Influence of Breed and Hormones on Production of Milk Proteins by Mammary Explants from Prepubertal Heifers

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ABSTRACT

Effects of hormone treatment and breed on lactogenic responses were studied in organ culture of mammary tissue obtained from prepubertal Angus or Holstein heifers. Donor animals were treated with injections of estradiol and progesterone for 7 d and were killed on d 15 after initial injection. Explants were cultured for up to 96 h in a basal medium containing insulin, hydrocortisone, and triiodothyronine or a stimulatory medium, further supplemented with prolactin. Concentrations of α-lactalbumin and αs1-casein were measured in media and explant homogenates by radioimmunoassay, as an index of lactogenesis. In selected cultures, 3H-labeled amino acids were added to assess de novo protein synthesis. Addition of prolactin to medium elicited marked increases in accumulation of αs1-casein but had less effect on content of α-lactalbumin or [3H]protein in media or explant homogenates. Explants from Holstein heifers consistently produced more α-lactalbumin, casein, and total protein than those from Angus heifers, reflecting their inherent superiority in ability to produce milk. Breed differences were more readily detected among cultures exposed to prolactin. Demonstration of breed differences in biosynthetic capacity of mammary tissue suggests a possible means for early selection of dairy heifers.

INTRODUCTION

Many of the basic hormonal requirements for lactogenesis in cultured mammary tissue have been established (25). In general, biosynthesis of milk components in response to lactogenic hormones and the specific hormonal requirements to elicit these responses are remarkably consistent across species examined. However, deviations from these basic requirements and responses have been noted. For example, a striking feature of some studies has been the marked differences in mammary responses to hormones during different stages of ontogeny (3, 19). Furthermore, variation in biosynthetic responses of mammary tissue from individual animals within a given developmental stage has been attributed to genetic differences among individuals in response to hormones (8). Further study of these phenomena may reveal basic mechanisms of development that relate ultimately to the functional capacity of the mature mammary gland.

The value of developing reliable criteria for selection of dairy heifers at an early age has long been recognized (27). Unfortunately, attempts to relate measures of mammary development (23, 26) or blood hormone concentrations (12) early in life to subsequent milk-producing ability have not resulted in applicable procedures for selection. However, Naito et al. (16) found it possible to predict accurately future lactational performance of heifers from yields obtained during hormone-induced lactation. These results show promise for early prediction of performance, but ability to predict at a much younger age is desirable.

We have recently demonstrated breed differences in hormone-induced production of milk...
proteins by mammary explants from beef and dairy bulls (13). This indicates the opportunity for developing a bioassay for genetic merit to produce milk. Such a procedure would allow for early selection of animals based on projected milk production potential.

Our objectives were to study the effects of hormones on production of milk proteins by mammary tissue from prepubertal heifers and to determine differences in protein synthesis between breeds intensively selected for high milk yield (Holstein) or relatively unselected for milk (Angus).

MATERIALS AND METHODS

Six Angus (H) and six Holstein (H) heifers (6 to 8 mo of age) were obtained for this experiment. Holstein heifers were purchased at auction, so age was estimated from the standard growth curve for H heifers (15) based on mean BW of about 145 kg. Angus heifers were obtained from the Virginia Polytechnic Institute and State University (VPI) beef research herd. Age of these heifers ranged from 6 to 8 mo and BW averaged ca. 150 kg.

Angus heifers were housed in separate pens in a research barn, exposed essentially to natural photoperiod and temperature. Holstein heifers were housed in metabolism crates in the Animal Sciences Building, VPI to avoid severely cold January and February temperatures. Photoperiod was maintained at 12 h light and 12 h dark to approximate the photoperiod to which A heifers were exposed. During the 2 wk prior to being killed, heifers were allowed free access to alfalfa hay and water and received 1.5 kg of ground corn daily. Blood samples were obtained by puncture of the coccygeal vessels every 2 or 3 d during this period.

