

Role of Prolactin, Growth Hormone and Insulin-Like Growth Factor 1 in Mammary Gland Involution in the Dairy Cow

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ABSTRACT

Bovine mammary involution, an important process for subsequent lactations, is characterized by loss of epithelial cells by apoptosis, but its hormonal regulation is still not well defined. Prolactin (PRL) and growth hormone (GH) play a specific role on rat mammary gland apoptosis, through insulin-like growth factor 1 (IGF-1) and the IGF binding protein (IGFBP) system. The purpose of our investigation was to determine the possible role of PRL, GH, and IGF-1 on cell survival and on IGFBP-5 expression in the bovine mammary gland.

Mammary gland explants were cultured in the presence of cortisol, 17 β -estradiol, progesterone, insulin, PRL, GH, and IGF-1 and with the same treatment but without PRL, GH or IGF-1, respectively. After 24 h of culture, we determined the level of apoptosis through evaluation of DNA laddering in the oligonucleosomal fraction and examined IGFBP-5 messenger RNA (mRNA) expression. The results show a high level of DNA laddering and an increase in IGFBP-5 mRNA content in mammary explants cultured in the absence of PRL, GH, or IGF-1 with respect to explants treated with all hormones. Moreover, explants cultured in presence of PRL, GH, or IGF-1 show a low level of DNA laddering and IGFBP-5 expression with respect to explants cultured without any hormones. These data demonstrate a relationship between levels of apoptosis and IGFBP-5 mRNA expression in the bovine mammary gland and confirm the involvement of this binding protein programmed cell death and its relationship with the main lactogenic hormones.

(Key words: dairy cow, mammary gland, involution)

Abbreviation key: GH = growth hormone, I = initial tissue, IGFBP = IGF binding protein, NH = no hormones, PRL = prolactin, RT-PCR = reverse transcriptase-PCR, SHI = involuting hormonal set.

INTRODUCTION

Involution of mammary gland as part of the reproductive cycle in dairy cows is a very important remodeling transformation of the gland for the subsequent lactation. It is mainly characterized by two contemporary processes: loss of mammary epithelial cells (even though more moderate than the intensive loss of cells in rodent mammary gland) and proteolytic degradation of the extracellular matrix (Talhouk et al., 1992; Quarrie et al., 1996; Wilde et al., 1997a). During this transformation, cell removal occurs through programmed cell death or apoptosis (Jaggi et al., 1996).

The apoptotic process has several hallmarks, but one of the last stages is DNA fragmentation. Apoptosis is accompanied by the activation of endonucleases, the enzymes responsible for DNA fragmentation into oligonucleosomal fragments. Thus DNA laddering is considered to be an indicator of mammary epithelial cells apoptosis (Quarrie et al., 1996).

Although the intracellular events that determine the beginning of apoptosis in mammary epithelial cells are well known, the hormonal control and regulation of this process resulting in the immediate modification of survival equilibrium or consecutive activation of programmed cellular death pathways, are still not well defined (Quarrie et al., 1996). Until now, knowledge of mammary gland apoptosis has been based on observations mainly from the mouse, rat (Atwood et al., 1995), and goat (Quarrie et al., 1994). Observations on the hormonal regulation and control of mammary gland epithelial cells apoptosis during lactation and involution in cows have not been studied extensively. Thus, an understanding of the role and action of such factors in controlling apoptosis is essential to understand and to modify the cellular survival in order to have the possibility of controlling the intensity and duration of lactation in dairy cows.

The most important hormones playing an interactive role in maintaining mammary gland function and cell survival in rodents are growth hormone (GH) and prolactin (PRL) (Barber et al., 1992; Flint et al., 1992; Travers et al., 1996).

