Domestic Animal Endocrinology 51 (2015) 8–18

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Ovariectomy in young prepubertal dairy heifers causes complete suppression of mammary progesterone receptors

B.T. Velayudhan [a], B.P. Huderson [a], S.E. Ellis [b], C.L. Parsons [a], R.C. Hovey [c], A.R. Rowson [c], R.M. Akers [a,*]

[a] Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[b] Department of Animal and Veterinary Sciences, Clemson University, SC 29634, USA
[c] Department of Animal Science, University of California, Davis, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 30 September 2014
Accepted 31 October 2014

Keywords:
Estrogen receptor
Mammary
Ovariectomy
Progesterone receptor
Bovine

ABSTRACT

Mammary growth and development depends on ovarian steroids and particularly interaction of estrogen and progesterone with their intracellular receptors. The objectives of this study were to determine the effect of ovariectomy on the expression of protein and messenger RNA for estrogen receptor-alpha (ESR1) and progesterone receptor (PGR) and their relation to mammary ductal development and cell proliferation. Prepubertal Holstein heifers 2, 3, or 4 mo of age were randomly assigned to one of 2 treatments, ovariectomized (OVX; n = 8) or sham operated (INT; n = 12). Mammary parenchymal (PAR) tissue samples were harvested 30 d after surgery. Localization and quantitation of ESR1 and PGR in PAR were determined by immunohistochemistry and quantitative multispectral imaging. Relative messenger RNA expression of ESR1 and PGR in PAR was measured by quantitative real time polymerase chain reaction. We observed the complete absence of PGR-positive epithelial cell nuclei and reduced PGR transcript abundance in mammary parenchyma of OVX heifers. The percent of epithelial cells expressing ESR1 did not differ by treatment but was decreased with age. However, average intensity of ESR1 expression per cell was reduced in OVX heifers. The abundance of Ki67 labeled epithelial cells and stromal cells was reduced after ovariectomy. These data suggest that reduced mammary development after ovariectomy may be mediated by loss of PGR expression and reduced ESR1 expression in positive cells. A presumptive relationship with ovarian-derived circulating estradiol remains unresolved, but data suggest other ovarian-derived agents may play a role. Use of specific antagonists to manipulate expression or action of PGR and ESR1 receptors should provide direct evidence for roles of these receptors in prepubertal bovine mammary development.

1. Introduction

Prepubertal mammary gland development is important because the growth of the gland at this time creates the rudimentary structures required for future alveolar development [1]. It is well recognized that the ovary and especially estrogen and progesterone are critical for mammary growth and development with the onset of puberty and during gestation. However, the significance of these steroids and their receptors in neonatal and early prepubertal bovine mammary development is not well defined. In general, estrogen and growth hormone are recognized as the classic mammogenic hormones impacting ductal development with progesterone believed to be most important with the onset of alveolar development during gestation [2–5]. Prior ablation studies have shown

* Corresponding author. Tel.: +1 540 231 6331; fax: +1 540 231 5014.
E-mail address: rma@vt.edu (R.M. Akers).

0739-7240/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.domaniend.2014.10.002

that overall prepubertal mammary growth in dairy heifers is impaired by the removal of ovary [6–9].

