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Expression of activin pathway genes in granulosa cells of dominant and subordinate bovine follicles

B. Sisco, P.L. Pfeffer *

AgResearch, Ruakura Campus, East Street, Hamilton, New Zealand

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Abstract

We examined the association between the expression profiles of genes of the activin signalling pathway and ovarian follicular dominance in cattle. In monovular species such as cattle, one ovarian follicle of a cohort is selected to become dominant, whereas all others (i.e. the subordinate follicles) eventually succumb to apoptosis. We showed that *Inhibin-βA*, coding for the βA chain found in the A isoforms of activin, *Inh-α* coding for the inhibin-specific α chain, and the activin antagonist *follistatin* were expressed at higher levels in dominant follicle granulosa cells from Day 3.5 (ovulation = Day 0). Before selection, *Inh-βA* but not *Inh-α* was significantly correlated with potential dominant follicles, as defined by high *aromatase* expression and follicular fluid estrogen concentrations. *Follistatin* expression marked the largest follicles at Day 1.5, but displayed large variation in levels among cows. The third inhibin gene, *Inh-βB*, could only be detected at very low levels from Day 7 and thus was unlikely to play a prominent role in activin/inhibin signalling in cattle during these stages. There was a decrease in activin tone ($P = 0.07$) specifically in the aromatase-high/dominant follicles, as measured by the ratio of *Inh-βA* to *Inh-α* plus *follistatin* transcripts between Days 1.5 and 7. Messenger RNA for both *activin type II receptors* and the nuclear effector *Smad2* were detected in granulosa cells, consistent with an autocrine role for activin signalling. Additionally, expression of the putative activin target genes *Smad2* and *FSHreceptor* were, respectively, either strongly ($P < 0.001$) or weakly ($P = 0.09$) associated with dominant follicles.
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Keywords: Activin; Inhibin; Follistatin; Dominant follicle; Ovary; Bovine

1. Introduction

In monovular species such as cattle, a transient rise in systemic FSH concentrations nearly coincident with the time of ovulation recruits a cohort of antral ovarian follicles smaller than 4 mm to grow [1,2]. While the FSH concentrations decline, follicles continue to grow but within 48 h of the FSH peak, selection has occurred. One follicle, ~8 to 9 mm in diameter (generally slightly larger than the next-biggest follicles), has been selected as the dominant follicle and it alone continues to grow [3,4]. In contrast, the remaining follicles, termed subordinate follicles (SF), have a reduced rate of growth and undergo programmed cell death as FSH concentrations decrease below a critical threshold.

Genes responsible for the establishment of dominance are presently unknown. However, in our previous experiments in which we performed a differential subtraction of cDNA from the granulosa cells of bovine dominant and subordinate follicles near the time point of selection, we isolated the gene coding for one of the three inhibin genes, namely *Inh-βA* [5]. *Inh-βA* was upregulated in dominant follicles at Day 3.5, that is, after selection had occurred. *Inh-βA* codes

* Corresponding author. Tel.: +64 7 838 5002; fax: +64 7 838 5628.
E-mail address: peter.pfeffer@agresearch.co.nz (P.L. Pfeffer).

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for the inhibin-βA peptide which can homodimerise to form activin A or heterodimerise with inhibin-βB or inhibin-α to generate activin AB or inhibin A, respectively. Activin, which belongs to the TGF-β superfamily of growth factors, functions by binding to type I and type II activin receptors leading to signal transduction and gene regulation via the Smad 2/3 pathway. Inhibin acts antagonistically to activin by binding to the type II activin receptors in the presence of β-glycan [6].

