Expression profile of caseins, estrogen and prolactin receptors in mammary glands of dairy ewes

Monica Colitti,1 Giuseppe Pulina2
1Dipartimento di Scienze Animali, Università di Udine, Italy
2Dipartimento di Scienze Zootecniche, Università di Sassari, Italy

Abstract

In this study, we analyzed the developmental expression of estrogen receptors (ESR1 and ESR2), prolactin receptors (PRLR) and casein genes (CSN1S1, CSN1S2, CSN2 and CSN3) in the ewe mammary glands from prepubertal stage to involution. Using Real-time PCR we showed that the activation of casein genes transcription was up regulated during lactation and significantly down regulated before lambing and at involution in comparison to the expression measured in the prepubertal group. The highest expression of ESR1 and ESR2 genes occurred in prepubertal group compared to adult group. The PRLR expression of the short and long forms was up regulated before lambing and down regulated during lactation and involution.

Thus, the mRNA expression data for ESRs and PRLR show clear regulatory changes suggesting involvement of these receptors in sheep mammary glands during development to involution. Casein genes transcription could be primed through PRLR signal, but other factors may be necessary for milk protein long-term expression during lactation.

Introduction

In dairy sheep, genetic selection has caused deep morphological changes in the udder and physiological changes in the whole body of the animal. The former is identified by the higher mammary cistern volume, the latter by neuro-hormonal changes. These changes allow the alveoli to have a longer life span and maintain a metabolic status that favours the switch of energy and nutrients to the mammary gland instead of body reserves (Pulina et al., 2007). The pattern of the lactation curve is influenced by the number of secretory cells in the mammary gland at each day in milking (DIM) and by the synthetic activity of each secretory cell. Growth and differentiation of the glandular epithelium during puberty and pregnancy are important determinants of the total area of secretory epithelium and consequently of milk yield (Pulina et al., 2009).

These physiological changes are orchestrated by systemic and local factors, which control synthetic and secretory mechanisms by transcriptional control of key mammary genes (Stefanoni et al., 2002).

In dairy species, it is generally believed that there is normally little mammary growth during established lactation (Akers 2002); however, in human breast the onset of secretory activity is accompanied by glandular-alveolar growth and expansion of acini (Battersby and Anderson, 1988). Moreover, in many tissues it exists a dividing transit population of cells in which signs of proliferation, phenotypic differentiation and functional differentiation are displayed simultaneously (Potten and Loeffler, 1990). In a recent paper, we reported for the first time the turnover of mammary cells and the interaction of their signals during the complete lactation cycle in sheep (Colitti and Farinacci, 2009); we concluded that mammary glands of dairy ewes seem to operate in a much more dynamic state than other lactating animals and this is particularly important in the construction of mechanistic models of lactation. In general, these models are based on the assumption that milk production at each time of lactation depends on the number of active cells and on the secretory activity (Dimaruo et al., 2007). Therefore, mechanistic models of lactation could represent a useful tool to evaluate possible effect of selection for increasing lactation persistency in different breed and production scenarios. In a complementary paper (Pulina et al., 2009), based on the same experimental units, we concluded that the milk production around lactation peak (30 L) is sustained by the higher epithelium volume and higher milk secretion rate per secretory tissue unit.

The estrogen receptor-α (ESR1) is a critical transcription factor that regulates epithelial cell proliferation and ductal morphogenesis during postnatal mammary gland development (Feng et al., 2007). Between the two isoforms α and β (ESR1 and ESR2, respectively), ESR1 is considered the primary receptor for mammary gland development and function; it induces proliferation of the mammary tissue, but the mechanism is not clear, since the proliferating mammary cells do not contain this receptor (Clarke et al., 1997). Moreover, ESR2 modulates ESR1 action in tissues where they are co-expressed (Hall and McDonnell, 1999).

