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MOLECULAR AND CELLULAR INVESTIGATION OF LEPTIN HORMONE AND ITS RECEPTORS IN LARGE RUMINANTS

Doctoral Thesis

OF

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Made in eight copies
Ahmed Saber Ibrahim Sayed-Ahmed

Dedication

To my parents,
my wife, Neveen
and my daughter, Nada

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INTRODUCTION

Leptin was discovered in 1994, as a protein found deficient in the genetically obese mice. This discovery has generated enormous interest in how the periphery signals the status of nutritional stores to specific hypothalamic nuclei involved in regulating feeding and energy balance. The obesity of genetically obese mice can be cured by leptin treatment, associating with marked improve in the energy expenditure, fertility, immune response, brain development, and wound healing. Thus, a complex physiological role is attributed to the leptin, which replaces the original view describing it as a hormone with metabolic effects only. The primary role of leptin was originally described as to decrease appetite and increase energy expenditure. Therefore, in the beginning of the present work we will describe a general review about leptin and its complex physiological roles.

LEPTIN A MULTI-FUNCTION HORMONE

Leptin Hormone

Leptin, the hormone encoded by obesity (Ob) gene, is a 146 aa protein playing 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; and Ahima and Flier, 2000). Leptin was first identified, as gene product found deficient in obese (ob/ob) mice. A single base mutation of the leptin gene at the codon 105, as observed in the ob/ob mouse involved C T mutation and replacement of arginine by a premature stop codon and a subsequent production of an inactive form of leptin (Zhang et al., 1994). The genetically obese ob/ob mouse exhibits obesity, infertility, hyperglycemia, impaired thyroid function and hyperinsulinemia with insulin resistance (Dubuc, 1976). Treatments of the ob/ob mice with recombinant leptin reduce feeding and body weights (Weigle et al., 1995; Halaas et al., 1995).

A homozygous frame-shift mutation involving the deletion of a single guanine nucleotide in codon 133 of the gene for leptin is found in two severely obese children with very low leptin level (Montague et al., 1997). Genetic screening of morbidly obese patients revealed a polymorphism in Exon 1 of the ob gene (A G, base 19), which resulted in lower BMI-corrected leptin concentrations compared to obese patients with no mutation (Hager et al., 1998). Four polymorphisms in the porcine leptin are associated with fatness (Jiang and

Gibson, 1999). The leptin gene polymorphisms are also reported in the bovine with association with the fat deposition in beef cattle (Fitzsimmons et al., 1998). A missense mutation (C T) that encoded an amino acid change of an arginine to a cysteine in exon 2 of the bovine leptin gene is associated with fatter carcasses and higher levels of leptin mRNA (Buchanan et al., 2002). The leptin gene polymorphisms of the dairy cow are associated with genetic variations in the energy balance, milk yield, live weight, and fertility traits (Liefers et al., 2002). A recent study in bovine reports an association between the polymorphisms in the exon 2 and the feed intake, that the individuals with genotype A/T has 19% greater mean feed intake than individuals with genotype A/A (Lagonigro et al., 2003).

The leptin gene has been cloned in most domestic animal species such as sheep (Dyer et al., 1997), cow (Ji et al., 1998), swine (Ramsay et al., 1998), dog (Iwase et al., 2000), cat (Sasaki et al., 2001), and horse (Buff et al., 2002). The leptin gene is highly conserved among vertebrates (Houseknecht and Portocarrero, 1998).

The original studies thought that leptin is exclusively expressed in white adipose tissue, while extensive research on leptin over the last years has shown that leptin is expressed in several peripheral tissues, such as placenta, fetal tissue (Hoggard et al., 1997; Henson et al., 1998), stomach (Bado et al 1998; Konturek et al., 2001), skeletal muscle (Wang et al., 1998), brain, pituitary (Morash et al., 1999), mammary gland tissues (Smith-Kirwin et al., 1998; Aoki et al., 1999; Chilliard et al., 2001; Bonnet et al., 2002b; and Smith and Sheffield, 2002), human follicular papilla cells (Iguchi et al., 2001), calf rumen, abomasum, duodenum (Yonekura et al., 2002), and intralobular ducts of human major salivary glands (De Matteis et al., 2002).

Leptin has been classified as a cytokine based upon the structural similarity between leptin receptor and gp130, a member of the interleukin-6 family of receptors (Baumann et al., 1996: Tartaglia, 1997). Threading analysis of leptin sequence indicated a cytokine-folding pattern similar to interleukin-2 and growth hormone (Madej et al., 1995). Nuclear magnetic resonance (NMR) revealed that leptin is a four-helix bundle cytokine (Fig.1 Kline et al., 1997). Additionally, NMR analyses revealed that leptin contains a single disulfide linkage (Cys 96 and Cys 146) connecting the CD loop to the carboxyl terminus. This disulfide linkage is crucial for leptin function, since disruption of the cysteine leads to loss of bioactivity (Rock et al., 1996; Kline et al., 1997).

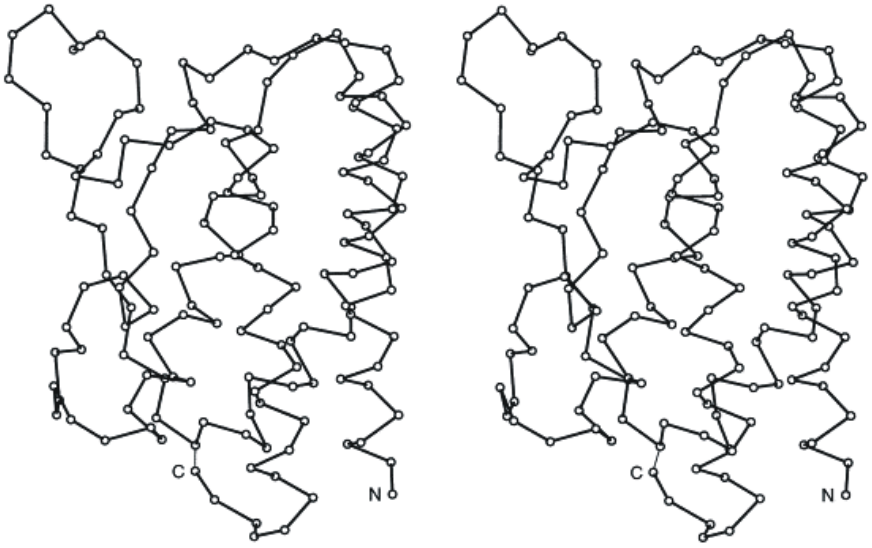

Fig. 1. A stereo C fold drawing of the mouse leptin model based on the NMR helix assignments. C atoms are shown as open circles; termini are labeled near the bottom of the structure. The 96-146 disulfide is represented by a thin line near the C-terminus (Kline et al., 1997).

Leptin Receptor

Expressional cloning studies revealed that the db gene encodes the leptin receptor (Ob-R). Leptin receptor is a single membrane spanning receptor that has strong sequence homology to the class I cytokine receptor family (Tartaglia, 1997). db/db mice possess a mutant allele of the leptin receptor (Ob-R) gene, which encodes a non-functional leptin receptor due to a 106 amino acid insertion, resulting in a premature truncation of the receptor cytoplasmic domain (Chen et al., 1996). Because of this mutation, the long form transcript of the leptin receptor in db/db mice encodes a portion in which the majority of the intracytoplasmic domain has been truncated and will be identical to the short forms (Tartaglia, 1997). db/db mice exhibits obese and diabetic phenotype due to the absence of leptin signaling (Brann et al., 2002) Similar mutation has also been identified in the Ob-R gene of the Zucker fa/fa rat and are believed to contribute to the obese phenotype exhibited by this animal (Takaya et al., 1996).

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, Tartaglia, 1997; Friedman and Halaas, 1998). Ob-Rb, the so-called long isoform is thought to be the most important for transmitting the leptin signal in cells and is predominantly localized in hypothalamus yet it is also found in many peripheral tissues at lower levels (Fei

et al., 1997; Vernon et al., 2001). The short isoform (Ob-Ra) is expressed ubiquitously and represented the major isoform in many peripheral tissues (Mercer et al., 1996; Fei et al., 1997; Laud et al., 1999; and 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. RT-PCR studies indicate the expression of the short (Ob-Ra) isoform in the bovine adrenal medulla (Yanagihara et al., 2000) and both of long (Ob-Rb) and short (Ob-Ra) isoforms of leptin receptor are found in ovine mammary tissue during pregnancy and lactation (Laud et al., 1999).

Class I cytokine receptors contain highly conserved Box 1 and Box 2 motifs proximal to the transmembrane domain. Box motifs mediate the association of Janus-activated kinase (JAK) family members initiate an intracellular signaling cascade (Brann et al., 2002). While leptin receptor isoforms contain a Box 1 motif, and only the Ob-Rb isoform contains an additional Box 2 motif. The Ob-Rb isoform is also the only leptin receptor that contains intracellular tyrosine residues, which upon phosphorylation, allows interaction with signal transducers and activators of transcription (STAT). STAT protein then translocates to the nucleus and interacts with specific DNA elements in the promoters of target genes to regulate gene expression (Tartaglia, 1997; Sweeney, 2002).

Signal transduction of leptin receptor is not limited to JAK-STAT system but is linked to mitogen activated protein kinase (MAPK) pathways, which eventually induces expression of several genes involved in the transcriptional regulation and signaling (Houseknecht et al., 1998). Leptin induces transient expression of suppressor of cytokine signaling-3 (SOCS-3) protein and forced expression of SOCS-3 result in inhibition of leptin induced tyrosine phosphorylation of JAK2 (Bjorbaek, et al., 1999). SOCS proteins act in a typical negative feedback loop and can inhibit leptin signaling (Zabeau et al., 2003).

A strong correlation is assumed between the leptin and insulin signaling pathways since leptin and insulin resistances occur coincidentally in the majority of obese humans. Phosphorylation of the insulin receptor substrates 1 and 2 (IRS1 and 2) as well as their interaction with phosphatidylinositol 3-kinase (PI3K) show clear modulation by leptin (Zabeau et al., 2003). There are several other signaling proteins regulated by leptin such as protein kinase B (PKB), protein kinase C (PKC), cyclic AMP, nitric oxide (NO), Rho family proteins and actin cytoskeleton, reactive oxygen species (ROS), prostaglandin E2 and

prostaglandin F2 (Sweeney, 2002). The majority of signaling proteins regulated by leptin is summarized in Fig. 2.

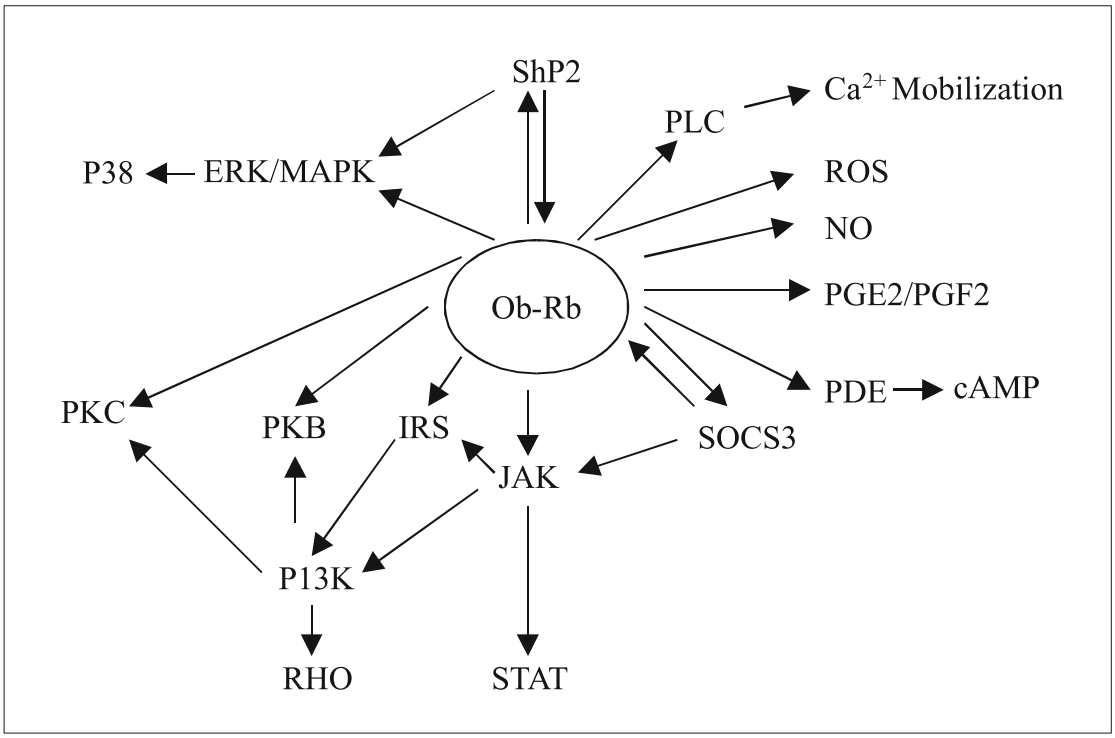

Fig. 2. Signaling pathways that have been demonstrated to be regulated by leptin. Including P38 (p38 MAP kinase), ERK (extracellular regulated kinase), SHP-2 (Src-like homology 2 (SH2) domain containing protein tyrosine phosphatase), PLC (phospholipase C), ROS (reactive oxygen species), NO (nitric oxide), PGE2/PGF2 (prostaglandins E2/F2), PDE (phosphodiesterase), cAMP (cyclic AMP), SOCS-3 (suppressor of cytokine signaling 3), JAK (Janus-family tyrosine kinase), STAT (signal transducers and activators of transcription), PI3K (phosphatidylinositol 3-kinase), IRS (insulin receptor substrates), PKB (protein kinase B), and PKC (protein kinase C).

Leptin and Energy Balance

The original overall metabolic role of leptin whose primary effects are to decrease appetite and increase energy expenditure was based on the following observations.

1. Leptin (ob/ob mice) and leptin receptor mutants (db/db mice, fa/fa rats) have lower core body temperatures, are more susceptible to cold stress soon after birth, and are hyperphagic and obese by the time of weaning (Leibel et al., 1997).

2. Daily intraperitoneal (ip) injections of recombinant leptin reduce food intake and body weight and increase energy expenditure in ob/ob mice and in wild type rodents but no effect on db/db mice (Halaas et al., 1995). Intracerebroventricular (icv) injection cause rapid decrease in food intake and body weight in ob/ob mice, wild type mice, rat, monkey, pigs

indicating that the satiety effects of leptin are central (Ingvartsen and Boisclair, 2001). The satiety effects of leptin are lost or attenuated in mice with lesions of the ventromedial hypothalamus (VMH) or the arcuate nucleus (ARC), demonstrating that these regions are involved (Satoh et al., 1997; Dawson et al., 1997). The satiety effects of leptin are also observed in ruminants that icv administration of leptin to ovariectomized ewes inhibits food intake (Henry et al., 1999).

3. The long isoform of leptin receptor is predominately expressed in the hypothalamus especially the arcuate, paraventricular, and ventromedial nuclei that control of both food intake and sexual behavior (Fei et al., 1997; Hakansson et al., 1998).

4. The high affinity leptin transport in the hypothalamus and choroid plexus is playing a key role in regulating leptin entry into the CNS and CSF. These high affinity transport systems mediate leptin entry into the hypothalamus and across the blood-CSF barrier at rates 18 to 53- fold faster than across the blood brain barrier in other CNS-regions (Zlokovic et al., 2000). Leptin is transported intact from blood to brain by a saturable system this saturable transport does not occur out of the brain (Banks et al., 1996). Leptin transport across blood-CSF barrier can also be via saturable and non-saturable mechanism in which the choroid plexus involved in the regulation of leptin availability to the brain (Thomas et al., 2001). The leptin receptor is involved in leptin entry to brain and this is supported by the studies in rats lacking the short form of leptin receptor (Koletsky rats) in which there is a low entry rate of periphery-injected leptin to the brain (Kastin et al., 1999).

Regulation of feed intake, energy balance and neuroendocrine function by leptin is thought to be mediated by differential expression of a network of hypothalamic peptides include orexigenic (anabolic) and anorexiogenic (catabolic) neuropeptides. The orexigenic neuropeptides are neuropeptide Y (NPY), agouti-related peptide (AgRP), melanin-concentrating hormone (MCH), galanin, and orexins while anorexigenic peptides include corticotrophin-releasing hormone (CRH), proopiomelanocortin (precursor of -melanocyte stimulating hormone MSH), cocaine and amphetamine-regulated transcript (CART), and thyrotropin releasing hormone (TRH). This concepts is supported by the co-localization of many these neuropeptides neurons with leptin receptors (Ahima and Hileman, 2000; Ingvartsen and Boisclair, 2001).

