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BOVINE LEPTIN: EXPRESSION, CLONING AND PRODUCTION OF PURIFIED PROTEIN

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ABSTRACT

Leptin is an adipocyte-specific protein that functions as an adipostat. Leptin is implicated mainly in the regulation of feeding behaviour and the hypothalamic pituitary-adrenal axis (HPA). Leptin correlate closely with the degree of adiposity and decrease acutely with caloric restriction. In this study, we tested the bovine leptin expression in different tissues of cows using RT-PCR analysis. Moreover, bovine leptin cloning and purification was carried out throughout this study. The obtained results indicated that bovine leptin was highly expressed in the adipose tissue mammary gland and not expressed in any other organs tested. Also bovine leptin was cloned into pGEM-T easy vector and expressed into expression vector then transfected into expression host cells. The protein from the induced cells was purified and confirmed using western blot analysis and SDS-PAGE. The results in this study suggest that bovine leptin besides its role intheregulation of energy homeostasis plays a role in mammary gland growth and development

INTRODUCTION

Leptin is a 16 kDa protein secreted mainly by adipocytes and is the major regulator of appetite and metabolism **(Freidman and Halaas 1998)** Recently leptin has been found to be expressed in the tissues other than the adipose tissue **(Aoki et al., 1999, Ashworth et al., 2000 and Chilliard et al., 2001)**, and its receptors have also been shown to be distributed in various cell types **(Chien et al., 1997 and Laud, et al ., 1999)**. Since leptin is capable of influencing cell growth **(Takahashi et al., 1997)**. it is strongly suggested that leptin has a vital role in reproduction and fetal growth **(Ashworth et al., 2000 and, Moschos et al., 2002)**. Furthermore, leptin is expressed in the parametrial adipose tissue and epithelial cells of human and mouse mam-

mary glands **(Smith-Kirwin et al., 1998, Aoki et al., 1999, Laud. et al., 1999 and Chilliard et al., 2001)**. Consequently, leptin may have both autocrine and paracrine actions in the mammary gland by acting as a local factor to mediate adipocyte-epithelial cell interactions. To elucidate the regulation of leptin production in ruminants, we first confirmed the expression of leptin gene in various bovine tissues using the RT-PCR method and analysed its cDNA sequence. In addition, we produced recombinant protein to test its biological activity in the future.

MATERIALS AND METHODS

Tissue collection and RNA extraction :

Two adult Holstein cows were euthanized by being exsanguinated after giving sedative dose of sodium pentobarbital. Within 30 min of euthanasia, various tissue samples were collected and frozen immediately in liquid nitrogen. Total RNA was isolated by the guanidine-isothiocyanate method **(Chomczynski and Sacchi 1987)** using Trizol reagent **(Gibco BRL, Rochville, MD, USA)**. The yield of RNA was assessed by measuring the absorbance at 260 nm.

RT-PCR analysis :

The total RNA (1 μg) from various tissues (adipose tissue, mammary gland, liver, spleen, lung, pancreas, kidney, jejunum, ileum and colon) was denatured at 72°C for 5 min and reverse transcribed using 100 units of Moloney murine leukaemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10 μl at 37°C for 1 hr (Chilliard et al., 2001). After heating at 94°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Perkin-Elmer, Foster City, CA, USA), 3 mM $MgCl^2$ and 50 pmol of forward and reverse primers (5/-AACAGAGGGTCACTGGTTT-3/ and 5/ -CAACATGTCCTGTAGTG-3/, respeetive ely) in a total volume of 50 μl. PCR was conducted for 35 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and DNA extension at 72°C for 1 min. The PCR product was then analysed in 2% agarose gel electrophoresis with ethidium bromide staining.

Cloning of bovine leptin :

According to the methods explained previously **(Chilliard et al., 2001, Fawzi, et al., 1996 and Iwase et al., 2000)**, for molecular cloning of bovine leptin, primers containing the nucleotide sequence for restriction enzyme digestion were designed as follow: forward 5/ CTAGTCTAGAATG GTGCCCATCCGCAAGG3/T; reverse 5/ CCGCTCGAGTCA GCACCCGGGACTG AGGTC-

3/ (the underlined sequences indicate Xba I and Xho I digestion sites, respectively). PCR was performed using cDNA from bovine adipose tissue as a template. The cDNA fragment around 460 bp was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and then excised by using the restriction enzymes. Xba I and Xho I. The gel-purified fragment was inserted into pET14β vector (Novagen, Madison. WI, USA), and subsequently transfected into NovaBlue cells (Novagen) as a host for cloning and maintenance of plasmid. The plasmid was extracted from some colonies and subjected to sequencing using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan) with DNA sequencing kit (Applied Biosystems).

