An Investigation of the Role of Insulin in Bovine Milk Protein Gene Expression in Mammary Explant Culture.

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ABSTRACT: Experiments have shown that administration of insulin to dairy cattle during lactation may increase the concentration of milk protein. In the experiments reported here the requirement for insulin to stimulate expression of milk protein genes, in the presence of hydrocortisone and prolactin, was examined in a bovine mammary explant culture model, using tissue from late pregnant multiparous Holstein–Fresian cows. Stimulation of κ-, β-casein and β-lactoglobulin gene expression, in response to the addition of prolactin, was observed in media which included hydrocortisone in either the presence or absence of insulin. The magnitude and persistence of the response observed following stimulation with prolactin, was enhanced in explants cultured in the absence of insulin when preceded by a 4 day incubation in media with insulin and hydrocortisone. This response was consistent between all three milk protein genes examined. We suggest that either insulin is not required for expression of milk protein genes or that the requirement for insulin may be substituted by endogenous insulin-like growth factors/hormones produced by the bovine mammary explants.

Keywords: Insulin, Milk Proteins, Gene Expression.

INTRODUCTION

The role often attributed to insulin in in vitro culture of mammary epithelial cells is the maintenance of a viable and hormonally-responsive state to support milk protein synthesis and secretion (Djiane et al. 1975). However, insulin, in the presence of glucocorticoids and prolactin, has been shown to be essential for milk protein mRNA accumulation in both the mouse and rat (Bolander et al. 1981, Nicholas et al. 1983, Kulski et al. 1983, Chomczynski et al. 1984). The role of insulin in this process is to stimulate milk protein gene transcription rather than to increase stabilisation of specific mRNA transcripts (Chomczynski et al. 1984). Insulin has been shown to enhance efficacy of prolactin in the induction of milk protein gene expression (Houdebine et al. 1985).

The role of insulin for milk protein gene expression in bovine mammary tissue is less clearly defined. Some reports suggest that insulin does play an essential role in bovine milk protein gene expression (Andersen and Larson 1970, Djiane et al. 1975), while others report insulin-independent expression of bovine milk protein genes (Goodman et al. 1983, Houdebine et al. 1985).

This experiment investigates the requirement for insulin in a bovine mammary explant model, which is stimulated to express milk protein mRNAs through the addition of prolactin in the presence of hydrocortisone.

MATERIALS AND METHODS

Two cows (at 31 and 32 days pre-partum) were utilised to examine the effect of insulin (I), in the presence of hydrocortisone (F) and prolactin (P), on the expression of bovine milk protein genes in mammary explant culture. Mammary tissue was obtained by biopsy of pre-parturient cows anaesthetised by the administration of xylazine as an epidural analgesic. The use of ultrasound to localise vasculature assisted in limiting blood loss and 7-10g of mammary tissue was recovered surgically from the dorsal third of the fore quarter. A maximum of 20 explants (each of 1mg), maintained on siliconised lens paper, were initially incubated with Medium 199 which included I (5ug/mL) and F (500ng/mL) for the first 2 or 4 days of culture. Following this first incubation, treatments included media containing both I and F, F and P (500ng/mL) or F, P and I alone for a further 2 or 4 days. Histological examination indicated that the tissue at the time of biopsy had a morphology consistent with this stage of gestation and gram stain analysis showed no subclinical mastitis.

Total RNA was extracted and purified with ‘Tri-Reagent’ as per manufacturers instructions (Sigma). Sufficient RNA was recovered from approximately 100 explants (per treatment) to analyse for κ-, β-casein and β-lactoglobulin gene expression by Northern and Slot Blot analysis. Chemiluminescent probes for these genes (Digoxigenin, Boehringer Mannheim) were labelled by PCR from cDNA clones (Alexander et al. 1988, Stewart et al. 1984, Stewart et al. 1987). Quantitative estimates of gene expression were determined from band intensity following exposure to x-ray film as previously described (Krueger and Williams 1995). Values were expressed as a percentage of levels observed in tissue collected from a control animal during mid lactation.

The rate of DNA synthesis was determined by incubating explants with methyl-3H Thymidine (1μCi/mL of culture media) for 4 hours. Explants were then collected, washed, decolourised and solubilised.
prior to the addition of scintillation fluid and measurement of radioactivity using a liquid scintillation spectrophotometer.

RESULTS

κ-Casein Gene Expression

The level of κ-casein gene expression observed in mammary tissue obtained from the pre-parturient cows declined slightly when the tissue was cultured as explants for 4 days in media with I and F. However, a marked increase in κ-casein gene expression was observed following the addition of P to the culture after either 2 or 4 days of culture in I and F. (figure 1). This increase was not dependent on the inclusion of I when P was applied at day 2 of culture. In contrast, the addition of P at day 4 of culture exhibited a greater elevation in κ-casein gene mRNA in the absence of I. The persistence of elevated levels of κ-casein gene expression was similarly affected by the presence of I as explants cultured in the absence of I showed a sustained elevation of expression irrespective of the timing of addition of P to culture.

