Detection of leptin and leptin receptor mRNA expression in the bovine testis

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Abstract: Leptin, hormonal product of the ob gene, is known to regulate food intake, energy metabolism and reproductive functions in mammals. The mechanism by which leptin affect male reproductive system, in contrast to its well proven effects in female fertility, has been a matter of debate. Expression of leptin and its receptor in some reproductive organs suggest that leptin has both endocrine and paracrine/autocrine effects on reproduction. Various evidences have pointed to a direct role of leptin in the control of rodent testicular function such as steroidogenesis and spermatogenesis. So, detection of leptin and leptin receptor mRNA in bovine testis will be the first crucial step to an understanding of its paracrine/autocrine effect on testes in cattle. In the present study, we showed the expression of leptin mRNA as well as its functional receptor (Ob-Rb) mRNA in whole testis of Holstein cattle using reverse transcription and polymerase chain reaction (RT-PCR) analysis. To confirm the first results, RT-PCR products were amplified with Nested PCR using inner leptin primer pairs designed on different exons. Based on our results, although we could not determine the exact cell source of leptin in testis, it suggests that besides its primary actions at the hypothalamic-pituitary level, leptin can also involved in autocrine and/or paracrine mechanisms in testicular physiology in cattle.

Key words: cattle, leptin, leptin receptor, testis, RT-PCR.

Introduction

Leptin, the hormone encoded by obesity (Ob) gene, is a 146 aa protein with a tertiary structure similar to cytokines (Zhang et al., 1994). Leptin with diverse biological functions has been shown to contribute to the regulation of energy, metabolism, feeding behavior, and reproduction in mammals by a large variety of neuroendocrine, paracrine, and autocrine actions (Messinis and Milingos, 1999; Baratta, 2002; Williams et al., 2002; Zeiba et al., 2005; Cervero et al., 2006).

This hormone acts through five receptor isoforms. Among these isoforms, only the long form (Ob-Rb) is functional and is responsible for the physiological effects of leptin (Tartaglia, 1997; Kawachi et al., 2007). Signal transduction pathways affected by leptin/leptin receptor interaction are the activation of janus kinase/signal transducer and activator of transcription (JAK/STAT) via the full length Ob-Rb and mitogen-activated protein kinase (MAPK) via Ob-Rb and also via the truncated Ob-Ra (Bjorbaek et al., 1997).

Although the expression of functional leptin receptor is highest in the central nervous system, the widespread distribution of the receptor in bovine various tissues has been demonstrated over the last decade, including fat, mammary parenchyma, liver, muscle, adrenal cortex, testis, lung, spleen, kidney, abomasum, small intestine, and this could be understand as an evidence for multiple peripheral
effects of leptin (Frunbeck, 2001; Silva et al., 2002; Smith and Sheffield, 2002; Chelikani et al., 2003; Sayed-Ahmed et al., 2004; Bartha et al., 2005; Kawachi et al., 2007).

In comparison, the expression of bovine leptin has been only detected in adipose tissue, mammary parenchyma (Silva et al., 2002; Smith and Sheffield, 2002; Chelikani et al., 2003; Sayed-Ahmed et al., 2004; Bartha et al., 2005) and calf rumen, abomasum, and duodenum (Yonekura et al., 2002). Based on these findings, it is believed that leptin mainly acts through endocrine route. Nevertheless, based on an extensive growing numbers of recent publications, leptin also expresses in some other tissues such as mouse testis (Herrid et al., 2007), mouse placenta and fetal tissue (Hoggard et al., 1997), stomach (Bado et al., 1998), skeletal muscle (Wang et al., 1998), brain and pituitary (Morash et al., 1999), and human spermatozoa (Jope et al., 2003; Aquila et al., 2005). So, it increases the probability of paracrine and/or autocrine role of leptin, at least in some organs.