NPY potently stimulates food intake, inhibits the thermogenesis, and increase plasma insulin and glucocorticoid concentrations. The NPY expression is increased in the arcuate nucleus of the hypothalamus of the obese (ob/ob) mice (Houseknecht and Portocarrero, 1998). Injected NPY induce hyperphagia (Miner et al., 1989 McMinn et al., 1998) while icv injection of NPY-antibody has the opposite effect (Dube et al., 1994). Leptin acts centrally to inhibit the effects of NPY by inhibiting its synthesis in the arcuate nucleus of the hypothalamus. Leptin administration to the hyperphagic ob/ob mice, wild type mice, and sheep results in a rapid reduction in NPY mRNA abundance, protein secretion, and reduced food intake before any change in body weight (Schwartz et al., 1996; Henry et al., 1999). Although the role of NPY is crucial, the leptin administration to mice in which the NPY gene has been disrupted reduces the food intake (Considine and Caro, 1997). Taken together, these observations provide evidence that the central regulation of energy balance is not mediated exclusively by NPY and, furthermore that NPY is not an absolute requirement for leptin action.

Regarding to other orexigenic peptides, central administration of leptin decrease hypothalamic MCH and galanin expression and prevent MCH or galanin-induced increase in food intake (Sahu, 1998). Both NPY- and galanin-induced feeding behaviors were completely inhibited by pre-administration of leptin (3 g), while the same or a higher dose (10 g) of leptin only partially inhibited orexin-A or -B-induced increase of food intake (Zhu etal., 2002).

The anorexigenic neuropeptides includes:

1. The corticotrpin-releasing hormone (CRH) which is a potent anorexigenic peptide secreted in the hypothalamus, primarily from the PVN (Hillebrand et al., 2002). Administration of CRH reduces food intake in rodent, monkeys, and cattle (Ingvartsen and Boisclair, 2001). Leptin administrations increase the secretion of CRH from hypothalamic explants (Costa et al., 1997) and icv infusion of leptin increase the expression of hypothalamic CRH (Arvaniti et al., 2001). Besides the effect on food intake, CRH increases sympathetic nervous system activity, thereby increasing thermogenesis, energy expenditure, and lipolysis (Hillebrand et al., 2002).

2. Melanocortins (MC) is peptides that are cleaved from the precursor molecule POMC that is expressed in the ARC of the hypothalamus and pituitary gland. The role of

melanocortins in feeding behavior is strengthened by the presence of POMC neurons expressing the Ob-Rb leptin receptor in the ARC. When leptin levels are high, POMC neurons in the ARC are stimulated which leads to a decrease in food intake and increase in sympathetic activity that is prevented by melanocortin antagonists (Vrang et al., 2000; Hillebrand et al., 2002; Munzberg et al., 2003). The effects of melanocortins on the food intake are mediated mainly by the melanocortin receptors specially MC3 & 4 receptor which are mainly distributed in the brain (Gantz et al., 1993).

The agouti-related protein AgRP is other orexigenic peptides secreted in the ARC and act as melanocortin receptor antagonist. The central injection of -MSH or melancortin agonists decrease food intake (Hansen et al., 2001), while the central injection of AgRP increase food intake, increase fat deposition and reduce energy expenditure (Wirth and Giraudo, 2000). Leptin administration inhibits the expression and synthesis of AgRP (Mizuno and Mobbs, 1999; Arvaniti et al., 2001).

3. CART-containing neurons are widespread throughout the brain, including the ARC, LH and PVN. Recombinant CART injected icv in rats tended to inhibit normal and starvation-induced feeding, and blocked the feeding response induced by NPY and peripheral leptin injection increases the expression of CART (Kristensen et al., 1998).

4. Thyrotropin-releasing hormone (TRH) is an anorexigenic peptide that plays a major role in the hypothalamus–pituitary–thyroid (HPT) axis by stimulating the release of thyroid-stimulating hormone (TSH). In addition, TRH is an important stimulator of energy expenditure through an increase in thermogenesis (Hillebrand et al., 2002). Administration of leptin to food-deprived rats has been reported to restore to normal decreased proTRH mRNA levels in neurones of the paraventricular nucleus, medial parvocellular neurones (Friedman and Halaas, 1998; Seoane et al., 2000; Nillni et al., 2000). -MSH has an important role in the activation of proTRH gene expression (Fekete et al., 2000). Thus leptin activate directly TRH neurons in the paraventricular nucleus or indirectly by increasing the production of the MC4R ligand, (-melanocyte stimulating hormone -MSH), to regulate TRH expression on the level of TRH promoter (Harris et al., 2001). Other indirect pathway is the inhibition of the NPY synthesis that has inhibitory action on the hypothalamic-pituitary-thyroid axis (Fekete et al., 2001 & 2002). Central leptin administration produced marked increases in monodeiodinase type II mRNA expression and activity in brown

adipose tissues, changes that were likely responsible for increased plasma T3 and decreased plasma T4 levels (Cettour-Rose et al., 2002).

The effects of leptin via its receptors on energy balance of peripheral tissues are apparent in skeletal muscle, liver, pancreas, and adipose tissue (Muoio and Lynis Dohm, 2002). Leptin has direct thermogenic effects in skeletal muscle, and these effects require both the long form of leptin receptors and phosphatidylinositol 3-kinase signaling (Dulloo et al., 2002). In hepatocytes, leptin has complex effects on the insulin response. It stimulates glucose transport and turnover consistent with an insulin-like response (Houseknecht and Portocarrero, 1998; Baratta, 2002). Leptin administration in ob/ob mice resulted in decreased expression of genes involved in fatty acid and cholesterol synthesis, glycolysis, gluconeogenesis, and urea synthesis, and increased expression of genes mediating fatty acid oxidation, ATP synthesis, and oxidant defenses. The changes in mRNA expression are consistent with a switch in energy metabolism from glucose utilization and fatty acid synthesis to fatty acid oxidation and increased respiration (Liang and Tall, 2001). Leptin inhibition of hepatic gluconeogenesis is mediated by insulin receptor substrate-2 (Anderwald et al., 2002). Recently most of intention is directed toward pro-fibrogenic activity of leptin through its activation of hepatic stellate cells (Saxena et al., 2002; Leclercq et al., 2002; Saxena et al., 2003). Leptin inhibits cortisol secretion from human and rat adrenal glands (Pralong et al., 1998). Additionally, leptin increases catecholamine synthesis in the bovine adrenal medullary cells (Yanagihara et al., 2000; Shibuya et al., 2002). The central and peripheral effects of leptin on the energy balance are summarized in Fig. 3.

Leptin and Tissue Development

Leptin, through its receptors in the peripheral tissues, has been implicated in numerous other roles, including, hematopoiesis, angiogenesis, brain and bone development, wound healing, and cell differentiation and proliferation (Ahima and Flier, 2000). Thus the original view of leptin as a metabolic hormone its role as a circulating satiety factor has been replaced by a more complex one (Harvey and Ashford, 2003).

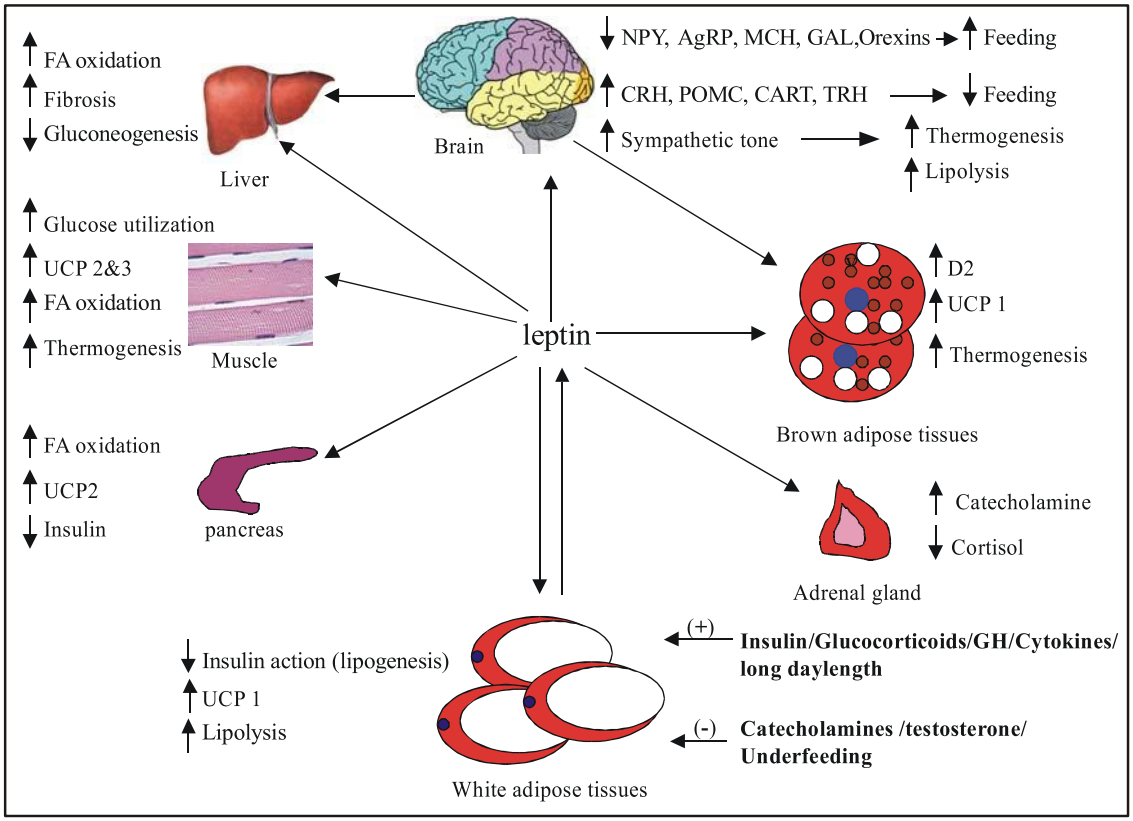

Fig. 3. The central and peripheral effects of leptin on the energy balance.

Leptin deficiency or insensitivity to its action leads to decreased brain weight, decreased whole brain protein content and a reduction in the expression of several synaptic, glial and myelin basic protein which are restored by exogenous leptin administration (Ahima et al., 1999). Intraperitoneal administration of leptin for 2 weeks daily to 4-week-old ob/ob mice resulted in a maximal 10% increase in both wet and dry brain weights and 19% increase in total brain DNA (Steppan and Swick, 1999). Recent studies indicate that leptin receptor immunoreactivity and mRNA are expressed in areas of the CNS that are not directly associated with the regulation of energy balance, such as the cerebellum, pyriform cortex, cerebral cortex, thalamus, hippocampus, amygdala, olfactory tract and substantia nigra. Thus, it is likely that leptin has additional functions in these brain regions (Harvey and Ashford, 2003).

Recently, leptin was demonstrated to induce differentiation of marrow stromal cells into osteoblasts, and not to adipocytes (Thomas et al., 1999). Leptin has also been demonstrated to have direct effects on proliferation, differentiation, mineralization, and to induce prolonged life span of human primary osteoblasts by inhibiting apoptosis (Gordeladze et al., 2002). The chondrocytes in the growth centers contain specific binding sites for leptin. Leptin stimulated the width of the chondroprogenitor zone at low

concentrations, whereas higher concentrations had an inhibitory effect (Maor et al., 2002). These results indicate that leptin acts as a skeletal growth factor with a direct peripheral effect on skeletal growth centers.

Leptin completely reversed the atrophied morphology of the migrating epithelial at the wound margins of leptin-deficient animals (ob/ob) into a well-organized hyper-proliferative epithelium. Furthermore, topically supplemented leptin accelerated normal wound-healing conditions in wild-type mice (Frank et al., 2000). The expression of leptin mRNA and protein was significantly increased at the ulcer edge and leptin reduce significantly the ulcer area as compared to vehicle-treated group by 50% (Konturek et al., 2001).

Leptin hormone via activation of the endothelial Ob-R generate growth signal and promotes the angiogenesis. Thus, the treatment of the cultured human umbilical venous endothelial cells with leptin led to increase in cell number with an enhanced formation of capillary like tube in an *in vitro* angiogenesis model. Leptin treatment is associated with the formation of new blood vessels in chick chorioallantoic membrane in an *in vivo* model of angiogenesis (Bouloumie et al., 1998). Leptin synergistically stimulates angiogenesis with fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), the two most potent and commonly expressed angiogenic factors (Cao et al., 2001).

Leptin mRNA was detected in blastocyst and hatched blastocyst, and Ob-Rb mRNA was detected in oocytes, 1-cell, 2-cell, morula, and blastocyst and hatched blastocyst. Supplementation of culture medium with leptin promotes the development of preimplantation embryos from 2-cell stage to the blastocysts and fully expanded blastocysts and hatched blastocysts. (Kawamura et al., 2002 & 2003). Injection of leptin in the incubated eggs of the Japanese quail result in earlier hatching with high body weight and significant changes in the T3 concentration and total lipids as well as high testosterone and high egg laying in leptin treated group (Macajova et al., 2002; Lamosova et al., 2003).

Leptin and Immune System

The circulating concentration of leptin is proportional to fat mass and reduced body fat or nutritional deprivation associated typically with hypoleptinaemia (Houseknecht et al., 1998), which is a direct cause of secondary immunodeficiency and increased susceptibility

to infection (Matarese, 2000). The reason for this association was not apparent until recently, when administration of leptin to mice reversed the immunosuppressive effects of acute starvation (Lord et al., 1998; Matarese, 2000). Both naturally leptin-deficient (ob/ob) and leptin-receptor-deficient (db/db) mice despite their great body-fat mass have thymic and lymph-node atrophy reduced numbers of bone-marrow precursors and impaired cell-mediated immune responses (Mandel and Mahmoud, 1978; Chandra, 1980). These defects are normalized when leptin is injected in ob/ob mice (Howard et al., 1999). Leptin increase the phagocytically active macrophages in normal and ob/ob mice but not in db/db mice, in addition to increase of TNF- , IL-6 and IL-12 response to LPS (Loffreda et al., 1998). Leptin enhanced cytokine production and phagocytosis of leishmania parasites by murine macrophages (Gainsford et al., 1996). The leptin receptor is expressed on a diverse range of hemopoietic cells and leptin itself appears to enhance proliferation of hemopoietic cells (Gainsford and Alexander, 1999).

Leptin and Reproduction

The first indication, that leptin is involved in the regulation of the reproduction was the finding that the ob/ob mice which is infertile rescued their fertility by exogenous leptin treatment (Chehab et al., 1996; Mounzih et al., 1997). Leptin was reported to play a role in timing or accelerate the onset of puberty in several species (Cunningham et al., 1999; Spicer, 2001; Almog et al., 2001). The Ob-Rb isoform is highly expressed in the arcuate and ventromedial hypothalmic nuclei which control both sexual behavior and food intake (Fei et al., 1997; Hakansson et al., 1998). Thus, it was believed that leptin may facilitate the GnRH secretion directly or via indirect mechanisms through inter-neurons secreting neuropeptides. A hypothalamic site of action was suggested by studies demonstrating that leptin significantly increase GnRH secretion in vitro from hypothalamic ARC/ME explants (Yu et al., 1997). A hypothalamic site of action of leptin is further supported by the findings that leptin injected into the third ventricle stimulates LH and PRL release. While administration of leptin antiserum into the third ventricle of female rats induces a decrease in LH pulsatility, disrupts cyclicity, and delays the onset of the PRL surge (Carro et al., 1997).