Heifers were treated for 7 d with injections (6 ml/d; s.c.) of estradiol-17β and progesterone (.1 and .25 mg/kg BW per d) as previously described (13). In a pilot study we found that amount of mammary epithelium available for explant cultures were limited in untreated animals of this age. However, priming with steroids allowed for enough growth of the mammary tissue to obtain sufficient epithelial tissue for replication of cultures. Steroid treatment elicited palpable increases in amount of mammary epithelium in the udder. Because the treatment was not intended to induce lactation, but rather to stimulate proliferation of tissue, animals were slaughtered on d 15 after initial injection.

Mammary glands were excised immediately after slaughter and transported to the laboratory (5 to 15 min). Mammary parenchyma was identified and aseptically diced into explants (about 3 to 4 mm³) in sterile Medium 199 (Gibco, Grand Island, NY). Explants were rinsed twice in sterile medium and cultured (4 to 5 explants/culture dish) as previously described (13).

Final culture media were supplemented with hormones as described by McFadden et al. (13). Basal medium (B) included bovine insulin, hydrocortisone, and L-triiodothyronine (T₃) at final concentrations of 5 µg/ml, .5 µg/ml, and .65 ng/ml, respectively (9). Stimulatory medium (P) was further supplemented with bovine prolactin (PRL; NIH-B₆, National Institutes of Health, Bethesda, MD) at 1 µg/ml final concentration.

Incubations began about 30 to 45 min post-slaughter. Media were harvested and renewed at 24 h intervals, with the final collection of media after 96 h. Harvested media were frozen at −20°C until assayed for α-lactalbumin (α-lac) and αₛ₁-casein (cas) by radioimmunoassay (RIA). The number of culture dishes per heifer depended on the amount of mammary tissue available to be explanted. At least 12 and up to 20 cultures per heifer were conducted.

Media supplemented with [³H]amino acids replaced unlabeled media in two randomly selected dishes of each treatment (B and P) at 24 and 72 h. Media and explants were collected after 24 h of incubation in the presence of labeled amino acids. Explants were blotted semidry, weighed, and stored at −20°C.

Because cultures receiving labeled amino acids were terminated prior to 96 h, dish numbers declined over time. For example, 12 dishes/animal provided 12 media samples at 24 and 72 h. Media and explants were collected after 24 h of incubation in the presence of labeled amino acids. Explants were blotted semidry, weighed, and stored at −20°C. Incorporation of [³H]amino acids into an acid-insoluble protein fraction was determined
TABLE 1. Mean content of α-lactalbumin in medium after incubation of mammary explants from Angus (A) and Holstein (H) heifers.

<table>
<thead>
<tr>
<th>Time of culture</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed Treatment</td>
<td>A Basal</td>
<td>H Basal</td>
<td>A Stimulatory</td>
<td>H Stimulatory</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>--------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>A Basal</td>
<td>50.3**</td>
<td>52.4</td>
<td>32.5</td>
<td>49.0</td>
</tr>
<tr>
<td>H Basal</td>
<td>2098.9</td>
<td>124.8</td>
<td>12.0</td>
<td>16.2</td>
</tr>
<tr>
<td>A Stimulatory</td>
<td>65.2**</td>
<td>21.3**</td>
<td>74.3*</td>
<td>60.2</td>
</tr>
<tr>
<td>H Stimulatory</td>
<td>2196.3</td>
<td>638.4</td>
<td>321.5</td>
<td>152.0</td>
</tr>
</tbody>
</table>

1 Units are in picograms per milligram tissue/24 h; pooled SE = 103.9.

*Breed means within same time and treatment differ (P<.10).

**Breed means within same time and treatment differ (P<.001).

in media and homogenates of explants incubated in the presence of labeled amino acids ([3H]amino acid mixture #444K, Amersham, Arlington Heights, IL; 43 Ci/mM; final concentration 4 μCi/ml) as described (13). Precipitation of labeled protein was 85 to 94% efficient, based on recoveries of various quantities (.05 to 100 ng) of [125I]α-lac added to fresh medium.

