Regulation of milk protein synthesis in the bovine mammary gland: A proteomic approach

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ABSTRACT

The molecular mechanisms that regulate milk protein synthesis in the dairy cow are not well understood. We used a proteomic approach to identify potential regulators of milk protein synthesis among the endoplasmic reticulum (ER) proteins. Mammary tissue was collected from mid-lactation dairy cows treated with atropine (Atr; decreased milk production), growth hormone (GH; increased milk production) or vehicle alone. Fractions enriched for ER associated proteins were prepared by differential centrifugation and then resolved by 2-dimensional electrophoresis (2-DE). Proteins were visualised using colloidal coomassie G-250 and the phosphor-protein specific stain, Pro-Q Diamond. We identified 4 significant ($P<0.05$) and 9 marginally significant ($P<0.15$) spots that were differentially expressed between treatment groups. Six spots were subjected to LTQ ion trap tandem mass spectrometry and 2 proteins were identified with a high level of confidence: major vault protein (MVP) (27% of the control level after GH treatment, $P<0.05$), and heat shock 70Kda protein 8 (HSPA8) (41% of the control level after Atr treatment, $P <0.01$). Western blotting showed a 24% decrease in HSPA8 following Atr treatment ($P<0.10$) and a 55% decrease in MVP following GH treatment ($P<0.10$). These results provide a focus for further investigation of the role of MVP and HSPA8 in influencing milk production.

Keywords: proteomics; milk protein synthesis; growth hormone; atropine.

INTRODUCTION

Milk proteins constitute one of the most valuable components of milk for both dairy producers and processors. Although knowledge of the endocrine and molecular pathways involved in mammary gland physiology has greatly improved (reviewed by Hennighausen & Robinson, 2001; Suchyta *et al.,* 2003), knowledge of the mechanism(s) by which milk protein synthesis in the mammary gland is regulated is far from complete. Understanding this at the molecular level may enable us in the future to manipulate milk protein synthesis to enhance its commercial value.

Growth hormone (GH) is an important control in the co-ordination of nutrients to support higher milk production. GH is produced naturally in the pituitary gland of cattle and regulates milk production in lactating cows. GH administered to lactating cows increases milk production (milk volume and protein yield). Several mechanisms may explain the effects of GH on milk production including directing more nutrients toward milk production (Bauman, 1992), indirect effects via IGF-1 (Bauman & Vernon, 1993; Molento *et al.,* 2002; Sakamoto *et al.,* 2006), enhanced capacity for cell proliferation (Capuco *et al.,* 2001) and potentially direct effects of GH on the mammary gland via GH receptors and signalling pathways such as the JAK/STAT pathway (Sinowatz *et al.,* 2000). In contrast, the muscarinic cholinergic antagonist atropine (Atr), reduces milk production as well as protein and lactose concentration (Auldist *et al.,* 2003; McCoard *et al.,* 2004). This occurs, at least in part, by inhibiting gut peristalsis, thereby attenuating the supply of nutrients to the mammary gland. Nutrient supply is thought to affect milk protein synthesis through an intracellular signalling pathway that ultimately modulates the protein synthesis machinery in the endoplasmic reticulum (ER). This treatment model is therefore useful for investigating the underlying mechanisms regulating milk protein synthesis in the bovine mammary gland.

The objective of this study was to use proteomic technologies to profile the changes in ER proteins in the mammary gland of cows treated with GH or Atr.

MATERIALS AND METHODS

Experimental design

Twelve Jersey cows, in mid to late lactation (190 ±12 days in milk), were randomly assigned to three treatment groups of four animals. All cows were housed indoors, fed a nutritionally balanced total mixed ration *ad libitum* and milked twice daily. Milk yield was measured and milk samples taken for compositional analysis at each milking.

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Average daily milk production per cow was 9.9±0.73 *L*. Treatments included a subcutaneous injection of a slow-release formulation (commercially designed for a 2-week slow-release period) of GH (Lactatropin®, Elanco Animal Health), an 8-h intravenous infusion of Atropine (Atr; Atropine sulphate 120 $ug/kg^{-0.75}.h^{-1}$; Sigma Aldrich). Atropine sulphate was prepared fresh on the day of the experiment, weighed for each cow individually and solubilised in physiological (0.9%) saline or vehicle (physiological saline) alone (controls).

All animal manipulations and sampling procedures were approved by the Palmerston North CRI Animal Ethics Committee in accordance with the regulations of the New Zealand Animal Welfare Act, 1999.