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PRL is of fundamental importance in the development and differentiation of the mammary gland and is widely considered to be responsible for the maintenance of mammary gland function especially in rodents (Ginsburg et al., 1997), whereas GH is important in milk production and mammary growth for ruminant lactation. While PRL seems to act directly on mammary tissue, the effects of GH are considered to be indirect, mediated through stimulation of insulin-like growth factor 1 (IGF-1) production (Flint and Knight, 1997; Keys and Dijane, 1988; Shkreta et al., 1997). IGF-1 is involved in the regulation of somatic growth and cellular proliferation, but its action is controlled by a family of proteins, the insulin-like growth factor binding proteins (IGFBP), that bind to IGF with different affinities. They have been shown to either inhibit or enhance biological effects of IGF-1 (Cohick, 1998).

It has been shown that milk from lactating animals contained very low levels of all the IGFBP. However, there was an increase in one IGFBP during mammary gland involution that was identified as IGFBP-5 (Tonner et al., 1997). IGFBP-5 expression increased in the involuting thyroid (Phillips et al., 1994), in ovarian follicles undergoing atresia (Liux et al., 1993) and in regressing prostate (Guenette, 1996). Therefore, it has been proposed that the secretion of IGFBP-5 is involved in the initial stages of apoptosis, inhibiting IGF-1-mediated cell survival (Tonner et al., 1997). Regarding the role of hormones on mammary gland apoptosis, studies have demonstrated that PRL is capable of repressing expression of IGFBP-5 mRNA in the rat mammary gland, indirectly increasing the available amount of free IGF-1 in tissue and so enhancing the survival effect on mammary epithelial cells (Sheffield and Kotolski, 1992; Travers et al., 1996; Flint and Knight, 1997).

As all the current studies have been performed in rodents, and there is no comparable information on the endocrine control of ruminant mammary involution, the aim of this work was to evaluate the effects of PRL, GH, and IGF-1 on DNA fragmentation index of apoptosis and on the expression of IGFBP-5 in bovine mammary gland explants.

MATERIALS AND METHODS

Reagents

Medium 199 was obtained from Gibco BRL Life Technologies (Carlsbad, CA); sheep prolactin, bovine insulin, hydrocortisone, progesterone, and 17β -estradiol were purchased from Sigma Chemical Co. (St. Louis, MO); bovine growth hormone was obtained from ICN Biomedicals Inc. (Costa Mesa, CA) and human insulin-like growth factor 1 was purchased from Boehringer Mannheim (Basel, Switzerland). Reagents for reverse

transcriptase-PCR (RT-PCR) assay were purchased from Promega (Madison, WI). All other reagents were of the highest purity commercially available.

Source of Tissues and Treatment

Mammary explants were prepared using a modification of the methods previously described (Dils and Forsyth, 1981). Briefly, tissue samples were obtained at slaughter from 10 nonpregnant Italian-Friesian cows in the last stage of lactation (7 to 10 mo of lactation) and placed in sterile solution until explants were prepared (approximately 30 min). About 50 mg of mammary tissue from every cow was immediately frozen in liquid nitrogen and stored at -80°C until analysis (I). Tissues were chopped to obtain pieces of about 5 to 10 mg and about three to four explants were cultured in each well of a plastic culture plate, on a stainless-steel grid support at the surface of the liquid medium. Explants were immediately incubated at 37°C (95% air; 5% CO_2) in medium 199 (1-ml volume) supplemented as follows: treatment 1) medium 199 alone (no hormones: **NH**); treatment 2) medium 199 + hydrocortisone (20 ng/ml) + 17β -estradiol (12 ng/ml) + progesterone (5 ng/ml) + insulin (0.5 ng/ml) + GH (100 ng/ml) + PRL (200 ng/ml) + IGF-1 (50 ng/ml) (**SHI**: hormonal set like the bovine plasma at the end of lactation); treatment 3) SHI without PRL (SHI-PRL); treatment 4) SHI without GH (SHI-GH); treatment 5) SHI without IGF-1 (SHI-IGF-1).

