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# EXPRESSION AND LOCALISATION OF LEPTIN AND LEPTIN RECEPTOR IN THE MAMMARY GLAND OF THE DRY AND LACTATING NON-PREGNANT COW

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Leptin and leptin receptor were studied in the mammary gland of non-pregnant dry and lactating cows. Using RT-PCR it was demonstrated that leptin and its short (Ob-Ra) and long (Ob-Rb) receptor isoforms are expressed both in the dry and the lactating mammary gland tissue. Tissue distribution of leptin and its receptor mRNA transcripts were examined by *in situ* hybridisation, while the leptin protein was localised by immunohistochemistry. Although *in situ* hybridisation is semiquantitative, our morphological data suggest that the epithelial leptin mRNA expression of the lactating gland is higher than that of the dry gland. To compare the leptin mRNA levels between dry and lactating udders competitive PCR was used, which showed no difference in leptin expression for the whole mammary tissues. The lack of difference in total leptin mRNA levels is explained by the high adipose tissue content of the dry mammary gland. Leptin and its receptor transcripts are expressed mainly in the epithelial cells of lactating cows, while in dry mammary tissue the signal is found in the stromal tissues as well. The results provide additional evidence that locally produced leptin takes part in the regulation and maintenance of mammary epithelial cell activity.

**Key words:** Cow, leptin, leptin receptor, mammary gland

Zhang et al. discovered leptin, a 16 KDa polypeptide hormone in 1994 as the product of the obese (ob) gene. Leptin has an important role in body weight homeostasis via its effects on food intake and energy expenditure (Halaas et al., 1995; Houseknecht et al., 1998; Houseknecht and Portocarrero, 1998; Ahima and Flier, 2000). Leptin transcripts appear to be expressed fairly specifically in adipose tissue (Zhang et al., 1994). However, recent studies have demonstrated that leptin mRNA transcripts are also detectable in several peripheral tissues such as

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the mammary gland (Smith-Kirwin et al., 1998; Aoki et al., 1999; Chilliard et al., 2001; Bonnet et al., 2002; Smith and Sheffield, 2002).

Leptin acts through interaction with the Ob-R receptor (leptin receptor), a single membrane spanning receptor that has strong sequence homology to the class I cytokine receptor family (Tartaglia, 1997). Several forms of leptin receptors are produced by the alternative splicing of Ob-R mRNA, which have a common extracellular domain and either lack the transmembrane domain (Ob-Re isoform) or have different lengths of cytoplasmic domain (Ob-Ra, b, c, d, f, Moschos et al., 2002). The so-called long isoform (Ob-Rb) is predominantly expressed in the hypothalamus and can mediate, via the Janus Kinase (JAK2), the activation of signal transducer and activator of transcription (STAT) proteins (Baumann et al., 1996). Ob-Rb is also found in many peripheral tissues at low levels (Fei et al., 1997; Vernon et al., 2001). The short isoform (Ob-Ra) is expressed ubiquitously and represents the major isoform in many peripheral tissues (Mercer et al., 1996; Fei et al., 1997; Laud et al., 1999; Sweeney, 2002). In prepubertal heifers (Silva et al., 2002) it was reported that the long isoform of leptin receptor mRNA was present in all bovine tissues, while the short isoform (Ob-Ra) was detected only in bovine liver, pituitary gland, and spleen. Reverse transcription-polymerase chain reaction (RT-PCR) studies indicate the expression of the short (Ob-Ra) isoform in the bovine adrenal medulla (Yanagihara et al., 2000). Both long (Ob-Rb) and short (Ob-Ra) isoforms of leptin receptor were found in ovine mammary tissue during pregnancy and lactation (Laud et al., 1999).

Leptin has been demonstrated in the milk of rats (Casabiell et al., 1997), humans (Smith-Kirwin et al., 1998), pigs (Estienne et al., 2000), and cattle (Smith and Sheffield, 2002). In addition, using RT-PCR technique the presence of leptin mRNA was indicated in cultured bovine mammary epithelial cells (Smith and Sheffield, 2002) and in the mammary tissue (Smith-Kirwin et al., 1998; Aoki et al., 1999; Smith and Sheffield, 2002). The last mentioned study did not indicate the type of cell in which leptin was present. Immunohistochemical staining of breast tissue, cultured mammary epithelial cells, and secretory epithelial cells present in the human milk revealed the production of leptin by human mammary epithelial cells (Smith-Kirwin et al., 1998). The site of expression of leptin in the ovine mammary gland varies with the stage of pregnancy as determined by immunohistochemical analysis. It was first traced in adipose cells during early stages of pregnancy, then in epithelial cells just before parturition, and finally in myoepithelial cells after parturition (Bonnet et al., 2002).

Leptin appears to be produced in secretory epithelial cells of the active human mammary tissue (Smith-Kirwin et al., 1998) and in ovine mammary epithelial cells just before parturition in addition to myoepithelial cells after parturition (Bonnet et al., 2002). So far there is no information about the localisation of leptin or leptin receptor in cow mammary tissue. Leptin appears to be able to control the proliferation of both normal and malignant mammary epithelial cells

(Hu et al., 2002). No information is available about the alteration of leptin level between quiescent mammary gland and lactating mammary gland in cows. In the present study dry and lactating non-pregnant cows were selected to examine the expression of leptin and its receptor in the mammary gland and to study the alteration of mammary leptin levels using competitive RT-PCR. Paraffin-embedded mammary gland sections from dry and lactating cows were subjected to *in situ* hybridisation procedure for leptin and leptin receptor mRNA transcripts and immunohistochemical staining for leptin protein, to specify the type of the cell in which leptin and its receptor were present.