The role of prolactin in milk synthesis is probably related to the fact that it inhibits mammary apoptosis by suppressing the actions of IGF binding protein (IGFBP-5), which antagonizes the effects of IGF-1 on the survival of mammary epithelial cells (Tonner et al., 2000). Proliferation and differentiation of secretory mammary epithelium are also dependent on the presence of the prolactin receptor (Ormandy et al., 1997) and the downstream Jak2-Stat5 pathway (Liu et al., 1998). The prolactin receptor (PRLR) belongs to the superfamily of cytokine receptors (Kelly et al., 1991) and exists in different isoforms, generated by alternative splicing, that are identical in their extracellular ligand-binding domain, but differ in the length and sequence of their intracellular domains (Bole-Feyssot et al., 1998). cDNAs encoding a long and a short form of PRLR have been isolated from different species (Bignon et al., 1997; Shirota et al., 1990) and are differentially expressed in different tissues, suggesting that they can activate distinct signalling pathways (Schuler et al., 1997). The long form of PRLR activates Jak2, a cytoplasmic protein tyrosine kinase, which in turn can serve as docking sites for the SH2 domains in STAT5 (Groner, 2002). Activated STAT5 binds to DNA sites in the nucleus known as GAS elements and modulates the activity of target genes, as the β-casein gene (Kazansky et al., 1995; John et al., 1999). However, the lack of correspondence of STAT5a gene expression and β-casein gene expression suggests that STAT5 activation may facilitate the interaction of other factors binding within composite response elements identified recently in the milk protein gene promoters. Responsive
elements are responsible for the stable expression of milk protein genes in terminally differentiated mammary epithelial cells (Kazansky et al., 1995). In lactating animals, STAT5a induces expression of milk protein genes, largely in response to prolactin (Nevalainen et al., 2002) together with laminin-1, which is a major basement membrane component required for milk protein expression (Streuli et al., 1995; Xu et al., 2009).

Moreover, in a recent paper it has been demonstrated that in ruminants, the increased milking frequency enhances the expression of the long and short isoform of prolactin receptors and β casein on the mammary epithelial cells and reduces cell apoptosis by modulating hormone sensitivity (Bernier-Donner et al., 2010).

Caseins comprise a group of four proteins (κs1, κs2, β, and α) resulting from the expression of four structural genes (CSN1S1, CSN1S2, CSN2, and CSN3, respectively) (Bevilacqua et al., 2006). These proteins represent on average 82% of sheep milk Total Nitrogen (N x 6.38; Pulina and Nudda, 2004), but there is a large variability from one species to another (Miranda et al., 2004).

Since a surprisingly very high proliferation index, measured by Ki-67 immunostaining, was observed during lactation in mammary glands of Sardinian sheep (Colitti and Farinacci, 2009), the goal of this study was to investigate in the same mammary tissues the expression of CSN1S1, CSN1S2, CSN2, and CSN3, markers of functional differentiation. The expression patterns of ESR1, ESR2, and PRLR long and short forms, which play an active role in morphogenesis, growth, and functional differentiation, were also studied in mammary glands of sheep prior to lambing to involution.

**Materials and methods**

**Animals**

Tissue was collected from mammary glands of thirty Sardinian sheep that were slaughtered at different developmental stages: prepubertal (30±5 days, group P), 10 days before lambing (group LateP), 50, 60, 150 DIM (groups 30L, 60L, 150L, respectively) and 8 days after the end of lactation (group 8IN). At each sampling periods, five animals were randomly selected from a flock of grazing sheep and a clinical examination was conducted in vivo to ascertain animal health and to exclude mastitis. Sardinian sheep are a breed primarily used to produce milk; the typical breeding system involves one lambing per year, with the mating season starting in late spring for mature ewes and in early autumn for maiden ewes and with lactation starting in autumn and in late winter, respectively. Dry-off occurs simultaneously in mid summer for yearlings and mature ewes (lactation length 150 DIM and 240 DIM, respectively) when nutritional value of pastures collapses due to lack of rain in this season. In this study milk yield ranged from 1600 g/d to 900 g/d at 150 DIM. Ewes at 30 DIM were allowed to suckle their lamb; the other groups (60L, 150L) were mechanically milked twice daily and manually ten minutes before slaughtering, therefore just before tissue collection.