In a subsequent investigation, we collected mammary tissue from other 10 nonpregnant Italian-Friesian cows in the last stage of lactation (7 to 10 mo of lactation). Explants were prepared like the previous experiment but treated as follows: 1) medium 199 alone (NH); treatment 2) medium 199 + PRL (200 ng/ml); treatment 3) medium 199 + GH (100 ng/ml); treatment 4) medium 199 + IGF-1 (50 ng/ml).

In vitro hormones concentration were based on our previous in vitro research (data not published) and from hormonal pattern of cows in the last phase of lactation (Chiesa et al., 1991).

In both experiments, after 24 h of culture 3-well explants for each treatment were collected and processed for subsequent analysis.

DNA Analysis

Oligonucleosomal fraction of cellular DNA was extracted from mammary explants after 24 h of culture using a modification of method of Hughes and Gorospe (Hughes and Gorospe, 1991).

Briefly, tissue was homogenized in 5 mM Tris-HCl pH 8, 20 mM EDTA pH 8 and 0.5% Triton (1:10 wt/vol)

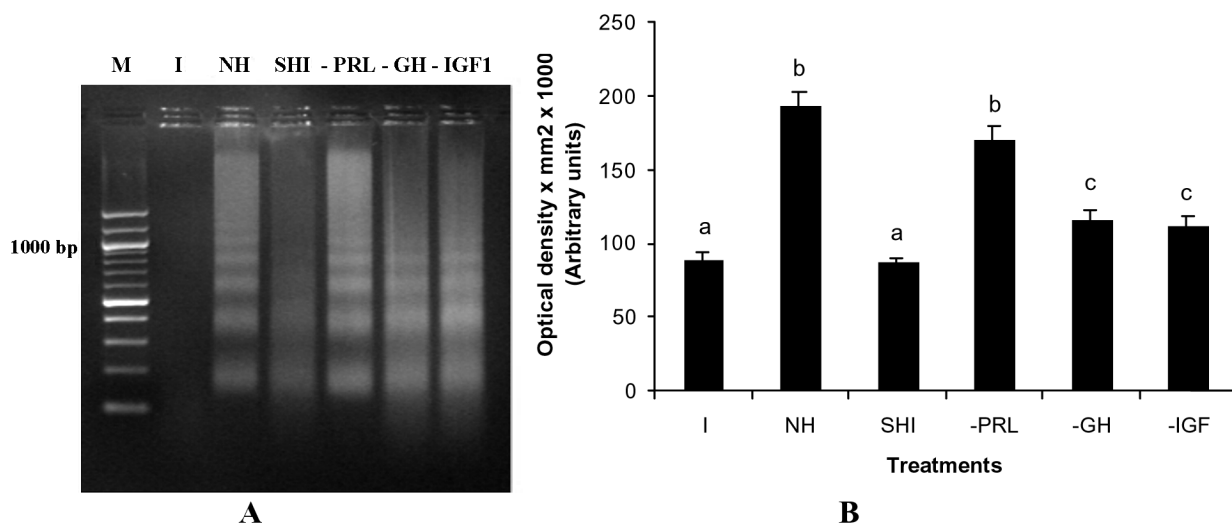


Figure 1. A. Photo of agarose gel of low molecular weight DNA extracted from bovine mammary explants treated in different hormonal concentrations (M: standard weight marker). B. Graphics of means \pm SEM of optical density of the electrophoretic DNA fragment lower than 1000 bp from 10 replicates of explants treated in different hormonal concentrations. I: mammary tissue conserved in liquid nitrogen at the moment of explants preparation, NH: explants cultured with medium 199 without hormones, SHI: medium 199 + hydrocortisone + 17β -estradiol + progesterone + insulin + GH + prolactin (PRL) + IGF-1, -PRL: explants treated as SHI without PRL, -GH: explants treated as SHI without GH, -IGF-1: explants treated as SHI without IGF-1. Means with different letters are significantly different ($P < 0.01$).