![Figure 1. The effect of the presence of I on the P-dependent stimulation of κ-casein in bovine mammary explants.](image)

β-Casein Gene Expression

The response of β-casein gene expression to the inclusion of P in media in either the presence or absence of I was similar to that observed for κ-casein. The stimulation of β-casein gene expression following the addition of P at day 4 of culture was more pronounced than that observed following the addition of P after only 2 days in culture (figure 2). In a similar fashion to κ-casein responses, the P-dependent stimulations of β-casein gene expression was more pronounced in the absence of I irrespective of timing of P addition.

![Figure 2. The effect of the presence of I on the P-dependent stimulation of β-casein in bovine mammary explants.](image)

β-Lactoglobulin Gene Expression.

The importance of I status on the magnitude of prolactin-dependent stimulation and persistence of β-lactoglobulin gene expression was similar to that shown for both the κ-casein and β-casein genes. (figure 3). This response was observed regardless of timing of the addition of P to culture.

![Figure 3. The effect of the presence of I on the P-dependent stimulation of β-lactoglobulin in bovine mammary explants.](image)

The Rate of DNA Synthesis

The addition of P at day 2 of culture resulted in a small increase in the rate of DNA synthesis in the presence of I but little change in its absence (figure 4). Following the first 2 days of P-dependent stimulation of tritiated thymidine incorporation, a 50% decrease was observed regardless of the presence of I. The addition of P at day 4 of culture resulted in little change regardless of I status.
DISCUSSION

The most significant finding of this experiment was that bovine milk protein gene expression was stimulated in mammary explants in the absence of I. A number of in vivo studies utilising hyperinsulinemic-euglycemic clamps in lactating dairy cows have shown that when accompanied with an infusion of amino acids, elevated circulating I levels can produce an increase in milk protein yield (Grinari et al., 1997, Mackle et al. 1999). A hyperinsulenic-euglycemic clamp alone, however, has only moderate effects on milk protein yield (McGuire et al., 1995), has little effect on glucose uptake and only a small effect on amino acid uptake in the mammary gland (Laarveld et al. 1981). Similarly, the stage of lactation in which an elevated circulating concentration of I occurs coincides with decreased milk yield and increasing feed intake (Vasilatos and Wangsness 1981), further complicating the issue of mammary responsiveness to I during lactation.

The results suggest that bovine milk protein genes do not require I, in addition to F and P for expression. Similar results have been reported for in vitro expression of specific milk protein genes from the rabbit (Devinoy et al. 1978) and the Tammar Wallaby (Nicholas and Tyndale-Biscoe 1985).

Alternatively, a plausible explanation for this apparent insulin-dependent expression of bovine milk protein genes is that either the I activity may be supplied by endogenous insulin-like growth factor-I (IGF-I). Previous studies have indicated that IGF-I may substitute for I in mammary explant culture, from a variety of species, in the maintenance or induction of milk protein gene expression (Prosser et al. 1988). Previous researchers have found that IGF-I as well as Insulin-Like Growth factor II (IGF-II) and Insulin-Like Growth Factor Binding Proteins (IGFBPs) are expressed and secreted from bovine mammary tissue in vitro (Campbell et al. 1991). Similarly IGF-I mRNA transcripts were observed by PCR in all samples from this experiment (data not shown). Insulin, IGF-I and their receptors are structurally similar and exhibit similar biological action in a range of tissues; similarly Prosser et al. (1987) found that in a murine model, IGF-I was able to substitute for I in β-casein gene expression in the presence of lactogenic hormones, although a 6-fold higher concentration was required. IGF-I has also been indicated in the regulation of DNA synthesis in mammary epithelial cells (Baumrucker and Stenberger 1989, Winder et al. 1989). Late-pregnant mammary tissue as used in the current study has also been shown to be more responsive to IGF-I administration for the regulation of DNA synthesis even though receptor number increases at the onset of lactation (Baumrucker and Stenberger 1989).

CONCLUSIONS

The observation that I is not essential for the stimulation and maintenance of milk protein gene expression in bovine mammary explants is consistent with previous studies. Although in vivo studies have concluded that I has a role to play in milk protein gene expression, its function in these studies may be at the level of cell bio-energetics rather than in the direct molecular regulation of bovine milk protein genes. Alternatively, the actions of I may be replaced by other insulin-like molecules, thereby providing a further example of endocrine redundancy.

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