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Adam F. Summers
University of Nebraska–Lincoln

William E. Pohlmeier
University of Nebraska–Lincoln

Vanessa M. Brauer Brauer
University of Nebraska–Lincoln

Kevin M. Sargent
University of Nebraska–Lincoln

Renee M. McFee
University of Nebraska–Lincoln, rmcfee3@unl.edu

See next page for additional authors

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Authors
Adam F. Summers, William E. Pohlmeier, Vanessa M. Brauer Brauer, Kevin M. Sargent, Renee M. McFee, Scott G. Kurz, Robert A. Cushman, Jennifer R. Wood, and Andrea S. Cupp

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Androgen Excess in Beef Cows Results in Altered Theca Cell Gene Expression and Fertility

Adam F. Summers
William E. Pohlmeier
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Kevin M. Sargent
Renee M. McFee
Scott G. Kurz
Robert A. Cushman
Jennifer R. Wood
Andrea S. Cupp[1]

Summary

Within the University of Nebraska–Lincoln physiology herd, two sub-populations of cows with different concentrations of androstenedione have been identified. Androstenedione is a precursor for estradiol production, and androstenedione concentration is increased 24.5-fold in the high androstenedione cows. Our objective was to determine the cause of increased androstenedione production in high androstenedione cows and the effects on theca cell and oocyte gene expression. High androstenedione cows had increased steroidogenic enzyme abundance in theca cells and altered oocyte mRNA abundance. Increased androgen production in high androstenedione cows is associated with altered gene expression and/or mRNA stability during oocyte growth and maturation, which may reduce fertility.

Introduction

Profitability is directly related to the ability of a cow to maintain a 365-day calving interval and wean a marketable calf each year. Consequently, the main reason cows are removed from the production herd is the inability to maintain pregnancy. Early embryonic mortality results in loss of 20 to 44% of pregnancies in beef cattle. Thus, development of tools or markers to help predict fertility in beef cattle could decrease the number of low fertility heifers developed and placed in the herd. Many factors can impact fertility, including follicle quality and ovarian environment.

Steroidogenesis, or the conversion of cholesterol to estradiol (E2), occurs within the theca and granulosa cells of the ovarian follicle through actions mediated by specific steroidogenic enzymes. Previous studies have reported altered steroidogenic enzyme expression, which results in increased androgen hormone production, leads to increased androstenedione (A4) production and reduced fertility in women (polycystic ovary syndrome; PCOS). Differential production of A4 in sub-populations within the UNL physiology herd has been previously reported (*2012 Nebraska Beef Cattle Report*, pp. 28-29). The objective of this study was to identify differences in mRNA abundance of theca steroidogenic enzymes and oocyte maternal effect genes collected from these two cow sub-populations.

Procedure

All procedures were approved by the University of Nebraska–Lincoln Institutional Animal Care and Use Committee. Non-lactating, composite [25% MARC III (¼ Angus, ¼ Hereford, ¼ Pinzgauer, ¼ Red Poll) and 75% Red Angus] beef cows from the beef physiology herd at the University of Nebraska Agricultural Research and Development Center (ARDC), near Mead, Neb., were used in this study.

Estrus was synchronized in (n = 64) utilizing a Co-Synch + CIDR protocol for timed artificial insemination, with ovariectomy performed after. Cows received a single injection (100 µg/cow; i.m.) of GnRH (Cystorelin, Merial Limited, Duluth, Ga.) on treatment day 0 to induce ovulation and, thus, initiate a new follicular wave. Also on day 0, an intravaginal insert [controlled internal drug release device (CIDR), Zoetis, Florham Park, N.J.] containing 1.38 g of progesterone (P4) was inserted. Approximately 84 hours prior to ovariectomy, cows were transported to the UNL 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 hours 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, the cumulus-oocyte complex (COC) was retrieved, and the theca cells were removed via microdissection.

