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Developmental and Nutritional Regulation of the Prepubertal Bovine Mammary Gland: II. Epithelial Cell Proliferation, Parenchymal Accretion Rate, and Allometric Growth

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ABSTRACT

It is well documented that elevated nutrient intake prior to puberty reduces prepubertal mammary development in the bovine. The companion paper demonstrated that age at harvest is a primary determinant of parenchymal (PAR) mass and that any effects of elevated energy intake on mechanisms regulating mammary development are dwarfed by this effect of time. Therefore, it is hypothesized that while causing a decrease in prepubertal PAR mass, elevated nutrient intake will have no effect on growth characteristics of the mammary gland. The objectives of this experiment were to evaluate the effects of increased nutrient intake from early in life on 1) mammary epithelial cell proliferation, 2) mammary PAR DNA accretion rates, and 3) the dynamics of prepubertal allometric PAR growth. Holstein heifers (n = 78) were fed from 45 kg of body weight either elevated (E) or restricted (R) levels of nutrients to support 950 (E) or 650 (R) g/d of body weight gain. Six heifers per treatment were harvested at 50-kg increments from 100 to 350 kg of body weight. Heifers on the E plane of nutrition had higher plasma leptin and less PAR DNA than their body weight-matched R-intake cohorts. Despite this reduction in PAR DNA, treatment did not negatively influence mammary epithelial cell proliferation or the PAR DNA accretion rate. Dynamics of allometric and isometric mammary growth were also unaffected by the level of nutrient intake, as was exit from allometric growth. This work represents the first demonstrating that the level of nutrient intake and the concomitant increase in plasma leptin have no measurable influence on 1) the rate of PAR DNA accretion, 2) mammary epithelial cell proliferation, or 3) total PAR mass and, by default, the local or systemic controls that coordinate these processes.

Key words: heifer, mammary development, allometric growth

INTRODUCTION

In the bovine, postnatal mammary growth occurs at an allometric rate prior to puberty and returns to an isometric rate after puberty (Sinha and Tucker, 1969). It is well documented that elevated nutrient intake during this allometric growth phase results in reduced parenchymal (**PAR**) mass and DNA (Sejrsen et al., 1982; Petitclerc et al., 1984; Capuco et al., 1995). Several hypotheses have been proposed to explain this phenomenon. Elevated nutrient intake affects plasma concentrations of a host of homeorhetic signals, including growth hormone (Sejrsen et al., 1983) and leptin (Block et al., 2003). Both hormones have been proposed as mediators of diet-impaired prepubertal mammary development. Growth hormone is required for prepubertal mammary development (Cowie et al., 1966) and systemic levels are reduced in animals on high nutrient intakes (Sejrsen et al., 1983). Plasma leptin is increased with elevated nutrient intake (Block et al., 2003), and intramammary leptin infusions have blocked epithelial cell proliferation in the prepubertal bovine mammary gland (Silva et al., 2003). These observations have led to the hypotheses that elevated nutrient intake reduces prepubertal mammary development by impairing epithelial cell proliferation because of reduced circulating growth hormone (Sejrsen, 1978; Sejrsen et al., 1999) or elevated circulating levels of leptin (Silva et al., 2002).

In a companion paper (Meyer et al., 2006), we suggested that elevated energy intake does not directly impair mammary growth. Instead, the difference in age at harvest, which is often an artifact of growing heifers at divergent rates of BW gain, is the primary cause for the observed differences in PAR mass. Therefore, we hypothesized that while experiencing decreased PAR mass at harvest, prepubertal heifers raised on an elevated plane of nutrition would have mammary epithelial cell proliferation, PAR DNA accretion rates, and dynamics of prepubertal allometric mammary growth similar to those of their intake-restricted cohorts. Ef-

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fects on PAR and fat pad mass, DNA, and composition are described in a companion paper (Meyer et al., 2006).

MATERIALS AND METHODS

Animals and Tissue Collection

The Cornell University Animal Care and Use Committee approved all procedures used in this study. The experimental design is described in detail in the companion paper (Meyer et al., 2006). Briefly, 78 Holstein heifers (44.2 kg of BW, 9.9 d of age) were assigned to an elevated (**E**) or restricted (**R**) level of nutrient intake supporting 950 or 650 g of daily BW gain.