on ice. Homogenates were incubated overnight at $+4^{\circ}\text{C}$ then centrifuged twice at $27,000 \times g$ for 20 min at $+4^{\circ}\text{C}$. The supernatant was extracted with 1 volume of phenol, pH 8.0 and 1 volume of chloroform:isoamylalcohol (24:1 vol/vol) and centrifuged at $27,000 \times g$ for 10 min at $+4^{\circ}\text{C}$. The aqueous phase was precipitated with 3 M sodium acetate (1:10 vol/vol) and isopropanol (1:1 vol/vol), incubated for 30 min at $+4^{\circ}\text{C}$ and centrifuged $12,000 \times g$ for 20 min at $+4^{\circ}\text{C}$. Then the pellet was washed with ethanol 75% (1:1 vol/vol) and centrifuged $12,000 \times g$ for 20 min at $+4^{\circ}\text{C}$. At the end, the pellet was resuspended with dH_2O and treated with RNase (20 μg) for 30 min at 37°C .

After electrophoresis on 2% agarose gel, extracted DNA was stained with ethidium bromide (Gel Doc 1000, Bio-Rad) and DNA oligonucleosomal fragments smaller than 1000 pb for each treatment were quantified by densitometry and expressed in arbitrary units.

RT-PCR Assay

Oligonucleotide primers used in the RT-PCR assay were derived from the sequence of rat insulin-like growth factor binding protein 5 (IGFBP-5) mRNA (accession M62781, GenBank DNA sequences) (Hahnel and Schultz, 1994). Primers were: 5'-GTT CAA AGC CAG CCC ACG CAT-3' (sense) and 5'-GTC GAA GGC GTG GCA CTG AA-3' (antisense), synthesized by Gibco BRL, chosen in the conserved region aligning Rat IGFBP-5 mRNA and *Bubalus arnee bubalis* insulin-like

growth factor binding protein type 5 mRNA (accession AF045566, GenBank DNA sequences).

To detect the IGFBP-5 expression, 1.5 μg of total RNA were reversed transcribed. Then 9 μl of the RT reaction mixture were used in each PCR reaction which contained 45 pmol each specific primer, 50 μM MgCl_2 , 200 μM deoxy-NTP and 2.5 units *Taq* DNA polymerase, in a 50- μl total volume reaction. Samples were amplified for 35 cycles. Integrity of the sample was tested in a parallel assay in which the target cDNA was amplified using the primers for β -actin. Each PCR reaction was subjected to 2% agarose gel electrophoresis, and the amplified products were visualized by staining with ethidium bromide. Optical density of each gel-lines was measured (Gel Doc 1000, Bio-Rad), expressed in arbitrary units and normalized using the signals generated with β -actin. The presence of possible contaminants was checked by control reactions in which amplification was carried out on samples without RT in the PCR mixture.

Statistical Analysis

Statistical analyses were undertaken using ANOVA. Where significant differences were found, Duncan's test was used to determine significant difference between individual treatment.

RESULTS

Mean values of optical density of gel lines obtained from 10 different cows used as replicates and grouped