## Materials and methods

*Tissue samples*

Mammary gland tissues were collected from dry and lactating non-pregnant cows immediately after slaughtering. Nearly 5-g pieces of the parenchymal tissue of each sample were quickly frozen in liquid nitrogen and stored at –70 °C until RNA extraction. In parallel, about two-gram pieces of each sample were fixed in 50 ml of diethyl pyrocarbonate (DEPC, Sigma) treated phosphate-buffered saline (PBS) containing 4% paraformaldehyde for *in situ* hybridisation and immunohistochemistry.

*RNA isolation*

Total RNA was isolated using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA yields and purity were assessed by absorbency at 260 and 280 nm. In order to eliminate the residual genomic DNA from the RNA samples, one unit DNaseI (Roche) was added per each microgram of RNA and incubated at 37 °C for 30 min followed by heat inactivation of the enzyme at 75 °C for 5 min.

*Partial cloning of leptin and leptin receptors*

Oligonucleotide primers for amplification of cow leptin were designed based on the known sequences of the cow leptin reported in the GenBank. Primers for amplification of cow short and long isoforms of leptin receptor were selected on the basis of regions of high sequence identity between known sequences of water buffalo, sheep, pig, mouse, and the human. The primers were designed to span the junction of two exons to be RNA specific and were synthesised by Creative Labor Ltd: Ap1 5′- caggatgacaccaaaaccctcatc -3′ and Ap14 5′- ggagtagagtgaggcttccaggac -3′ for amplification of 342 base pair (bp) fragment of Ob cDNA, Ap20 5′- ttgagaagtaccagttcagt c-3′ and Ap21 5′- caaagaatgtccgttctctt

c-3′ for amplification of 280 bp of Ob-Ra and Ap22 5′- agggttctatttgtattagt gacc-3′ and Ap23 5′- gaaatttccc tcaagtttcaaaag -3′ for amplification of 353 bp of Ob-Rb.

The reverse transcription reaction was performed using 2 µg of total RNA with 200 units of MMLV reverse transcriptase (Promega), 1 µg Oligo(dT)15 and 10 mM dNTPs for 60 min at 42 °C in a final volume of 25 µl. Two µl of RT reaction product was amplified by PCR in final volume of 30 µl with 200 µM dNTPs, 25 pmol of each primer, 1.5 units Red Taq Polymerase (Sigma) and 10X Red Taq PCR buffer. PCR thermal cycling parameters were as follows: 1 cycle 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 50 sec, annealing at 55 °C for 40 sec, and extension at 72 °C for 1 min. The program was terminated with a final extension step at 72 °C for 5 min.

The PCR products were detected by electrophoresis on 1% agarose gel containing ethidium bromide. The specificity of the amplified products was confirmed by Southern blot analysis using water buffalo digoxigenin-11-dUTP (DIG) labelled cDNA probe for Ob, Ob-Rb, and Ob-Ra (GenBank accession numbers AY177609, AY177610 and AY177611).

Other PCR products were isolated from the gel using Qiaquick gel extraction kit (Qiagen), then ligated into PGEM-T vector (Promega) and used to transform *Escherichia coli* cells. Plasmids from resultant cultures were isolated with QiAprep Miniprep kit (Qiagen) and used for production of RNA probes and cow leptin internal competitor.

*Competitive PCR*

A competitive PCR procedure according to Tu et al. (1997) was used to compare the abundance of mammary tissue leptin mRNA between lactating and dry cows. Our clone for cow leptin partial cDNA was used for the construction of a cow leptin internal competitor that can be primed for PCR amplification using the same primer site of leptin target amplicon. The plasmid containing cow leptin cDNA was digested with the restriction enzyme *Eco*01091 (BioLabs Inc) which cut a 55-bp fragment from cow leptin cDNA. The plasmid was separated in agarose gel and isolated from the gel using Qiaquick gel extraction kit (Qiagen), then finally religated by T4 DNA ligase (Promega). The procedure of competitive PCR was achieved by co-amplification of our RT reaction derived from mammary tissue with different dilutions of leptin internal competitor in one PCR reaction. Firstly, each 20 µl PCR reaction contained 3 µl of RT reaction and 1 µl of 10-fold dilutions of the internal competitor with the leptin specific primer. The starting concentration of the competitor was 70 pg/µl. PCR thermal cycling parameters were as described previously but for only 30 cycles to be sure that we are in the exponential phase of PCR. Secondly and according to the result obtained with the 10-fold dilution of internal competitor a narrower range (twofold dilution) of internal competitor concentration was used in new PCR reactions. PCR products were detected by electrophoresis on 1% agarose gel con-

taining ethidium bromide and the location of the predicted products was confirmed by using 100-bp molecular ladder (Bio-Rad Laboratories) as a standard size marker. The comparison between lactating and dry mammary leptin mRNA was achieved by comparing the intensity of bands obtained with different competitor dilutions.

In situ *hybridisation*

Paraffin embedded tissue was used to localise the mRNA transcripts for Ob, Ob-Ra, and Ob-Rb genes within mammary epithelial cells of the dry and lactating cows by DIG-labelled RNA probes using DIG RNA Labelling Kit (Roche). The procedures were used according to the current protocols in molecular biology (Knoll and Lichter, 1995) and the non-radioactive *in situ* hybridisation application manual (Roche). Deparaffinised and hydrated sections were treated by 200 µl of 200 mM HCl for 10 min to denature the proteins, then washed by DEPC water for 10 min. The sections were permeabilised for 30 min with DEPC-treated PBS containing 10 µg/ml Rnase-free proteinase K (Boehringer M.), followed by post-fixation for 10 min at 4 °C with DEPC-treated PBS containing 4% paraformaldehyde. The sections were washed by DEPC water and overlaid with 30 µl of hybridisation buffer containing 10 ng of DIG-labelled RNA probe and covered by 18 × 18 coverslip.