Experimental procedure

Immediately after each treatment period had concluded mammary alveolar tissue was removed immediately post mortem, snap-frozen in liquid nitrogen and stored at -80°C. The ER enriched fraction was obtained by homogenising an aliquot of powdered tissue (Broadhurst *et al.,* 2005), and then centrifuging the lysate at 10,000 × G for 20 minutes at 4°C to remove nuclei and cellular debris. The ER enriched pellet was then obtained by centrifuging an aliquot of the supernatant at 105,000 × G x 2 hoours at 4°C. The pellet was resolubilised in iso-electric focusing sample buffer containing 7 M urea, 2 M thiourea, 4% ASB-14 and 80 mM DTT. Protein quantitation was performed using the 2D Quant Kit (GE Healthcare, Uppsala, Sweden).

For two-dimensional electrophoresis (2-DE), solubilised ER proteins (400 µg) were separated in the first dimension on IPG strips (pH 3.5-10, GE Healthcare) and then resolved in the second dimension by discontinuous SDS-polyacryamide gel electrophoresis (SDS-PAGE) (Smolenski *et al.,* 2007). Each gel was stained firstly with Pro-Q Diamond (Molecular Probes, Eugene, U.S.A.) to visualise the phosphoproteins, and then scanned using an FX molecular imager (Bio-Rad, Hercules, U.S.A.). The gels were then stained for total protein using colloidal Coomassie G-250 and then re-scanned using a calibrated densitometer (Bio-Rad).

Spot detection, gel alignment and spot quantification were performed using the PDQuest 2-D Analysis Software, Version 6.2 (Bio-Rad). Quantitative differences between untreated and treated samples were found by comparing the average intensity of resolved spots using statistical and quantitative functions of the PDQuest software.

Protein spots of interest were excised manually and digested, as previously described (Smolenski *et al.,* 2007), to produce a tryptic peptide pool for tandem mass spectroscopy (MS) analysis using an LCQ Deca ion trap mass spectrometer. Each individual peptide was analysed using TurboSEQUEST (Bioworks v3.2, Thermo Finnigan, San Jose, U.S.A.) using an indexed *'mammalian'* subset of the NCBI non-redundant protein sequence database and were accepted as being valid if met a list of criteria that is generally accepted in the proteomics field. These criteria are cited in Smolenski *et al.* (2007).

For Western blot analysis, protein (50 µg) for each mammary tissue lysate was denatured and separated under reduced conditions using a 12.5% Tris-glycine buffered polyacrylamide gel and transferred to nitrocellulose by electroblotting. The membrane was probed with polyclonal anti-Hsc70 (1:200, Abcam, Cambridge, U.K.) and monoclonal anti-MVP (1:200, Abcam) primary antibodies prior to detection by enhanced chemiluminescence (ECL) following a previously described procedure (Beaton *et al.,* 2003). The images were captured using a GS-800 calibrated densitometer (Bio-Rad), and quantified using the QuantityOne v4.2.1 software package (Bio-Rad).