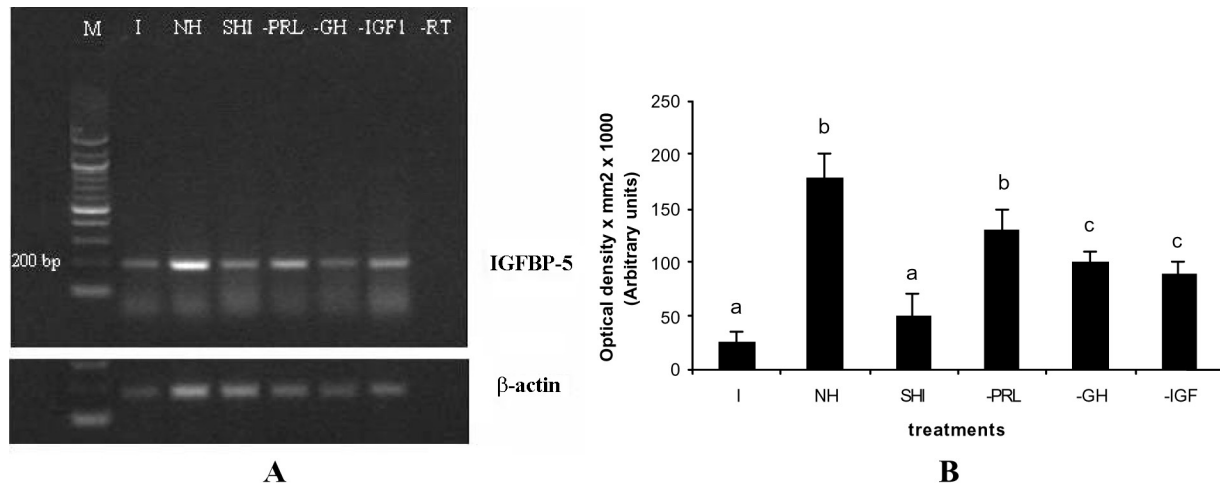


Figure 2. **A.** Photo of agarose gel electrophoresis of the reverse transcriptase (RT)-PCR analysis of IGFBP-5 and β -actin mRNA from bovine mammary gland explants cultured with different hormonal treatments (M: standard weight marker, -RT: without cDNA). **B.** Graphics of means \pm SEM of 10 replicates of optical density of electrophoretic bands expressed in arbitrary units and normalized using the signals generated with β -actin. I: mammary tissue conserved in liquid nitrogen at the moment of explants preparation, NH: explants cultured with medium 199 without hormones, SHI: Medium 199 + hydrocortisone + 17β -estradiol + progesterone + insulin + GH + prolactin (PRL) + IGF-1, -PRL: explants treated as SHI without PRL, -GH: explants treated as SHI without GH, -IGF-1: explants treated as SHI without IGF-1. Means with different letters are significantly different ($P < 0.05$).

according to the hormonal treatment and one representative gel photographed under UV illumination are presented (Figure 1). Distinct ladder-like patterns of DNA fragmentation, characteristic of apoptosis, were evident after 24 h of culture without hormones (NH). Treatment with hormonal set (SHI) suppressed cellular apoptosis, while the absence of a single hormone (PRL, GH, or IGF-1) from the hormonal set caused an increase in apoptotic DNA fragmentation after 24 h of culture. DNA laddering of the explants cultured without PRL (SHI-PRL), without GH (SHI-GH) and without IGF-1 (SHI-IGF) were statistically different ($P < 0.01$) from DNA laddering in mammary tissue prepared immediately (I) and explants treated with the complete hormonal set (SHI). The size of these fragments was consistent with generation by endonuclease cleavage of DNA into oligonucleotides. The lack of DNA fragmentation in SHI explants comparable with I explants demonstrates the validity of the culture conditions.

Amplification by RT-PCR confirmed the presence of IGFBP-5 mRNA in the lactating mammary tissue. As expected a 196-bp DNA fragment was amplified, the product extracted was sequenced (M-medical GENENCO srl, Firenze, Italy) and showed a 98% homology with rodent, bubalus, and *Homo sapiens* related sequences.

The IGFBP-5 expression on bovine mammary gland explants is shown in Figure 2. The lowest value of IGFBP-5 expression has been observed in mammary tissue frozen immediately (I) and in treatment with the

complete hormonal mix (SHI), while the highest was in the explants cultured with only medium 199 (NH) and SHI-PRL.

