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

Doctoral Thesis

OF

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1. INTRODUCTION

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. The genetically obese ob/ob mouse exhibits obesity, infertility, hyperglycemia, impaired thyroid function, and hyperinsulinemia with insulin resistance. The obesity of genetically obese mice can be cured by peripheral leptin treatment, associating with marked improvement in the energy expenditure, fertility, immune response, brain development, and wound healing. Thus, the role of leptin is not limited to regulate the appetite and the energy expenditure rather it has complex physiological effects on various regulatory systems.

1.1 Leptin hormone

Leptin, the hormone encoded by obesity (Ob) gene, is a 146 aa protein expressed mainly in the white adipose tissue. Extensive research on leptin over the last years has shown, however, that leptin is expressed in several peripheral tissues, such as placenta, stomach, skeletal muscle, brain, pituitary, mammary gland tissues, human follicular papilla cells, calf rumen, abomasum, duodenum, and intralobular ducts of human major salivary glands.

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. Threading analysis and nuclear magnetic resonance of leptin sequence indicated a cytokine-folding pattern similar to interleukin-2 and growth with 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.

1.2 Leptin receptor

Leptin receptor (Ob-R) is a product of the db gene and is a single membrane spanning receptor that has strong sequence homology to the class I cytokine receptor

family. 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,). 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 the hypothalamus yet it is also found in many peripheral tissues at lower levels. The short isoform (Ob-Ra) is expressed ubiquitously and represented the major isoform in many peripheral tissues. The db/db mice possess a mutant allele of the leptin receptor (Ob-R) gene, which encodes a non-functional leptin receptor. 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. The db/db mice exhibit obese and diabetic phenotype due to the absence of leptin signaling.

1.3 Leptin and energy balance

Leptin (ob/ob mice) and leptin receptor mutants (db/db mice) have lower core body temperatures, are more susceptible to cold stress soon after birth, and are hyperphagic and obese by the time of weaning. Treatments with 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.

The metabolic role of leptin is thought to be mediated centrally at the level of hypothalamic nuclei. The satiety effects of leptin are lost or attenuated in mice with lesions of the ventromedial hypothalamus (VMH) or the arcuate nucleus (ARC). Additionally, 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.

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 α-melanocyte stimulating hormone αMSH), cocaine and amphetamine-regulated transcript (CART), and thyrotropin releasing hormone (TRH)

Leptin acts centrally through inhibiting the synthesis of the NPY 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. Also central administrations of leptin decrease hypothalamic MCH and galanin expression and prevent MCH or galanin-induced increase in food intake.

Leptin stimulated CART and POMC neurons in the ARC that leads to a decrease in food intake and increase in sympathetic activity. Leptin activate directly TRH neurons in the paraventricular nucleus or indirectly by increasing the production of the MC4R ligand, (α-melanocyte stimulating hormone α-MSH), to regulate TRH expression on the level of TRH promoter.

The effects of leptin via its receptors on the energy balance of peripheral tissues are apparent in skeletal muscle, liver, pancreas, and adipose tissue. Leptin has direct thermogenic effects in skeletal muscle and white and brown adipose tissue. 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 T_3 and decreased plasma T_4 levels.

1.4 Leptin, tissue developments and immune system

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. Leptin acts as a skeletal growth factor with a direct peripheral effect on skeletal growth centers. Additionally, the Supplementation of embryo culture medium with leptin promotes the development of preimplantation embryos from 2-cell stage to the blastocysts, fully expanded blastocysts and hatched blastocysts.

Administration of leptin to mice reverses the immunosuppressive effects of acute starvation. Leptin increase the phagocytically active macrophages in normal and

ob/ob mice but not in db/db mice, in addition to increase of TNF-α, IL-6 and IL-12 response to LPS. In addition, leptin enhances cytokine production and phagocytosis of leishmania parasites by murine macrophages.

1.5 Leptin and reproduction

The first indication, that leptin is involved in the regulation of the reproduction was the finding that the infertile ob/ob mice rescued their fertility by exogenous leptin treatment. Leptin was reported to play a role in timing or accelerate the onset of puberty in several species.

