (NanoDrop ND-1000 spectrophotometer), bromophenol blue and β-mercaptoethanol were added to the samples to give final concentrations of 0.02% (w/v) and 5% (v/v) respectively. The samples were then stored at −20 °C until analysed.

Protein lysates (100 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Watford, Hertfordshire, UK). Membranes were blocked in Tris-buffered saline (TBS–T; 50 mM Tris, pH 7.4, 150 mM NaCl and 0.02% Tween-20) containing 10% (w/v) non-fat milk for 2 h at room temperature with gentle agitation before incubating overnight in TBS–T–10% BSA containing antibodies to p-AKT (dilution 1:1000) with gentle agitation at 4 °C or p-ERK (dilution 1:1000) with maximum agitation at room temperature. Membranes were then washed, incubated with HRP-conjugated secondary antibodies in TBS–T for 1 h at room temperature and washed. A chemiluminescent signal was generated using enhanced chemiluminescence reagent (ECL Amersham Biosciences), and membranes were exposed to X-ray film (ECL Amersham Biosciences). After detection of p-AKT and p-ERK, membranes were stripped by immersing in stripping buffer (6.25 mM Tris–HCl, pH 6.7, 2% (w/v) SDS and 0.7% (v/v) β-mercaptoethanol) and incubated for 30 min at 50 °C. Stripped blots were washed, blocked and re-probed with antibodies for total AKT (dilution 1:1000) with gentle agitation at 4 °C or ERK2 (dilution 1:1000) with maximum agitation at room temperature overnight. Signals were quantified by densitometry of digitalized images using Bio-Rad Molecular Quantity One software, version 4.4.0, and the ratios of p-kinases to total kinases were calculated.

Table 4 Specific cycling temperatures used for real-time PCR assays.

	Temperature (°C)			
Gene	Denaturation	Annealing	Extension	Fluorescence acquisition
CYP11A1	95	52.5	72	77
HSD3B1	95	54.2	72	78.5
CYP19A1	95	52.5	72	77
BCL2	95	52.5	72	77
BAX	95	52.5	72	82.5
IGF1R	95	55.9	72	78.5
FSHR	95	52.5	72	81.5

Statistical analysis

Data were analysed by mixed model analysis using SPSS v 17 (SPSS Inc., Chicago, IL, USA) using a random block design. This model assumes the variance within the block (in this case, each experimental batch of cultured granulosa cells) is homogeneous, whereas the variance between blocks may be heterogeneous. In this model, the treatments were taken as fixed effects and the batches (blocks) as random effects for correction of the difference between the batches. Within each batch, the experimental replicates were taken as repeated measurements. In time-course experiments, both treatment and time were included as fixed effects and their interaction was also tested. Bonferroni tests were used for comparison of means when overall significance (treatment effect of $P<0.05$) was observed. Each treatment was repeated on several separate batches of granulosa cells as reported in the figure legends. The values are presented as mean±s.e.m.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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