Two days of total feed restriction in 11–12-month-old heifers markedly reduced leptin mRNA in adipose tissue, as well as circulating concentrations of leptin IGF-I, and insulin, and reduced the frequency of LH pulses compared to controls (Amstalden et al., 2000). In contrast to the prepubertal heifer, short-term fasting (60 h) did not attenuate

pulsatile LH release in the mature cow. However, a nearly identical pattern of reduction in leptin gene expression, and circulating leptin, insulin and IGF-I to that observed in heifers was observed (Amstalden et al., 2002). Fasting has reduced circulating leptin in heifers by about 30% (to less than 1 ng/ml). In mature cows after 60 h of fasting, baseline concentrations of circulating leptin were over 7 ng/ml and declined after fasting to just over 5 ng/ml. Hence, the failure of short-term fasting to suppress LH secretion in mature cows could be the result of the failure to reduce circulating concentrations of leptin below "critical" levels (Williams et al., 2002). Central administration of leptin increased plasma LH in fasted but not in control-fed cows (Amstalden et al., 2002). Short-term (72-h) fasting and fasting-mediated reductions in LH pulse frequency were attenuated by peripherally administered recombinant leptin (Nagatani et al., 2000). Therefore, metabolic state appears to be an important determinant of hypothalamic–pituitary responses to leptin.

Not all the downstream mediators of leptin action on GnRH release are known. Recent reports have suggested that leptin action may be mediated in part, by CART (cocaine and amphetamine-regulated peptide). In support of this contention, treatment with a CART antibody was shown to attenuate leptin stimulation of GnRH pulsatility, while in contrast, a neuropeptides Y-5 (NPY-5) receptor antagonist was without effect on leptin action (Parent et al., 2000). Recently also it has been shown that leptin exerts its action at both hypothalamus and pituitary level by activating nitric oxide synthase (NOS) which activate the release of LHRH, LH and FSH (McCann et al., 1999 & 2003). In sheep (Iqbal et al., 2001b) it was reported that leptin can influence reproductive neuroendocrine function through its effect on orexins A and B-containing neurons.

Further downstream effectors of leptin on GnRH secretion remain less well defined including the control of feeding conditions. Thus, neuropeptide Y (NPY) can have stimulatory or inhibitory effects on GnRH secretion, depending upon several factors such as the site of NPY administration, species, gender, acute versus chronic exposure, and the degree of sexual maturation (Moschos et al., 2002).

Gonadotropes in the pars tuberalis and pars distalis of the pituitary express Ob-Rb. Thus leptin has direct effects on the anterior pituitary as well (Iqbal et al., 2001a; Moschos et al., 2002). Leptin may directly stimulate LH and to a lesser extent FSH, release by the pituitary via NO synthase activation in gonadotropes (Yu et al., 1997).

The endocrine and/or direct paracrine effects of leptin on the peripheral reproductive organ are suggested after localization of functional leptin receptor (Ob-Rb) in the ovary (Ruiz-Corttes et al., 2000), the uterus (Gonzalez et al., 2000; Kitawaki et al., 2000; Lin et al., 2000), the testis germ cells (El-Hefnawy et al., 2000), and the Lyedig and Sertoli cells of the testis (Tena-Sempere and Barrerio, 2002). Furthermore, the placenta (Henson et al., 1998), the endometrium (Gonzalez et al., 2000; Wang et al., 2003), the murine ovary (Ryan et al., 2002), and even the embryos (Kawamura et al., 2002) express both the leptin and the long form leptin receptor.

Leptin may be important in regulating maternal nutrition and the metabolic adaptation of nutrient partitioning during the energy-consuming processes of pregnancy and lactation. More specifically, pregnancy with its associated hormonal changes (especially insulin, glucocorticoids, estrogens, and prolactin) appears to be a state of physiologic hyperleptinemia and leptin resistance, with uncoupling of eating behavior and metabolic activity (Mukherjea et al., 1999; Moschos et al., 2002). Leptin is dramatically decreased to pre-pregnancy levels around parturition in human (Butte et al., 1997; Henson and Castracane, 2000), rodent (Gavrilova et al., 1997; Aoki et al., 1999; Brogan et al., 1999), ewe (Ehrhardt et al., 2001) and after parturition in cow (Liefers et al., 2003) and mare (Heidler et al., 2003). The increase in blood leptin level during pregnancy could result from the increased leptin synthesis by the adipose tissue, the excess leptin synthesis by the placenta, or the reduced leptin clearance due to the increase level of the hormone-binding soluble form of leptin receptor, which is also produced by the placenta (Bonnet et al., 2002a)

During zero energy balance (in steady-state conditions) leptin expression and secretion reflect the body fat mass (Maffei et al., 1995; Considine and Caro, 1997) and the circulating hormone levels can highly be correlated with the average adipocyte size (Houseknecht et al., 1998). This correlation is drastically altered in energy disbalance. Thus, leptin not only function as an adipostat to signal the status of body energy stores to the brain but also function as a sensor of energy balance (Houseknecht et al., 1998). In cows the leptin concentration reflects the state of energy balance during lactation as the plasma leptin concentration is lower in cows with a negative energy balance. These cows usually produce more milk, consume less feed, and have a lower body weigh compared with cows having a positive energy balance (Liefers et al., 2003). Reduced leptin concentration may promote feed intake and allow lactating mares to avoid energy deficit (Heidler et al., 2003).

Leptin is demonstrated in the colostrum of the porcine (Estienne et al; 2000), cow (Bonnet et al., 2002a) as well as in the milk of rat (Casabiell et al; 1997), human (Smith-Kirwn et al; 1998), porcine (Estienne et al; 2000), and bovine (Smith and Sheffield, 2002). Furthermore maternal leptin is transferred to the milk, then to the stomach of the suckling pup, and afterwards to the neonatal circulation (Casabiell et al; 1997).

RT-PCR technique revealed the presence of leptin mRNA in the mammary gland tissues (Smith-Kirwin et al., 1998; Aoki et al., 1999; Smith and Sheffield, 2002; Bonnet et al., 2002b). Latter study did not indicate the type of the cell in which leptin was present. Moreover immunohistochemical staining revealed the production of leptin by human active mammary epithelial cells (Smith-Kirwin et al., 1998). On the other hand Bonnet et al (2002b) reported the expression of leptin in the ovine mammary gland throughout pregnancy and lactation associated with alteration in the localization of leptin protein within mammary tissue. 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.

Leptin deficient mice (ob/ob) can be induced to become pregnant with exogenous leptin treatment. The induced mice deliver normal litter but stop lactating upon withdrawal of the exogenous leptin (Mounzih et al., 1998). Both blood and mammary leptin, via leptin receptors could exert endocrine, paracrine and/or autocrine control over mammogenesis and maintenance of alveolar structures (Bonnet et al., 2002a). This hypothesis is further supported by the reports describe the ability of leptin to control the proliferation of both normal and malignant mammary epithelial cells (Dieudonne et al., 2002; Hu et al., 2002; Silva et al., 2002; Baratta et al., 2003).

The long (Ob-Rb) and short (Ob-Ra) isoforms of leptin receptors are expressed in the gastric mucosa (Goiot et al; 2001) as well as the absorptive intestinal cells (Barrenetxe et al; 2002). Leptin induce increase in the proliferation of gastric mucosal cell and contribute to mucosal integrity and gastroprotection (Schneider et al; 2001). The secretion of leptin into colostrum and milk and its production by the mammary epithelial cells suggests a possible role of maternal leptin in regulating neonatal appetite or metabolism, although this role has been debated and require more investigations (Ucar et al; 2000; Bonnet et al., 2002a).

AIMS OF THE THESIS

It was described in the previous chapter that both the blood origin and the locally produced leptin could play an important role in the mammogenesis and the maintenance of the lactation. The site of the expression of mammary-gland-leptin varies with the stages of pregnancy and parturition in the ovine. So far, there is no information about the localization of leptin or leptin receptors in the mammary gland of the large ruminant animals. In the first experiment of the present work, the Egyptian water buffalo was selected as an example of large ruminants to examine the leptin gene and its receptor expression in the mammary gland. In this species, so far there is no information available about leptin. Leptin can be in focus of interest in water buffalo because nutritional and metabolic disorders are major problems in this species. cDNA cloning is necessary to indicate the expression of leptin and its receptor in buffalo mammary tissue. In order to examine whether buffalo leptin is expressed in the epithelial cell or myoepithelial cell of lactating mammary gland, we are adapting and/or developing *in situ* hybridization procedure for leptin and its receptor mRNA transcripts, to specify the type of the cell in which leptin and its receptors are present in the mammary gland tissues of lactating buffalo.

During pregnancy a larger amount of energy is required to sustain pregnancy, fetal growth, and development. Blood leptin increases during pregnancy and then decreases dramatically after parturition. During early lactation, most of the high producing dairy cows show a negative energy balance associated with low leptin level since leptin reflects the state of energy balance in lactating animals. In the second experiment of the present work, the non-pregnant dry and lactating cows were selected. According to the health condition of the animals our samples were collected from animals showing a good nutritional condition. These animals were selected to examine the leptin and its receptor expression in the mammary gland in addition to study the alteration of mammary leptin level using competitive RT-PCR without any effect of pregnancy or negative energy balance. Paraffin embedded mammary gland sections from dry and lactating cows were subjected to *in situ* hybridization 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.

In hot arid areas, maintenance of internal homeostasis is closely related to endocrine system, particularly to thyroid hormone economy. Leptin hormone is involved in the regulation of food uptake and whole-body energy homeostasis. As mentioned above leptin also interacts with thyroid hormone economy at the level of central regulation (TRH production in the hypothalamus) or the peripheral hormone activation (up-regulation of monodeiodinase type II mRNA expression and activity in brown adipose tissues and indirectly increasing of the plasma T_3). In the third experiment of the present work the one-humped camel selected as an example of the desert animals, which is exposed to several harsh environmental factors. We investigated the expression of leptin and its receptors in this species. Partial cDNA clones were prepared in order to localize the leptin and leptin receptors in the mammary gland and in liver tissue of the one-humped camel.

MATERIALS AND METHODS

Tissue Samples

The tissue samples of the Egyptian water buffalo and one-humped camel were collected from Cairo slaughterhouse. Subcutaneous adipose tissue and mammary gland tissues were collected from two lactating buffalo in advanced stage of lactation. Hump adipose tissue, liver and mammary gland tissues were collected from two dry one-humped female camels. The tissue samples were collected immediately after slaughtering. Nearly 500 mg of each sample was kept in 5 ml of ice cold RNALATER (Qiagen) and stored at -20 C until RNA extraction. Parallely about 5 g of each tissue was fixed in 100 ml of Diethyl Pyrocarbonate (Sigma) treated PBS containing 4% paraformaldehyde for *in situ* hybridization and immunohistochemistry.

The mammary gland tissues of the dry and lactating non-pregnant cows were collected immediately after slaughtering at Budapest slaughterhouse. Nearly 5 g of the parenchymal tissue of each sample was quickly frozen in liquid nitrogen and stored at -70 C until RNA extraction. At the same time, about 5gm of each sample was fixed in 4% paraformaldehyde for *in situ* hybridization and immunohistochemistry.

RNA Isolation

Total RNA was isolated by TRIzol reagent (Life Technologies). After homogenization, lysis, and incubation at room temperature (20 C), 200 l chloroform per millilitre of TRIzol was added. Samples were centrifuged at 12000-x g for 15 minutes at 4 C and RNA was precipitated with 500 l isopropyl alcohol per millilitr of TRIzol, washed with 75% ethanol, dried and resuspended in DEPC treated water. RNA yields and purity were assessed by absorbency at 260 and 280 nm. Accepted ratios of absorption (260/280) were between 1.7 and 2.0. Aliquots of RNA samples were subjected to electrophoresis to verify their integrity.

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 (Ren et al., 2002)

Partial cDNA Cloning of Leptin and Leptin Receptors

Oligonucleotide primers for amplification of buffalo and one-humped camel leptin as well as short and long isoforms of leptin receptor were selected on the bases of regions of high sequence identity between known sequences of cow, sheep, pig, mouse, and the human. The primers were designed to span the junction of two exons to be RNA specific and were synthesized by (Creative Labor KFT). Ap15 5 - gcagtccgtc tcctccaaac agag-3 and Ap16 5 - catgtcctgt agtgacccct gcag-3 for amplification of 328 bp fragment of Ob cDNA, Ap20 5 - ttgagaagta ccagttcagt c-3 and Ap21 5 - caaagaatgt ccgttctctt c-3 for amplification of 280 bp of Ob-Ra and Ap22 5 - agggttctat ttgtattagt gacc-3 and Ap23 5 - gaaatttccc tcaagtttca aaag - 3 for amplification of 353 bp of Ob-Rb.

Oligonucleotide primers for amplification of cow leptin were designed based on the known sequences of the cow leptin reported in the GenBank. Ap1 5 - caggatgacaccaaaaccctcatc -3 and Ap14 5 - ggagtagagtgaggcttccaggac -3 for amplification of 342 bp fragment of Ob cDNA. While the Oligonucleotide primers for amplification of cow leptin receptors were the same to that used in buffalo and one-humped camel.

The reverse transcription reaction was performed using 2 g of total RNA with 200 units of MMLV reverse transcriptase (Promega), 1 g Oligo(dT)15 and 10 mM dNTPs for 60 minutes at 42 C in a final volume of 25 l. 2 l of RT reaction product was amplified by PCR in final volume of 30 l with 200 M 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 minutes, followed by 35 cycles of denaturing at 94 C for 50 seconds, annealing at 56 C (Ob and Ob-Rb) or 54 C (Ob-Ra) for 40 seconds, and extension at 72 C for 1 minute. The program was terminated with a final extension step at 72 C for 5 minutes.

PCR products were detected by electrophoreses on 1% agarose gel containing 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. Based on the expected size and southern blot verification the PCR products were isolated from the gel using Qiaquick gel extraction kit (Qiagen), then cloned into PGEM-T vector (Promega) and used

to transform Escherichia coli cells. Plasmids from resultant cultures were isolated with QiAprep Miniprep kit (Qiagen) and the cDNA inserts were then sequenced by (Genotype GmbH Germany). The entire sequences of buffalo and one-humped camel were reported to Gen Bank. The accession numbers of buffalo are AY177609, AY177610, and AY177611 and for camel are AY247404, AY247405, and AY247406.

Southern Blot

The specificity of the amplified products of the Egyptian water buffalo were confirmed by Southern blot analysis using cow DIG labeled cDNA probe for Ob and Ob-Rb and human DIG labeled cDNA probe for Ob-Ra. Latter on the partial sequenced clones of the buffalo were used to prepare DIG labeled cDNA probes to examine the specificity of the amplified products of one-humped camel and cow.

After electrophoreses run, the gels were equilibrated in 0.4 M NaOH for 20 minutes, then transferred by 0.4 M NaOH for 5 hours onto nylon membranes. The blots is cross-linked to the nylon membranes by 5 minutes exposure to UV then washed with distilled water for 20 minutes. The DIG labeled cDNA probes was prepared by PCR, in which the specific fragments were amplified by PCR as mentioned above and then purified from the gel. Purified fragments were digoxigeninized by linear PCR (200 M dATP, dCTP, dGTP, 134 M dTTP and 66 M DIG-dUTP) using the antisense primers only.

The hybridization buffer contains 50% formamide, 5X SSPE pH 7.4, 5X Dernhardt, 100 g salmon sperm DNA, and 0.1% SDS. The blots were prehybridized at 42 C for 3 hours then hybridized at 42 C overnight with 25 ng probe per milliliter of hybridization buffer. The blots were washed with 2X SCC containing 0.1% SDS 3 times at room temperatures for 20 minutes, then 2 times by 0.5 X SSC containing o.1 SDS at 50 C. Detection of hybridization signals was achieved using DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions.

Competitive PCR

A competitive PCR procedure according to (Tu et al., 1997) was used to compare the amount of mammary tissue leptin mRNA between lactating and dry cows. Our clone for cow leptin partial cDNA (ap1+ap14 fragment) 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 (ap1+ap14 fragment) was digested with the restriction enzyme Eco01091 (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 l PCR reaction contains 3 l of RT reaction and 1 l of 10-fold dilutions of the internal competitor with the leptin specific primer (ap1+ap14). The starting concentration of the competitor was 70 pg/ l. 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 10-fold dilution of internal competitor a narrower range (2X dilution) of internal competitor concentration was used in new PCR reactions. PCR products were detected by electrophoresis on 1% agarose gel containing 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 bands intensity with different competitor dilutions.

