Journal of Dairy Research (2010) **77** 199–204. © *Proprietors of Journal of Dairy Research 2010*
doi:10.1017/S0022029910000087

Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line

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Received 27 May 2009; accepted for publication 11 November 2009

The MAC-T cell line has been used extensively to investigate bovine mammary epithelial cell function. A lactogenic phenotype is generally induced in this cell line by a combination of dexamethasone, insulin and prolactin and has typically been assessed by milk protein production. Few studies have focused on identifying other factors that may affect milk protein synthesis in the MAC-T cell line, and none have considered the lipid class distribution of MAC-T cells as a component of the lactogenic phenotype. Growth hormone (GH) has been shown to increase milk protein synthesis both in vivo and in mammary cell models, and has been shown to alter the lipogenic profile of mammary explant models. We tested the hypothesis that MAC-T cells would respond directly to GH and that the response would include alterations to the lipid class distribution as well as to milk protein gene expression, leading to a more appropriate model for mammary cell function than treatment with dexamethasone, insulin and prolactin alone. Differentiated cells expressed GH receptor mRNA, and addition of GH to the differentiation medium significantly induced production of α-$_{s1}$ casein and α-lactalbumin mRNA. GH also significantly affected the proportion of triacylglycerol and sphingomyelin. These results indicate that GH is an important factor in inducing a lactogenic phenotype in the MAC-T cell line, and support the possibility of a direct effect of GH on milk synthesis *in vivo*.

Keywords: Growth hormone, somatotropin, MAC-T, mammary, lipid, milk protein.

The mammary epithelial cell is responsible for the synthesis and secretion of milk components and therefore is a primary focus for the study of mammary function. The mammary alveolar cell-T (MAC-T) is a continuous cell line derived from bovine mammary tissue by stable transfection with the SV-40 large T-antigen (Huynh, 1991) and has been used extensively for the study of mammary epithelial cell function. Induction and maintenance of a lactogenic phenotype in mammary cell and explant models, including the MAC-T cell line, has commonly been achieved using a combination of dexamethasone, insulin and prolactin (PRL; e.g. Huynh, 1991; Matitashvili, 1997).

Growth hormone (GH), also termed somatotropin, is known to have stimulatory effects on milk production *in vivo* (reviewed by Bauman, 1992; Bauman & Vernon, 1993; Bauman, 1999) and while it is well accepted that GH acts through somatomedins such as the insulin-like growth factors produced locally and by the liver, it remains somewhat in question whether the effects of GH include direct influence on the mammary epithelium (e.g. see Bauman & Vernon, 1993; Svennersten-Sjaunja & Olson, 2005; Akers, 2006). GH receptor (GHR) has been detected in mammary tissue (e.g. Hauser et al. 1990; Plath-Gabler et al. 2001) and investigation into the effects of GH administration on the mammary epithelium in particular have subsequently been pursued in cell models of lactation. Sakamoto et al. (2005) observed GHR expression on the surface of a novel bovine mammary cell line cloned from a Holstein heifer, and found that GH administration to these cells enhanced their secretion of α-casein both independently and in synergy with the classical combination of dexamethasone, insulin and PRL (DIP). Additionally, these investigators noted that treatment with DIP but without GH enhanced the expression of GHR in the mammary cells. More recently, both the GHR and the signal transducer and activator of transcription 5 (STAT5) were ectopically expressed in MAC-T cells, and

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Table 1. Primer sequences used for quantitative PCR analysis in this study

Target	Sense Primer (5′–3′)	Antisense Primer (5′–3′)	Source†
GHR	CTAACTAGCAATGGCGGT	GGGTGGATCTGGTTGTACTA	NM_176608
LALBA	AAAGACGACCAGAACCCTCA	GCTTTATGGGCCAACCAGTA	Zhou et al. 2008
CSN1S1	AATCCATGCCCAACAGAAAG	TCAGAGCCAATGGGATTAGG	Zhou et al. 2008
ACTB	GCGTGGCTACAGCTTCACC	TTGATGTCACGGACGATTTC	Bionaz & Loor, 2007

† GHR primers were designed from GenBank accession number NM_176608 using PrimerQuest software available at www.idtdna.com and primer quality was further assessed using Beacon Designer (Premier Biosoft International)

subsequent treatment with GH led to significant increases in the abundance of α_{s1}-, α_{s2}- and β-casein, as well as α-lactalbumin mRNA (Zhou et al. 2008). While these investigators were able to detect GHR mRNA in MAC-T cells both by PCR and ribonuclease protection assay, the overexpression of GHR and STAT5 was intended to exaggerate any response to GH that may have been observed; no data were reported on the effects of GH in the untransfected cells.