The hybridisation buffer consists of 50% formamide, 0.3 M NaCl, 10 mM Tris/HCl (pH 8), 1 mM EDTA, 5 × Denhardt, 500 µl/ml yeast tRNA (Invitrogen Life Technologies), 10% PEG (MW 6000, Fluka), 10 mM Vanadyl-Ribonucleoside complex (BioLabs Inc.) and 100 µg/ml salmon testis DNA (Sigma) which was denatured at 99 °C for 10 min before being added to the hybridisation buffer. The slides were placed on hot plate at 94 °C for 4 min, then incubated at 45 °C for 20 h.

Post-hybridisation slide processing included 2 × 10-min wash with 2 × saline sodium citrate (SSC) at room temperature, followed by 30-min incubation with 20 µg/ml RNase A in 2 × SSC at 37 °C. The slides were then subjected to 1 × SSC for 20 min at 37 °C and 2 × 15-min washes with 0.1 × SSC at 46 °C. Detection of hybridisation was achieved using DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions. Finally the sections were air-dried, mounted by Entellan (Merck) and photographed by light microscopy.

*Immunohistochemistry*

Sections from dry and lactating mammary gland were used for immunohistochemistry using polyclonal rabbit anti-bovine leptin. After inactivation of endogenous peroxidases the sections were placed in 0.01 mol/L citrate buffer (pH 6) and heated in microwave (700 watt) for 10 min. The sections were

blocked by TBS containing 5% bovine serum albumin for 1 h, then incubated with rabbit anti-bovine leptin (1000× dilution in 1% bovine serum albumin, BSA) at 4 °C overnight and for 1 h at room temperature. The sections were washed with Tris-buffered saline (TBS) for 5 min and incubated with biotinylated goat anti-rabbit IgG for 30 min at room temperature. The secondary antibody was detected with Vectastain ABC kit (Vector Laboratories Inc.) and the colour was developed using 3,3´-diaminobenzidine (DAB, Sigma).

## Results

*Partial cloning of Ob, Ob-Ra and Ob-Rb cDNA*

RT-PCR analysis of total RNA from dry quiescent and lactating mammary gland tissue of the non-pregnant cows revealed the presence of leptin mRNA in these tissues. In addition, both the Ob-Ra and Ob-Rb mRNA were expressed in the mammary gland (Fig. 1A). Southern blot hybridisation of RT-PCR products revealed the specificity of the amplified products, which hybridised with specific water buffalo DIG-labelled cDNA probes for Ob, Ob-Ra, and Ob-Rb (Fig. 1B).

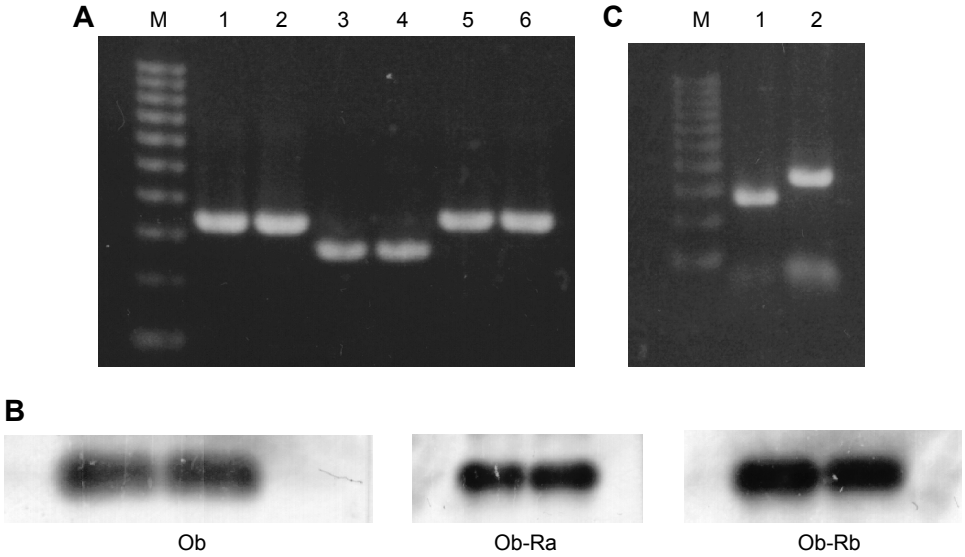


*Fig. 1.* RT-PCR of leptin and leptin receptors. Panel **A.** Lanes 1 and 2: RT-PCR detection of Ob mRNA; lanes 3 and 4: detection of Ob-Ra mRNA; lanes 5 and 6: detection of Ob-Rb mRNA in the dry and lactating mammary gland tissue of non-pregnant cows. M is a 100-bp molecular ladder. Panel **B.** Southern blot analysis of RT-PCR products using specific DIG-labelled cDNA probes indicating the specificity of amplified fragments of leptin (Ob) and both short (Ob-Ra) and long (Ob-Rb) isoforms of leptin receptor in the dry and lactating mammary gland tissue. Panel **C.** RT-PCR detection of both short (1) and long (2) isoforms of leptin receptor in the adipose tissue of cow

*Competitive PCR*

The comparison between lactating and dry mammary gland leptin mRNA of non-pregnant cows did not show difference in the leptin mRNA level. Ten-fold dilution of the internal competitor starting with 70 pg/µl concentration in the first PCR tube was used to find the optimal dilution (Fig. 2A). Using a narrower range of competitor concentration (2× dilution) beginning from the second tube in the first PCR which contained 7 pg/µl of the internal competitor revealed the absence of clear difference. The band intensities of our target were similar to the internal competitor intensity at the third tube which contained 1.75 pg/µl of the internal competitor in both dry and lactating cows (Fig. 2B).