Incorporation of labeled thymidine into explant DNA was determined at the initiation of the culture period. Explants (200 to 300 mg) were incubated for 4 h at 37°C in 2.5 ml medium 199 containing [3H]thymidine (#TRK-637, Amersham; 49 Ci/mM) at a final concentration of 10 μCi/ml. Radioactivity was determined by liquid scintillation spectrometry. Incorporation of thymidine was not determined for one subject. Concentrations of DNA in explants were quantified in two randomly selected homogenates per treatment group per heifer by the method of Labarca and Paigen (11), using a TKO mini-fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Quantification of α-lac (1) and cas (14) in media and explant homogenates was by double-antibody RIA as previously described. Intraassay and interassay coefficients of variation averaged less than 15% for α-lac and cas in standard medium and serum pools. Concentrations of PRL in serum were determined by the RIA method of Koprowski and Tucker (10) in a single assay. Intraassay coefficient of variation averaged less than 10% in two serum pools.

Data were subjected to split plot analysis of variance using the general linear models (GLM) procedure of SAS (22). Because a major interest of the study was in breed differences, least squares means for breeds were compared within each treatment-period using orthogonal contrasts. Statistical analysis of α-lac and cas concentrations in medium was performed on means of all dishes within a treatment-period for each animal. All other analyses were based on individual dish means.

RESULTS

On average, medium from cultures of H heifer tissue contained greater overall quantities of α-lac across all times and treatments than that from A cultures (695.0 vs. 50.6 pg/mg per 24 h: Table 1). This difference did not reach statistical significance (P>.10) due to variation introduced by exceptionally high values at the 24-h period. However, comparison of breed means for each treatment-period revealed that content of α-lac in medium from H cultures exceeded that from A cultures at 24 h, regardless of treatment, and at 48 h, in the presence of PRL (P<.001). Medium from H cultures also contained four times more α-lac at 72 h in the presence of PRL than that from A cultures (P<.10). Overall, addition of PRL to medium did not affect mean α-lac content in medium (304.5 vs. 441.2 pg/mg per 24 h; P>.10; for B and P, respectively), although exclusion of data from the 24-h period revealed fourfold higher α-lac content in the presence of PRL (211.3 vs. 47.8 pg/mg per 24 h; Table 1). Unexpectedly, medium α-lac concentrations progressively declined during culture, particularly among cultures of H tissue, although apparent rate of decline was less in presence of PRL.

Content of cas in medium did not differ overall by breed (P>.10) but was higher for H
TABLE 2. Mean content of casein in medium after incubation of mammary explants from Angus (A) and Holstein (H) heifers.  

<table>
<thead>
<tr>
<th>Breed</th>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Basal</td>
<td>4.2*</td>
<td>2.2*</td>
<td>4.0</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>H Basal</td>
<td>27.4</td>
<td>8.6</td>
<td>4.1</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>A Stimulatory</td>
<td>4.1*</td>
<td>3.7*</td>
<td>4.5*</td>
<td>3.8*</td>
<td></td>
</tr>
<tr>
<td>H Stimulatory</td>
<td>22.0</td>
<td>17.1</td>
<td>21.7</td>
<td>18.2</td>
<td></td>
</tr>
</tbody>
</table>

1Units are in nanograms per milligram tissue/24 h; SE = 1.3 to 2.6.  
*Breeds means within same time and treatment differ (P<.01).

explants in 6 of 8 treatment periods (P<.01; Table 2). Addition of PRL to culture medium increased content of casein in medium (11.9 vs. 7.1 ng/mg per 24 h; P<.01), and magnified differences between breeds (P<.05). Furthermore, unlike α-lac, addition of PRL to cultures of mammary tissue from H heifers maintained apparent secretion of casein throughout the culture period.

Mean content of α-lac in medium or in explant homogenate from the subset of cultures incubated in the presence of [3H]amino acids are depicted in Figure 1. These data include only those cultures exposed to labeled amino acids, and thus are not directly comparable with those in Table 1. Medium values are presented to facilitate comparison with quantities in homogenate from the same culture dish. Overall, tissue from H heifers released more α-lac into medium (142.2 vs. 28.1 pg/mg per 24 h; P<.05) and contained higher quantities in homogenates (205.3 vs. 90.9 pg/mg; P<.01) than that from A heifers. Quantities of α-lac in medium and homogenate were not affected by hormone treatment (P>.10).

