Granulosa Cell Exposure to Excess Androgens Inhibits Their Ability to Proliferate in the Cow Which May Cause or Perpetuate Androgen Excess

Andrea S. Cupp, Sarah Romereim, Adam F. Summers, William E. Pohlmeier, Renee M. McFee, Renata Spuri-Gomes, Scott G. Kurz, Anthony K. McNeel, Robert A. Cushman, John S. Davis and Jennifer R. Wood

Summary

Within the UNL physiology herd, a group of cows have been identified with excess androgen (androstenedione, A4) in their dominant follicle (30 fold higher than controls) and a 17% reduction in calving rate, suggesting subfertility. The objective was to identify altered granulosa cell gene expression that could be preventing these cells from converting excess androgen into estrogen. Microarray analysis suggests these granulosa cells experience inhibited proliferation resulting in a reduced total population of cells. Improved understanding of the causes of this phenotype may provide beef producers with tools to identify potentially subfertile cattle and improve reproductive efficiency.

Introduction

Profitability of a beef herd is linked to a heifer or cow's ability to become pregnant within the first 21 d of the breeding season, allowing her to maintain a 365-d calving interval and wean a marketable calf each yr (*1988 Journal of Animal Science*, Hohenboken, pp. 1885–1891). Achieving this timing is largely dependent on cows ovulating each estrous cycle, which is largely dependent on the ovarian environment. In the UNL Physiology herd a group of cows with increased androgen production have been identified (*2012 Nebraska Beef Cattle Report*, pp. 28–29). This population of cattle produces calves with greater weaning weights (26 lb heavier) but tends to have reduced pregnancy rates (17% lower) compared with cows with low androstenedione (A4) concentrations.

Most steroid hormone production (steroidogenesis) occurs in the gonad. Increased follicular androgens are associated with decreased ovulation efficiency and fertility in cattle. Ovarian steroidogenesis, the process of creating steroid hormones within the ovary, occurs within the theca and granulosa cells of the follicle. These cell layers surround the oocyte; therefore, increased concentrations of steroid hormones likely affect oocyte quality (*2014 Nebraska Beef Cattle Report*, pp. 11–13), impacting fertility rates. Site specific enzymes within the granulosa cells are responsible for the conversion of androstenedione to estrogen, thus gene expression patterns within the granulosa cells could affect these enzymes or the genes responsible for producing these enzymes resulting in altered steroid hormone concentrations, creating an adverse environment for the developing follicle and oocyte. The objective was to determine if granulosa cell gene expression or function was altered in cows classified as High A4 compared with Low A4 cows and assess how these changes impact fertility.

Procedure

Estrus was synchronized in cows utilizing a Co-Synch + CIDR protocol for timed AI, with ovariectomy performed after. Cows received a single injection (100 µg/cow, i.m.) of GnRH (Cystorelin, Merial Limited, Duluth, GA) on treatment d 0 to induce ovulation and thus, initiate a new follicular wave. Also on d 0, an intravaginal insert (controlled internal drug release device [CIDR], Zoetis, Florham Park, NJ) containing 1.38 g of progesterone (P4) was inserted. Approximately 84 h prior to ovariectomy, cows were transported to the University of Nebraska-Lincoln Animal Science building for holding and surgery. The CIDR was removed on day 7 and cows received a single injection (25 mg/cow; i.m.) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; ProstaMate, AgriLabs, St. Joseph, MO). Thirty-six h after CIDR removal and $PGF_{2\alpha}$ administration, ovaries were removed via right flank laparotomy. Following removal, ovaries were measured and dominant follicles collected. Follicular fluid was aspirated from these follicles, and granulosa cells were removed via microdissection and messenger RNA was extracted. Messenger RNA was sent to University of Nebraska Medical Center to their microarray core and placed on Affymetrix chips to determine differences in genes expressed in High A4 classified cows (n = 5; excess A4 where A4 greater than 40 ng/ml in follicular fluid) vs. Low A4 classified cows (n-4; control; A4 less than 40 ng/ml in follicular fluid of dominant follicle).

Results

Statistics were performed (Analysis of Variance) and genes increased or decreased in granulosa cells from High A4 cows vs. Low A4 cows were selected based on statistical criteria ($P < 0.005$, False Discovery Rate < 0.05, fold-change > 1.5 or < -1.5). These criteria ensure the differences in expression of selected genes are not due to random variation between measurements or sampling error. The messenger RNAs for 166 genes were decreased and 90 genes were increased in granulosa cells from High A4 cows compared with Low A4.