Production of recombinant bovine leptin :

To produce recombinant bovine leptin. the pET14b vector containing bovine leptin cDNA was transfected into BL21 (DE3) pLysS cells (Novagen) and the cells were cultured in the Luria broth media containing 30µg/ml chloramphenicol and 50 µg/ml ampicillin at 37µC with shaking. When the cell density determined by absorbance at 600 nm reached 0.6-1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM and the cells were further cultured for up to 7 hr. As shown in Fig. 1, leptin was detected 2 to 5 hr after the addition of IPTG. In the further analysis, the cells were harvested at 2 hr after the addition **(Fawzi et al., 1996)**.

To purify the recombinant leptin, cells were collected by centrifugation at 2.770 xg for 5 min at 4°C and resuspended in the Bug Buster (Novagen) protein extraction reagent. The suspension was then treated with DNAase and lysozyme (Wako Pure Chemical. Osaka, Japan) followed by centrifugation. The pellet was washed 3 times with the X10 diluted Bug Buster reagent, and finally dissolved in the buffer A [50 mM Tris-HCl (pH. 8.0), 1 mM EDTA and 10 mM DTT] containing 8 M urea. Following the incubation at room temperature for 1 hr. the sample was centrifuged at 21.700 xg for 15 min and the supernatant was dialyzed against buffer A containing 4 M urea, buffer A containing the lower urea concentrations, and finally buffer A alone. The dialysate was applied onto the Q Sepharose fast flow column (Amersham Bioscience Co., Piscataway, NJ, USA) equilibrated with buffer A and unbound fraction containing bovine leptin was recovered **(Iwase et al., 2000)**.

SDS-PAGE and Western blot analysis :

Proteins were analysed by 15% SDS-poly acrylamide gel electrophoresis (PAGE), and stained with Coomassie Brilliant Blue R 250 **(Fawzi et al., 1996)**. For Western blot analysis **(Laud et al., 1999)**, after electrophoresis, leptin from the gel was blotted onto PVDF membranes (Immobilon, Millipore, Bedford, MA, USA) without staining. The membranes were incubated first in a

blocking buffer [20 mM Tris-HCl (pH7.5), 150 mM NaCl] containing 0.1% Tween 20 and 5% skim milk overnight at 4°C, then incubated for 1 hr at room temperature in a buffer containing anti-bovine leptin antibody raised in goat (Diagnostic Systems Laboratories, Inc. Webster, TX, USA). The bound antibody was detected with peroxidase-conjugated affinity purified rabbit anti-goat Immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and an enhanced chemiluminescence system (Amersham Bioscience).

RESULTS AND DISCUSSION

Tissue distribution of bovine leptin mRNA

To determine the mRNA expression of bovine leptin, RT PCR analysis was performed for various tissues of Holstein cows. As shown in Fig.2, leptin mRNA was detected in the adipose tissue, and also to less extent in the mammary gland, but not in other tissues tested. These results are in good agreement with those reported in sheep and human (**Smith-Kirwin et al., 1998, Chilliard et al., 2001 and Moschos et al., 2002**). Considering greater masses of total adipose tissue in the body, it is considered that blood leptin is produced and secreted primarily from the adipose tissue.

Blood leptin acts on the central nervous system (CNS) as a satiety factor and also plays roles in fetal growth and reproduction (**Masuzaki et al., 1997 and Chilliard et al., 2001**). Leptin in the mammary gland may be related to the organ development (**Chilliard et al., 2001**). The expression level of the leptin receptor in mammary gland epithelial cells increases during pregnancy and decreases before delivery and during lactation in sheep (**Laud et al., 1999**). The increase in leptin receptor expression in the mid-pregnancy coincides with the initiation of mammary epithelial cell proliferation and the development of the mammary gland. In addition, leptin can inhibit growth factor-induced mammary epithelial cell growth (**Silva et al., 2002**), thus the presence of leptin mRNA in the tissue supports the hypothesis that leptin probably acts as an autocrine or paracrine factor of mammary epithelial cells. Moreover, leptin is found in milk (**Chilliard et al., 2001**), therefore, leptin production in the mammary gland possibly plays a role in regulating neonatal appetite and metabolism.

Cloning of bovine leptin and its production by E. coli

Using the PCR technique, a mature form of bovine leptin gene was amplified and subcloned into the pGEM-T easy and pET14b vectors. After confirming the presence of an insert, the nucleotide sequence of bovine leptin was determined by the cycle-sequence method. The sequence was identical with reported bovine leptin sequence (GeneBank accession number U50365).