Ob-Rb isoform is highly expressed in the arcuate and ventromedial hypothalmic nuclei which control both the sexual behavior and the food intake. So it was believed that leptin might facilitate the GnRH secretion directly or via indirect mechanisms through inter-neurons secreting neuropeptides. A hypothalamic site of action was supported by studies demonstrating that leptin significantly increase GnRH secretion in vitro from hypothalamic ARC/ME explants.

During zero energy balance leptin expression and secretion reflect the body fat mass and it is highly correlated with the adipocyte size. This correlation is drastically altered with changes in energy balance. 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. In the cow leptin concentration reflects the state of energy balance during lactation: plasma leptin concentration is lower in cows with a mean negative energy balance during lactation. Those cows usually produced more milk, consumed less feed, and have a lower live body weigh compared with cows having a positive energy balance. Reduced leptin concentration may promote feed intake and allow lactating animals to avoid energy deficit.

Leptin is demonstrated in the colostrum of the porcine and cow as well as in the milk of rat, human, porcine, and cow. In rats, leptin is transferred from maternal circulation to the milk, then to the stomach of the suckling pup, and afterwards to the neonatal circulation. Besides the presence of leptin protein, leptin mRNA was indicated using RT-PCR technique in cultured bovine mammary epithelial cells and in the mammary tissue. Latter study did not indicate the type of the cell in which leptin was present.

Immunohistochemical staining of breast tissue cultured mammary epithelial cells and secretory epithelial cells present in the human milk revealed the production of leptin by human mammary epithelial cells. On the other hand leptin is expressed 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) induced to become pregnant after exogenous leptin treatment, the mice deliver normal litter but stop lactating upon withdrawal of exogenous leptin. Thus, both blood and mammary leptin could exert endocrine, paracrine, and/or autocrine control over mammogenesis and the maintenance of lactation. Additionally, 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.

2. AIMS OF THE THESIS

- To adapt and/or develop *in situ* hybridization procedure for leptin and its receptor mRNA transcripts in order to specify the type of the cell in which leptin and its receptors are present in the mammary gland tissues of the large ruminants.
- To clone the cDNA of leptin and leptin receptors of the Egyptian water buffalo.
- To study the mammary leptin gene expression in dry and lactating non-pregnant cow. This investigation includes the study of the alteration of the gene expression level and the detection of the producing cells.
- Molecular and cellular investigation of leptin hormone and its receptors of the one-humped camel as an example of the desert animals.
- To clone the cDNA of leptin and leptin receptors of the one-humped camel.

3. MATERIALS AND METHODS

3.1 Partial cDNA Cloning of Leptin and Leptin Receptors

The tissue samples of the Egyptian water buffalo and one-humped camel were collected from Cairo slaughterhouse. Approximately 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 gm 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.

Total RNA was isolated by TRIzol reagent (Life Technologies). 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.

Oligonucleotide primers for amplification of buffalo and one-humped camel were 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 into the GenBank. Ap1 5′- caggatgaca ccaaaaccct catc -3′ and Ap14 5′- ggagtagagt gaggcttcca ggac -3′ for amplification of 342 bp fragment of Ob cDNA.

The reverse transcription reaction was performed using 2 μg of total RNA with 200 units of MMLV reverse transcriptase (Promega). 2 μl of RT reaction product was amplified by PCR in final volume of 30 μl with 200 μM dNTPs, 25 pmol of each primer, 1.5 units Red Taq Polymerase (Sigma) and 10X Red Taq PCR buffer.

Based on the expected size and southern blot verification the PCR products were cloned into PGEM-T vector (Promega). The cDNA inserts were then sequenced

and 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.

We analyzed the partial sequences of the buffalo and camel with the nucleotide and amino acid sequences of the other species available in the GenBank by GCG PILEUP program and prepared the consensus sequence for each gene and protein using GCG PRETTY program.