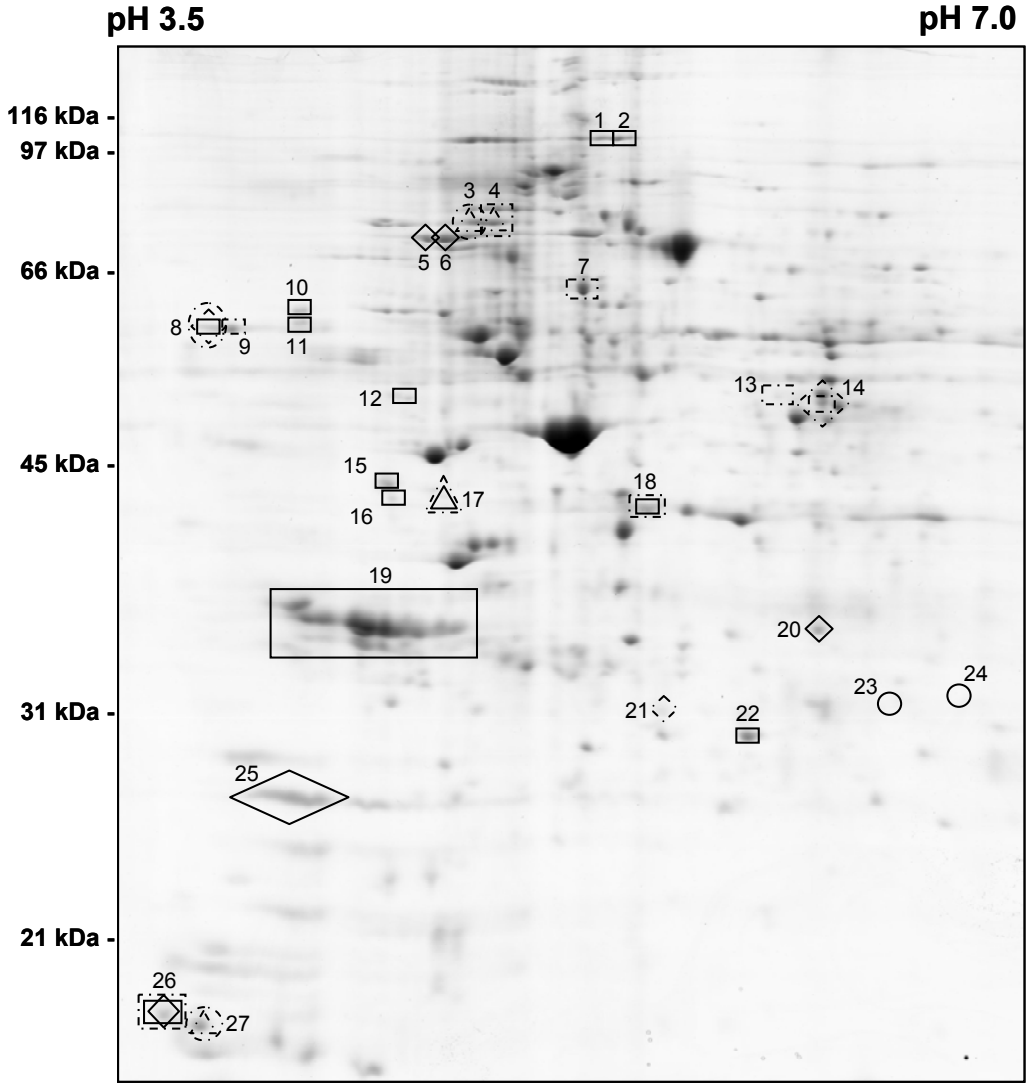
Statistical analysis

For both band identification from the Western blot analysis and spot detection from 2-DE gels the degree of variability was calculated for each spot within and between treatment groups, and the significance of apparent treatment differences was calculated using the Students *t*-test (Microsoft Excel, Seattle, U.S.A.). The degree of treatment effect was expressed as a fold change, *i.e*. the within-group mean of the treatment group divided by the within group mean of the control group. Significant effects for all analysis were declared at $P<0.05$ and trends at $P<0.15$.

RESULTS

Milk yield was increased by 32% following 6 days of GH treatment with a concomitant increase in protein, fat and lactose yields as reported previously (McCoard *et al.,* 2004). In contrast, milk yield was decreased 50% and protein percentage decreased 23% following 8 hours of Atr treatment (S.A. McCoard, unpublished data).
Cytosol and ER fractions were prepared from mammary tissue from all of the trial animals and enrichment was verified by Western analysis for the well known translation factors eEF-2 and p70S6K. These identities were confirmed by MS (results not shown).

Figure 1: Representative 2-DE map of endoplasmic reticulum (ER) proteins, showing the location of spots with differing abundance after growth hormone (GH) and atropine (Atr) treatment to increase and decrease milk production, respectively. Rectangles represent proteins detected by Coomassie that decreased in expression with GH. Spots circled represent proteins detected by Coomassie that increased in expression with GH. Diamonds represent proteins detected by Coomassie that decreased in expression with Atr. Triangles represent proteins detected by Coomassie that increased in expression with Atr. Corresponding changes in phospho-protein status are represented using the same symbols, except with dashed outlines.



To identify changes in protein expression between the treatment and control groups, solubilised ER fractions from each animal were subjected to 2-DE and the proteins visualised by either Coomassie blue (all proteins) or Pro-Q Diamond (phospho-proteins) staining. On Coomassie stained gels we observed 18 protein spots whose abundance was altered by either agent (Figure 1). Two spots were up-regulated and 11 spots were down-regulated by GH, and only a single spot was up-regulated and five were down-regulated by Atr treatment. On Pro-Q Diamond stained gels, 12 spots were altered in abundance by either agent, however, only one of these could be matched to a Coomassie stainable spot (spot #27, up-regulated by GH). Using PDQuest software, four of these spots were identified as having significant changes in abundance ($P<0.05$), while another nine were of marginal significance ($P<0.15$). The results of all quantitations are summarised in Table 1.

Six of these spots, exhibiting differential expression in response to treatment and of sufficient abundance to be identified by MS, were identified by LTQ ion trap tandem MS (Table 2).