In situ Hybridization

Paraffin embedded tissues were used to localize the mRNA transcripts for Ob, Ob-Ra, and Ob-Rb genes within mammary epithelial cells of lactating Egyptian water buffalo, dry, lactating non pregnant cows, dry one-humped female camel as well as the liver tissue of the one-humped camel using DIG labeled RNA probes. The procedures were used according to the current protocols in molecular biology (Knoll and Lichter, 1995) and the non-radioactive *in situ* hybridization application manual (Roche). The RNA probes were generated by in vitro transcription of a linearized plasmid with NcoI or NotI (BioLabs Inc) for transcription with T7 or SP6 RNA polymerases to generate antisense and sense probes using DIG RNA Labeling Kit (Roche). Deparaffinized and hydrated sections were treated by 200 l of 200 mM HCl for 10 minutes to denature the proteins then washed with DEPC water for 10 minutes. The sections were permeabilized for 30 minutes with DEPC treated PBS containing 10 g/ml RNase free Proteinase K (Boehringer M.), followed by post-fixation for 10 minutes at 4 C with DEPC treated PBS containing 4 % paraformaldehyde.

To reduce the non-specific binding of probes to positively charged amino acids; the slides were placed in freshly mixed 0.25 % acetic anhydride in 0.1-M triethanolamine HCl, pH 8 for 10 minutes at room temperature. Finally the sections were washed with DEPC water and overlaid with 30 l of hybridization buffer containing 10 ng of DIG labeled RNA probe and covered by 18 x 18 coverslip.

The hybridization buffer consists of 50 % formamide, 0.3 M NaCl, 10 mM Tris / HCl (PH 8), 1 mM EDTA, 5 X Denhardt, 500 l/ml yeast tRNA (Invitrogen life technologies), 10 % PEG (MW 600, Fluka), 10 mM Vanadyl-Ribonucleoside complex (BioLabs Inc) and 100 g/ml Salmon testis DNA (Sigma) which was denatured at 99 C for 10 minutes before being added to the hybridization buffer. The slides were placed on hot plate at 94 C for 4 minutes then incubated at 45 C for 20 hours.

Post-hybridization slide processing included 2 X 10 minutes wash with 2 X SSC at room temperature, followed by a 30 minutes incubation with 20 g / ml RNase A in 2 X SSC at 37 C. The slides were then subjected to 1 X SSC for 20 minutes at 37 C and 2 X 15 minutes washes with 0.1 X SSC at 46 C. Detection of hybridization was achieved using DIG Nucleic Acid Detection Kit (Roche). Briefly, the sections were immersed for 30 minutes with blocking solution and then each section was covered by 75 l of 1:100 diluted Anti-Digoxigenin-AP in blocking solution for 1 hour at room temperature, then 2 X 15 minutes washes with washing buffer. Each slide was equilibrated 4 minutes in detection buffer and then covered by 200 l of freshly prepared color substrate for color development. The reaction was stopped by washing the section with TE buffer and finally air-dried and mounted by Entellan (Merck) and photographed by light microscopy.

Immunohistochemistry

We used immunohistochemistry procedures to localize the leptin protein in the mammary epithelial cells of the dry and lactating non pregnant cows as well as the mammary epithelial cell and liver tissue of the one-humped camel. Paraffin sections were used for immunohistochemistry using polyclonal rabbit anti-bovine leptin. Deparaffinized and hydrated sections were incubated in chloroform for 15 minutes to extract the lipid membrane components followed by several time washing by distilled water. After inactivation of endogenous peroxidases the sections were placed in 0.01 mol/l citrate buffer

(pH 6) and heated in microwave for 10 minutes. The sections were blocked by PBS containing 5% bovine serum albumin for an hour, and then incubated with rabbit anti-bovine leptin (2000X dilution in 1% BSA) at 4 C overnight and for an hour at room temperature. The sections were washed with PBS for 5 minutes and incubated with biotinylated goat anti-rabbit IgG for 30 minutes at room temperature. The secondary antibody was detected with Vectastain ABC kit (Vector Laboratories Inc.) and the color was developed using DAB (Sigma). Finally the sections were counter-stained by hematoxylin, washed with distilled water, air-dried, mounted by Entellan (Merck), and photographed by light microscopy.

I. PARTIAL CLONING AND LOCALIZATION OF LEPTIN AND LEPTIN RECEPTOR IN THE MAMMARY GLAND OF THE EGYPTIAN WATER BUFFALO

Introduction

Leptin is transferred from maternal circulation to the milk, then to the stomach of the suckling pup, and afterwards to the neonatal circulation (Casabiell et al; 1997). The immunoreactive leptin is present in both whole and skim milk (Smith-Kirwin et al., 1998; Estienne et al., 2000). The level of leptin in either whole or skim milk are higher than levels occurring in the circulation, reflecting a local leptin production by the sow mammary glands (Estienne et al., 2000). 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). RT-PCR indicate the presence of leptin mRNA in the mammary gland tissue without indicating the type of the cell in which leptin was present (Smith-Kirwin et al., 1998; Aoki et al., 1999; and Smith and Sheffield, 2002; Bonnet et al., 2002b). However, ovine mammary leptin expression within the mammary tissues changes with the reproductive cycle. First it was located 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., 2002b).

So far, there is no information about the source of the local leptin production in the mammary gland of lactating large ruminants. In the present study, Egyptian water buffalo was selected to examine the leptin gene and its receptor expression in the mammary gland. cDNA cloning is necessary to indicate the expression of leptin and its receptor in buffalo mammary tissue. To examine whether buffalo leptin is also expressed in the epithelial cell or myoepithelial cell of lactating mammary gland, paraffin embedded sections of lactating mammary gland samples were subjected to *in situ* hybridization procedure in order to visualize leptin mRNA transcripts and thus to identify the cell types in which leptin is present.

Results

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

RT-PCR analysis of total RNA from adipose and mammary gland tissue of the Egyptian water buffalo revealed the presence of leptin mRNA in these tissues. In addition, both the Ob-Ra and Ob-Rb mRNA are expressed in the mammary gland (Fig.4. A). Southern blot hybridization of RT-PCR products revealed the specificity of the amplified products, which hybridized with specific cow DIG labeled cDNA probes for Ob and Ob-Rb, and human DIG labeled cDNA probe for Ob-Ra (Fig.4. B).

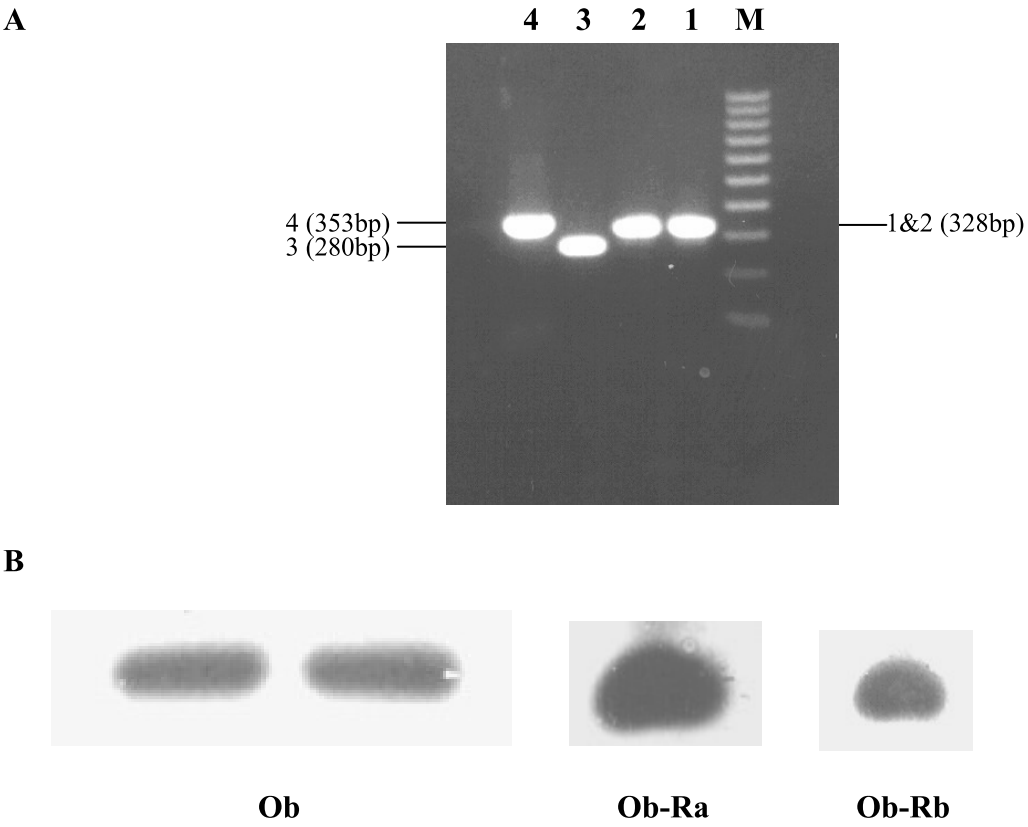

Fig. 4. RT-PCR of leptin and leptin receptors. Panel **A**. Lanes 1 and 2: RT-PCR detection of Ob mRNA in the adipose tissue and mammary gland tissue of buffalo. Lanes 3 and 4: detection of Ob-Ra and Ob-Rb mRNA in the mammary gland tissue, respectively. M is a 100 bp molecular ladder. Panel **B.** Southern blot analysis of RT-PCR products using specific DIG labeled cDNA probes indicating the specificity of amplified fragments of leptin in adipose tissue and mammary gland and both short and long isoforms of leptin receptor in mammary gland tissue of buffalo.

Subsequent partial sequencing of buffalo cDNAs for leptin and both isoforms of the leptin receptor revealed high percentage of nucleotide and amino acid similarity with partial sequences of cow, sheep, pig, human, and mouse. The results of similarity were summarized in Table 1 and Figures 5, 6, and 7. We compared the nucleotide and amino acid sequences of the species available in the GenBank by GCG PILEUP program and prepared the consensus sequence for each gene and protein using GCG PRETTY program.

A- Ob (328 bp.GenBank accession no. AY177609)

Accession No	species	nucleotides identity %	amino acids identity %	amino acids similarity %
AJ132764	Cow	99	99	99
U84247	Sheep	96	97	98
U59894	Pig	92	92	97
U18915	Human	86	83	90

B- Ob-Ra (277 bp.GenBank accession no. AY177611)

Accession No	species	nucleotides identity %	amino acids identity %	amino acids similarity %
Yanagihara et al; 2000	Cow	97	96	99
AF092422	Pig	90	90	94
U66495	Human	90	91	94
U58863	Mouse	83	83	89

C- Ob-Rb (353 bp.GenBank accession no. AY177610)

Accession No	species	nucleotides identity %	amino acids identity %	amino acids similarity %
U62385	Cow	100	100	100
U62124	Sheep	94	92	96
AF036908	Pig	86	78	88
U66497	Human	84	79	84

Table I. Nucleotide and Amino Acid Homology of Buffalo Ob, Ob-Ra, and Ob-Rb with other sequences reported in GenBank

```
          CAGTCCGTCTCCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCTCTCCTGAGTTTGTCC
buffalo   Q  S  V  S  S  K  Q  R  V  T  G  L  D  F  I  P  G  L  H  P  L  L  S  L  S
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  V  -  -  -  -
human     -  -  -  -  -  -  -  K  -  -  -  -  -  -  -  -  -  -  -  -  I  -  T  -  -
consen    Q  S  V  S  S  K  Q  R  V  T  G  L  D  F  I  P  G  L  H  P  L  L  S  L  S

          AAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGTCTGCCTTCCAGAAATGTGGTCCAAATATCC
buffalo   K  M  D  Q  T  L  A  I  Y  Q  Q  I  L  T  S  L  P  S  R  N  V  v  Q  I  S
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  V  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
human     -  -  -  -  -  -  -  V  -  -  -  -  -  -  -  M  -  -  -  -  -  -  -  -  -
consen    K  M  D  Q  T  L  A  I  Y  Q  Q  I  L  T  S  L  P  S  R  N  V  I  Q  I  S

          AATGACCTAGAGAACCTCCGGGACCTTCTCCACCTGCTGGCCGCCTCCAAGAGCTGCCCCTTGCCGCAGGTCAGG
buffalo   N  D  L  E  N  L  R  D  L  L  H  L  L  A  A  S  K  S  C  P  L  P  Q  V  R
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  S  -  -  -  -  -  -  -  A  -
human     -  -  -  -  -  -  -  -  -  -  -  V  -  -  F  -  -  -  -  H  -  W  A  S  G
consen    N  D  L  E  N  L  R  D  L  L  H  L  L  A  A  S  K  S  C  P  L  P  Q  V  R

          GCCCTGGAGAGCTTGAAGAGCTTGGGCGTTGTCCTGGAAGCTTCCCTCTACTCCACCGAGGTGGTGGCCCTGAGC
buffalo   A  L  E  S  L  k  S  L  G  V  V  L  E  A  S  L  Y  S  T  E  V  V  A  L  S
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  T  -  -  -  -  -  G  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
human     -  -  -  T  -  D  -  -  -  G  -  -  -  -  -  G  -  -  -  -  -  -  -  -  -
consen    A  L  E  S  L  E  S  L  G  V  V  L  E  A  S  L  Y  S  T  E  V  V  A  L  S

          CGGCTGCAGGGGTCACTACAGGACATG
buffalo   R  L  Q  G  S  L  Q  D  M
cow       -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -
pig       -  -  -  -  A  -  -  -  -
human     -  -  -  -  -  -  -  -  -
consen    R  L  Q  G  S  L  Q  D  M
```

Fig. 5. Partial sequence of buffalo Ob cDNA and its deduced amino acid sequence. Buffalo deduced amino acid sequence was compared with those of cow, sheep, pig, human, and the consensus sequence.

Cellular localization of leptin, and its receptors in mammary tissues

We used *in situ* hybridization analysis to localize leptin and its receptor isoforms in paraffin embedded mammary gland sections of lactating buffalo using antisense riboprobes. *In situ* hybridization signals localizing the mRNA transcripts of leptin and its receptor isoforms were observed within the secretory alveolar epithelial cells of the acini. We could not demonstrate any clear signals in cells surrounding the secretory acini, in other words we could not localize mRNA transcripts of leptin and its receptors within the myoepithelial cells, either. On the other hand, due to complete regressed mammary adipose tissue during lactation, we could not observe clear signals for leptin mRNA transcripts within the connective tissue between acini (picture A, B, and C on Figure 8). The specificity of

hybridization was demonstrated by using the sense riboprobes for each gene as negative controls (picture D, E, and F on Figure 8).

Although the detection of mRNA by *in situ* hybridization is only semiquantitative, a difference existing in the intensity of signals for Ob-Ra and Ob-Rb after two times repeat in two different samples suggests that the Ob-Ra is the major isoform in the mammary gland.

```
          GAGAAGTACCAGTTCAGTCTTTACCCAGTATTTACGGAAGGAGTAGGGAAACCGAAGATAATTAATAGTTTTACT
buffalo   E  K  Y  Q  F  S  L  Y  P  v  F  t  E  G  V  G  K  P  K  I  I  N  S  F  T
cow       -  -  -  -  -  -  -  -  -  -  -  T  -  -  -  -  -  -  -  -  -  -  -  -  A
pig       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
human     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
mouse     -  -  -  -  -  -  -  -  -  V  -  -  -  -  -  -  -  -  -  -  -  -  G  -  -
consens   E  K  Y  Q  F  S  L  Y  P  i  F  M  E  G  V  G  K  P  K  I  I  N  S  F  T

          CAAGATGA...TGAAAAACACCAGCATGATGCAGATCTGTATGTAATCGTGCCAATAATAATCTCCTCCTCAATC
buffalo   Q  D  D  .  E  K  H  Q  h  D  A  d  L  Y  V  I  V  P  I  I  I  S  S  S  I
cow       -  -  -  .  -  -  -  -  H  -  -  D  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  G  .  -  -  -  R  N  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
human     -  -  -  -  -  -  -  -  S  -  -  -  -  -  -  -  -  -  V  -  -  -  -  -  -
mouse     K  -  A  -  D  -  Q  -  N  -  -  -  -  -  -  -  -  -  -  -  -  -  C  V
consens   Q  D  D  I  E  K  H  Q  ?  D  A  G  L  Y  V  I  V  P  I  I  I  S  S  S  I

          TTATTGCTTGGAACATTGTCAGTATCACATCAAAGAATGAAAAAGTTGTTTTGGGAAGATGTTCCAAACCCCAAG
buffalo   L  L  L  G  T  L  s  v  S  H  Q  R  M  K  K  L  F  W  E  D  V  P  N  P  K
cow       -  -  -  -  I  -  S  V  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  -  -  -  -  M  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
human     -  -  -  -  -  -  -  I  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
mouse     -  -  -  -  -  -  -  I  -  -  -  -  -  -  -  -  -  -  D  -  -  -  -  -  -
consens   L  L  L  G  T  L  L  ?  S  H  Q  R  M  K  K  L  F  W  E  D  V  P  N  P  K

          AACTGTTCCTGGGCACAAGGACTTAATTTTCAGAAGAGAACGGACATTCTT
buffalo   N  C  S  W  A  Q  G  L  N  F  Q  K  R  T  D  I  L
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  -  -  -  -  -  -  -  -  -  -  .  .  .  .  .
human     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
mouse     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
consens   N  C  S  W  A  Q  G  L  N  F  Q  K  R  T  D  I  L
```

Fig. 6. Partial sequence of buffalo Ob-Ra cDNA and its deduced amino acid sequence. Buffalo deduced amino acid sequence was compared with those of cow, pig human, mouse and the consensus sequence.