In normal animals leptin serves as a metabolic signal to the reproductive system (Barash et al., 1996; Cunningham et al., 1999). This appears to be a tightly regulated action, carried out at different levels of the hypothalamic-pituitary-gonadal system (Tena-Sempere, 2002). Although the participation of leptin in female reproduction is well established, the contribution of leptin to the proper functioning of the male reproductive system has been less clear (Camina et al., 2002; Tena-Sempere, 2002). The leptin deficient Ob/Ob mouse, exhibit atrophied testes with hollow seminiferous tubules (Barash et al., 1996; Mounzih et al., 1997); increased apoptosis (Bhat et al., 2005); elevated steroidogenesis (Tena-Sempere, 2002); and small ovaries with reduced numbers of primary and Graafian follicles (Barash et al., 1996). However, the underlying mechanisms are not still clear as to how leptin acts through the hypothalamic-pituitary axis to exhibit effects on the reproductive organs or whether leptin can directly affect their development or either the combination of direct and indirect effects (Baratta, 2002; Tena-Sempere, 2002).

As a first step to identify the paracrine or autocrine effects of leptin in cattle, especially in reproduction, it is necessary to demonstrate gene expression and its receptor in reproductive organs. Thus, the aim of the present study was to determine the presence of leptin mRNA and functional leptin receptor (Ob-Rb) mRNA in whole bovine testis using RT-PCR.

Materials and Methods

Tissue samples: Non-lactating mammary gland tissue of female adult Holstein cows and Pericardial adipose tissue, liver and testis tissues of male adult Holstein cows were collected immediately after slaughtering. Samples were transferred to the laboratory next to ice cold at 5 °C and subsequently about 30 mg of each sample was used directly for RNA isolation or kept in 1.5ml tubes and stored at -80°C until RNA extraction.

RNA isolation and DNAse I treatment: Total RNA was isolated by TRIzol reagent (Invitrogen). Briefly, the samples were first lysed and homogenized in 1 ml TRIzol using vortexing and repeatedly drawing up and down through a 26 gauge needle. The homogenates were incubated at room temperature (20 °C) for 5 minutes. In the case of the fat samples, insoluble material was first removed from the homogenate before incubation. After that, 200 μl chloroform per milliliter of TRIzol was added. Samples were centrifuged at 12,000 × g for 15 min at 4 °C and upper phase containing RNA was precipitated with 500 μl isopropyl alcohol per milliliter of TRIzol, washed with 75% ethanol (diluted with diethyl pyrocarbonate-treated water), air dried and resuspended in 50 μl DEPC treated water. In order to eliminate the possible residual genomic DNA from the RNA samples, one unit RNasefree DNase I (Fermentas) was added per each 8 μl of RNA sample and incubated at 37 °C for 30 min followed by adding 1 μl 25 mM EDTA (Fermentas) and heat inactivation of the enzyme at 65 °C for 10 min.

Reverse Transcription -Polymerase Chain Reaction: The reverse transcription reaction was performed using 10 μl of total RNA as template with 200 units of MMLV reverse transcriptase, 0.5 μg Oligo(dT) 18, 1 mM dNTP mix, 4 μl 5X reaction
buffer and 20U Ribonuclease Inhibitor for 60 min at 42 °C in a final volume of 20 μl followed by heating at 70 °C for 10 min to stop the reaction. All used reagents were from Fermentas. The RT-PCR were carried out in a DNA thermocycler (MWG, Germany).

Oligonucleotide primers for amplification of bovine leptin and leptin receptor as well as beta actin (forward and reverse primers) were designed from GenBank sequence information (table 1). The forward and reverse leptin as well as the beta-actin primer pairs were designed to span the junction of two exons to be RNA specific. So, the amplification of the cDNAs and DNAs of the same gene resulted in the different PCR products in length (as shown in table 1). For the control of DNA contamination, 1 μl DNase I treated RNA from each sample was amplified directly with the primer pairs listed in table 1. One μl of RT reaction product was amplified by PCR in a final volume of 50 μl with 0.2 mM dNTP mix, 20 pmol of each primer, 1.25 units SmarTaq DNA Polymerase, 1.5 mM MgCl2 and 5 μl 10X PCR buffer. PCR thermal cycling parameters were as follows: 1 cycle 94 °C for 5 min, followed by 40 cycles of denaturing at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 45 s. The program was terminated with a final extension step at 72 °C for 10 min. For nested PCR, using inner leptin primers, 1 μl of PCR reaction product amplified with leptin primers was amplified by second PCR similar to above mentioned condition. Fat and mammary gland tissues were used as positive control for leptin and liver was positive control for leptin receptor. The housekeeping gene beta actin was used as a control for successful isolation of RNA and preparation of cDNA.