To determine the biological relevance of these differences in gene expression, a software package called Ingenuity Pathway Analysis was used to categorize the genes and how they may affect normal cellular functions. Overwhelmingly, the most inhibited functions in High A4 granulosa cells involve cell cycle regulation. The analysis indicated granulosa cells from the High A4 cows experienced inhibition of proliferation. The expected decrease in total numbers of granulosa cells may explain why the follicle as a whole is not efficiently converting androgens to estrogens.

The major categories of genes with decreased expression included cell cycle, cell proliferation, and cellular growth and

Table 1. Categories of genes that are either increased or decreased in granulosa cells from High A4 vs. Low A4 cows

Categories	Diseases or functions annotation	*P*-value	Predicted activation state	Number of genes
Cancer	Incidence of malignant tumor	2.50E−04	Increased	9
Cancer	Incidence of tumor	3.63E−04	Increased	12
Cell cycle	Ploidy	6.01E−05	Increased	9
Embryonic development, organismal survival	Death of embryo	3.04E−05	Increased	9
Organismal survival	Organismal death	2.43E−05	Increased	54
Cancer	Cell transformation	4.68E−04	Decreased	15
Cancer	Transformation of fibroblasts	9.56E−03	Decreased	5
Cell cycle	M phase	1.14E−16	Decreased	24
Cell cycle	Cell cycle progression	2.78E−18	Decreased	52
Cell cycle	M phase of tumor cell lines	1.12E−15	Decreased	16
Cell cycle	Mitosis	2.13E−19	Decreased	37
Cell cycle	Interphase	3.23E−07	Decreased	25
Cell cycle	Entry into mitosis	1.48E−05	Decreased	5
Cell cycle	M phase of cervical cancer cell lines	1.99E−12	Decreased	12
Cell cycle, cellular movement	Cytokinesis	1.18E−10	Decreased	16
Cell cycle, cellular movement	Cytokinesis of tumor cell lines	1.33E−11	Decreased	11
Cell death and survival	Cell survival	2.67E−03	Decreased	30
Cell death and survival	Cell viability	3.15E−03	Decreased	28
Cell death and survival	Cell viability of tumor cell lines	8.80E−04	Decreased	20
Cell death and survival	Cell viability of myeloma cell lines	9.12E−03	Decreased	4
Cellular assembly and organization	Organization of cytoskeleton	6.23E−04	Decreased	32
Cellular assembly and organization	Organization of cytoplasm	1.41E−03	Decreased	33
Cellular assembly and organization	Microtubule dynamics	3.63E−04	Decreased	29
Cellular assembly and organization	Formation of microtubules	7.77E−04	Decreased	5
Cellular growth and proliferation	Proliferation of tumor cell lines	1.76E−08	Decreased	45
Cellular growth and proliferation	Proliferation of breast cancer cell lines	2.47E−05	Decreased	16
Cellular growth and proliferation	Proliferation of fibroblasts	2.28E−03	Decreased	11
Cellular growth and proliferation	Proliferation of cells	4.70E−05	Decreased	73
DNA replication, recombination, and repair	Alignment of chromosomes	5.49E−16	Decreased	11

proliferation (Table 1). A wide variety of growth factor gene potential networks were down regulated in granulosa cells from the High A4 cows including Epidermal Growth Factor (EGF), Platelet Derived Growth Factor BB (PDGF BB), Leukocyte Inhibitory Factor (LIF), Vascular Endothelial Growth Factor A (VEGFA), and Hepatocyte Growth Factor (HGF). A major function of these factors is to stimulate growth and proliferation by regulating the cell cycle and promoting increases in cell size and number.

Understanding what is different about the granulosa cells from High A4 cows compared with Low A4 cows might allow us to develop techniques to enhance the fertility of affected cattle. This could be accomplished by treatments to increase granulosa cell proliferation survival to ensure conversion of A4 to estrogen thus preventing androgen excess. Better estrous synchronization techniques to ensure ovulation occurs in the affected females may also be developed.

Andrea S. Cupp, professor Animal Science, Lincoln

Sarah Romereim, postdoctoral research associate

Adam F. Summers, former postdoctoral research associate

William E. Pohlmeier, research technician

Renee M. McFee, graduate student

Renata Spuri-Gomes, graduate student

Scott G. Kurz, research technician

Anthony K. McNeel, former US Meat Animal Research Center postdoctoral research associate

Robert A. Cushman, research physiologist U.S. Meat Animal Research Center, Clay Center, NE

John S. Davis, Professor, Department of OBGYN, University of Nebraska Medical Center, Omaha, NE

Jennifer R. Wood, associate professor Animal Science, Lincoln