The influence of lactogenic hormones on milk protein production is an important means of assessing the quality of a mammary cell model, though the lipogenic profile of mammary cells could also be used to determine the pertinence of such a model. Milk lipids consist mainly of triacylglycerol (TAG) and while freshly dispersed mammary cells of ruminant origin synthesize predominanty TAG (Hansen & Knudsen, 1987a,b) the MAC-T cell line produces predominantly polar lipids under the traditional lactogenic hormone milieu (present work and D G Peterson, unpublished observations). Treatments that increase the proportion of TAG synthesized by cultured mammary cells could be useful, particularly when studying mammary lipogenesis.

The recent findings with respect to GH treatment of mammary cell lines led us to the hypothesis that GH may act directly to alter milk protein synthesis and the lipid class distribution of MAC-T cells to better mimic the mammary epithelium *in vivo*. Our objectives were to determine whether the cells expressed GHR mRNA, and whether the addition of GH to the differentiation medium would affect the abundance of α-lactalbumin and α_{s1}-casein mRNA, as well as the lipid class distribution as determined by thin-layer chromatography.

Materials and Methods

All reagents were acquired from Sigma Aldrich (St Louis MO, USA) unless otherwise stated.

Cell culture and treatments

MAC-T cells were grown to confluence in 100-mm diameter CellBIND-treated plastic cell culture dishes (Corning Inc., Corning NY, USA) using a proliferation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS; Innovative Research, Novi MI, USA), 1% penicillin/streptomycin, 5 μg/ml insulin, 1 μg/ml progesterone. To ensure that any effect of serum was consistent between treatments, all FBS used was from a single lot. Once confluent, MAC-T cells were treated for 7 d with differentiation medium consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 10 μg/ml dexamethasone, 5 μg/ml insulin, 5 μg/ml prolactin and either 0 or 10 ng/ml bovine GH [generously provided by G Bogosian (gregg.bogosian@monsanto.com) at Monsanto, St. Louis MO, USA]. Each experiment was conducted in triplicate.

mRNA abundance

For determination of mRNA abundance, cells were harvested from one confluent 100-mm plate per replicate (n=3 replicates) by scraping, total RNA was extracted using the RNeasy kit (Qiagen, Valencia CA, USA) and 500 ng was reverse transcribed using oligo dT primed iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules CA, USA). Abundance of GHR, α-lactalbumin (LALBA) and α_{s1}-casein (CSN1S1) mRNA was determined by quantitative PCR using Fast SYBR Green Master Mix (Applied Biosystems, Foster City CA, USA) and the primers specified in Table 1. All primers were obtained from Integrated DNA Technologies (Coralville IA, USA). Reactions were carried out using a 7500Fast PCR system (Applied Biosystems) with an initial denaturing step of 20 s at 95 °C followed by 55 cycles of 3 s at 95 °C and 30 s at 60 °C. Initial target mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method with abundance of β-actin mRNA serving as an internal control to correct for the efficiency of reverse transcription and data for each treatment were expressed relative to the undifferentiated cells (Livak & Schmittgen, 2001). PCR product quality and specificity were verified by melt curve analysis and subsequent agarose gel electrophoresis.

Lipid composition

For lipid separation, cells were harvested by scraping from two confluent 100-mm plates of cells per replicate (n=3 replicates) and subsequently pelleted by centrifugation at 500 ***g*** at 4 °C for 10 min. Total lipid was extracted from the cell pellet using 2 : 1 chloroform-methanol (Folch et al. 1957) and separated on the basis of polarity by thin layer chromatography (TLC) using a 65 : 25 : 4

chloroform-methanol-water mobile phase (Christie, 1982). TLC plates were placed in a sealed chamber with iodine crystals for 24 h followed immediately by scanning and quantification by densitometry using Molecular Analyst (Bio Rad, Hercules CA, USA). Relative abundance was calculated as the density of each band normalized to the total density of all bands for each lane on each TLC plate. Bands were identified by comparison with standards representing TAG, cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and sphingomyelin (SM) that were included with each plate; two bands did not correspond to any of the chosen standards and are designated Unknown.