Estrogen and its receptors are unequivocally critical to mammary ductal development and progesterone receptor (PGR) is a well-recognized ER-responsive gene. However, the significance of progesterone and PGR in prepubertal bovine mammary development is unclear. Estrogen and progesterone produce their effects on mammary tissue by acting through their intracellular receptors ER and PGR, respectively. The 2 isoforms of ER namely, estrogen receptor-alpha (ESR1) and ESR2 belong to a nuclear receptor superfamily and are encoded by different genes. These isoforms have been identified in many tissues in various species [10,11]. ESR1 is the major isoform of ER which mediates the effects of estrogen, including the induction of PGR expression (for review [12]). Schams et al [13] and Connor et al [14] reported predominance of ESR1 in bovine mammary gland compared with ESR2. Progesterone receptor also has 2 isoforms, A and B which are transcribed from the same gene [15]. Estrogen enters the target cells and binds to the ER in estrogen-responsive cells. Much research has been done with primates and rodents to understand the regulation of expression of steroid receptors during mammary gland development. Remarkable impairment in ductal development was noted in the mammary glands of ESR1-knockout mice, and these changes were not reversible with estradiol injections [16]. However, the action of progesterone in prepubertal mammary gland differs considerably with species. Progesterone increases ductal branching and terminal end bud formation thereby augmenting ductal morphogenesis in prepubertal mice [17]. However, considerable ductal branching occurs in prepubertal heifers well before the increased production of progesterone linked with the onset of puberty. Neither are there end buds in the bovine mammary gland [18]. Thus, the role of progesterone and PGR in the neonatal and prepubertal bovine with respect to mammary development and relation with estrogen and ESR1 is unexplored. Moreover, PGR knock-out mice develop normal mammary glands [19] implying that progesterone is not an absolute necessity for prepubertal mammary growth even in mice. Regardless, estrogen and ESR1 together play an important role in proliferation of mammary epithelial cells [20]. Progesterone receptor is also a known estrogen induced target gene and is proposed as an indicator of ER function in human breast cancer [21]. We hypothesized that ovariectomy would down regulate the expression of both progesterone and estrogen receptors in the prepubertal bovine mammary gland. When linked with impaired mammary development after ovariectomy this would support the idea that these receptors are drivers of prepubertal mammary growth as well. We also wished to determine if changes in receptor expression (proportion of positive cells and/or level of receptor expression per cell) would be associated with clusters of proliferating cells or correspond with altered rates of epithelial cell proliferation generally. The main objective of our study was to determine if ovariectomy affects the proportion of mammary epithelial cells that express PGR or ESR1 or the degree of receptor expression per cell. A specific objective was to determine if staged ovariectomy well before the onset of puberty affects these parameters. We reasoned that the impact of ovariectomy might well be related to the timing of ovariectomy, this then explained our design for ovariectomy at 30 d of intervals during the early prepubertal period of development.

2. Materials and methods

2.1. Animals and treatments

All the procedures and animal care protocols followed in this study were approved by the Clemson University Institutional Animal Care and Use Committee, as described previously [9]. Briefly, the heifers were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions before weaning and fed with grains and hay thereafter. Heifers were randomly assigned to either ovariectomy (OVX; $n = 8$) or sham operation (INT; $n = 12$) when they were either 60 ± 1, 93 ± 3, or 122 ± 2 d of age. Surgeries were performed by laparoscopic vertical incision of about 10 cm on the left flank after desensitizing the area with an injectable solution of 2% lidocaine HCl (AGRI Laboratories, St. Joseph, MO, USA). Removal of ovaries was achieved by using a Meagher Ovary Flute (c/o Harry Disney DVM, 2582 Bear Creek Road, Libby, Montana 59923; www.spaytool.com). A sham operation was conducted in intact heifers in which the ovaries were left intact. Mammary tissues were harvested after 30 d for histologic and gene expression analyses. Removal of ovaries was confirmed at slaughter. Any suspect tissue found at slaughter was subjected to histologic evaluation to rule out the presence of ovarian tissue remnants. This explains unequal numbers of heifers in each of the treatment groups as previously described [9]. Blood samples were collected from both OVX and INT heifers before surgery and 30 d after surgery at the time of tissue harvest.

2.2. Estradiol assay

Plasma concentrations of estradiol were determined using a commercially available RIA kit (Ultra-sensitive estradiol assay; DSL-4800, Diagnostic Systems Laboratory, Webster, TX) which is currently sold by Beckman Coulter (Brea, CA 92822). Procedures were essentially as described by Gibbons et al [22] for the bovine. Twenty-five milliliter glass tubes with Teflon-lined screw caps were acid washed before the adding the plasma samples. Five milliliter of plasma was added to assay tubes, followed by 10 mL of diethyl ether (E138–4, Fisher Scientific, Pittsburgh, PA 15275). The tubes were vortexed for 2 min (VWR Model DVX-2500 Multi-tube vortex, VWR Scientific, Radnor, PA 19087) operating at 2500 rpm. The tubes were then placed in a dry ice ethanol bath, and the lower aqueous layer was frozen, and the upper organic phase was decanted into acid washed 15 mL Teflon-lined screw top glass tubes. The decanted tubes were placed into an N-EVAP 111 nitrogen evaporator (Organomation Associates, Inc Berlin, MA 01503), and the organic phase was allowed to evaporate under a stream of nitrogen and gentle heating (38°C –40°C). The extraction process was repeated 3 times, and the dried tubes were capped until further processing. Once all the