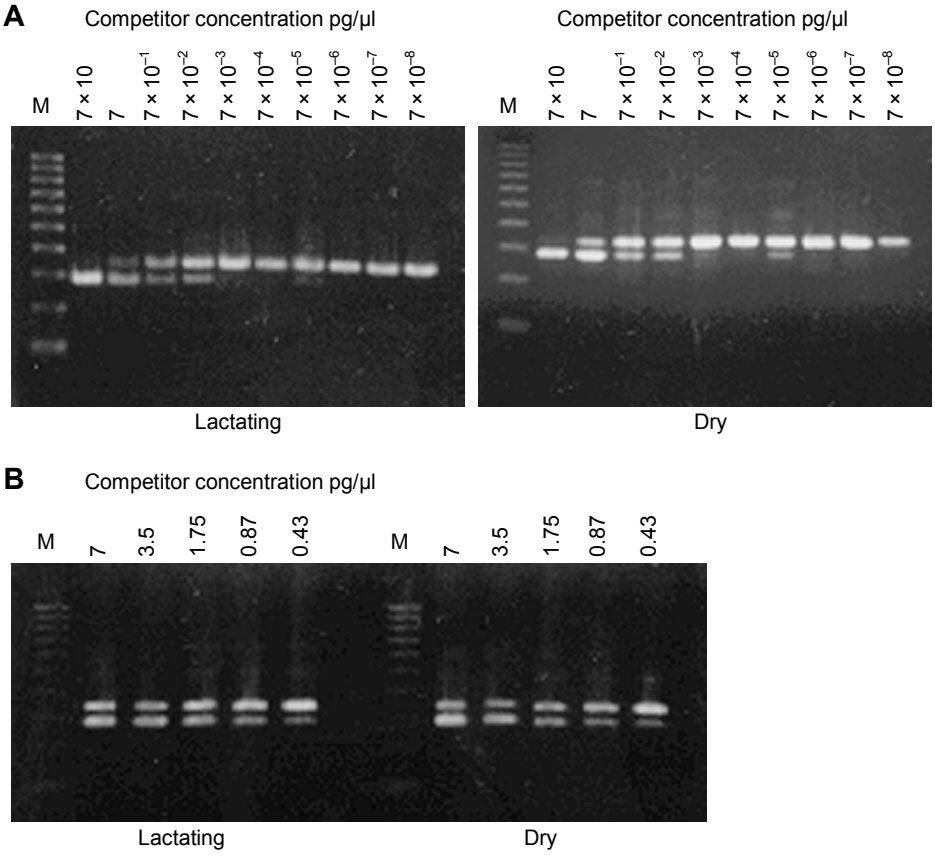


*Fig. 2.* Competitive PCR of mammary leptin for lactating and dry non-pregnant cows. Panel **A** revealed the difficulty to compare between lactating and dry mammary gland leptin using 10-fold dilution of cow leptin internal competitor starting with 70 pg/µl of the competitor in the first tube. Panel **B** revealed the competitive PCR using 2-fold dilution of the internal competitor starting from the second tube of panel A which contained 7 pg/µl of the internal competitor. The band intensities of leptin target are similar to the competitor band intensities in the third tube in the lactating and dry cows where the competitor concentration is 1.75 pg/µl

*Cellular localisation of leptin, and its receptors in mammary tissues*

*In situ* hybridisation signals localising the mRNA transcripts of leptin and its receptor isoforms were observed within the secretory alveolar epithelial cells of the acini (pictures A, B and C in Figs 3 and 4). We could not demonstrate any clear signals in cells surrounding the secretory acini; in other words we could not localise mRNA transcripts of leptin and its receptors within the myoepithelial cells. Due to the completely regressed mammary adipose tissue during lactation, we could not observe clear signals for leptin or its receptor mRNA transcripts within the stromal tissue between acini in the mammary gland of the lactating cows (pictures A, B and C in Fig. 3). In the dry quiescent mammary gland the stromal connective tissue which contained many fat cells, we could see *in situ* hybridisation signals for leptin and it receptor between acini beside the signals localised in the epithelial cells (pictures A, B and C in Fig. 4). The specificity of Ob-Ra and Ob-Rb signals localised between acini of dry mammary gland was confirmed by RT-PCR analysis for both short and long isoforms of leptin receptor in the adipose tissue of the dry cow. The result showed the expression of both isoforms in the adipose tissue (Fig. 1C). The specificity of *in situ* hybridisation was demonstrated by using the sense riboprobes for each gene as negative controls (picture D in Figs 3 and 4).

Although the detection of mRNA by *in situ* hybridisation is only semiquantitative, a difference existing in the intensity of signals for Ob, Ob-Ra, and Ob-Rb within the epithelial cell could be demonstrated between lactating and dry cows. The expression of leptin and its receptor in the lactating mammary gland epithelial cell seems to be higher than that of the dry quiescent mammary gland epithelial cell.