Cell viability

Cell viability was assessed in parallel cultures grown as described above. Viability was assessed in cells grown to confluence in proliferation medium, as well as in cells differentiated with DIP alone and DIP+10 ng/ml GH using flow cytometry with the ViaCount cell viability assay (Guava Technologies, Hayward CA, USA) according to the manufacturer's recommendation in a Guava EasyCyte Plus flow cytometer (Guava Technologies). Prior to incubation with the ViaCount reagent, cells were detached from the culture substrate by incubation with trypsin-EDTA solution and washed in complete medium before pelleting and resuspension in PBS.

Statistical analysis

Lipid composition data were analysed using the general linear model of SAS (SAS Institute, Cary NC, USA) for differences between treatments. Data were transformed by arcsine-square root for analysis owing to a tendency for non-normality at the extremes of values represented as a proportion (between 0 and 1; Freeman & Tukey, 1950). Pairwise comparisons between means were made using the Tukey test. For statistical analysis of mRNA abundance and cell viability data, normalized abundance for each treatment was analysed using the general linear model of SAS for differences between treatments. Tukey's simultaneous test was used to compare all means with each other. In all cases, differences were considered significant at $P<0{\cdot}05$.

Results

To determine whether any effect of GH could be due to direct, receptor-mediated effects, we used the classical DIP differentiation protocol with either 0 or 10 ng/ml GH and quantified the abundance of GHR mRNA, as well as that of two milk protein genes, *LALBA* and *CSN1S1*. Differentiation with DIP in the absence of GH led to a 14-fold increase in GHR mRNA, while DIP with 10 ng/ml GH led to a 35-fold increase in GHR mRNA compared with the undifferentiated cells (Fig. 1A). Differentiation

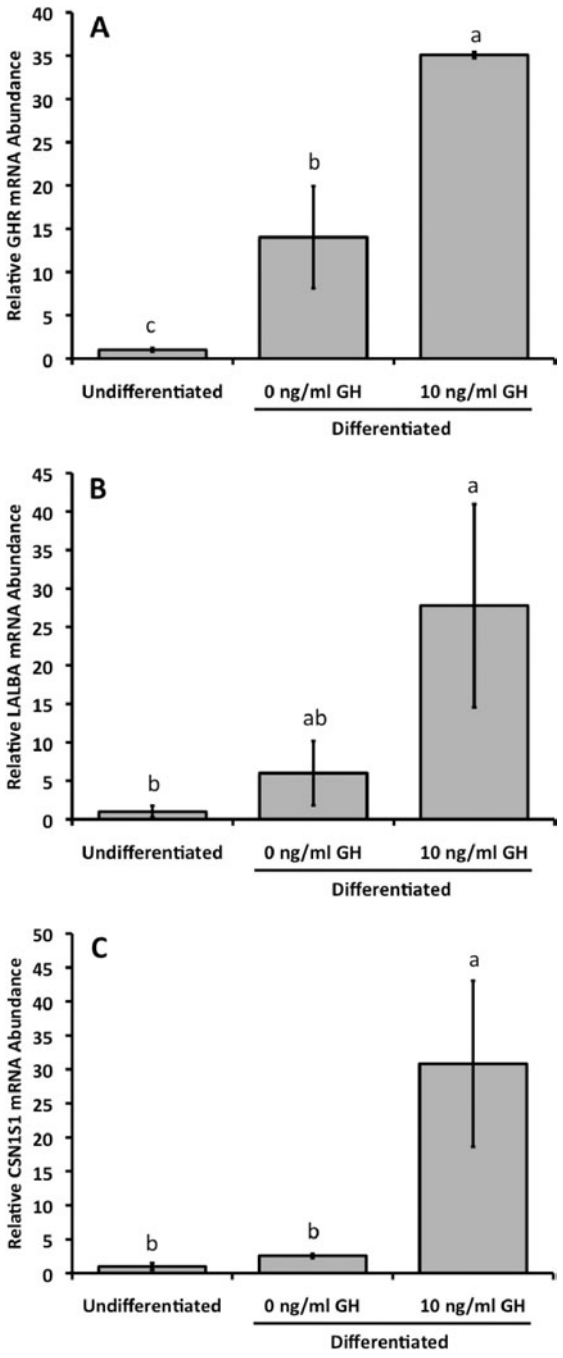

Fig. 1. Abundance of growth hormone receptor (GHR; panel A), ∝-lactalbumin (LALBA; panel B) and $\propto_{s1}$-casein (CSN1S1; panel C) mRNA in undifferentiated MAC-T cells as well as those differentiated with either 0 or 10 ng/ml GH. Undifferentiated cells were grown to confluence in DMEM supplemented with 10% FBS, 5 μg/ml insulin and 1 μg/ml progesterone; differentiated cells were grown to confluence as the undifferentiated cells then cultured for 7 d in DMEM supplemented with 10% FBS, 5 μg/ml insulin, 10 μg/ml dexamethasone, 5 μg/ml PRL, and the GH concentrations noted. Relative mRNA abundance was assessed by quantitative PCR and normalized to the expression of β-actin. Values represented are means ($n=3$); error bars represent SEM; values without a common letter differ ($P<0{\cdot}05$).