The discovery of increased *Inh-βA* RNA levels (contributing to inhibin A *and/or* activin A or AB) in dominant follicles was interesting, as a clear endocrine role for inhibin in the negative feedback regulation of FSH release by the pituitary has been demonstrated [7]. Indeed, the decline in FSH prior to selection is caused by the FSH-dependent follicles themselves [8]. After selection, the dominant follicle assumes this negative feedback control, as shown by the increase in circulating FSH concentrations after dominant follicle ablation [9,10]. The negative feedback may be mediated by inhibin A [11–13], as well as estradiol [9,14] produced by the dominant follicle. Notably, activin, in contrast to inhibin, appears to act predominantly in an autocrine/paracrine fashion within ovarian follicles [15–17]. In vitro studies using granulosa cells demonstrated that activin could regulate cell proliferation, FSH receptor number, FSH-mediated gene expression, as well as steroid production [18]. In summary, local action of activin produced by the dominant follicle would be expected to aid follicle growth as well as mediating the reduced requirement for FSH that is characteristic of the dominant follicle [10], whereas systemic action of inhibin produced by the dominant follicle would reduce FSH concentrations, to the detriment of subordinate follicles.

Whereas several studies have addressed the follicular peptide levels of activin and inhibin during folliculogenesis and dominance [19–26], measurements of RNA levels of the corresponding genes are rare [27]. Building on our previous work, in the present study we examined the expression of all three *inhibin* genes in granulosa cells of dominant and subordinate follicles before and after selection. Furthermore, we analysed *follistatin* expression as this gene codes for a monomeric glycoprotein that can bind activin with high affinity (via the β-inhibin subunits), neutralising its biological activity [28]. Lastly, we explored the possibility of autocrine signalling in granulosa cells by measuring expression of activin signalling pathway genes and potential transcriptional targets.

2. Materials and methods

2.1. Animals

All procedures were conducted following approval by the Ruakura Animal Ethics Committee (number RAEC 3237), in accordance with the 1999 Animal Welfare Act of New Zealand. Synchronization of estrous cycles and collection of follicular material were done as previously explained [5]. Briefly, two intramuscular injections of 250 μg prostaglandin F2α ($PGF_{2\alpha}$) (Estroplan; Parnell Laboratories, NZ) were given 14 d apart to synchronise the estrous cycle. Twelve hours after the onset of estrus, all follicles ≥ 4 mm were ablated by ultrasound-guided transvaginal aspiration of follicle contents with a 7.5 MHz transducer (Aloka DX210, Medtel, Auckland), thus avoiding the inclusion of follicles from the previous follicular wave. The day of ovulation (Day 0) was defined by either natural ovulation or, in cases where the largest follicle had not yet ruptured, as the time of follicle ablation. Heifers were slaughtered at the local abattoir on Day 1.5 ($n = 3$), Day 2.5 ($n = 3$), Day 3.5 ($n = 4$) and Day 7 ($n = 2$). Follicles ≥ 4 mm were dissected out of all ovaries and external diameters measured. Follicular fluid (FF) was collected, centrifuged at 2000 × g for 3 min, and flash-frozen in liquid nitrogen. The granulosa cell pellet was washed in cold PBS and the pellet flash frozen.

2.2. Hormone assay

Estradiol 17-β concentrations in FF were measured by a double-antibody RIA kit (Estradiol-2 kit; DiaSorin, Saluggia, Italy). The assay sensitivity was reported to be <5 pg/mL at the 95% confidence limit. The coefficient of variation was 6%.

2.3. RNA extraction

Total RNA was extracted from frozen granulosa cells using TRIzol (Invitrogen, Auckland, New Zealand), according to the manufacturer's instructions. Granulosa cells from individual follicles were used for RNA extraction. RNA samples were DNase treated as follows: 1 μL 0.1 M DTT, 2 μL 10× DNase reaction buffer, 1 μL RNasin and 1 μL DNase I were added to each 15 μL RNA sample and incubated for 30 min at 37 °C, followed by a phenol:chloroform extraction and ethanol precipitation, and resuspension in 11 μL DEPC-treated water. One microliter of RNA was kept as reverse transcription negative control for use

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