Immunohistochemistry staining for leptin protein confirmed the result of *in situ* hybridisation. The immunostaining was mainly localised in the epithelial cells of the acini in the lactating tissue and mainly in epithelial cells in addition to few signals in the stromal tissue of the dry mammary gland (Fig. 5A and B)

## Discussion

In this article we report the first information about leptin localisation in the mammary tissue of the dry and lactating non-pregnant cow. Using *in situ* hybridisation and immunohistochemistry techniques we could demonstrate leptin mRNA and protein in the alveolar epithelial cells in the dry and lactating mammary gland in addition to stromal adipose cells in the dry mammary gland. This observation about alveolar epithelial cell as a source of mammary leptin is in agreement with the result reported in human breast tissue (Smith-Kirwin et al., 1998), and with that in cultured bovine mammary epithelial cells using RT-PCR (Smith and Sheffield, 2002).

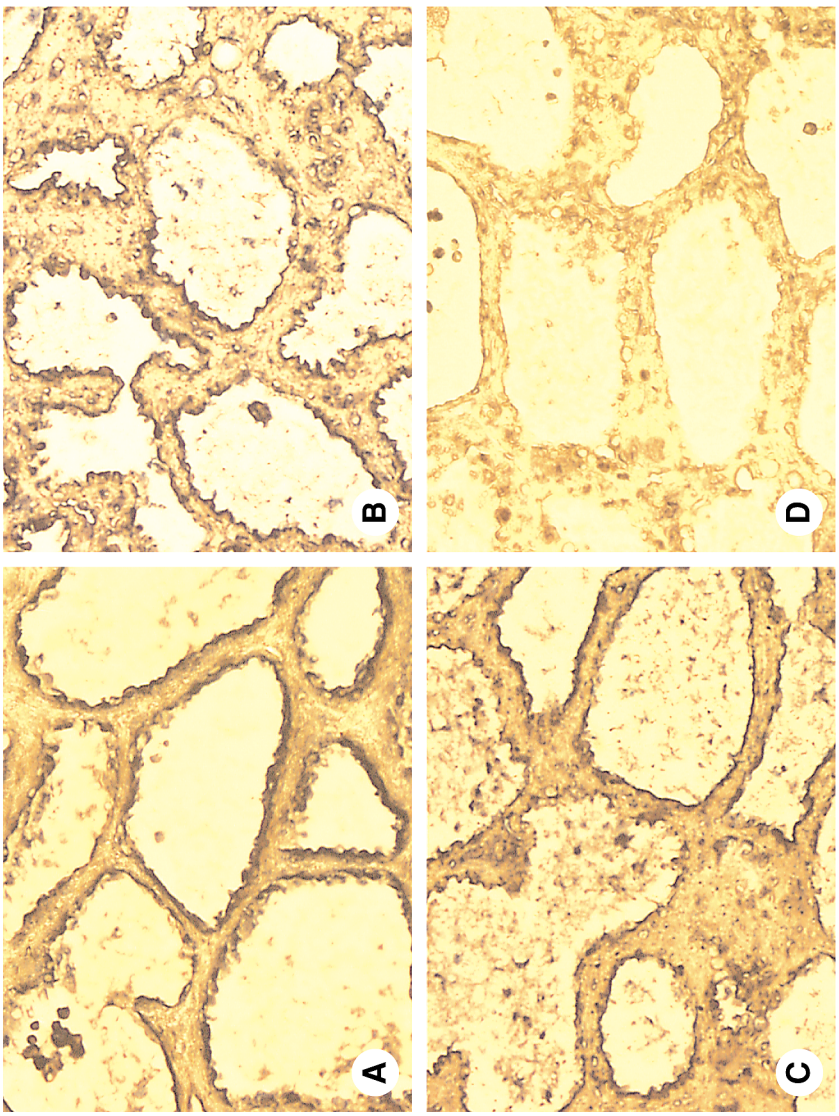


*Fig. 3.* Light microscopy photomicrograph representing *in situ* hybridisation analysis of leptin (A) and its short (B) and long (C) isoforms of receptor within mammary gland tissue of lactating cow (500×). Photomicrographs A, B and C show hybridisation with antisense probes for Ob, Ob-Ra and Ob-Rb mRNA, respectively. Positive signals are located in the secretory epithelial cells of the acini. Photomicrograph D is the negative control and is hybridised with the sense riboprobe of leptin. The sense riboprobe for both isoforms of leptin receptor showed the same result as photomicrograph D