Samples of tissue were collected in TRIzol® (Invitrogen, Milano, Italy), frozen in liquid nitrogen and kept one week at -80°C till RNA extraction. The experiment was carried out in accordance with state and local laws and ethical regulations (Italian Regulation, 1992).

**RNA extraction and primer design**

Total RNA was extracted from about 30 mg of mammary tissues using TRIzol® Plus RNA Purification System (Invitrogen, Milano, Italy), following the manufacturer’s instructions. The concentration of the extracted total RNA was quantified using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo-Scientific, Wilmington, DE, USA) and the assessment of the purity of RNA samples ranged between 1.8-1.9. The RNA integrity was evaluated through the observation of 18S and 28S ribosomal bands after electrophoresis on 1% agarose gel, in the presence of ethidium bromide. In sample analysis, the β-actin (U39357) expression was used as an internal control, confirming thorough integrity of the RNA.

A Primer3 Input software (Rozen and Skaletsy, 2000) was used to design the primer sequences encoding for: CSN1S1 (X03237), CSN1S2A (X03238), CSN2 (X79703), CSN3 (AY237637), PRLR long form (AF041257), PRLR short form (AF041977), ESR1 (AY033393), ESR2 (AF177936) and 18S rRNA (AY753190). Primers and product lengths for each gene are listed in Table 1 according to the HUGO Gene Nomenclature Committee.

**Reverse transcription**

Reverse transcriptions were performed with 2 μg of extracted total RNA by using Improns-II Reverse Transcriptase (Promega, Milano, Italy) as following described. Two micrograms of total RNA with 1 μL oligo(dT)18 primers (0.5 μg/μL MBI Fermentas, Italy) and nuclease

| Gene     | Primer sets                                      | Product length, bp | cDNA concentration, ng | Primers, nM |
|----------|--------------------------------------------------|--------------------|------------------------|-------------|
| CSN1S1   | F: 5’AGACCAAGAGCTACTCTCCA 3’ R: 5’CACCAGGACAGGTCTGCTCA 3’ | 186                | 0.1                    | 300         |
| CSN1S2A  | F: 5’AGACCAAGAGCTACTCTCCA 3’ R: 5’TCCACTGAGAAGGTAAGGA 3’ | 189                | 0.1                    | 200         |
| CSN2     | F: 5’AGACCTCCACAAAGACATC 3’ R: 5’ATCCCATGTTGCTTACAAATC 3’ | 206                | 0.1                    | 300         |
| CSN3     | F: 5’TTGTGAGGAGCATCCACACCA 3’ R: 5’GACATCAGTTGCTGGAAGG 3’ | 156                | 0.1                    | 200         |
| PRLR long| F: 5’TCCCAAGTGGAAGGTAACAC 3’ R: 5’GTTCCCTGGAGGTTGAGG 3’ | 310                | 10                     | 200         |
| PRLR short| F: 5’TCCCAAGTGGAAGGTAACAC 3’ R: 5’CATTAAAACACAGAACAGG 3’ | 207                | 10                     | 600         |
| ESR1     | F: 5’CCACAGGATGCCAATGCCTC 3’ R: 5’AGGAACCGAGAGCACTGAG 3’ | 193                | 10                     | 200         |
| ESR2     | F: 5’TCCCTGCTCCAGCTGCTTTC 3’ R: 5’GACCTTGTGCTGGAAGAC 3’ | 213                | 10                     | 300         |
| 18S rRNA | F: 5’AAACAGCTACCCATACCAAG 3’ R: 5’TCCCTGCTCCAGCTGCTTTC 3’ | 90                 | 0.1                    | 100         |
| ACTB     | F: 5’TTGGACAGGAAGGGAAGAC 3’ R: 5’AGACCCGCTTTGCTGGAAGG 3’ | 102                | 5                      | 200         |