Table 2. Lipid class distribution of MAC-T cells grown to confluence in DMEM supplemented with 10% FBS, 5 μg/ml insulin and 1 μg/ml progesterone, and then differentiated for 7 d in DMEM supplemented with 10% FBS, 5 μg/ml insulin, 10 μg/ml dexamethasone and either 0 or 10 ng/ml GH. Values are means (n=3) with SEM, the pooled standard error of the mean for each dependent variable

	Growth hormone (ng/ml)		
	0	10	P
Triacylglycerol	10·3±1·0	18·6±0·2	<0·01
Cholesterol	18·8±1·7	24·5±5·9	0·16
Phosphatidylethanolamine	7·0±0·6	6·7±0·7	0·80
Phosphatidylcholine+phosphatidylinositol†	7·3±0·3	6·0±0·4	0·07
Sphingomyelin 1‡	24·3±2·2	24·6±1·7	0·92
Sphingomyelin 2‡	13·4±0·3	3·7±0·4	<0·01
Unknown 1§	4·2±0·4	2·8±0·6	0·13
Unknown 2§	9·6±1·7	7·2±1·8	0·39
Origin	5·3±0·9	6·0±1·0	0·66

† Co-eluted and are expressed as the combined value
‡ Spingomyelin eluted as two separate bands as has been previously observed (Ramstedt et al. 1999) and each band is represented individually
§ Two bands did not correspond to any of the chosen standards, and are represented as Unknown

with DIP led to small numerical increases in LALBA and CSN1S1 mRNA that were not significant, while GH addition to the differentiation media led to significant 28- and 32-fold increases in LALBA and CSN1S1 mRNA, respectively, as compared with the undifferentiated controls (Figs 1B, C).

Observations of our TLC plates indicate that PC and PI migrated as one band with the current protocol, and SM eluted as two separate bands as has been described previously (Ramstedt et al. 1999). Semi-quantitative analysis of lipid class distribution was achieved by densitometry and the results are shown in Table 2. Differentiation with 10 ng/ml GH led to an 80% increase in the proportion of TAG and a more than three-fold decrease in the proportion of the SM2 fraction (P<0·05). It is noteworthy that 10–14% of the total lipid did not correspond to any of our chosen standards (designated Unknown) and while these fractions were not significantly affected by GH, it would be of interest to identify these lipid classes. Based on their migration in relation to the known lipid classes, these are polar lipids with Unknown 1 being more polar than SM, while Unknown 2 had a polarity in between SM and the PC+PI fraction.

To ensure that any differences observed were not due to differences in cell number due to GH addition to the differentiation media, we analysed cell viability and found no significant effect of GH on viable cell number or percent viable cells (P>0·20; data not shown).

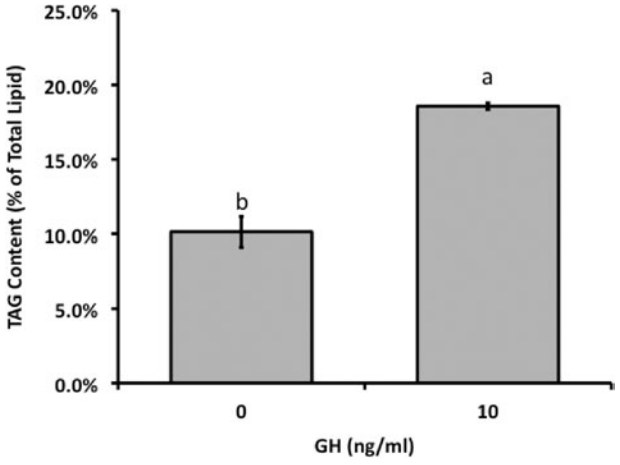

Fig. 2. Effect of growth hormone (GH) on triacylglycerol (TAG) content in MAC-T cells. Cells were grown to confluence in DMEM supplemented with 10% FBS, 5 μg/ml insulin and 1 μg/ml progesterone, and then differentiated for 7 d in DMEM supplemented with 10% FBS, 5 μg/ml insulin, 10 μg/ml dexamethasone and either 0 or 10 ng/ml GH. Total lipids were extracted and lipid classes separated by TLC. Each band resulting from the TLC separation was quantified by densitometry and TAG content was calculated as a percent of the sum of all band densities for that lane. Values represented are means (n=3); error bars represent SEM; values with different letters differ (P<0·01).