By contrast, presence of PRL in medium increased overall content of casein in medium, homogenate, and total (medium plus homogenate), averaging 13.8 vs. 2.9, 27.4 vs. 4.1, and 41.2 vs. 7.0 ng/mg per 24 h, respectively (P<.05; Table 3). Data are derived only from cultures incubated in the presence of labeled amino acids, thus media values do not correspond directly with those in Table 2. Quantity of casein in medium did not differ overall by breed (P>.10), but in the presence of PRL, explants from H heifers released more cas into medium at 48 and 96 h than those from A heifers (P<.05). Content of cas in homogenates (28.2 vs. 3.3 ng/mg) and total accumulation of cas in cultures (40.7 vs. 7.5 ng/mg) was greater for H than for A heifers (P<.05). However, examination of individual treatment-period means indicated that breed differences were significant only among cultures treated with PRL (Table 3).

As illustrated in Figure 2, quantities (cpm/mg per 24 h) of [3H]protein in medium (130.8 vs. 71.0; P<.10) and homogenate (131.0 vs. 47.4; P<.01) were greater in cultures for H than for A heifers. Addition of PRL to medium had no effect on amount of [3H]protein appearing in medium or retained within explants (P>.10).

Evaluation of total synthesis (medium and homogenate quantities combined) of [3H]protein, and percent of total recovered in medium [(amount in medium/total × 100%)] revealed no effect of PRL treatment (P>.10; Table 4). Protein synthesis was greater in explants from H heifers overall (261.8 vs. 118.4 cpm/mg per 24 h; P<.001), and in each treatment period (P<.0001), compared with synthesis by A explants. The percentage of newly synthesized protein appearing in medium did not differ overall by breed (P>.10), but the increase in percent apparently secreted by A tissue at 96 h in both treatments resulted in a breed by period interaction (P<.05).
Figure 1. Mean content of α-lactalbumin in medium and explant homogenates after incubation of mammary tissue from Angus and Holstein heifers. Data are derived only from those cultures exposed to [3H]amino acids. Panel A: α-lactalbumin in medium, pooled SE = 13.8, units are in picograms per milligram/24 h. Panel B: α-lactalbumin in homogenates, SE = 18.7, units are in picograms per milligram. AB = Angus, basal medium; AP = Angus, stimulatory medium; HB = Holstein, basal; HP = Holstein, stimulatory. Data from one Holstein heifer at 96 h are excluded due to extremely high values. Breed means for the same time and treatments differ: \( ^aP < .01 \), \( ^bP < .001 \).

Data in this study are presented on a tissue weight basis. To evaluate the possibility that breed or treatment effects were due to differences in number of cells per milligram of tissue, DNA concentration was determined in selected homogenate samples. Concentration of DNA in explants did not differ by treatment \( (P > .10) \) but was higher in tissue from H heifers than in
TABLE 3. Mean content of casein in medium and explant homogenates after incubation of mammary tissue from Angus (A) and Holstein (H) heifers.1

<table>
<thead>
<tr>
<th>Breed</th>
<th>Time of culture</th>
<th>48 h</th>
<th>96 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Media Homo Total</td>
<td>Media Homo Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>.6</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>H</td>
<td>B</td>
<td>7.3</td>
<td>7.0</td>
<td>14.3</td>
</tr>
<tr>
<td>A</td>
<td>P</td>
<td>3.9*</td>
<td>3.4**</td>
<td>7.3**</td>
</tr>
<tr>
<td>H</td>
<td>P</td>
<td>17.0</td>
<td>41.8</td>
<td>58.8</td>
</tr>
</tbody>
</table>

1Units are in nanograms per milligram tissue/24 h; SE = 3.1 to 3.3 for media. For homogenate units are nanograms per milligram; SE = 5.0 to 5.3 or SE = 2.8 for total. Data are derived only from those cultures exposed to [3H]amino acids.