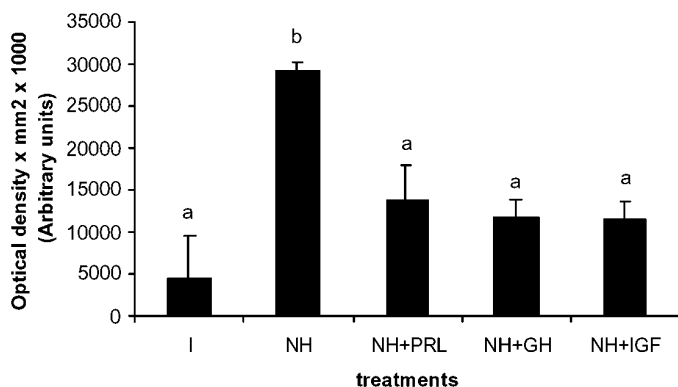
Explants treated with the complete hormonal set (SHI) showed IGFBP-5 expression statistically ($P < 0.01$) lesser than explants cultured without PRL (SHI-PRL) and, to a lesser extent, in explants cultured without GH (SHI-GH) and without IGF-1 (SHI-IGF).

The results in Figure 3 show that DNA laddering and IGFBP-5 expression were significant ($P < 0.05$) decrease in explants treated with PRL, GH, or IGF-1 add separately to NH samples compared with explants cultured without any hormones.

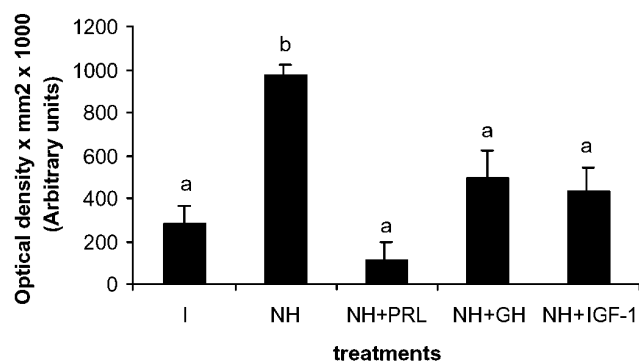
DISCUSSION

In dairy cows, genetic selection has greatly increased two interrelated factors, peak yield, and lactation persistency. Peak milk yield is determined by secretory cell and by secretory activity per cell. Studies in goats show that parenchyma cells increase in number during pregnancy and into early lactation, then the hypertrophy of the secretory cells ensure the persistence of lactation. After peak lactation, cell loss is largely responsible for the decline in milk yield, but activity per cell is maintained (Forsyth, 1996).

The decrease in mammary cell number in goats is the result of programmed cell death. The endocrine regulation of this mammary apoptosis is partially defined in rodents but awaits detailed analysis in rumi-



A



B

Figure 3. A. Graphics of means \pm SEM of optical density of electrophoretic bands of low molecular weight DNA extracted from 10 replicates of bovine mammary explants cultured with different hormonal treatments. **B.** Graphics of means \pm SEM of optical density of electrophoretic bands of reverse-transcriptase-PCR of IGFBP-5 from 10 replicates of bovine mammary gland explants cultured with different hormonal treatments expressed in arbitrary units and normalized using the signals generated with β -actin. I: mammary tissue conserved in liquid nitrogen at the moment of explants preparation, NH: explants cultured with Medium 199 without hormones, NH+prolactin (PRL): explants cultured with only prolactin, NH+GH: explants cultured with GH, NH+IGF-1: explants cultured with IGF-1. Means with different letters are significantly different ($P < 0.05$).

nants (Wilde et al., 1997b). In this study, we have found that during involution of bovine mammary gland, the loss of mammary epithelial cells by apoptosis, demonstrated by typical DNA fragmentation, is correlated to the declining of PRL, GH, and IGF-1 concentration as already seen in rodents (Wilde et al., 1997a).

Many studies have shown that PRL is stimulatory to mammary growth and lactogenesis. The importance of PRL in lactogenesis is well known (Akers, 1985), but

in the last years studies in rodents and in goat show that PRL seems to be one of the principal endocrine signals in controlling cell death during involution. In fact, the deficiency of this hormone resulted in a consistent reduction of milk yield and loss of around 20 to 25% of the secretory cell population of the mammary gland in 48 h (Flint and Knight, 1997). Moreover, prolactin treatment following litter removal delayed mouse mammary apoptosis (Sheffield and Kotolski, 1992), and PRL depletion with bromocriptine during lactation also rapidly induced DNA laddering and cell loss, even in the absence of milk stasis (Travers et al., 1996, Wilde et al., 1999). Our results show that PRL, until now considered a survival factor only in rodents, seems to have an important role in the control of involution of mammary epithelial cells also in the bovine.