Discussion

There has been much speculation about the exact nature of the effects of GH on lactation *in vivo*, and it remains unclear whether GH has any direct effect on the mammary epithelium. The identification of GHR expression both in the bovine mammary gland (Plath-Gabler et al. 2001) and in bovine mammary cell lines including the MAC-T (Sakamoto et al. 2005; Zhou et al. 2008) indicate a possible direct influence of GH on the mammary epithelial cell. Accordingly, Sakamoto et al. (2005) showed a positive influence of GH on milk protein production in a mammary cell line. More recently, Zhou et al. (2008) demonstrated that GH administered to MAC-T cells that were overexpressing GHR and STAT5 also led to increased production of milk proteins.

The demonstration of very low GHR abundance in undifferentiated native MAC-T cells by Zhou et al. (2008) led us to determine the abundance of GHR mRNA in undifferentiated cells as well as those differentiated with either DIP or DIP and GH. We found that differentiation led to a significant increase in the abundance of GHR mRNA, and that this was even more dramatic in the GH treated cells. A similar pattern was observed for two major milk protein genes, *CSN1S1* and *LALBA*, though it was surprising to find a much greater difference between the DIP differentiated cells with and without GH than between undifferentiated cells and those differentiated with DIP alone.

The lipid component of milk represents the major energy cost for milk production, the primary energy source for the consumer of whole milk, and a natural source of bioactive compounds such as conjugated linoleic acids. As such, milk fat synthesis is of interest to many research groups, and a convenient cell model for the study of milk fat synthesis would be of great value. While Hansen & Knudsen (1987a,b) have reported that freshly dispersed mammary cells of bovine and caprine origin predominantly produce TAG, the major lipid class of secreted milk fat, there is no report of the actual secretion of a milk fat globule by a mammary cell model. Fekry et al. (1989) found that milk lipid synthesis was enhanced in bovine mammary explants treated with GH, though only when co-cultured with adipose and liver explants, leading to the conclusion that the effects of GH on mammary lipid synthesis were indirect, possibly through IGF production by the liver explants.

Here, we have demonstrated that the addition of GH to differentiation medium shifts the lipid class distribution of MAC-T cells toward greater TAG content, a phenotype that more closely resembles lactation *in vivo*, and that this effect was observed without overexpression of GHR or STAT5. It is unclear from this experiment whether the change in TAG content as a proportion of total lipid represented an increase in actual amount of lipid synthesized. Our results indicate that changes in cell number due to proliferative or toxic effects of GH are not responsible for the alterations in lipid composition. Observations *in vivo* of increased output of milk with normal composition without any change in mammary DNA content indicate that GH acts to increase the overall synthetic capacity of the mammary gland (reviewed in Bauman, 1999). This overall change, whether direct, indirect, or both, would require a coordinated response within the cell that involved many different pathways, and our results demonstrate that GH affects not only lipid metabolism but also the mRNA abundance of two milk proteins in MAC-T cells. It is important to note that based on our analysis, the changes in mRNA abundance cannot be attributed specifically to increases in synthetic rate, but may instead represent changes in the stability of mRNA without any change in transcription rates.

The present results indicate that GH can directly affect the MAC-T cell line, and based on the presence and inducibility of GHR mRNA, that the effects of GH are likely mediated by GHR. Further, it appears that the effects of GH involve responses in milk protein mRNA abundance as well as lipogenic pathways, consistent with findings of GH treatment of lactating cows (reviewed by Bauman, 1999). It is possible that GH is important in attaining a more differentiated state in the MAC-T cells as opposed to enhancing the production of already differentiated cells. Either way, in developing cell culture models of lactation, it may be important to consider GH as an integral part of the hormone milieu that is required for maximal galactopoietic potential. Finally, these results support the developing hypothesis that GH may have a direct effect on mammary metabolism *in vivo*.

The authors would like to thank Dr Gregg Bogosian (Monsanto Co.) for providing the bST used in this project and Dr Matthew Burd, Dr Brooke Humphrey and Lisa McDonnell for critical review of the manuscript and technical assistance. Partial funding for this project was provided by the Department of the Navy, Office of Naval Research under award number N00014-06-1-1111, and by the California State University Agricultural Research Initiative (ARI) under award number 07-3-011.

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