Discussion

With the present study we were the first to report about leptin and its receptors in the Egyptian water buffalo. Leptin can be in focus of interest in water buffalo because nutritional and metabolic disorders are major problems in this species.

```
          GGTTCTATTTGTATTAGTGACCAATGCAGCAGTGCTCAATTCTCTGAGGCTGAGAGCACAGACATAACCTGTGAG
buffalo   G  S  I  C  I  S  D  Q  C  S  S  A  Q  F  S  E  A  E  S  T  D  I  T  C  E
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  E  -  -  -  -  -  -  -  -  -  -  -  -
pig       .  -  -  -  -  -  -  -  R  -  -  -  H  -  -  -  -  -  -  M  E  -  -  -  -
human     -  -  V  -  -  -  -  -  F  N  -  V  N  -  -  -  -  -  G  -  E  V  -  Y  -
consens   G  S  I  C  I  S  D  Q  C  S  S  A  Q  F  S  E  A  E  S  T  D  I  T  C  E

          GATGAGAGCAGGAGACAGCCCTCTGTTAAATATGCCACCCTGCTCAGCAACTCTAAATCAGGTGAAACTGAGGAG
buffalo   D  E  S  R  R  Q  P  S  V  K  Y  A  T  L  L  S  N  S  K  S  G  E  T  E  E
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  G  -  -  -  -  -  -  -  -  -  -  -  -  V  G  S  -  -  -  -  -  A  -  -
pig       -  -  N  -  -  -  -  -  I  -  -  -  -  -  -  -  S  P  -  -  -  -  -  -  Q
human     -  -  -  Q  -  -  -  F  -  -  -  -  -  -  I  -  -  -  -  P  S  -  -  G  -
consens   D  E  S  R  R  Q  P  S  V  K  Y  A  T  L  L  S  N  S  K  S  G  E  T  E  E

          GAGCAAGGACTTATAAATAGCTCAGTCAGCAAATGCTTTTTGAGCAACAATTCTCCACCAAAGGATTCTTCCTCT
buffalo   E  Q  G  L  I  N  S  S  V  S  K  C  F  L  S  N  N  S  P  P  K  D  S  S  S
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  H  -  -  -  -  E  -  -  -
pig       -  -  E  -  V  S  -  L  -  -  R  -  -  S  -  S  -  -  L  -  -  E  -  F  -
human     -  -  -  -  -  -  -  -  -  -  T  -  -  -  S  -  K  -  -  -  L  -  -  -  F  -
consens   E  Q  G  L  I  N  S  S  V  S  K  C  F  L  S  N  N  S  P  P  K  D  S  S  S

          AAGAGATCATGGGAAATAGAAACCCAGGCATTTTTTATATTGTCAGATCAACATCCCAATATAATTTCACCACAC
buffalo   K  R  S  W  E  I  E  T  Q  A  F  F  I  L  S  D  Q  H  P  N  I  I  S  P  H
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       N  S  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  M  -  -  -  -
human     N  S  -  -  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
consens   K  R  S  W  E  I  E  T  Q  A  F  F  I  L  S  D  Q  H  P  N  I  I  S  P  H

          CTTCCGTTCTCAGAAGGCCTGGATGAACTTTTGAAACTTGAGGGAAATTTC
buffalo   L  P  F  S  E  G  L  D  E  L  L  K  L  E  G  N  F
cow       -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
sheep     -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
pig       -  S  -  -  -  -  -  -  -  -  M  -  F  -  -  -  -
human     -  T  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
consens   L  P  F  S  E  G  L  D  E  L  L  K  L  E  G  N  F
```

Fig. 7. Partial sequence of buffalo Ob-Rb cDNA and its deduced amino acid sequence. Buffalo deduced amino acid sequence was compared with those of cow, sheep, pig, human, and the consensus sequence.

Using *in situ* hybridization technique, we could demonstrate the expression of leptin mRNA in alveolar epithelial cells of the lactating mammary gland. Therefore, alveolar epithelial cell might be an additional source of milk leptin, which is in agreement with the result reported in human, using immunohistochemical staining of breast tissue as well as RT-PCR analysis of total RNA from mammary gland (Smith-Kirwin et al., 1998). In the sheep, just before parturition, leptin is present in mammary epithelial cells, whereas during lactation leptin is located in myoepithelial cells as determined by immunohistochemical analysis (Bonnet et al., 2002b). RT-PCR indicates the presence of leptin mRNA in cultured bovine mammary epithelial cells as well as in the mammary tissue. In the latter study (Smith and Sheffield, 2002), however, the cell type specification was not confirmed for the leptin producing cells.

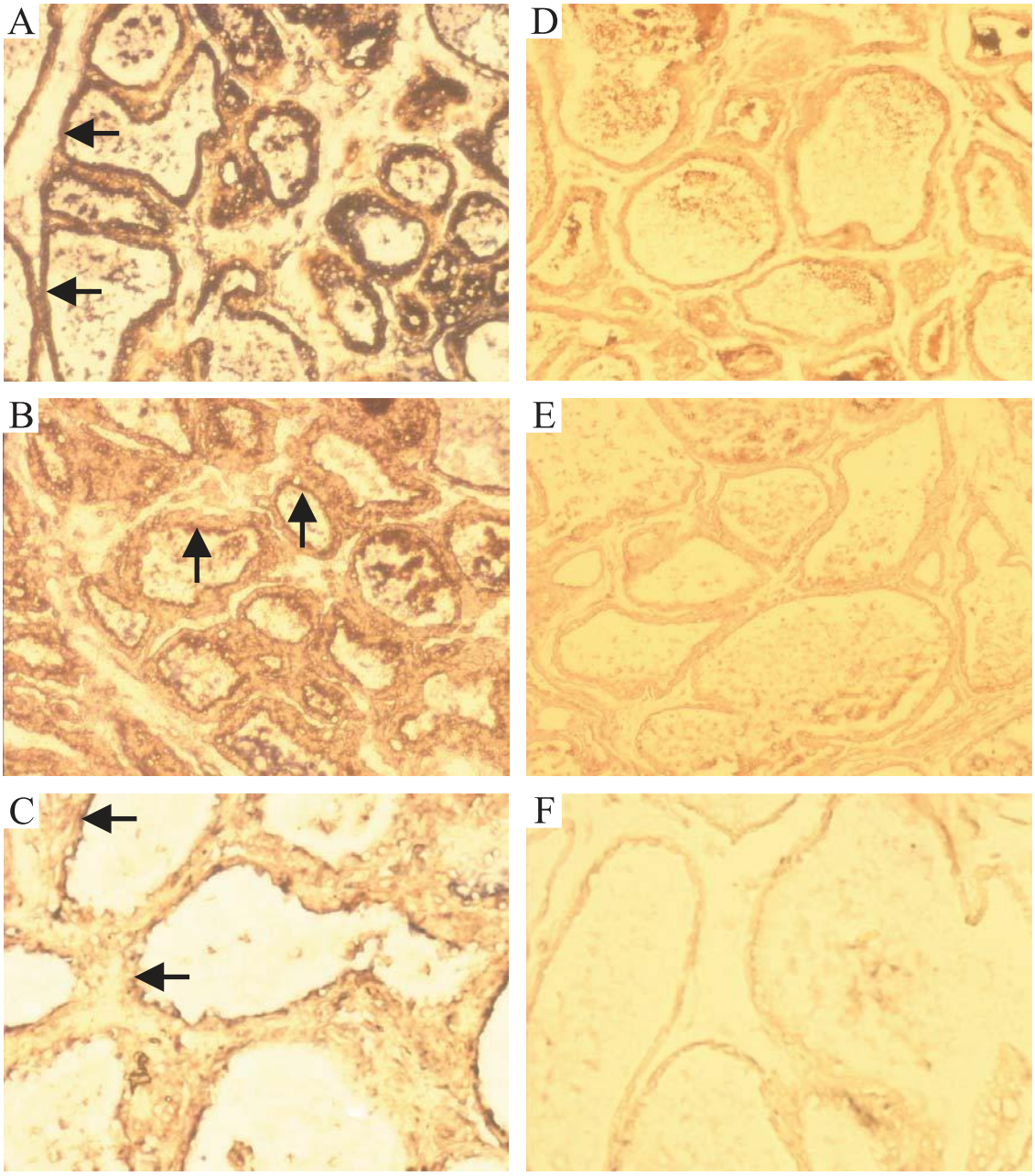

Fig. 8. Light microscopy photomicrographs represent *in situ* hybridization analysis of leptin and its short and long isoforms of receptor within mammary gland tissue of lactating buffalo. **A**, **B**, **D**, and **E** 500X, while **C** and **F** 1000 X magnification, due to low intensity of signals**. A, B,** and **C** photomicrographs show hybridization with the antisense probes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located in the secretory epithelial cells of the acini (black arrows). Photomicrographs **D, E,** and **F** are negative controls and were hybridized with the sense probes for Ob, OB-Ra, and Ob-Rb mRNA, respectively.

We have demonstrated that the long and short isoforms of leptin receptors are expressed in the mammary tissues of buffalo using RT-PCR. Furthermore we could localize both isoforms in the alveolar epithelial cells using *in situ* hybridization procedure. This result is in agreement with the result reported in sheep (Laud et al., 1999). Although the *in situ* hybridization is a semiquantitative procedure our data suggest that, the short isoform of leptin receptor Ob-Ra is the major isoform in the mammary gland tissue of the buffalo. This observation also coincide other reports (Mercer et al., 1996; Laud et al., 1999; Ingvartsen and Boisclair, 2001; and Sweeney, 2002).

Our partial cDNA clones of buffalo leptin and the two examined leptin receptor isoforms revealed a high percentage of nucleotide and amino acid similarity to the previously published sequences of other mammalian species especially to that of other ruminants. Our sequences are available in the GenBank.

The maternal leptin appears in the blood circulation of the newborn (Casabiell et al; 1997). Moreover the present results in buffalo, the detection of the leptin expression in the human mammary epithelial cells (Smith-Kirwin et al., 1998), and the detection of leptin protein in the myoepithelial cells of lactating sheep mammary gland (Bonnet et al., 2002) confirm the local production of leptin within the lactating mammary gland.

According to data in the literature (Bonnet et al., 2002a&b) leptin is produced exclusively by myoepithelial cells of the mammary gland of sheep after parturition, thus leptin could also act as paracrine factor on mammary cell growth and differentiation. Whereas the milk leptin, especially at the late stages of lactation, is not only transported from the maternal blood stream, but also locally produced and secreted by the mammary epithelial cells (Aoki et al., 1999). 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). These reports and our observations about leptin and its receptor expression in the mammary alveolar epithelial cells suggest that leptin could act also as an autocrine and paracrine mediator of mammary metabolism and might promote to maintain the alveolar epithelial activity during lactation.

Leptin is demonstrated in the milk of the rat (Casabiell et al., 1997), human (Smith-Kirwn et al., 1998), porcine (Estienne et al., 2000), and bovine (Smith and Sheffield, 2002). In addition mRNA encoding the long Ob-Rb and short Ob-Ra receptor isoforms are detected

in the gastric mucosa (Goiot et al., 2001) as well as the absorptive intestinal cells (Barrenetxe et al., 2002), supporting the hypothesis that leptin hormone is involved in regulation of gastrointestinal function (Barrenetxe et al., 2002). Leptin induces the proliferation of gastric mucosal cells and helps to maintain mucosal integrity and gastroprotection (Scheider et al., 2001). Our data about leptin secretion in mammary epithelial cells and the previous observations showing that leptin is secreted into the colostrum and milk suggest that there is a possible role of leptin in regulating neonatal appetite and metabolism, although this role has been debated (Ucar et al., 2000).

II. EXPRESSION AND LOCALIZATION OF LEPTIN AND LEPTIN RECEPTOR IN THE MAMMARY GLAND OF THE DRY AND LACTATING NON-PREGNANT COW

Introduction

Leptin appears to be produced in secretory epithelial cell of the active human mammary tissue (Smith-Kirwin et al., 1998) and in ovine mammary epithelial cells just before parturition in addition to myoepithelial cell after parturition (Bonnet et al., 2002b). The lactation induced down regulation of mammary leptin gene expression in rat (Aoki et al., 1999). In sheep the mammary leptin mRNA levels are high at the beginning and at the end of pregnancy while they are low at mid-pregnancy and through lactation (Bonnet et al., 2002b). Leptin appears to be able to control the proliferation of both the normal and the malignant mammary epithelial cells (Hu et al., 2002).

Leptin not only function as an adipostat to signal the status of body energy stores to the brain but also function as a sensor of energy balance (Houseknecht et al., 1998). In cows leptin concentration reflects the state of energy balance during lactation. The plasma leptin concentration are lower in the lactating cows with a mean negative energy balance during early lactation than those cows having a positive energy balance (Liefers et al., 2003).

The leptin expression is mainly related to the state of the energy balance of the animals. Since pregnancy induces large changes in the female metabolism leptin hormone economy might be interesting around parturition. In the present study dry and lactating non-pregnant cows showing a good health condition were selected to examine the leptin and its receptor expression in the mammary gland. In addition to that we decided to study the effect of lactation on the mammary leptin level using competitive RT-PCR. In our experiments the effects of pregnancy or negative energy balance were excluded. Paraffin embedded mammary gland sections from dry and lactating cows were subjected to *in situ* hybridization procedure for leptin and leptin receptor mRNA transcripts and to immunohistochemical staining for leptin protein, in order to specify the cell types in which leptin and its receptors were present.

Results

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

RT-PCR analysis of total RNA revealed the expression of leptin gene and both of the short and long isoforms of leptin receptor in the dry quiescent and lactating mammary gland tissues of the non-pregnant cows (Fig. 9. A). Southern blot hybridization of RT-PCR products revealed the specificity of the amplified products, which hybridized with specific water buffalo DIG labeled cDNA probes for Ob, Ob-Ra, and Ob-Rb (Fig. 9. B). RT-PCR analysis revealed also the expression of Ob-Ra and Ob-Rb in the white adipose tissue of the cow (Fig. 9. C).