The GH clearly plays an important role for maintaining lactation *in vivo*; in fact, exogenous administration of GH induces an increase in milk yield of 10 to 40% (Barber et al., 1992). Nevertheless, the induction of mammary apoptosis by antigrowth hormone serum in lactating rats (Tonner et al., 1997) suggests that growth hormone could be also a systemic mediator of the effect of litter removal on tissue involution (Wilde et al., 1999). Our results show that the absence of GH from the culture medium induces DNA fragmentation in ruminant tissues although it was less effective than PRL removal, as already seen in rodents (Wilde et al., 1997b).

The direct biological effect of GH on mammary gland is still controversial because it stimulates neither *in vitro* proliferation of mammary epithelial cells nor casein and fatty acid synthesis. Moreover to date there is no evidence for the presence of functional GH receptors on mammary epithelial cells, although expression of GH receptor mRNA has been reported (Akers, 1985; Hauser et al., 1990).

Therefore, GH action on mammary gland seems to be indirect and the hormone thought to mediate the effects of GH is IGF-1, mainly synthesized by the liver, but also produced by stromal mammary cells (Davis et al., 1987; Shkreta et al., 1997; Cohick, 1998). However, there are many contradictory results supporting or refusing this IGF-1 role (Flint and Knight, 1997).

Considerable data exist to support a role for IGF-1 in the regulation of lactation *in vitro*. It has a stimulatory effect on DNA synthesis and mammary cell proliferation (fibroblast and epithelial cell), casein gene expression and synthesis, glucose transport, (Baumrucker and Stemberger, 1989; Cohick, 1998; McGrath et al., 1991; Peri et al., 1992). Besides its mitogenic activity, IGF-1 could act as a survival factor, inhibiting apoptosis the mammary gland (Forsyth, 1996). In fact, transgenic mice expressing IGF-1 under the control of a mammary-

specific promoter failed to undergo postlactational involution (Neuenschwander et al., 1996).

IGF-1 is regulated by a family of proteins: IGFBP, which play an important role, activating or inhibiting IGF-1 action (Barber et al., 1992). Evidence shows that a number of IGFBP as well as IGF themselves are synthesized by the mammary epithelial cells (Quarrie et al., 1996). During lactation there is little or no expression of IGFBP mRNA. In contrast, during postlactational involution there is a fourfold increase in IGFBP-2 mRNA and dramatic six- and ten-fold increases, in the expression of IGFBP-4 and IGFBP-5, mRNA and protein within 24 h after weaning (Tonner et al., 1995; Tonner et al., 1997; Rosfjord and Dickson, 1999). IGFBP-5 has been proposed to bind to IGF-1, preventing its biological action and inducing the cascade events that lead to apoptosis (Guenette, 1996; Tonner et al., 1997).

Our results in dairy cows demonstrate that the presence of tropic hormones: PRL, GH, and IGF-1 repress the expression of IGFBP-5 mRNA. Thus, this study supports a possible explanation for an interactive role of GH and prolactin, whereby GH stimulates synthesis of a survival factor (IGF-1), whereas prolactin optimizes IGF-1 action by suppressing the expression of an inhibitor of IGF action, IGFBP-5 (Wilde et al., 1997b).

Aware of the limitation of in vitro cultures, the results of this paper demonstrate the involvement of IGFBP5 in apoptotic pathways. However, is important to take into account the presence of many other local factors regulating mammary cell death: the well-known milk stasis (Quarrie et al., 1996), milking frequency, and a novel milk protein named feedback inhibitor of lactation.

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