Prior to weaning, all heifers were fed twice daily at 0630 and 1800 h. E-heifers received a diet with 29% CP and 19% fat milk replacer at 0.32 Mcal intake energy/kg of $BW^{0.75}$, whereas R-heifers received a diet with 22% CP and 21% fat milk replacer at 0.20 Mcal intake energy/kg of $BW^{0.75}$. Weaning was initiated after approximately 6 wk on the treatment. A textured starter was offered from wk 3 of the study through wk 10 of the study, after which heifers were fed a TMR until the end of the treatment period. Heifers were weighed weekly and the amount of milk replacer or TMR offered was adjusted to meet the targeted rate of BW gain. Composition of the milk replacer, starter, and TMR were described previously (Meyer et al., 2006).

Once heifers reached 225 kg of BW, blood was collected twice weekly via jugular venipuncture and the plasma progesterone concentration was determined (Coat-A-Count progesterone radio immunoassay; Diagnostic Products Corp., Los Angeles, CA). Plasma progesterone concentrations greater than 1 ng/mL were interpreted to mean that the heifer possessed a functional corpus luteum and was therefore pubertal.

Six heifers were harvested at approximately 45 kg of BW to determine mammary development prior to initiation of the treatment. The remaining heifers (6 per treatment per harvest weight) were harvested at the following live BW: 100, 150, 200, 250, 300, or 350 kg. Harvest was conducted at the Cornell University abattoir by stunning with a captive bolt followed by exsanguination. Pubertal heifers were harvested in the luteal phase of the estrous cycle to minimize variation in PAR DNA associated with estrus (Sinha and Tucker, 1969). One to 2 h prior to harvest, each heifer was intravenously injected with 5-bromo-2-deoxyuridine (**BrdU**; 20 mg/mL in pH 8.5 saline) at a dose of 5 mg/kg of BW (Capuco et al., 2002). At harvest, the udder was removed and weighed. The left half was immediately dissected and tissue samples from the mid-PAR region were collected, fixed overnight in 10% neutral buffered formalin at 4°C, and stored in 70% ethanol until further processing for immunohistochemistry.

Following collection of PAR tissue from the left half, the udder was skinned and separated into right and left halves at the medial suspensory ligament. The skin and teats were weighed together and the skinned right half was weighed separately. The weight of the skinned left half was determined by the difference.

The skinned right half was frozen on dry ice and stored at –20°C until further processing. At a later date, the right half was partially thawed at 4°C and cut into 5-mm-thick slices using a meat slicer. From these slices, the PAR was quantitatively dissected by color, collected, and weighed. Total dissected PAR from each right udder-half was ground using a bowl chopper and subsampled. These PAR subsamples were frozen in liquid nitrogen and further ground to a fine powder in a commercial blender. The PAR DNA content was determined with these powered samples using the fluorometric bisbenzimidazole technique (Labarca and Paigen, 1980) as described in the companion paper (Meyer et al., 2006).

Plasma Leptin Assay

The plasma leptin concentration was determined in individual samples collected weekly for the final 4 wk before harvest. The leptin concentration was assayed in duplicate by a double antibody bovine radioimmunoassay (Ehrhardt et al., 2000).

Calculation of Daily PAR DNA Accretion Rates

To determine the daily PAR DNA accretion rates between harvest weights, the difference in the PAR DNA content between consecutive harvest weights was divided by the difference in age between the harvest weights. For example, when calculating the rate between 150 and 200 kg of BW, the difference in treatment mean PAR DNA content at 150 kg of BW and each heifer's individual PAR DNA content at 200 kg of BW was first determined, giving each 200-kg heifer a ΔPAR DNA value. The amount of time between these 2 harvest weights was then calculated for each 200-kg heifer (Δage) by taking the treatment mean age at 150 kg of BW minus each individual heifer's age at 200 kg of BW. The PAR DNA accretion rate was then calculated by dividing ΔPAR DNA by Δage. To ensure that using the mean ages of heifers per treatment weight did not bias the evaluation, the actual age of each heifer was also used to calculate the PAR DNA accretion rate for each treatment. Statistically, the results were similar; thus, only the mean age differences are reported.

Immunohistochemistry

After fixation, tissues were processed for immunohistochemistry as described previously (Capuco et al.,