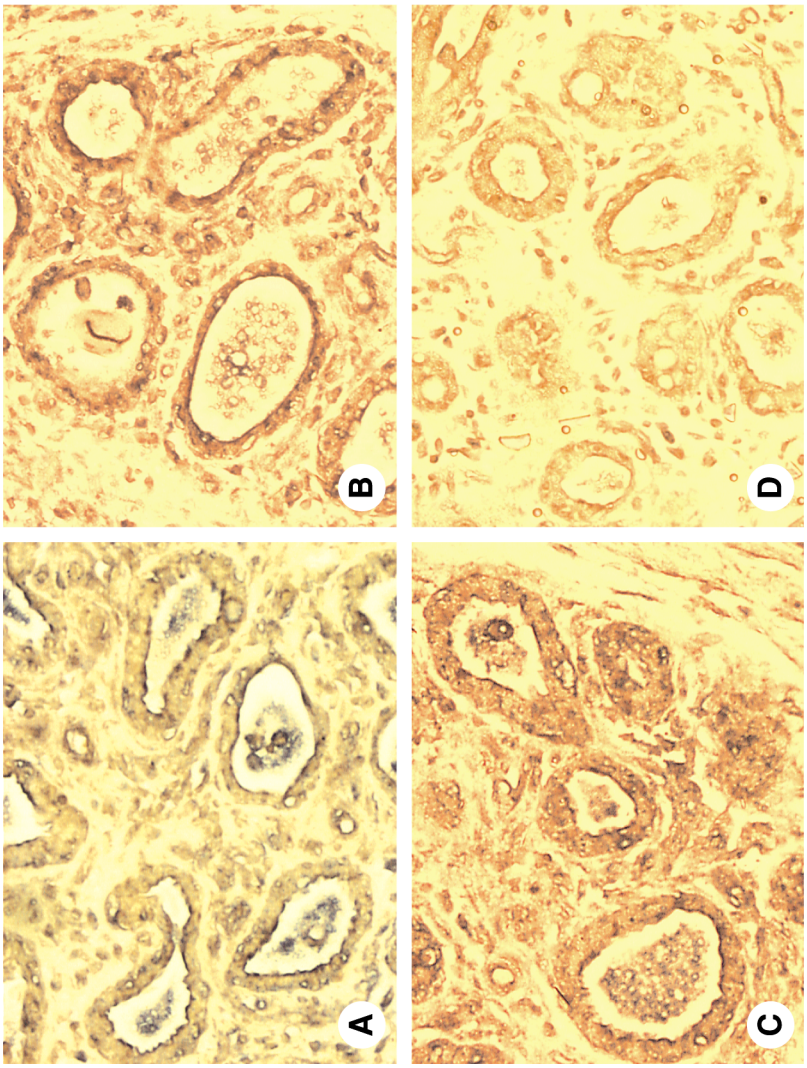


*Fig. 4.* Light photomicrograph representing *in situ* hybridisation analysis of leptin (A) and its short (B) and long (C) isoforms of receptor within mammary gland tissue of dry cow (500×). Photomicrographs A, B and C show hybridisation with the antisense riboprobes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located mainly in the epithelial cells in addition to moderate signals in the stromal adipose tissue which was tested positive for both leptin receptors using RT-PCR. Photomicrograph D is the negative control and is hybridised with the sense riboprobe of leptin. The same riboprobe for both isoforms of leptin receptor showed the same result as photomicrograph D

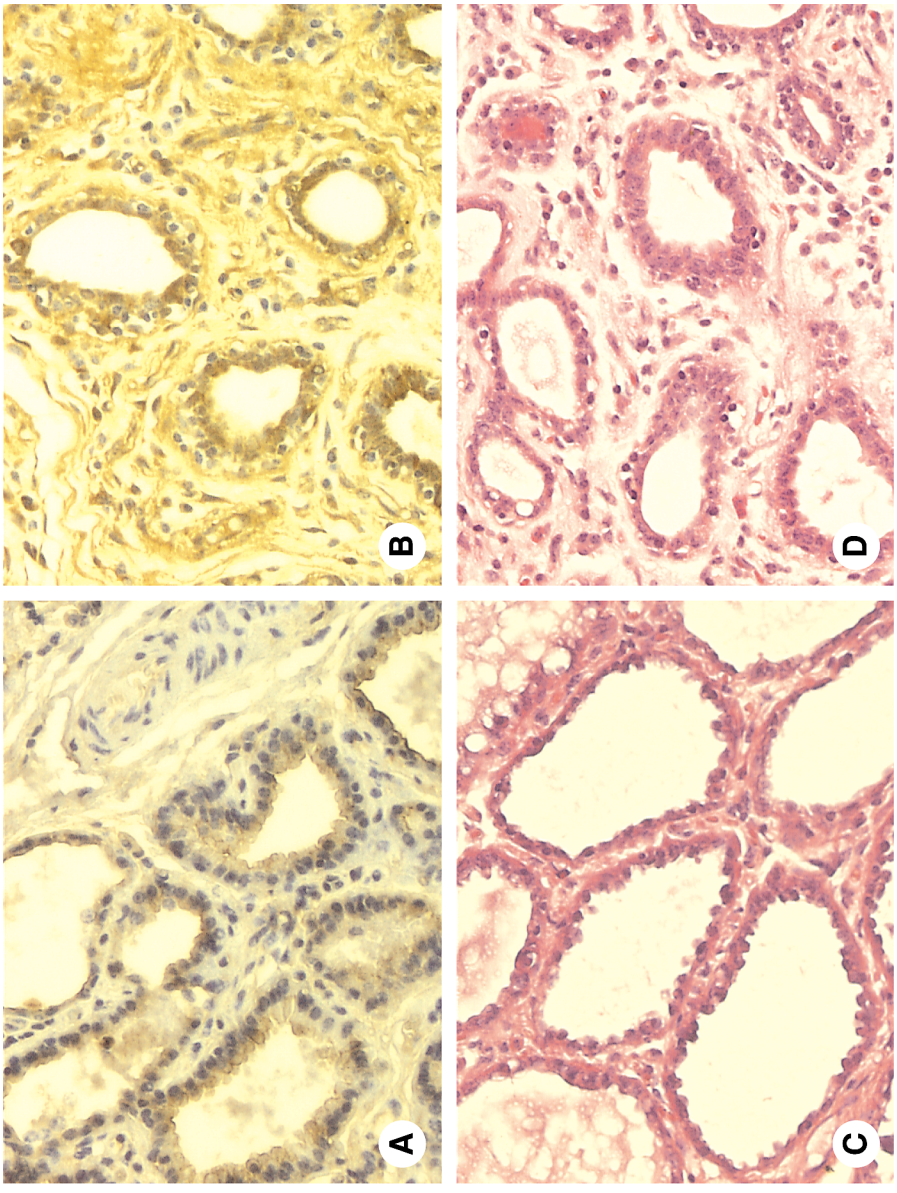


*Fig. 5.* Immunohistochemistry analysis of lactating (A) and dry (B) mammary gland leptin. The strong signals are mainly located in the acinar epithelial cell. Photomicrographs C and D show the lactating and dry mammary tissue stained with haematoxylin and eosin (HE), respectively

In this article we also report the expression and localisation of the long and short isoforms of leptin receptor in the mammary gland in both dry and lactating cows. This result is consistent with the result reported in sheep (Laud et al., 1999) and in human breast cancer cells (Laud et al., 2002). We have also demonstrated the expression of both Ob-Ra and Ob-Rb of leptin receptor in the mammary gland tissue of Egyptian water buffalo and one-humped camel (unpublished data). Using RT-PCR, Silva et al. (2002) have reported in prepubertal heifers that only Ob-Rb is expressed in the mammary gland tissue.