Follicular fluid E2 and P4 concentrations were determined by radioimmunoassay (RIA). Follicular fluid A4 and dehydroepiandrosterone (DHEA) concentrations were determined utilizing a human A4 ELISA kit (Alpha Diagnostics International, San Antonio, Tex.) and DHEA ELISA kit (Fitzgerald Industries International, Acton, Mass.), respectively. Follicles determined to be E2 active (E2:P4 ratio > 1) were utilized for data analysis. Cows were classified as high A4 (HIGH A4) or low A4 (LOW A4) based on follicular fluid A4 concentration (HIGH A4 > 40 ng/mL; LOW A4 < 20 ng/mL). Total RNA was extracted from theca cells and COCs for quantitative RT-PCR to evaluate mRNA abundance for steroidogenic enzymes, vascular endothelial growth factor A (*VEGFA*) receptors and isoforms, and maternal effect genes.

Primers were also designed for the constitutively expressed mRNAs, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L 15 (*RPL-15*), and ribosomal protein L 19 (*RPL-19*). The stability of the constitutively expressed mRNAs was

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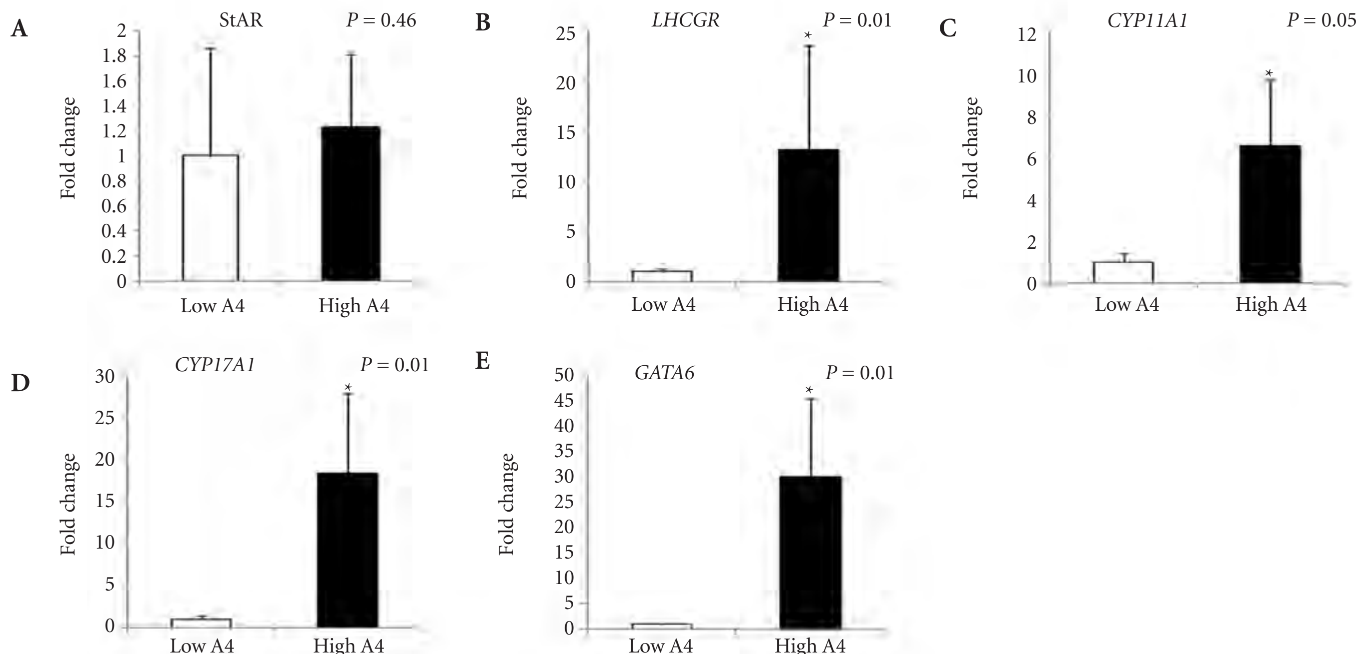

Figure 1. HIGH A4 Cows have increased steroidogenic gene expression. Quantitative RT-PCR results for steroid acute regulatory protein (*StAR*; A), luteinizing hormone/choriogonadotropin receptor (*LHCGR;* B), cholesterol side chain cleavage enzyme (*CYP11A1;* C), 17α-hydroxylase/17,20 lyase (*CYP17A1; D*), and transcription factor *GATA6* (E) in theca cells from dominant follicles of HIGH and LOW A4 cows. The geometric mean of *GAPDH* and *RPL-15* was used as an endogenous control to account for differences in starting material. Data for *CYP11A1*, *CYP17A1*, *LHCRG*, and *GATA6* were log transformed to meet normal distribution assumptions. Graphs were represented as a fold change with LOW A4 set as control (1). The mean ± SEM normalized values are presented from LOW A4 n ≥ 12 and HIGH A4 n ≥ 19. A $P \leq 0.05$ was considered significant.