CNVL1, α-S1-casein; CSN4A, α-S2-casein; CSN1, β-casein; CSN3, κ-casein. ACTB, β-actin; PRLR long, prolactin receptor long form; PRLR short, prolactin receptor short form; ESR1, estrogen receptor α; ESR2, estrogen receptor β. 18S rRNA, 18S subunit rRNA. ACTB, actin. F, forward primer; R, reverse primer.
free water to a final volume of 20 μL, were incubated at 70°C for 5 min in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA, USA). Then, a mix was prepared with 4 μL of Improm-II Reverse Transcriptase buffer (5X Promega, Milano, Italy), 1.2 μL MgCl₂ (50 mM), 1 μL of Improm-II Reverse Transcriptase and 1 μL of dNTP (10 mM) was added to the reaction and incubated at 37°C for 90 min and finally at 94°C for 5 min. The final concentration of cDNA was assumed as 100 ng/μL.

**Standard curves analyses**

For each gene, an aliquot of cDNA samples was pooled and standard curves with serial dilution of pool were used to optimize PCR conditions and to calculate the efficiency, fluorescence baseline and threshold. The expression of target genes was normalized using the 18S rRNA gene, which is known to be constitutively expressed (Robinson et al., 2007) and was retro transcribed also with 1 μL random hexamers (100 μM, MBI Fermentas, Milano Italy).

**Real time PCR quantitation**

Realtime PCRs were performed in triplicate form using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Milano, Italy). For these reactions, a master mix with the following components was prepared to the indicated end concentration: 1 μL of cDNA, 9.5 μL water, 1 μL of each primer and 12.5 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG for a total volume of 25 μL. cDNA concentrations and primers molarities were different for each gene and determined with standard curves analyses performed before Real time PCR reactions. cDNA and primers concentrations are showed in Table 1.

PCR amplifications were conducted applying 45 cycles (1 sec at 95°C, 30 sec at the specific annealing temperature, 30 sec at 72°C) in a 96-well spectrophotometric thermal cycler (DNA Engine Opticon 2; MJ Research, Inc., Waltham, MA USA). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression level of a given target gene in each experimental group was analyzed by the 2^-ΔΔCt method (Bustin, 2000; Pfaffl, 2001) where 2^-ΔΔCt represents the difference of a given target gene between each group before lambing and during lactation (groups from LateP to 8IN) vs. the group P. More precisely, individual ΔΔCt was calculated for each sample of group (LateP to 8IN) as ΔΔCt=ΔCt (sample group) – mean ΔCt (group P). The n-fold expression of a given target gene was calculated as log2 (2^-ΔΔCt) (Figure 1).

**Statistical analysis**

All the recorded variables were submitted to analysis of variance using the ANOVA model to assess significant differences between groups; Duncan’s least significant difference test was used to compare the means (SPSS Inc., 1997).

**Results**

18S rRNA expression was quantified in all samples and resulted in constant expression levels. No significant differences between the investigated ovine mammary tissues. Expression of ERS1, ERS2, PRLRs and caseins mRNA were normalised according to the relative 18S rRNA expression of each sample.

The n-fold values, reported in Figure 1 as log2(n-fold), indicate the relative abundance of each target gene in comparison with the P group (prepubertal).

The relative expression of CSN1S1, CSN1S2A, CSN2 and CSN3 genes in the sheep mammary glands indicated the same significant pattern of difference (P<0.05) among groups. These genes were down-regulated at LateP and 8IN and up-regulated during lactation (30L, 60L, 150L).

The relative transcription of ESR1 significantly increased (P<0.05) from LateP to 8IN; the same parameter for ESR2 significantly increased at 60L and remained constant and up regulated (even if not significantly for 150L) until 8IN (P<0.05). The PRLR expression of the short and long isoforms showed similar trend, being significantly up regulated
at LateP and down regulated during lactation and involution. Statistical analysis of the ratio between the long and the short form of PRLR did not significantly differ among groups, but showed a trend in which the mRNA encoding the long form of the ovine PRLR predominated on the short one, in every group of sheep (Figure 2).