Table 1: Quantification of 27 selected 2-DE spots of endoplasmic reticulum (ER) proteins with differing abundance after growth hormone (GH) and atropine (Atr) treatment to increase and decrease milk production, respectively. The fold changes observed with 2-DE analysis are indicated, where positive values specify an increase in protein expression and negative values decreased protein expression. Starred numbers indicate significant fold changes of pair wise comparisons (treatment vs. control), determined by Student's *t*-test, using P<0.05, as measured by either Coomassie (all proteins) or Pro-Q Diamond (phospho-protein) staining. § indicates marginal fold changes (P<0.15) whose significance was compromised by poor 2-DE resolution.

	Coomassie		Pro-Q-Diamond	
Spot No	Atr	GH	Atr	GH
1		-2.27§		
2		-3.7*		
3			1.10	-1.03
4			-1.19§	1.05
5	-2.13§			
6	-2.44*			
7				1.08
8		-2.78*	1.34	-1.25
9				-1.30
10		-1.75		
11		-1.35		
12		-1.07		
13				1.21
14			-1.11	1.00
15		-4.54§		
16		-4.54§		
17	2.89		1.96§	
18				-1.37
19		-4.54		
20	-4.88			
21			0.16	
22		-4.16§		
23		2.28		
24		1.72		
25	-5.55*			
26	-2.00	-2.56§		-1.49
27			-1.12	-2.66§

The two 100-kDa spots (spots #1 and #2), which were down-regulated in response to GH, were both identified as major vault protein (MVP). Both 71-kDa spots (spots #5 and #6), which were down-regulated in response to Atr, were identified as the stress response protein, cognate heat shock protein 70 (HSPA8). The 27-kDa spot (spot #25), which was down-regulated in response to Atr, shows high similarity to a fragment of the 60S ribosomal protein L9. The 17-kDa protein spot (spot #27), whose phosphorylation status was up-regulated in response to GH, was identified as a C-terminal fragment of Calmodulin.

Western blot analysis, on the mammary tissue lysate, was employed as an independent method to confirm the variations in abundance of both HSPA8 and MVP in the samples (Figure. 2). The abundance of MVP, after GH treatment, was decreased to 55% of the control level (P=0.08). The abundance of HSPA8 decreased to 24% of the control level (P=0.07) after Atr treatment.

DISCUSSION

This study has identified two proteins (MVP and HSPA8) whose abundance was decreased by GH and Atr, respectively, in the lactating bovine mammary gland.

The significance of these findings is open to speculation. MVP is the predominant component of a large cytoplasmic ribonucleoprotein particle, the vault complex (Rome *et al.,* 1991; Kickhoefer *et al.,* 1996). While the function of the vault complex is largely unknown (Suprenant, 2002; van Zon *et al.,* 2003), one suggestion is that MVP functions as a scaffold protein for both Src homology 2 (SH2) domain-containing tyrosine phosphatase (SHP-2) and extracellular-regulated kinases (Erks) and that it may play an important role in cell survival signalling (Kolli *et al.,* 2004).

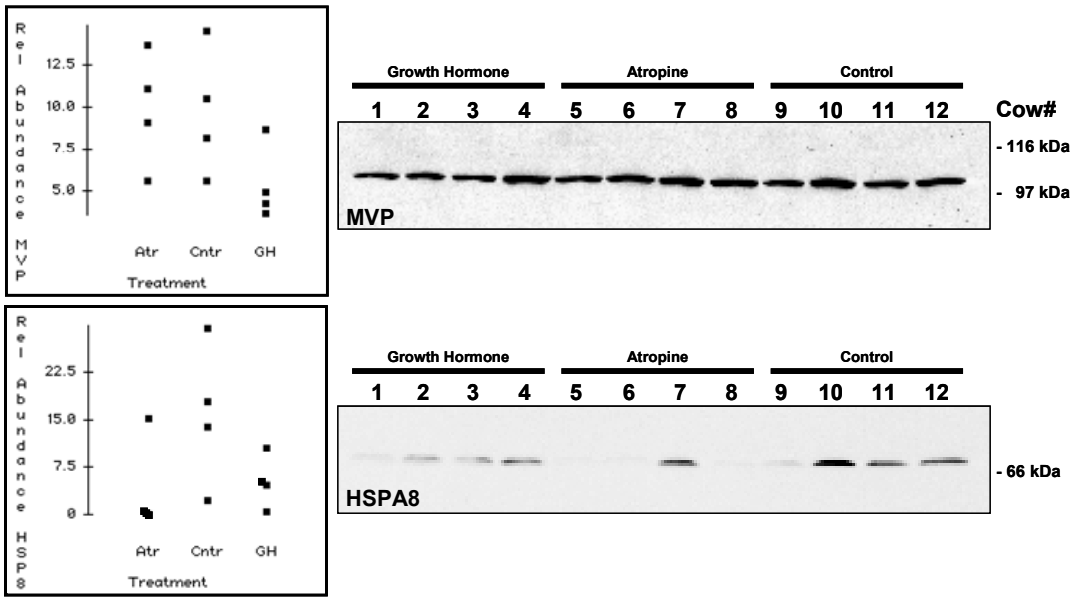
Table 2: Mass spectroscopy results of selected 2-DE spots of endoplasmic reticulum (ER) proteins with differing abundance after growth hormone (GH) and atropine (Atr) treatment to increase and decrease milk production, respectively.