RT-PCR analysis for both isoforms of leptin receptor using total RNA of adipose tissue indicates the expression of these isoforms of leptin receptor in the adipose cell. This result coincides with previous reports in sheep adipose tissue (Dyer et al., 1997), human white adipose tissue (Bornstein et al., 2000), and rat fat (Machinal-Quelin et al., 2002).

During pregnancy the metabolism in the female changes and larger amount of energy is required to sustain pregnancy and fetal growth and development. To avoid the effect of pregnancy we selected the non-pregnant cow to study the alteration of mammary leptin between dry and lactating animals. In the current study competitive PCR could not show a clear difference in leptin mRNA levels between dry and lactating mammary glands of non-pregnant cows. This finding is in contrast with the observation reported in mice by Aoki et al. (1999) who mentioned that throughout lactation the leptin expression in the mammary gland was significantly lower than that of non-pregnant mice. Ovine mammary leptin is high at the beginning and at the end of pregnancy and is low at mid-pregnancy and throughout lactation (Bonnet et al., 2002).

Although *in situ* hybridisation is semiquantitative, in the current study the signal intensity within the alveolar epithelial cell of lactating mammary gland appeared to be higher than that of the dry one. Leptin expression and secretion reflect the body fat mass (Maffei et al., 1995; Considine and Caro, 1997) and are highly correlated with adipocyte size (Houseknecht et al., 1998). During lactation the mammary adipose tissue is completely regressed. Thus our study suggests that the similarity of total leptin mRNA level between dry and lactating mammary gland tissues of non-pregnant cows may be related mainly to the high amount of adipose cells and large fat cell size in the dry mammary gland.

We demonstrated that the leptin is produced locally in the mammary epithelial cells. This observation well coincides with previous data which show a higher hormone concentration in the udder than in the circulation. Leptin concentration in the serum of cattle is normally between 5 to 10 ng/ml and increases with body fatness (Ehrhardt et al., 2000), while the aqueous extract prepared from mammary parenchymal tissue of prepubertal heifers contains about 10.4 to 12.1 ng of leptin in one gram of tissue (Silva et al., 2002). Leptin has a stimulatory effect on mammary epithelial cells as demonstrated in mouse cell lines (Hovey et al., 1998), which emphasises the importance of local effects of leptin

hormone. Leptin-deficient mice (ob/ob genotype) can be induced to become pregnant after exogenous leptin treatment. These mice deliver normal litter but stop lactating upon withdrawal of the exogenous leptin (Mounzih et al., 1998). According to this, local leptin production might also be important in maintaining lactation. Data of the literature and our present results demonstrate the possible physiological importance of the locally produced leptin in the regulation and maintenance of mammary epithelial cell activity.

In summary, we report here the expression and localisation of Ob, Ob-Ra, and Ob-Rb in the mammary gland of the dry and lactating cow, as well as the expression of Ob-Ra and Ob-Rb in cow adipose tissue. The competitive PCR revealed similar leptin mRNA levels in the dry and lactating mammary gland of non-pregnant cows.

## References

Ahima, R. S. and Flier, J. S. (2000): Adipose tissue as an endocrine organ. Trends Endocrinol. Metabol. **11,** 327–332.

Aoki, N., Kawamura, M. and Matsuda, T. (1999): Lactation-dependent down regulation of leptin production in mouse mammary gland. Biochim. Biophys. Acta **1427,** 298–306.

Baumann, H., Morella, K. K., White, D. W., Dembski, M., Bailon, P. S., Kim, H., Lai, C. F. and Tartaglia, L. A. (1996): The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc. Natl. Acad. Sci. USA **93,** 8374–8378.

Bonnet, M., Gourdou, I., Leroux, C., Chilliard, Y. and Djiane, J. (2002): Leptin expression in the ovine mammary gland: putative sequential involvement of adipose, epithelial, and myoepithelial cells during pregnancy and lactation. J. Anim. Sci. **80,** 723–728.

Bornstein, S. R., Abu-Asab, M., Glasow, A., Path, G., Hauner, H., Tsokos, M., Chrousos, G. P. and Scherbaum, W. A. (2000): Immunohistochemical and ultrastructural localization of leptin and leptin receptor in human white adipose tissue and differentiating human adipose cells in primary culture. Diabetes **49,** 532–538.

Casabiell, X., Pineiro, V., Tome, M. A., Peino, R., Dieguez, C. and Casanueva, F. F. (1997): Presence of leptin in colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. J. Clin. Endocrinol. Metab. **82,** 4270–4273.

Chilliard, Y., Bonnet, M., Delavaud, C., Faulconnier, Y., Leroux, C., Djiane, J. and Bocquier, F. (2001): Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentration. Domest. Anim. Endocrinol. **21,** 271–295.

Considine, R. V. and Caro, J. F. (1997): Leptin and the regulation of body weight Int. J. Biochem. Cell Biol. **29,** 1255–1272.