Discussion

In a previous paper (Colitti and Farinacci, 2009), where cell turnover and gene activity in mammary gland of Sardinian sheep was evaluated, we suggested that sheep mammary glands seem to operate in a much more dynamic state than those of other domestic ruminants. In particular, to explain the high apoptosis to proliferation ratio we suggested that, as reported by Potten and Loeffler (1990), there was a dividing transit population of cells in which signs of proliferation, phenotypic differentiation and functional differentiation were displayed simultaneously. This was in agreement with the results reported by Suzuki et al. (2000) that also found cells, in the breast tissue of pregnant women, positive to marker of proliferation, Ki-67 and to markers of mammary functional differentiation, β-casein and κ-casein. We evaluated markers of functional differentiation like CSN1S1, CSN1S2A, CSN2, CSN3 by Real time PCR analyses.

In this study, we showed that activation of casein genes transcription, relatively to the prepubertal group (P), is up regulated during lactation and significantly down regulated before lambing and at involution. This is concomitant to the enhancement, although not significantly, of the ratio between the long and short form of prolactin receptors (Figure 2). This is in agreement with the data found by Cassy et al. (1998) in which they suggested that the short form of the ovine PRLR may have a dominant negative action in the activation of milk protein gene transcription. In fact, the authors reported that the activation of caseins gene transcription was concomitant with the enhancement of the ratio of the long to the short form of the ovine PRLR, which may play a key role in the shift between growth and differentiation of the mammary gland.

Compared to P group, the long form of PRLR is up regulated before lambing and this is in agreement with the trend of STAT5a expression that was lower during lactation and higher after the end of lactation (Colitti and Farinacci, 2009). As already reported, STAT5a expression resulted negatively related to that of lactalbumin, a major milk protein gene for ruminants, which significantly increased from lambing to lactation and it is also related to the expression of caseins. Therefore, in agreement with Kazansky et al. (1995) the activation of STAT5a, induced by PRLR, may prime milk gene expression, but other factors are necessary for milk protein long-term expression during lactation.

Our mRNA expression results demonstrated a high expression of ESR1 and ESR2 genes in prepubertal group. In fact, at the beginning of lactation period and during lactation (lactogenesis and galactopoiesis) the n-folds were significantly lower for ERS1 gene and also for ESR2 gene (Figure 1). These is in agreement with Schams et al. (2003), who found the presence of high ERS1 and ESR2 levels before the start of lobulo-alveolar development and ESR1 significantly lower expression during pregnancy and lactogenesis. This is due to distinct regulatory mechanisms that involved the receptors, being the ESR1 regulated at post-transcriptional level and ESR2 at transcriptional level (Chang et al., 2005). In fact, the receptors present opposite expressions in presence of estradiol (Schams et al., 2003). This is in agreement with our results that showed a higher expression of ESR2 during lactation. Moreover, ESR2 is expressed, in mammary glands, not only in the luminal cells but also in myoepithelial and stromal cells, suggesting different roles for this gene within the glands (Speirs et al., 2002). In fact, the colocalization of ESR1 and ESR2 expressions with that of prolactin receptor (Ki-67) could be remarkable to clarify the nature of cells in which they are expressed and the pathways by which hormones modulate proliferation.

These observations suggest a possible and important role of these receptors for the initiation of alveolar development, maybe in cooperation with proliferative growth factors.

Conclusions

This study, designed to investigate in mammary tissues the expression of markers of functional differentiation as αs1, αs2, κ, β caseins, the expression patterns of α and β receptors for estrogen and prolactin receptors long and short forms, showed that: i) activation of caseins genes transcription, relatively to the P group, is upregulated during lactation and significantly downregulated before lambing and at involution; ii) a high expression of αs1 and β receptors for estrogen genes occurs in P group compared with adult group; iii) the prolactin receptor expression of the short and long isoform are upregulated at LateP in comparison with P group, and down-regulated during lactation and involution.

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