Spot no.[a)]	Protein Identification	Accession no.	observed MW[b)]	observed p*I*[b)]	No. of matched peptides	Sequence coverage (%)
1	Human Major Vault Protein	NP_059447	100	5.6	20	37.5
2	Human Major Vault Protein	NP_059447	100	5.7	20	34.5
5	heat shock 70kDa protein 8 (HSPA8)	NP_776770.2	71	4.7	40	67
6	heat shock 70kDa protein 8 (HSPA8)	NP_776770.2	71	4.8	32	67
25	similar to 60S ribosomal protein L9	XP_592843.1	27	4	12	36
27	Calmodulin fragment	XP_512771.2	17	3.6	7	43.9

a) Spot number corresponds to a protein spot labelled in Fig. 1 and quantified in Table 1.

b) Abbreviations: MW, molecular weight (kDa); p *I* , isoelectric point

Figure 2: Western blot analysis of bovine mammary tissue lysate obtained after growth hormone (n=4) and atropine (n=4) treatment to increase and decrease milk production, respectively, relative to controls (n=4). Each lane was loaded with 50 µg protein and probed with anti-MVP monoclonal (upper panel) and anti-HSPA8 polyclonal (lower panel) antibodies. The left hand panels display densitometric quantification of the bands shown to the right.



MVP has been implicated as a novel regulator of Src-mediated signalling cascades (Kim *et al.,* 2006) which regulate diverse cellular functions including proliferation, differentiation, motility, adhesion and architecture (Schwartzberg, 1998; Thomas *et al.,* 1998). Over-expression of MVP in Src over-expressing cells down-regulates epidermal growth factor (EGF)-dependent Erk activation (Kim *et al.,* 2006), which is required for activation of protein synthesis (Rolfe *et al.,* 2005). It is conceivable that down-regulation of MVP in the mammary gland of the lactating cow following GH treatment, may influence protein synthesis via the Erk pathways. This may be one mechanism that partially explains the increase in protein synthesis observed following GH treatment; however, further research is required to substantiate this.

The 71-kDa protein, HSPA8, functions as an ATP-dependent molecular chaperone that assists in the folding of newly synthesized polypeptides, the assembly of multiprotein complexes, transport of proteins across cellular membranes, and targeting of proteins for lysosomal degradation (reviewed in Kiang & Tsokos, 1998). Post-translational processing of milk proteins occurs in the ER and chaparonins may be involved in this. Therefore it is plausible that the decrease in HSPA8, in response to Atr treatment, is a secondary effect consequent on decreased milk protein synthesis. Additional research will be required to verify this.

A potential role for calmodulin in the regulation of milk protein synthesis could be via its involvement in the regulation of the elongation step of protein translation, through its effect on eukaryotic elongation factor 2 (eEF2) kinase which is normally completely dependent upon Ca^{2+} and calmodulin (Browne & Proud, 2004). eEF2 kinase is responsible for the phosphorylation of eEF2, which is a GTP binding protein that mediates the translocation step of elongation (Browne & Proud, 2002). Similarly, the involvement of translational regulation in the effect of Atr on milk protein synthesis is indicated by the down-regulation of the 60S ribosomal protein L9, which is the large subunit to the eukaryotic ribosome. Ribosomes contain specific sites that enable them to bind to the mRNA, the tRNAs, and specific protein factors required for protein synthesis. However, these results remain to be validated for their significance in relation to the regulation of milk synthesis.

CONCLUSION

The proteomic data obtained in this study has provided two novel targets, MVP and HSPA8, which may be involved in the regulation of milk protein synthesis in the lactating dairy cow. Further research is required to establish their functional role and their utility as targets for improving milk yield or milk characteristics.

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