2Treatments are basal (B) and stimulatory (P).

*Breed means within same time and treatment levels differ (P<.05).

**Breed means within same time and treatment levels differ (P<.001).

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**DISCUSSION**

Mammary explants from prepubertal heifers were capable of general protein synthesis and released measurable quantities of specific milk proteins during culture. Although synthesis of specific milk protein was not determined directly, comparison of the amount of α-lac and cas present in tissue at 48 h to that in tissue and medium at 96 h suggests that synthesis occurred during culture. Similarly, radiolabeled proteins appearing in medium are presumed to be secreted.

Effects of PRL varied depending on the protein fraction measured. Content of cas in medium and homogenate was markedly greater in the presence of PRL. By contrast, PRL-treatment had little effect on apparent synthesis or secretion of α-lac or acid-insoluble protein.

In agreement, Skarda et al. (20) reported that PRL specifically stimulated synthesis of cas (119 to 900%) in contrast to a comparatively minor increase in total protein synthesis (11 to 39%) in mammary explants from midpregnant goats. In a second study, PRL failed to increase synthesis of total protein, but enhanced lipid synthesis and was absolutely required for cas synthesis (21).

Others have shown a positive effect of PRL on lipid synthesis (5) and cellular differentiation (17) in cultured ruminant mammary tissue. In contrast to the present study, our previous findings in prepubertal bulls (13) and those of Goodman et al. (9) in pregnant cows indicate a marked stimulation of α-lac secretion in the presence of PRL. This discrepancy may be related to differential effects of hormones on biosynthesis of specific milk components. For example, T3 has been shown to synergize with PRL to enhance synthesis of α-lac but not cas in mice (28). Furthermore, cortisol exhibits differential actions on synthesis of cas and α-lac in cultured mouse mammary tissue (18). Differing effects of PRL on synthesis of total protein, lipid, and casein have also been reported (21).

In addition, the physiological status of the animals could affect response to PRL. The sensitivity of mouse mammary explants to inhibition of cas and α-lac synthesis by progesterone changes markedly from nonpregnant or early pregnant, to late pregnant status (24). Moreover, the two component proteins of the lactose synthetase enzyme complex are subject to differential hormonal induction during development (4). Other differences in hormone responsiveness of mammary tissue during various stages of ontogenesis have been reported in ruminants (19) and mice (3).

It may well be that differential responses of H and A mammary tissue to exogenous steroids were responsible for marked differences in milk protein content and secretion by mammary ex-
Figure 2. Incorporation of [3H]amino acids into acid-insoluble protein in medium and homogenate of mammary explants from Angus and Holstein heifers. Panel A: Tritiated protein in medium, SE = 17.4, units are in counts per minute per milligram/24 h. Panel B: Tritiated protein in homogenate, SE = 6.1, units are in counts per minute per milligram/24 h. AB = Angus, basal medium; AP = Angus, stimulatory medium; HB = Holstein, basal; HP = Holstein, stimulatory. Breed means for the same time and treatments differ: \(^{a}P<.10, \, ^{b}P<.005\).

plants in culture. In retrospect, measurement of tissue protein prior to culture would have determined if H heifers began with an advantage. Preliminary, histological evaluation (unpublished) of mammary tissue taken at initiation of culture indicates that the epithelium appears indistinguishable in A and H heifers. This would imply that differences between breeds are manifest after initiation of culture. This suggests that cells from H heifers were particularly “primed” by steroid treatment for differentiation in culture. Clearly, further experiments
TABLE 4. Total synthesis and secretion of $[^3]$H]protein by cultured mammary explants from Angus (A) and Holstein (H) heifers. 1, 2

<table>
<thead>
<tr>
<th>Breed</th>
<th>Tmt3</th>
<th>48 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total synthesis</td>
<td>Percentage secreted</td>
<td>Total synthesis</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>117.3**</td>
<td>54.3*</td>
</tr>
<tr>
<td>H</td>
<td>B</td>
<td>265.7</td>
<td>48.2</td>
</tr>
<tr>
<td>A</td>
<td>P</td>
<td>129.3**</td>
<td>53.8</td>
</tr>
<tr>
<td>H</td>
<td>P</td>
<td>258.0</td>
<td>51.1</td>
</tr>
</tbody>
</table>