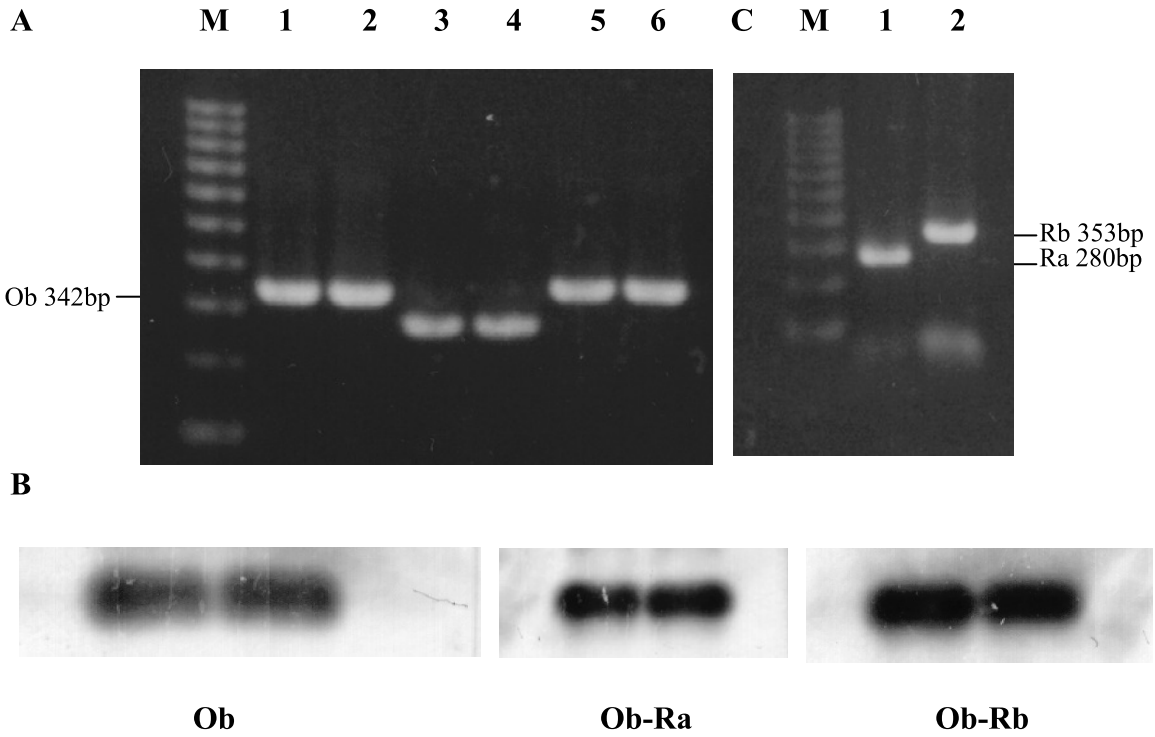

Fig. 9. RT-PCR of leptin and leptin receptors. Panel **A**. Lanes 1 and 2: RT-PCR detection of Ob mRNA, lanes 3 and 4: detection of Ob-Ra mRNA, and lanes 5 and 6 detection of Ob-Rb mRNA in the dry and lactating mammary gland tissue of non-pregnant cows. M is a 100 bp molecular ladder. Panel **B.** Southern blot analysis of RT-PCR products using specific DIG labeled 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/ l of concentration in the first PCR tube was used to find optimal dilution (Fig. 10. A). Using a narrower range of competitor concentration (2X dilution) beginning from the second tube in the first PCR which contain 7 pg/ l 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 contain 1.75 pg/ l of the internal competitor in both dry and lactating cows (Fig. 10. B).

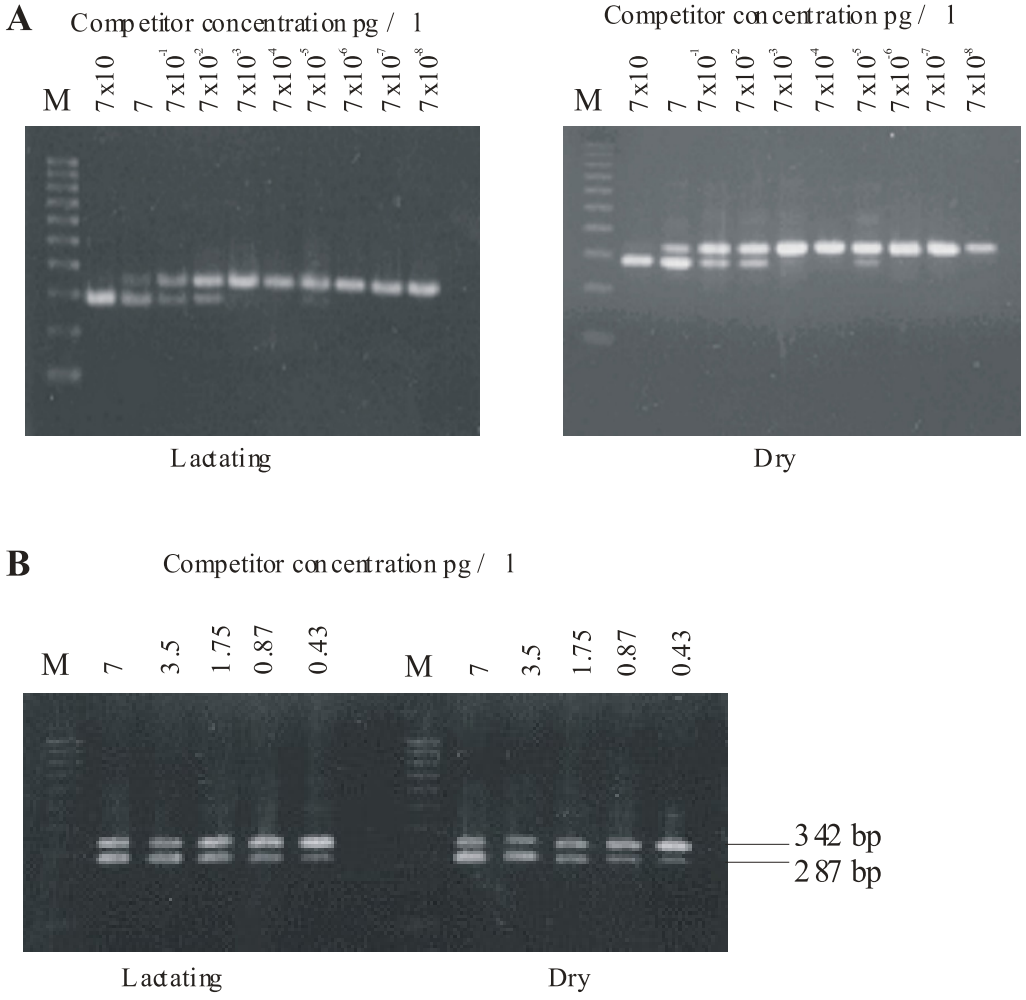

Fig. 10. Competitive PCR of mammary leptin for lactating and dry non-pregnant cows. Panel A revealed the difficulty to compare between lactating and dry mammary gland leptin using 10-fold dilution of cow leptin internal competitor starting with 70 pg/ml of the competitor in the first tube. Panel B revealed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

Cellular localization of leptin, and its receptors in mammary tissues

In situ hybridization signals localizing the mRNA transcripts of leptin and its receptor isoforms were observed within the secretory alveolar epithelial cells of the acini (picture A, B, and C on Figure 11&12). As previously mentioned in the Egyptian water buffalo we could not localize mRNA transcripts of leptin and its receptors within the myoepithelial cells that surrounding the mammary gland acini. Due to complete 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 (picture A, B, and C. Figure 11). In the dry quiescent mammary gland the stromal connective tissue that contains much of fat cells, we could see an *in situ* hybridization signals for leptin and it receptor between acini beside the signals localized in the epithelial cells (picture A, B, and C. Figure 12). To confirm the specificity of signals of Ob-Ra and Ob-Rb between acini of dry gland, an adipose tissue sample from dry animal was used in RT-PCR analysis for both short and long isoforms of leptin receptor. Our results show the expression of both isoforms in the adipose tissue (Fig. 9. C). The specificity of *in situ* hybridization was also demonstrated by using the sense riboprobes for each gene as negative controls (picture D Figure 11&12).

Although the detection of mRNA by *in situ* hybridization is only semiquantitative, a difference in the intensity of signals for Ob, Ob-Ra, and Ob-Rb within the epithelial cell between lactating and dry cow could be demonstrated. Signals in the epithelial cells of lactating mammary gland seem to be higher than that of dry mammary gland.

Immunohistochemistry staining for leptin protein confirmed the result of *in situ* hybridization. The immunostaining was mainly localized in the epithelial cell of the acini in the lactating tissue, while in dry mammary gland it was localized in both the epithelial and stromal cells (Fig. 13. A&B).

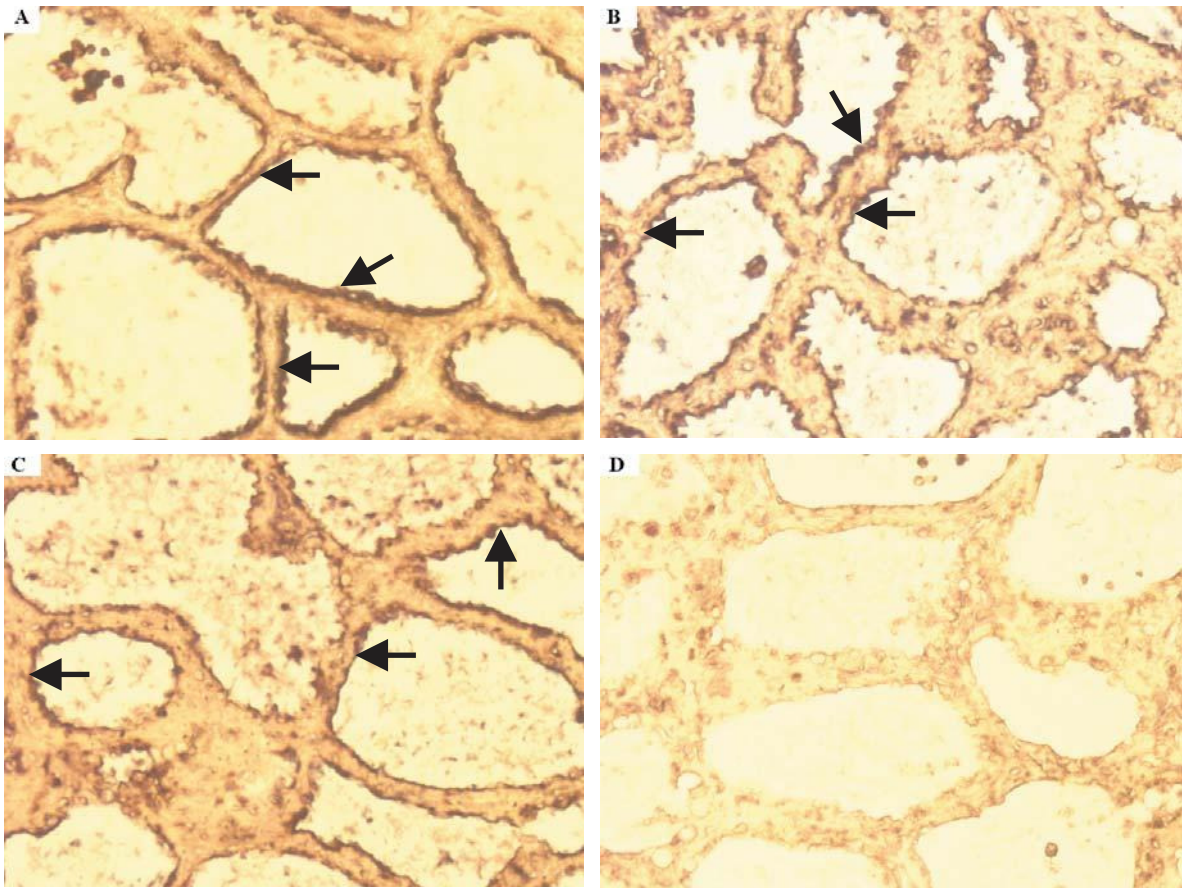

Fig. 11. Light microscopy photomicrograph represent in situ hybridization analysis of leptin (A) and its short (B) and long (C) isoforms of receptor within mammary gland tissue of lactating cow (500X). **A, B,** and **C** photomicrographs show hybridization with antisense probes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located in the secretory epithelial cells of the acini (black arrows). Photomicrograph D is the negative control and is hybridized with the sense riboprobe of leptin. The sense riboprobe for both isoforms of leptin receptor showed the same result of photomicrograph **D.**

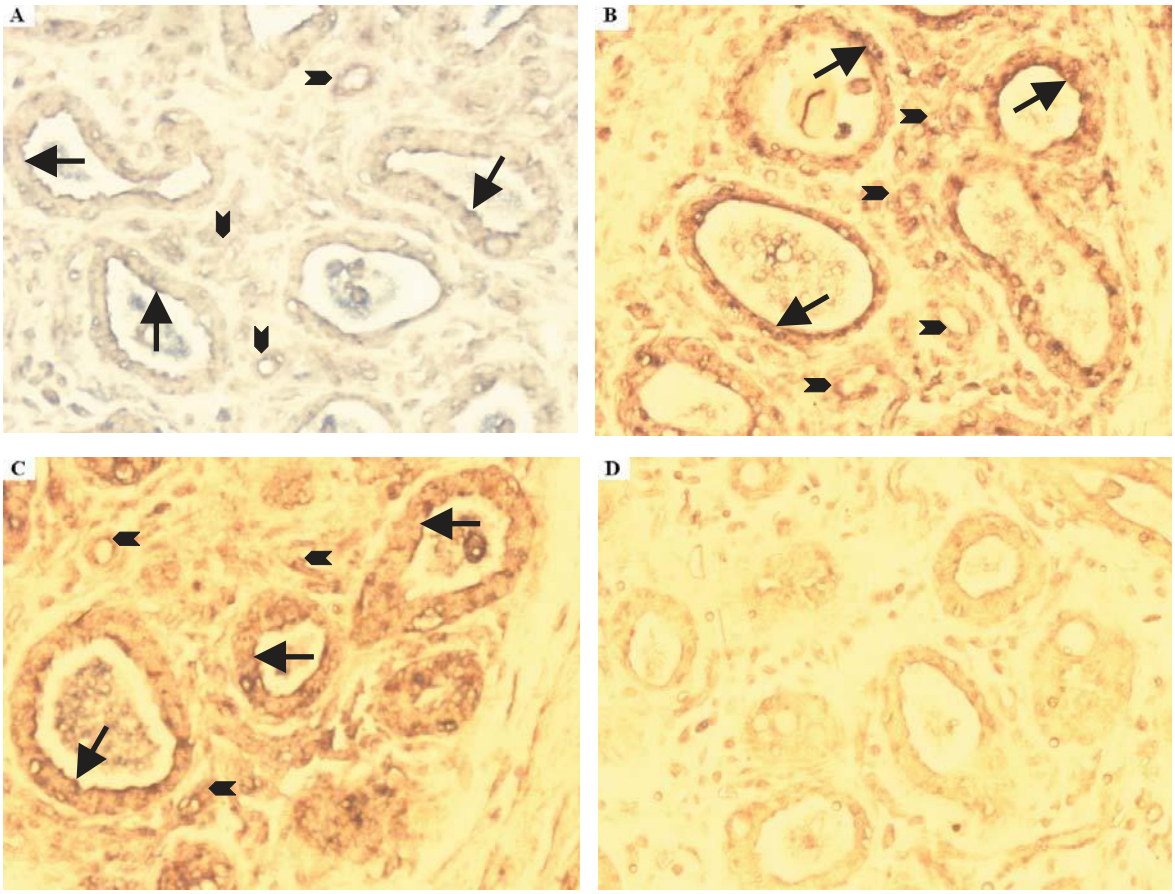

Fig.12. Light photomicrograph represent in situ hybridization analysis of leptin (A) and its short (B) and long (C) isoforms of receptor within mammary gland tissue of dry cow (500X). **A, B, and C** photomicrographs show hybridization with the antisense riboprobes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located mainly in the epithelial cells of the acini (black arrows) in addition to moderate signals in the stromal adipose tissue (arrow's heads) which was tested positive for both leptin receptor using RT-PCR. Photomicrograph **D** is the negative control and is hybridized with the sense riboprobe of leptin. The sense riboprobe for both isoforms of leptin receptor showed the same result of photomicrograph **D.**

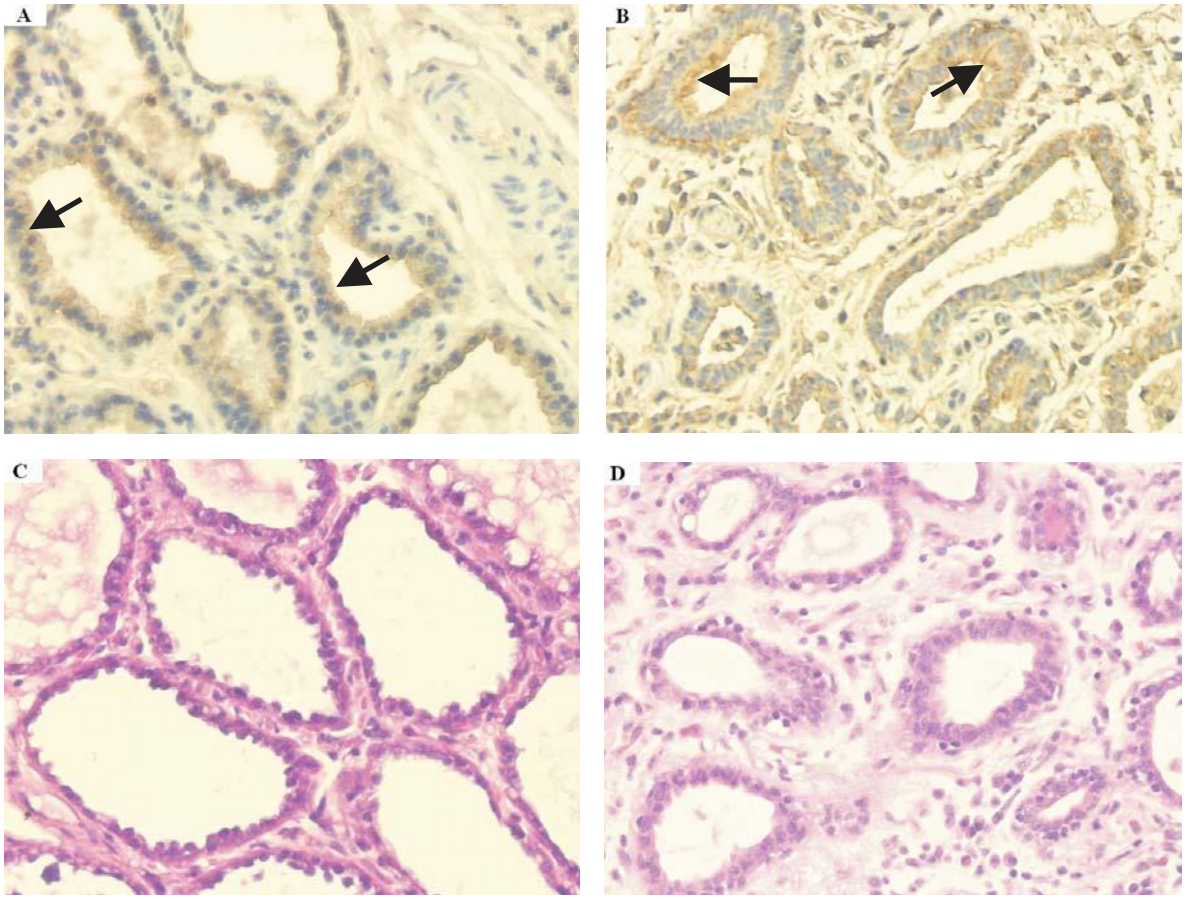

Fig. 13. Immunohistochemistry analysis of lactating (**A**) and dry (**B**) mammary gland leptin. The strong signals are mainly located in the acinar epithelial cell (black arrows). The photomicrographs **C,** and **D** show the lactating and dry mammary tissue stained with H&E, respectively.