Next, BL21 (DE3) pLysS cells were transfected with pET 14 containing leptin cDNA and cultured to an appropriate density, and then IPTG was added. After 2 hr several proteins were induced (Fig.3 A, lane 2) and a 16 kDa protein corresponding to bovine leptin was found in the inclusion body of the cells (Fig.3 A, lane 3). After refolding by dialysis (Fig,3 A, lane 4), bovine leptin was further purified by Q-Sepharose column chromatography (Fig.3 B). Bovine leptin did not bind to the column and recovered from the flow through fractions with approximately 65% recovery (buffer containing different NaCl concentrations). The purified protein was also immuno-stained with anti-bovine leptin antibodies as shown in Fig.1 1. So far, recombinant leptin proteins of rat, mouse, dog, human, sheep, rat and swine **(Fawzi et al., 1996, Freidman, J. M. and Halaas . 1998, Raver et al., 1998 and Park et al., 2001)** were produced. Since they are highly homologous in nucleotide and amino acid sequences to each other (Fig. 4), they have almost identical molecular weights and share the same characteristics not to bind to Q-Sepharose.

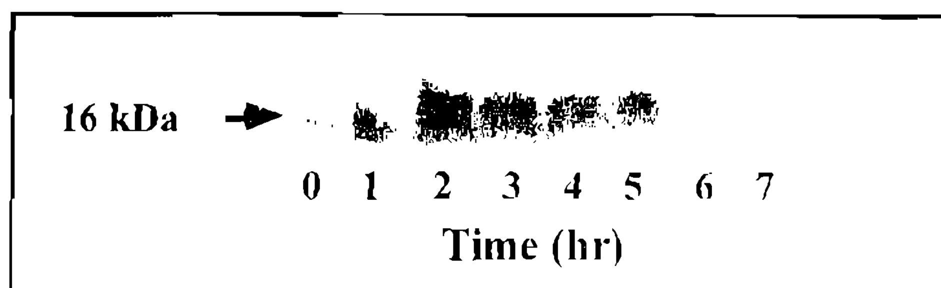

Fig. 1 : Induction of recombinant bovine leptin by IPTG. The cells (BL21 (DE3) pLysS) transfected with bovine leptin cDNA were treated with 0.4 mM IPTG for the indicated time periods. The cells were collected and subjected SDS-PAGE and then blotted into PVDF membrane and Western blot analysis was performed for determining bovine leptin expression. As shown in this figure, leptin was detected in the membrane using specific anti-bovine leptin antibody and no other bands detected.

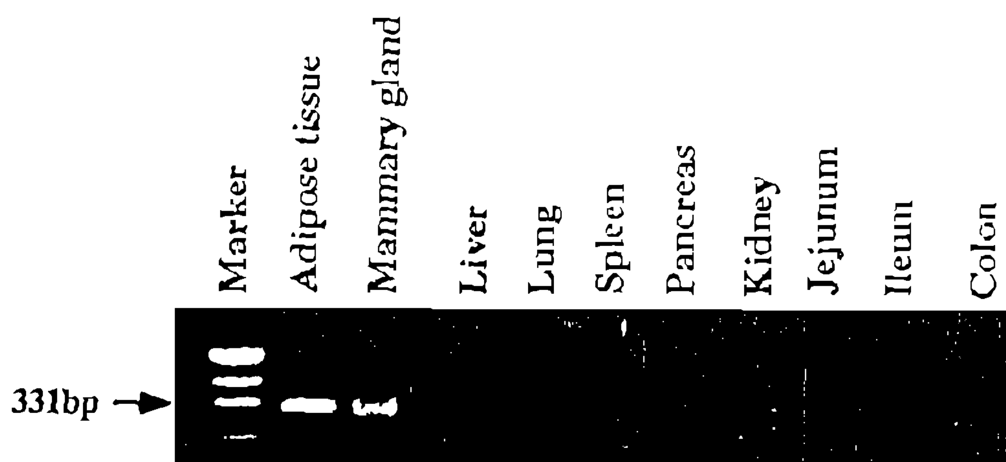

Fig. 2 : Leptin mRNA expression in bovine tissues. Total RNA was extracted from respective bovine tissue, 1 g RNA was reverse transcribed, amplified by PCR, and separated by 2% agarose gel with ethidium bromide. As shown, the leptin cDNA was expressed in adipose tissue and in mammary gland and not detected in any other tissues.