1Units are in counts per minute per milligram/24 h, or percent; SE for total synthesis = 17.6, for percent secreted = 2.3.
2Total synthesis = medium plus homogenate; percentage secreted = medium divided by total.
3Treatments are basal (B) and stimulatory (P).
*Breed means within same time and treatment differ (P<.10).
**Breed means within same and treatment differ (P<.001).

are needed to resolve this question and to determine if prior treatment with steroids is necessary for expression of presumptive genetic potential by mammary explants in culture.

Regardless, comparison of protein content in medium and homogenate revealed marked differences between dairy and beef heifers. These data indicate that differences in production of proteins by cultured mammary tissue from prepubertal heifers reflect the inherent differential in genetic potential for milk production between the two breeds. Naito et al. (16) reported that milk yields of heifers induced into lactation are highly correlated with future parturient production. We have extended this concept to testing of much younger animals. It has been suggested that variation attributed to individual animals in responses of cultured mammary tissue may reflect differences in intrinsic genetic potential to respond to lactogenic hormones and produce milk (7). The present data support this contention.

An important feature of the breed comparison in this study is the relative consistency of differences between breeds across measurements. Even in those cases where breeds did not differ overall, at least half of the breed contrasts within individual treatment periods favored the tissue from Holsteins. The high amount of protein released into medium by H explants at the 24-h period probably introduced greater variation into the measurement and precluded declaring overall breed differences. The large quantity of proteins secreted during the first 24 h of culture may represent release of preformed product (9) and may obscure breed or treatment differences (7).

In general, ability to discern breed differences was enhanced in the presence of PRL. Comparison of breeds within each treatment period for all parameters (excluding percent secretion, Table 4) revealed that 22 of 24 breed contrasts were significant (P<.10) in the presence of PRL, compared with 12 of 24 significant in basal medium.

Cultures of explants from H heifers consistently contained more protein than those from A heifers, although protein concentrations appeared better maintained over time in A cultures. Breed differences should be interpreted cautiously, however, since DNA concentration, and thus cell number, was greater in Holstein tissue. This reduces the apparent differences in productivity (on a per cell basis) between breeds. Because the difference in cell number was 50%, compared with differences of 4- to 30-fold in protein content, it is unlikely that cell number was the primary determinant of explant productivity. Because DNA content does not differentiate cell type, no conclusion can be made on relative numbers of secretory cells (26). Expression of results on a DNA basis would be expected to reduce variance but would probably not significantly alter overall results. It is likewise improbable that explant viability differed by breed or treatment, since

incorporation of labeled amino acids into total protein was virtually identical over time in each breed treatment group.

The elevated concentrations of PRL in serum of H heifers may also be a critical determinant of productivity in culture. Although photoperiod was similar between breeds, Holstein heifers were exposed to higher ambient temperatures, possibly causing the rise in serum concentrations of PRL (29). Elevated concentrations of PRL in serum have been associated with more successful induced lactations (6) and carryover of hormones bound in mammary tissue may influence response of tissue in culture (2).

Although substantial variation due to animal and sample was associated with measurements of protein content, it remained possible to distinguish breeds using these measures. Animal variation is requisite to a system intended to distinguish between individuals based on the performance of tissue samples. Sample (dish) variation could be reduced by optimizing homogeneity of tissue samples and choice of parameters measured.

CONCLUSIONS

Mammary tissue from prepubertal heifers can be induced to release measurable quantities of milk proteins. Breed differences in measured parameters appear to reflect inherent potential for milk production. Thus, detection of such differences within a breed could potentially be refined to provide a technique for early selection of dairy heifers. Further studies are needed to establish effects of ontogeny and hormones on lactogenic responses of ruminant mammary tissue.

REFERENCES