3.2 Competitive PCR

A competitive PCR procedure was used to compare the abundance of mammary tissue leptin mRNA between lactating and dry cows. 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 procedure of competitive PCR was achieved by co-amplification of our RT reaction derived from mammary tissue with different dilutions of leptin internal competitor in one PCR reaction. Firstly, each 20 μl PCR reaction contains 3 μl of RT reaction and 1 μl of 10-fold dilutions of the internal competitor with the leptin specific primer (ap1+ap14). 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.

3.3 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 she camel, as well as the liver tissue of the one-humped camel using DIG labeled RNA probes. The RNA probes were generated by *in vitro* transcription. The sections were permeabilized by 10 μg/ml RNase free Proteinase K and 200 mM Hcl, and then hybridized with (300-500 ng/ml) antisense and sense probe.

3.4 Immunohistochemistry

Immunohistochemistry procedures was used 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. The sections were treated with PBS containing 5-% bovine serum albumin for an hour, 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 by 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 reagent.

4. RESULTS

4.1 Partial cloning and localization of leptin and leptin receptor in the mammary gland of the Egyptian water buffalo

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. Additionally, both the Ob-Ra and Ob-Rb mRNA are expressed in the mammary gland. Southern blot hybridization of RT-PCR products revealed the specificity of the amplified products. 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.

In situ hybridization signals localizing the mRNA transcripts of leptin and its receptor isoforms were observed within the secretary alveolar epithelial cells of the acini. We could not demonstrate any clear signals in cells surrounding the secretary acini, in other words we could not localize mRNA transcripts of leptin and its receptors within the myoepithelial cells, either. Furthermore, 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.

4.2 Expression and localization of leptin and leptin receptor in the mammary gland of the dry and lactating non-pregnant cow

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. Additionally, both the Ob-Ra and the Ob-Rb mRNA were expressed in the adipose tissue of the cows. Southern blot hybridization of RT-PCR products proved the specificity of the amplified products.

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

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. 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, either. In the dry quiescent mammary gland the stromal connective tissue, which 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.

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

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.

4.3 Partial cloning and localization of leptin and leptin receptor of the one-humped camel

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. Southern blot hybridization of RT-PCR products revealed the specificity of the amplified products. 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 (98-%), Egyptian water buffalo (97-%), sheep (96-%), and pig (92-%).

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 using *in situ* hybridization. We could not localize mRNA transcripts of leptin and its receptors within the myoepithelial cells. *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.

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.

5. CONCLUSIONS

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. In summary, we can conclude that

- The leptin hormone is highly conserved in the mammalian species especially among ruminant animals.
- Local paracrine and/or autocrine effects are attributed to leptin, which gives the possibility for auto-regulation on the tissue level. This idea is supported by the observation, which describes the expression of leptin and its receptor in the same tissues.
- Leptin is expressed in the alveolar epithelial cells of the dry and lactating mammary gland. Thus, 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.
- Competitive PCR could not show a clear difference in leptin gene expression between dry and lactating mammary gland of non-pregnant cow.
- Leptin is expressed in the liver tissue of the one-humped camel. The hepatic leptin of the camel might be associated with the lipogenic activity of the liver in this species.

6. NEW RESULTS

- Partial cDNA cloning of leptin hormone and its receptors of the Egyptian water buffalo.
- Localization of the leptin and its receptor mRNA transcripts within the mammary alveolar epithelial cells of the Egyptian water buffalo.
- Using RT-PCR we observed the expression of the long form of leptin receptor and one of the short forms in the adipose tissue of the cows and one-humped camel.

- Localization of the leptin and its receptor mRNA transcripts within the mammary alveolar epithelial cells of the dry and lactating non-pregnant cow.

- Lactating epithelial cells produce the same amount of leptin as the epithelial cell and the fat cells together in the dry mammary gland.

- Partial cDNA cloning of leptin hormone and its receptors of the one-humped camel

- Localization of the leptin and its receptor mRNA transcripts within the mammary alveolar epithelial cells, liver hepatocyte, and the lining epithelium of the bile duct of the one-humped camel.

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