Discussion

We are the first to provide information about leptin localization in the mammary tissue of the dry and lactating non-pregnant cows. Using *in situ* hybridization and immunohistochemistry techniques we could demonstrate leptin protein and mRNA in the alveolar epithelial cells in both the dry and the lactating mammary gland. In dry mammary gland stromal adipose cells also give positive signals. 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 cell using RT-PCR (Smith and Sheffield, 2002).

We also examined the expression and localization of the long and short isoform of leptin receptor in the mammary gland in both dry and lactating cow. The results are consistent with the results reported in sheep (Laud et al., 1999), and human breast cancer cells (Laud et al., 2002). Similar results were found in other ruminants. For example in Egyptian water buffalo both the Ob-Ra and the Ob-Rb of leptin receptors expressed in the mammary gland (Sayed-Ahmed et al., 2003). Silva et al. (2002) using RT-PCR reported in prepubertal heifer, however, 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 indicate 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 changes, which reflects in hormonal changes including leptin hormone economy. To exclude the effect of pregnancy we selected the non-pregnant cow to study the alteration of mammary leptin between dry and lactating animal. The competitive PCR could not show clear difference in leptin mRNA levels between dry and lactating mammary gland of non-pregnant cows. This observation is in contrast with the observation reported in mice by Aoki et al. (1999) who mentioned that, throughout lactation the leptin expression in 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., 2002b).

Although *in situ* hybridization 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 dry one. As mentioned earlier leptin expression and secretion reflect the body fat mass (Maffei et al., 1995; Considine and Caro, 1997) and are highly correlated with the adipocyte size (Houseknecht et al., 1998). During lactation, the mammary adipose tissue is completely regressed. Thus, lactating epithelial cells produce the same amount of leptin as the epithelial cell and the fat cells together 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 that 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 increase with body fatness (Ehrhardt et al., 2000), while aqueous extract prepared from mammary parenchymal tissue of prepubertal heifers contain 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 emphasizes the importance of local effects of leptin hormone. Also exogenous leptin is necessary for maintenance of lactation of leptin deficient mice (ob/ob genotype Mounzih et al., 1998). According to this local leptin production might also be important in maintaining lactation. Data in literatures and our present results demonstrate the possible physiological importance of the locally produced leptin in the regulation and maintenance of the mammary epithelial cell activity.

III. PARTIAL CLONING AND LOCALIZATION OF LEPTIN AND LEPTIN RECEPTOR OF THE ONE-HUMPED CAMEL

Introduction

Camels constitute the majority of animal wealth in the desert and play an important role in maintaining human life. The camel adapt to desert environment of torrid heat and extreme desiccation. The efficiency of conversion of metabolizable energy intake to body tissue in the camel was 68 % that figure is higher than most values reported for other ruminants (Filali and Guerouali, 1994). In hot arid areas, the endocrine system particularly thyroid hormone could play a major role in the regulation of energy homeostasis of the desert animals (Wilson, 1989). As widely discussed in the introduction part leptin hormone contributes to the regulation of energy balance and feeding behavior by acting on the central nervous system (Houseknecht et al., 1998; Houseknecht and Portocarrero, 1998; Ahima and Flier, 2000; and Sainsbury et al., 2002), or peripheral tissues such as skeletal muscle, liver, pancreas, and adipose tissue (Muoio and Lynis Dohm, 2002; Dulloo et al., 2002; Baratta, 2002). Additionally, leptin hormone increases thyroid hormone level through activation of TRH or through increases the expression and activity of monodeiodenase type II in brown adipose tissue and indirectly increases the circulating T_3.

To date, numerous data on leptin physiology in various species, including experimental and livestock animals have been accumulated (for reviews, Houseknecht et al., 1998; Ahima and Flier, 2000; Roger and Unger, 2000; Chilliard et al, 2001). However, there is no information about leptin and / or leptin receptor in the camel even though leptin could play an important role in the camel acclimatization through maintenance of the internal homeostasis and regulation of feed intake and energy expenditure in this animal that is exposed to several harsh environmental factors. In this experiment we report partial cDNA cloning of camel leptin (Ob) and both of long (Ob-Rb) and short (Ob-Ra) isoforms of leptin receptors, in addition to localization of leptin and leptin receptors in the mammary gland and liver tissue of the camel.

Results

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

RT-PCR analysis of total RNA from the hump adipose tissue, liver, and mammary gland tissue of the one-humped camel revealed the presence of leptin mRNA transcripts as well as both the Ob-Ra and Ob-Rb mRNA in these tissues (Fig. 14. A). Southern blot hybridization of RT-PCR products revealed the specificity of the amplified products, which hybridized with specific buffalo DIG labeled cDNA probes for Ob, Ob-Ra, and Ob-Rb (Fig. 14. B).

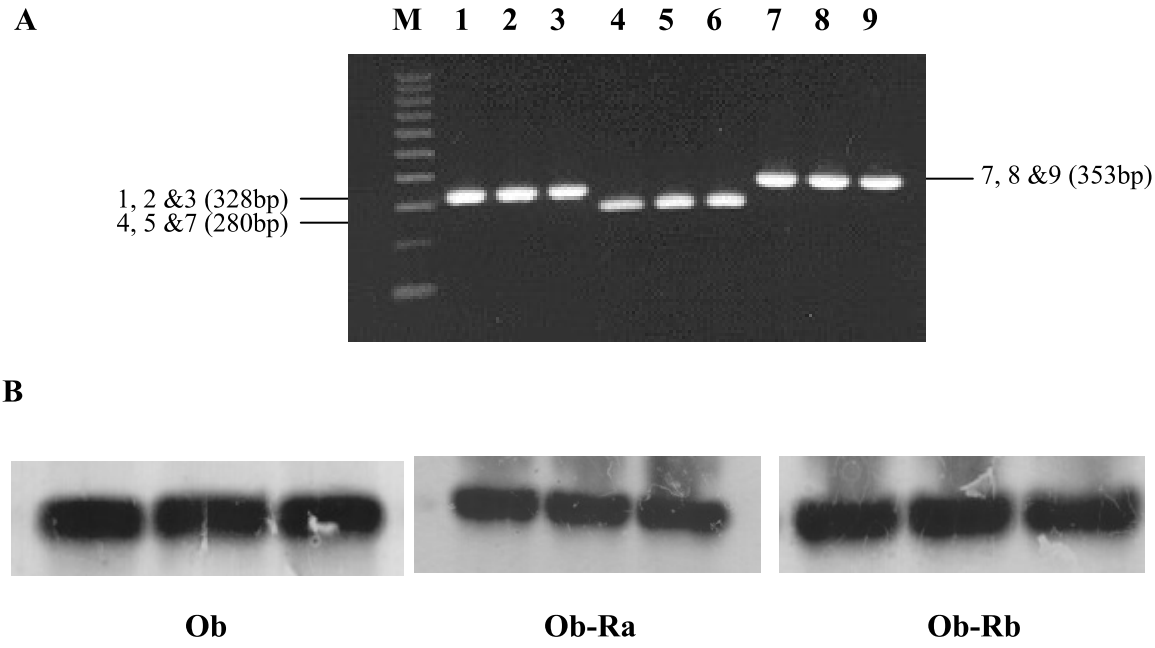

Fig. 14. RT-PCR of leptin and leptin receptors of the one-humped camel. Panel **A**. Lanes 1, 2, and 3: RT-PCR detection of Ob mRNA in the hump adipose tissue, liver, and mammary gland tissues. Lanes 4, 5, and 6 and lanes 7, 8, and 9: detection of Ob-Ra and Ob-Rb mRNA, respectively, in the same tissue. M is a 100 bp molecular ladder. Panel **B.** Southern blot analysis of RT-PCR products using buffalo DIG labeled cDNA probes indicating the specificity of amplified fragments of leptin and both of short and long isoforms of leptin receptor in adipose tissue, liver, and mammary gland of the one-humped camel

Subsequent partial sequencing of one-humped camel cDNAs for leptin and both isoforms of the leptin receptor revealed high percentage of nucleotide and amino acid similarity with partial sequences of cow, Egyptian water buffalo, sheep, pig, human, and mice. We compared and designed the dendrograms for the nucleotide and amino acid sequences of the species available in the GenBank using GCG PILEUP program (Fig. 15).

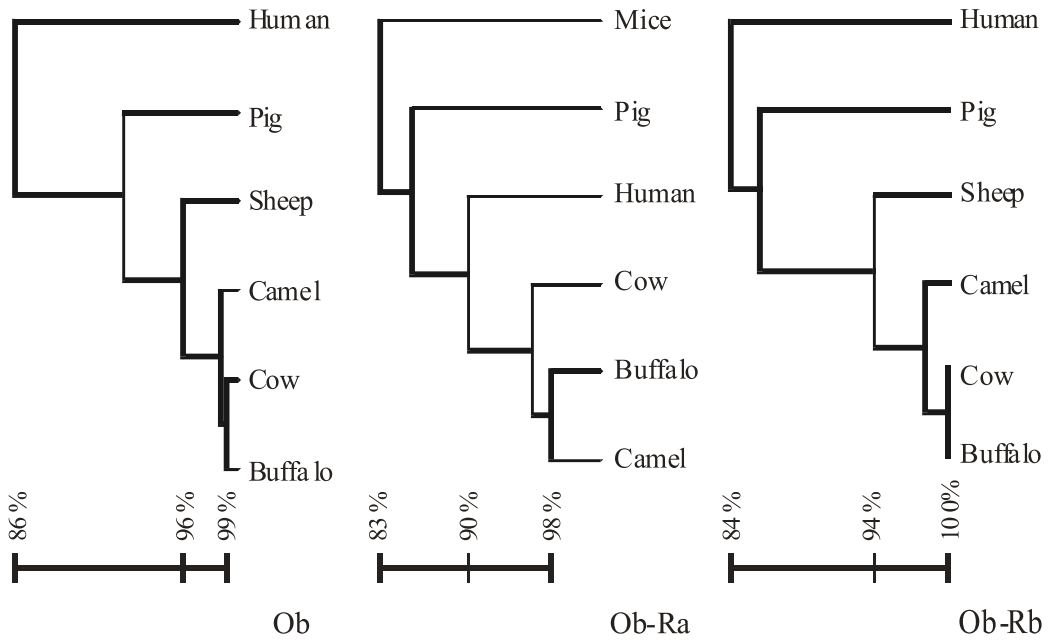

Fig. 15. Dendrograms of the leptin (Ob), short form of leptin receptor (Ob-Ra), and long form of leptin receptor (Ob-Rb) nucleotides showing the multiple sequence alignment between camel sequences and other sequences reported into GenBank for various species.

Cellular localization of leptin, and its receptors in mammary gland and liver tissues

In situ hybridization analysis was used to localize leptin and its receptor isoforms in paraffin embedded mammary gland and liver sections of the one-humped camel using antisense riboprobes. As previously shown in the buffalo and cow the mRNA transcripts of leptin and its receptor isoforms were observed within the epithelial cells of the mammary gland acini. We could not localize mRNA transcripts of leptin and its receptors within the myoepithelial cells (Fig. 16). *In situ* hybridization signal was observed mainly in the liver hepatocyte around the large fat vacuoles that is a normal histological character of camel liver. Low signals were also observed in the lining epithelium of the bile duct (Fig. 17). The specificity of hybridization was demonstrated by using the sense riboprobes for each gene as negative control (D pictures of Fig. 16 & 17).

Immunohistochemistry staining for leptin protein confirmed the result of *in situ* hybridization. The immunostaining was mainly localized in the alveolar epithelial cell of the mammary gland acini, as well as the liver hepatocyte, and the lining epithelium of the bile duct (Fig. 18).

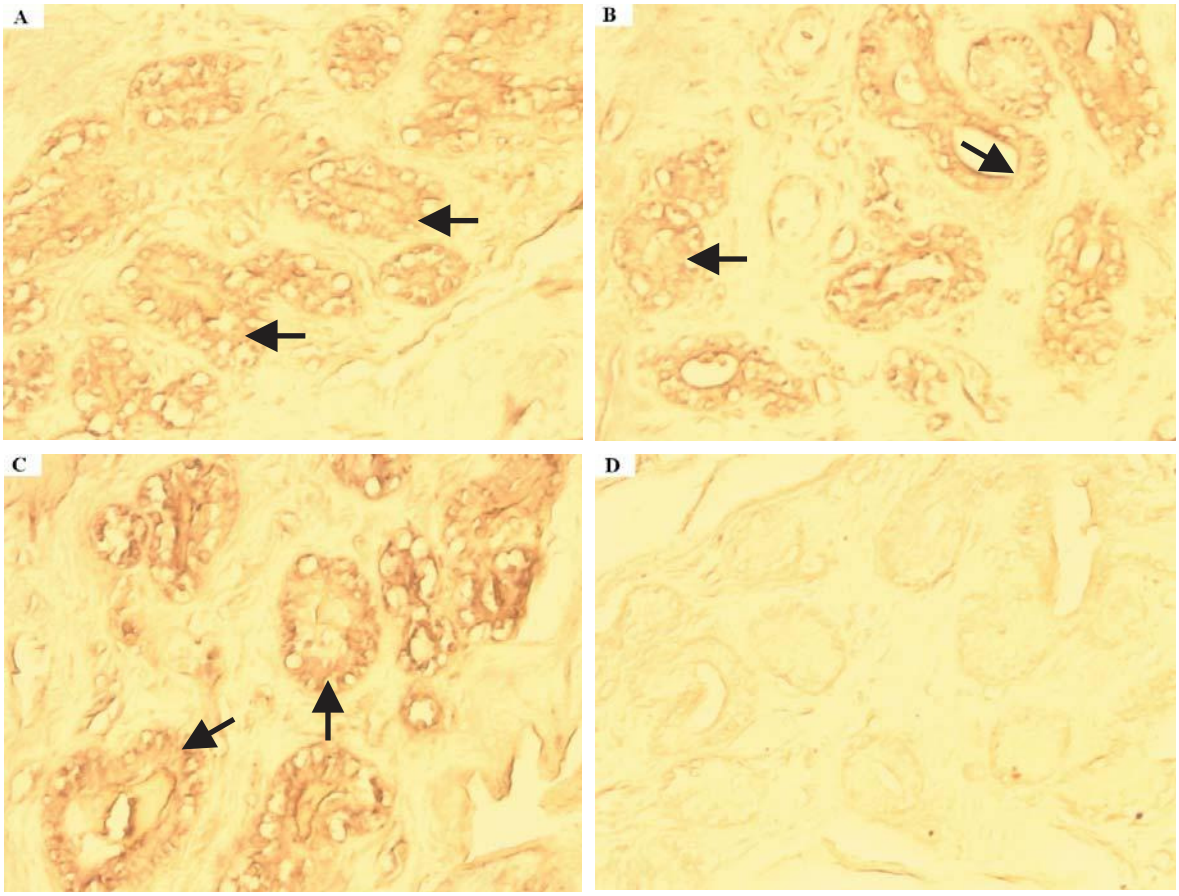

Fig. 16. Light microscopy photomicrograph represent in situ hybridization analysis of leptin (**A**) and its short (**B**) and long (**C**) isoforms of receptor within mammary gland tissue of the one-humped camel (500X). **A, B,** and **C** photomicrographs show hybridization with antisense probes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located in the secretory epithelial cells of the acini (black arrows). Photomicrograph **D** is the negative control and is hybridized with the sense riboprobe of leptin. The sense riboprobe for both isoforms of leptin receptor showed the same result of photomicrograph **D.**