calculated using Normfinder and based on this analysis, candidate gene mRNA abundance was normalized using the geometric mean of *GAPDH* and *RPL-15*. The resulting normalized data for each candidate mRNA was then compared to the mean normalized mRNA abundance in LOW A4 samples and expressed as a fold change.

Ovariectomies were performed over a 5-year period with approximately 10 to 14 cows ovariectomized during each replicate. Thus each surgery period was considered a replicate and animal was considered the experimental unit. Data were analyzed utilizing the MIXED procedure of SAS (SAS Institute, Inc., Cary, N.C.) with A4 classification considered the main effect and replicate a random effect. The original model included A4 classification and age as fixed effects with replicate as the random effect. Age was not significant, and thus was removed from the model. Data were log transformed where appropriate to meet normal distribution assumptions. A *P*-value ≤ 0.05 was considered significant.

Results

Concentrations of E2 in the follicular fluid tended ($P = 0.07$) to be greater for HIGH A4 compared with LOW A4 cows. However, there was no difference ($P = 0.15$) in P4 concentration based on A4 classification. Concentration of DHEA (a precursor of A4) was 2.7-times greater ($P < 0.0003$) in the follicular fluid of HIGH A4 cows compared with LOW A4 cows. Similarly, A4 concentration was approximately 19-times greater ($P < 0.01$) in the follicular fluid of HIGH A4 cows. Although the ratio of E2:A4 was 12.4 times greater ($P < 0.01$) for LOW A4 cows, the ratio of A4:P4 was greater ($P < 0.01$) in the HIGH A4 cows.

Theca cells are important in the regulation of steroidogenesis in the ovary. Steroidogenic enzyme gene expression, LH receptor (*LHCGR*), and growth factors regulating angiogenesis were analyzed. Binding of steroid acute regulatory protein (*StAR*) to the mitochondrial membrane resulting in cholesterol binding sites is the rate limiting step in steroidogenesis and is required to transport cholesterol into the mitochondria. There was no difference in *StAR* ($P = 0.46$, Figure 1A) mRNA expression between HIGH A4 cows and LOW A4. However, *LHCGR* ($P = 0.01$; Figure 1B) mRNA expression was increased 13.1-fold in theca cells of cows. *CYP11A1*, which is responsible for the conversion of cholesterol to pregnenolone, mRNA abundance was 6.5-fold greater

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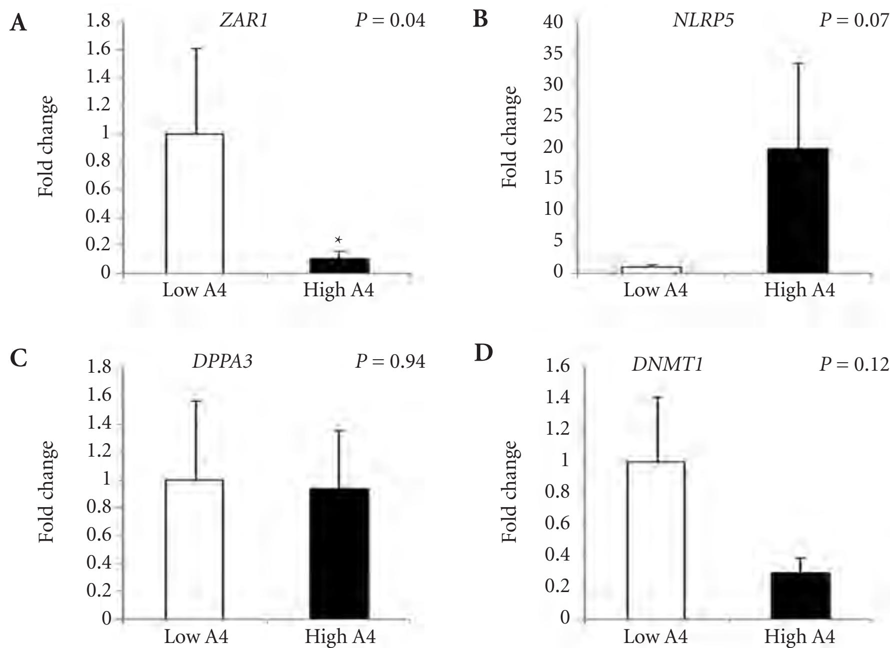