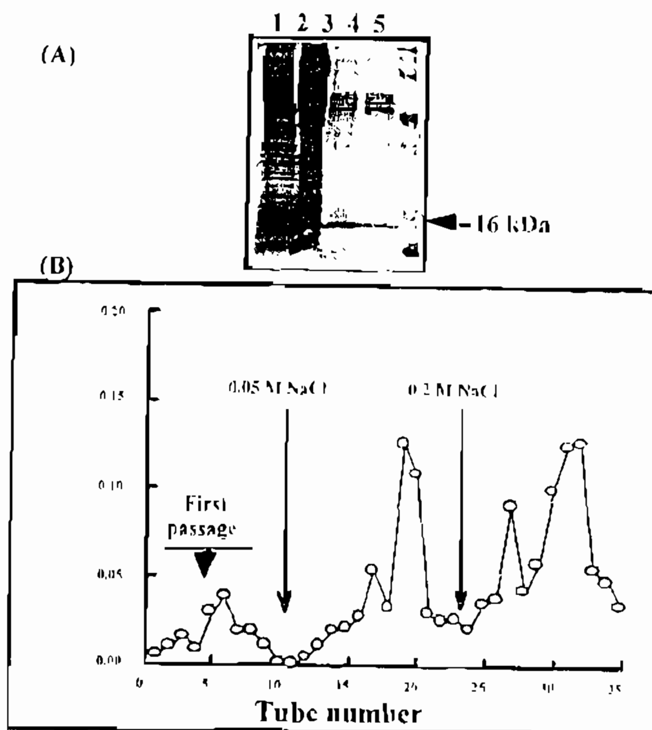

Fig. 3 : A is SDS-PAGE analysis of recombinant bovine leptin with Coomassie Brilliant Blue stain. Lane 1, whole cell lysate before IPTG induction; lane 2, whole cell lysate 2 hr after IPTG induction; lane 3, purified inclusion bodies; lane 4, refolded protein; lane 5, marker proteins. B indicates that the protein was passed through the Q-Sepharose column chromatography and the protein was eluted using 0.05 and 0.2 mM NaCl (peaked points). The fractions was taken and OD was measured. The peak points indicate the elution of protein by NaCl.

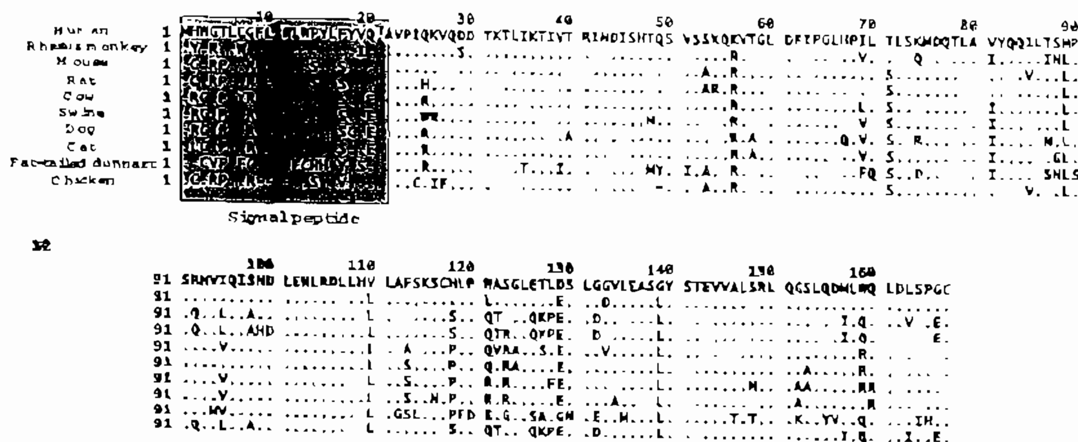

Fig. 4 : Deduced amino acid sequences of leptin in various specis.

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الملخص العربى

الليبتين البقرى : تعبيره، إستنساخه، إنتاجه وتنقية البروتين الخاص به

المشتركون فى البحث

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الليبتن هو هرمون يفرز أساساً من الخلاياص ٢ الدهنية ويعمل بصفة أساسيه فى المخ فى تحت سرير المخ كمثبط لكثرة تناول الطعام. فى هذا البحث باستخدام RT-PCR analysis وجد أن الليبتن يوجد بصفة أساسية فى النسيج الدهنى حول الكلى وكذلك فى خلايا الثدى، كذلك تم زرع الليبتن فى خلايا بكتيرية وتم إنتاج البروتين (الليبتن) وتنقيته باستخدام أحدث الوسائل المستخدمة فى مجال البيولوجيا الجزيئية مثل Western blot SDS PAGE analysis و وجود البروتين فى خلايا الثدى يوحى بأن الليبتن يلعب دوراً هاماً فى مجال التناسليات كعامل منشط للنمو بجانب دوره كمثبط لتناول الطعام.