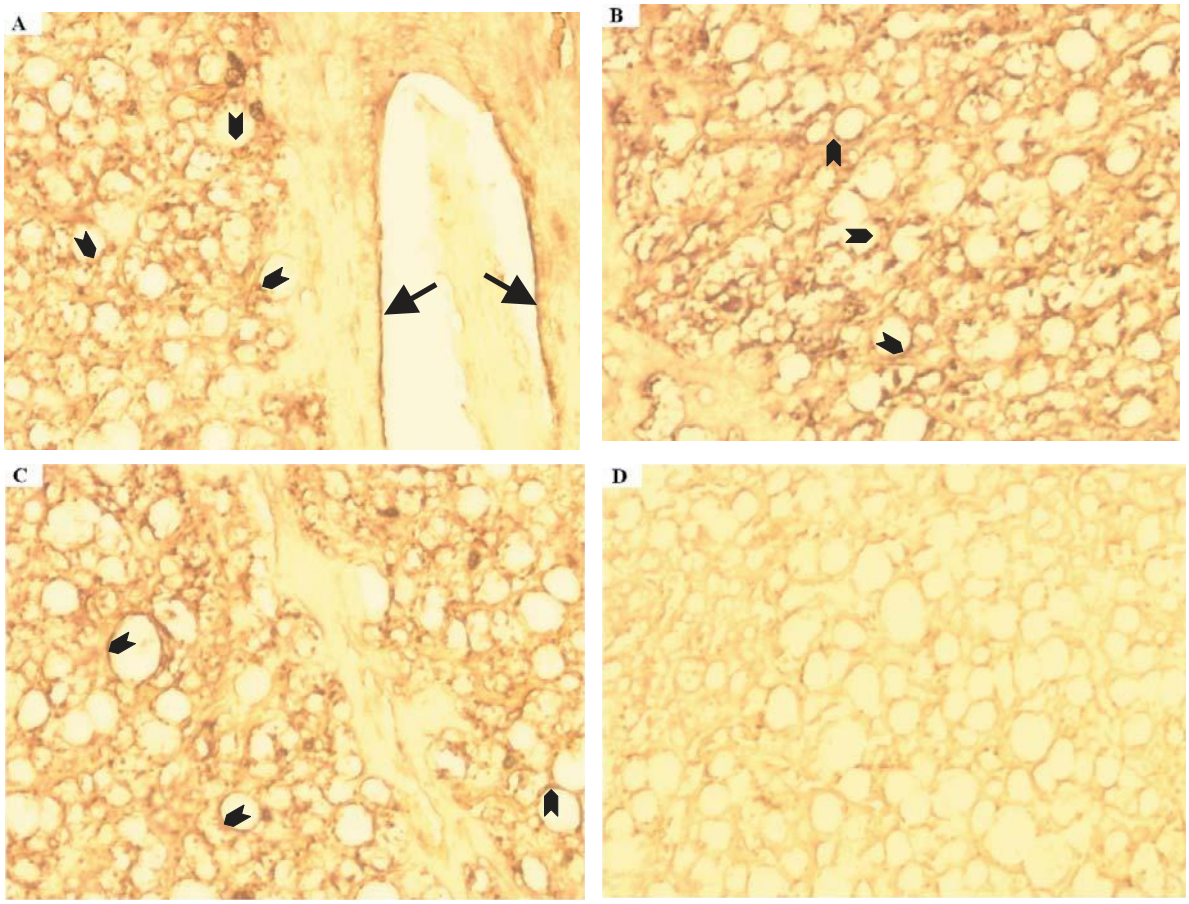

Fig. 17. Light microscopy photomicrograph represent in situ hybridization analysis of leptin (**A**) and its short (**B**) and long (**C**) isoforms of receptor within the liver tissue of the one-humped camel (500X). **A, B,** and **C** photomicrographs show hybridization with antisense probes for Ob, Ob-Ra, and Ob-Rb mRNA, respectively. Positive signals are located mainly in the hepatocyte around the fat vacuoles (arrow' s heads). Also the positive signal of leptin hybridization is clear in the lining epithelium of the bile duct (**A,** black arrows). Photomicrograph **D** is the negative control and is hybridized with the sense riboprobe of leptin. The sense riboprobe for both isoforms of leptin receptor showed the same result of photomicrograph **D.**

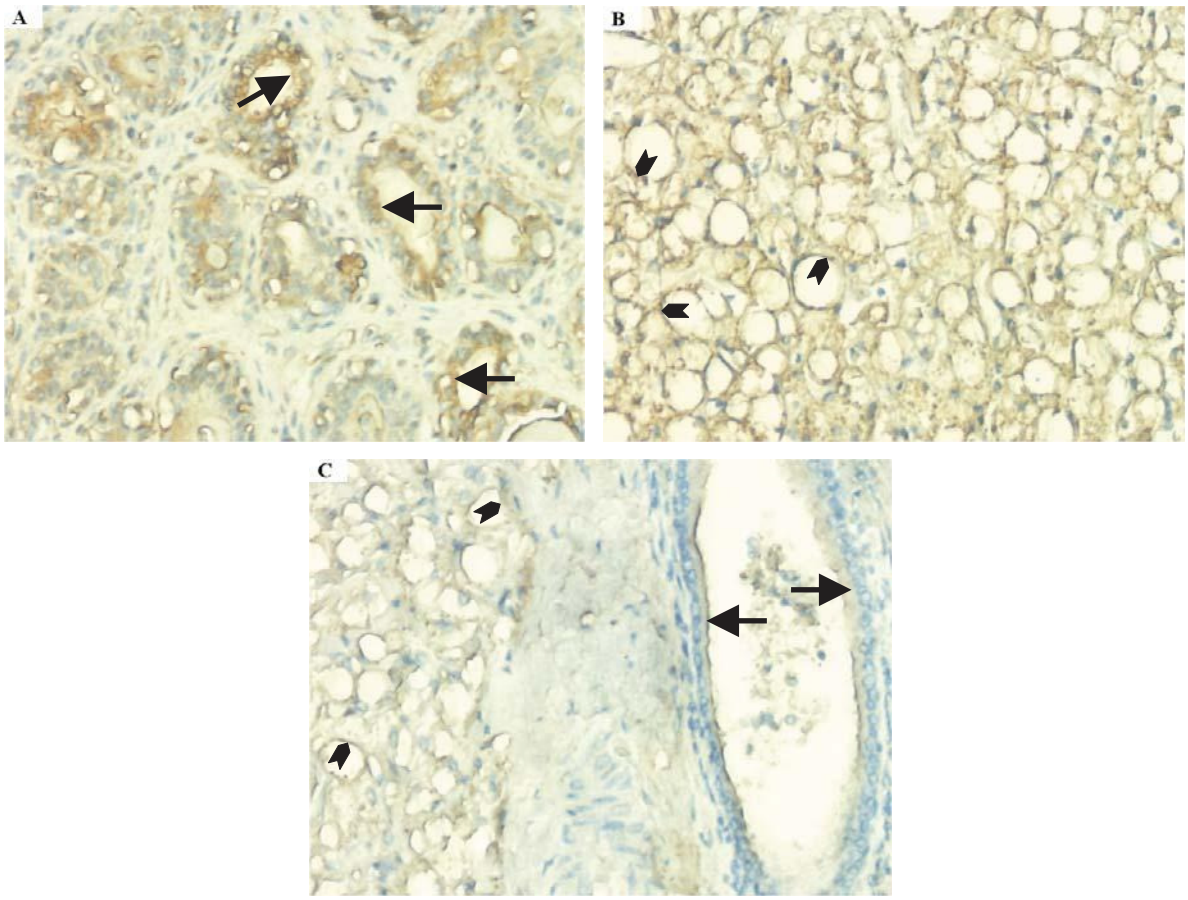

Fig. 18. Light microscopy photomicrograph represent immunohistochemistry analysis of leptin hormone in the mammary gland (**A**) and liver tissue (**B** & **C**) of the one-humped camel. The strong signals are mainly located in the acinar epithelial cell of the mammary gland (**A,** black arrows), in the liver hepatocytes around fat vacuoles (**B & C,** arrow' s heads), and the lining epithelium of the bile duct (**C,** black arrows**).**

Discussion

Our research group is the first to publish research data about the expression of leptin and its receptors in one humped camel. According to the available literatures, our observation in the liver tissue can be considered new in mammalian species. In chicken, Toauis et al (1998) reported the liver as a source of leptin. Using *in situ* hybridization and immunohistochemistry techniques, we could demonstrate the expression of leptin mRNA and leptin protein in alveolar epithelial cells of the one-humped camel mammary gland. In the previous chapters, data were shown to support the hypothesis that local leptin production in the udder contributes to milk leptin in the Egyptian water buffalo and cows (Sayed-Ahmed et. at., 2003 & 2004). We believe that this hypothesis is true for the one humped camel, as well. This is in agreement with the result reported in the human, using immunohistochemical staining of breast tissue as well as RT-PCR analysis of total RNA from breast tissue (Smith-Kirwin et al., 1998), cultured bovine mammary epithelial cells using RT-PCR (Smith and Sheffield, 2002), and sheep mammary gland, just before parturition using immuohistochemical analysis (Bonnet et al., 2002b).

As previously mentioned before for buffalo and cow, the long and short isoforms of leptin receptor are also expressed in the mammary gland of the one-humped camel. This result is consistent with (Laud et al., 1999 & 2002).

Our partial sequences of one-humped camel cDNAs for leptin and the two examined isoforms of the leptin receptor revealed high percentage of nucleotide and amino acid similarity with previously published sequences of other mammalian species especially to that of other ruminant animals which are reported in the GenBank using GCG program analysis.

Adipose tissue is not merely an inert form of connective tissue with the ability to store and release fat it is rather a dynamic cellular system that continuously uptakes up, stores, and releases lipids, and secretes hormones and cytokines, including leptin. In this chapter we demonstrate the presence of leptin and its receptors in the hump adipose tissue. 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). We also observed the leptin and its receptors expression in the liver and mammary gland tissue. Thus leptin could act not only as an endocrine signal in the brain and/or peripheral tissues, but also as an autocrine/paracrine signal within a tissue where it is produced.

Mild to moderate fatty infiltration of the liver was present in all one-humped camels and it is considered a normal histological structure in this species (Shahein et al., 1977; Lalla and Drommer, 1997). In our localization of leptin mRNA and leptin protein in the camel liver, we observed the *in situ* hybridization signals and immunostaining of leptin mainly in the liver hepatocyte around the fat vacuoles but the signal can be found in lining epithelium of the bile duct, as well. As in true ruminants, volatile fatty acids are produced in the fore-stomach of the camel too (Höller et al., 1989). At the same time camels maintain a high blood glucose level, which may be due to either the low rate of glucose elimination in camels (Elmahdi et al., 1997), or the higher level of glucagon in the camel than that in other ruminants or in human (Abdel-Fattah et al., 1999). The activity of lipogenic enzymes is much higher in the camel liver than in other ruminants (Mergani et al., 1987). Thus, the hepatic expression of leptin and leptin receptor in camel might be associated with this high lipogenic activity.

Based on the studies and results presented here, in one-humped camel leptin is expressed in mammary alveolar epithelial cells, liver hepatocytes, and lining epithelium of the bile duct. Our observations further support the biological importance of leptin in mammary gland. Our data also suggest an interrelationship between hepatic leptin expression and liver lipogenic activity in camel.

SUMMARY

The experimental data described in this thesis shows the first information about leptin hormone and its receptor in the Egyptian water buffalo and one-humped camel. Additionally our data shows the first investigation about the origin of locally produced leptin in the mammary gland of large ruminant animals. The study about the alteration of mammary leptin gene expression between dry and lactating gland also serves as new scientific data. The major achievements of the present PhD work are listed below:

Using RT-PCR we demonstrated the expression of leptin hormone in adipose tissue and mammary gland in the lactating Egyptian water buffalo, dry and lactating cow, and dry one-humped female camel. Leptin expression is also visualized in the liver tissue of the one-humped camel.

RT-PCR revealed the expression of long (Ob-Rb) and short (Ob-Ra) isoforms of leptin receptor in the mammary gland and adipose tissues of all the examined species, as well as in the liver of the on-humped camel.

We prepared partial cDNA clons of leptin hormone and the examined receptors for the examined species (cow, Egyptian water buffalo, and one-humped camel) using PCR cloning.

The result of cDNAs sequences and computer analysis using GCG program revealed that leptin hormone and the two examined isoforms of its receptor of both the buffalo and the camel show high homology to other mammalian species especially to ruminants.

In situ hybridization analysis revealed the localization of leptin and leptin receptor mRNA transcripts in the mammary gland epithelial cells of the of large ruminants

Immunohistochemical staining of leptin protein confirms the result of *in situ* hybridization analysis of leptin mRNA in the mammary gland.

In camel hepatocytes and lining epithelial cells of the bile duct leptin mRNA and protein was localized by *in situ* hybridization and immunohistochemistry procedures, respectively.

Competitive PCR method was used to compare the leptin mRNA levels between dry and lactating mammary gland of non-pregnant cows. Leptin expression for the whole mammary tissues showed no difference. However, during lactation, the mammary adipose tissue is completely regressed. Thus, lactating epithelial cells produce the same amount of leptin as the epithelial cell and the fat cells together in the dry mammary gland.

Finally, we can conclude that, the leptin hormone is highly conserved in the mammalian species especially among ruminants. Local paracrine and/or autocrine effects are attributed to leptin, which gives the possibility for auto-regulation on the tissue level. The idea is supported by the observation, which describes the expression of leptin and its receptor in the same tissues. Alveolar epithelial cells of the mammary gland might be an additional source of milk leptin. The epithelial cells of the mammary gland express much of leptin during lactation, which might help maintaining continuous milk production. The hepatic leptin of the camel might be associated with the lipogenic activity of the liver in this species.

LIST OF ABBREVIATIONS

AgRP	agouti-related peptide
ARC	Arcuate nucleus
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
CART	cocaine and amphetamine-regulated transcript
CNS	Central nervous system
CRH	corticotrophin-releasing hormone
CSF	Cerebrospinal fluid
DAB	3,3 -diaminobenzidine
DEPC	diethyl pyrocarbonate
DIG	digoxigenin-11-dUTP
ERK	extracellular regulated kinase
Gal	Galanin
GnRH	Gonadotropin releasing hormone
H&E	Haematoxylin and eosin
IRS	insulin receptor substrates
JAK	Janus-family tyrosine kinase
LH	Luteinizing hormone
MCH	melanin-concentrating hormone
NO	nitric oxide
NPY	neuropeptide Y
Ob	obese (leptin) gene
Ob-R	leptin receptor
p38	p38 MAP kinase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDE	Phosphodiesterase
PEG	Polyethyline glycol
PGE2/PGF2	prostaglandins E2/F2
PI3K	phosphatidylinositol3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
ROS	reactive oxygen species
RT	reverse transcription
SHP-2	Src-like homology 2 domain containing protein tyrosine phosphatase
SOCS-3	suppressor of cytokine signaling 3
SSC	saline sodium citrate
STAT	signal transducers and activators of transcription
TBS	tris-buffered saline
TRH	thyrotropin releasing hormone
UCP	uncoupling protein
VMH	Ventromedial hypothalamus

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