Figure 2. Maternal effect gene *ZAR1* mRNA abundance is reduced in HIGH A4 compared with LOW A4 cows. Quantitative RT-PCR results for zygote arrest-1 (*ZAR1*; A), NLR family, pyrin domain containing 5 (*NLRP5*; B), developmental pluripotency associated protein 3 (*DPPA3*; C), and DNA methyltransferases-I (*DNMT1*; D) in cumulus oocyte complexes from dominant follicles of HIGH and LOW A4. The geometric mean of *GAPDH* and *RPL-15* was used as an endogenous control to account for differences in starting material. Graphs were represented as a fold change with LOW A4 set as control (1). Data for *NLRP5, DPPA3,* and *WEE-1* were log transformed to meet normal distribution assumptions. The mean ± SEM normalized values are presented from LOW A4 n ≥ 5 and HIGH A4 n ≥ 3. *P* ≤ 0.05 was considered significant.

(*P* = 0.05, Figure 1C) in HIGH A4 cows compared with controls. Furthermore, *CYP17A1*, which is responsible for the conversion of pregnenolone to 17-OH pregnenolone and ultimately dehydroepiandrosterone (DHEA), mRNA abundance increased (*P* = 0.01, Figure 1D) 18.4-fold compared with LOW A4 cows. Expression of GATA-binding factor 6 (*GATA6*) has previously been reported to increase promoter activities of *CYP11A1* and *CYP17A1.* We report HIGH A4 cows have a 30-fold increase in expression of *GATA6* mRNA in theca cells compared with LOW A4 cows (*P* = 0.01, Figure 1E). Thus, it is likely the increased *GATA6* expression reported in the current study, although as a trend, increases regulation of the steroidogenic factors previously mentioned.

Maternal effect genes are important in promoting survival during early embryogenesis. Messenger RNA abundance of the maternal effect gene, *ZAR1*, was reduced 10-fold in HIGH A4 (*P* = 0.04, Figure 2A) compared with LOW A4 cows. There was no difference in *DNMT1* (*P* = 0.12, Figure 2D); however, *NLRP5* gene expression tended to be increased 19.8-fold in HIGH A4 cows (*P* = 0.07, Figure 2B). Whereas expression of *DPPA3* (Figure 2C, *P* = 0.94) mRNA was similar for HIGH and LOW A4 cows. The embryonic block coincides with the time that maternal genome activation is transferred to embryo genome activation; thus, alterations in maternal effect gene expression could be partially responsible for impaired fertility in the HIGH A4 cows.

Cows classified as HIGH A4, have altered steroidogenesis with increased expression of *CYP17A1* and *CYP11A1* steroidogenic enzyme mRNA abundance. Furthermore, these cows have increased concentrations of the E2 precursors, DHEA, and A4. These phenotypes are similar to a disorder in women with androgen excess and impaired fertility, PCOS. Theca cells from PCOS women have increased expression of the steroidogenic enzymes, *CYP11A1* and *CYP17A1.* Similarly, these patients also present increased expression of *GATA6.* Increasing our understanding of differential production of A4 in our subpopulations of cows will improve our knowledge regarding reduced fertility in beef cattle and potentially aid in developing improved synchronization protocols. Also, identifying specific genes associated with reduced fertility may aid in the development of genetic markers that will allow producers to cull potentially low fertility heifers at weaning.

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[1]Adam F. Summers, postdoctoral research associate; William E. Pohlmeier, research technician; Vanessa M. Brauer, former research technician; Kevin M. Sargent, graduate student; Renee M. McFee, graduate student; Scott G. Kurz, research technician; Robert A. Cushman, research physiologist, U.S. Meat Animal Research Center, Clay Center, Neb.; Jennifer R. Wood, associate professor; Andrea S. Cupp, professor, University of Nebraska–Lincoln Department of Animal Science, Lincoln, Neb.

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