Dyer, C. J., Simmons, J. M., Matteri, R. L. and Keisler, D. H. (1997): Leptin receptor mRNA is expressed in ewe anterior pituitary and adipose tissues and is differentially expressed in hypothalamic regions of well-fed and feed-restricted ewes. Domest. Anim. Endocrinol. **14,** 119–128.

Ehrhardt, R. A., Slepetis, R. M., Siegal-Willott, J., Van Amburgh, M. E., Bell, A. W. and Boisclair, Y. R. (2000): Development of a specific radioimmunoassay to measure physiological changes of circulating leptin in cattle and sheep. J. Endocrinol. **166,** 519–528.

Estienne, M. J., Harper, A. F., Barb, C. R. and Azain, M. J. (2000): Concentrations of leptin in serum and milk collected from lactating sows differing in body condition. Domest. Anim. Endocrinol. **19,** 275–280.

Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R. and Friedman, J. M. (1997): Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc. Natl. Acad. Sci. USA **94,** 7001–7005.

Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K. and Friedman, J. M. (1995): Weight-reducing effects of the plasma protein encoded by the obese gene. Science **269,** 543–546.

Houseknecht, K. L. and Portocarrero, C. P. (1998): Leptin and its receptors: regulators of whole-body energy homeostasis. Domest. Anim. Endocrinol. **15,** 457–475.

Houseknecht, K. L., Baile, C. A., Matteri, R. L. and Suprlock, M. E. (1998): The biology of leptin: a review. J. Anim. Sci. **76,** 1405–1420.

Hovey, R. C., MacKenzie, D. D. and McFadden, T. B. (1998): The proliferation of mouse mammary epithelial cells in response to specific mitogens is modulated by the mammary fat pad *in vitro*. In Vitro Cell. Dev. Biol. Anim. **34,** 385–392.

Hu, X., Juneja, S. C., Maihle, N. J. and Cleary, M. P. (2002): Leptin – a growth factor in normal and malignant breast cells and for normal mammary gland development. J. Natl. Cancer Inst. **94,** 1704–1711.

Knoll, J. H. M. and Lichter, P. (1995): *In situ* hybridization and detection using nonisotopic probes. In: Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds) Current Protocols in Molecular Biology. Volume 2. John Wiley & Sons, Inc., Etobicoke, Ontario, Canada. pp. 14.7.1–14.7.11.

Laud, K., Gourdou, I., Belair, L., Keisler, D. H. and Djiane, J. (1999): Detection and regulation of leptin receptor mRNA in ovine mammary epithelial cells during pregnancy and lactation. FEBS Lett. **463,** 194–198.

Laud, K., Gourdou, I., Pessemesse, L., Peyrat, J. P. and Djiane, J. (2002): Identification of leptin receptors in human breast cancer: functional activity in the T47-D breast cancer cell line. Mol. Cell. Endocrinol. **188,** 219–226.

Machinal-Quelin, F., Dieudonne, M. N., Leneveu, M. C., Pecquery, R. and Giudicelli, Y. (2002): Proadipogenic effect of leptin on rat preadipocytes *in vitro*: activation of MAPK and STAT3 signaling pathways. Am. J. Physiol. Cell Physiol. **282,** 853–863.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., Kern, P. K. and Friedman, J. M. (1995): Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat. Med. **11,** 1155–1161.

Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T. and Trayhurn, P. (1996): Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by *in situ* hybridization. FEBS Lett. **387,** 113–116.

Moschos, S., Chan, J. L. and Mantzoros, C. S. (2002): Leptin and reproduction: a review. Fertil. Steril. **77,** 433–444.

Mounzih, K., Qiu, J., Ewart-Toland, A. and Chehab, F. F. (1998): Leptin is not necessary for gestation and parturition but regulates maternal nutrition via a leptin resistance state. Endocrinology **139,** 5259–5262.

Silva, L. F., VandeHaar, M. J., Weber Nielsen, M. S. and Smith, G. W. (2002): Evidence for a local effect of leptin in bovine mammary gland. J. Dairy Sci. **85,** 3277–3286.

Smith, J. L. and Sheffield, L. G. (2002): Production and regulation of leptin in bovine mammary epithelial cells. Domest. Anim. Endocrinol. **22,** 145–154.

Smith-Kirwin, S. M., O'Connor, D. M., Johnston, J., De Lancey, E., Hassink, S. G. and Funanage, V. L. (1998): Leptin expression in human mammary epithelial cells and breast milk. J. Clin. Endocrinol. Metab. **83,** 1810–1813.

Sweeney, G. (2002): Leptin signalling. Cellular Signalling **14,** 655–663.

Tartaglia, L. A. (1997): The leptin receptor. J. Biol. Chem. **272,** 6093–6096.

Tu, H. M., Kim, S. W., Salvatore, D., Bartha, T., Legradi, G., Larsen, P. R. and Lechan, R. M. (1997): Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. Endocrinol. **138**, 3359–3368.

Vernon, R. G., Denis, R. G. P. and Sørensen, A. (2001): Signals of adiposity. Domest. Anim. Endocrinol. **21,** 197–214.

Yanagihara, N., Utsunomiya, K., Cheah, T. B., Hirano, H., Kajiwara, K., Hara, K., Nakamura, E., Toyohira, Y., Uezono, Y., Ueno, S. and Izumi, F. (2000): Characterization and functional role of leptin receptor in bovine adrenal medullary cells. Biochem. Pharmacol. **59,** 1141–1145.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994): Positional cloning of the mouse obese gene and its human homologue. Nature **372**, 425–432.