All PCR products were run on a 1.5% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide and visualized under UV transillumination. The location of the predicted products was confirmed using 100 bp or 200 bp molecular ladder as a standard size marker.

**Results**

**Expression of leptin in bovine testis:** The amplification of the cDNA prepared with the RNA isolated from fat tissue and non-lactating mammary gland revealed a predicted PCR product with the nucleotide length of 441 bp with the primer pair P1/P2, whereas the RT-PCR with the cDNA from testes (P1/P2) showed no detectable amount of PCR product on agarose gel (Fig.1A, lane 1, 2 and lane 3). Amplification of 1 μl of the above mentioned PCR products with primer pair P3/p4 (nested-PCR) resulted in a predicted PCR product of 384 bp in length for all three tissues (Fig. 1B, Lane 1, 2 and 3). These results confirmed that leptin mRNA expressed in adult testis as well but with low expression level compared to the fat and mammary gland tissues. The fragment size of PCR products using bovine DNA as template for PCR reaction with leptin primer pairs (P1/P2 and P3/P4) was detected as 2194 and 2131-bp band, respectively (data not shown). The mRNA for bovine beta actin acted as an internal positive control and was detected at all samples (Fig. 1C lane 2, 3 and 4). No amplification was observed in the negative controls (Fig.1A4, 1B5 and 1C5).

**Expression of functional leptin receptor (Ob-Rb) in bovine testis:** The RT-PCR analysis using isoform-specific primer pairs confirmed that Ob-Rb

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### Table 1: Nucleotide sequences of the primer sets used for RT-PCR. *For nested PCR, Leptin R.= Leptin Receptor.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Gene Bank Acc. No.</th>
<th>Forward</th>
<th>Reverse</th>
<th>Fragment size (cDNA), bp</th>
<th>Fragment size (DNA), bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>leptin (P1/P2)</td>
<td>U50365, AB003143</td>
<td>gtgccatcggcagaaggctca</td>
<td>tccagccgggactgagt</td>
<td>441</td>
<td>2194</td>
</tr>
<tr>
<td>Leptin (P3/P4)*</td>
<td>U50365, AB003143</td>
<td>tcatcaagacaaattgtccaggg</td>
<td>cagctgcggcaacatgtcctg</td>
<td>384</td>
<td>2131</td>
</tr>
<tr>
<td>Leptin R.(P5/P6)</td>
<td>NM-001012285, AJ580801</td>
<td>gtgccagcaactacagatgcctcag</td>
<td>aacctacggctcctgtagaagc</td>
<td>380</td>
<td>380</td>
</tr>
<tr>
<td>Beta actin(P7/P8)</td>
<td>NM-173979, NC007326</td>
<td>atcactgccctgcaaacccag</td>
<td>ccgggtgagc</td>
<td>509</td>
<td>639</td>
</tr>
</tbody>
</table>
mRNA is expressed in the adult bovine testis (Fig. 2, lane 2). The 380-bp fragment was not detected following PCR using total RNA incubated without reverse transcriptase as the template, demonstrating the absence of genomic DNA contamination (Fig. 2, lane 3). Liver was used as positive control for expression of Ob-Rb (Fig. 2, lane 1).

**Discussion**

This is the first study revealed the expression of leptin in bovine testis. Additionally, the present investigation also confirmed the expression of longform leptin receptor (Ob-Rb) in bovine testis in agreement with other reports in bovine (Chelikani *et al.*, 2003; Kawachi *et al.*, 2007) and in rodents (Caprio *et al.*, 1999; El-Hefnawy *et al.*, 2000; Tena-Sempere *et al.*, 2001). The specific amplified leptin fragment was not detectable in the first PCR using specific primer pairs but it was clearly amplified in nested PCR.

Although Leptin is able to pass the blood-testis barrier (Banks *et al.*, 1999), there is no correlation between leptin concentration in blood and seminal plasma (Camina *et al.*, 2002). Thus, it is postulated to be considered an independent production of leptin in testis. However, based on our results, the origin cells of leptin mRNA is not exactly defined.

The Ob-R isoforms are produced by different alternative splicing of the common transcript. Therefore, the patterns of alternative splicing of Ob-R transcript must differ among bovine tissues (Kawachi *et al.*, 2007). Ob-Rb has full function because this isoform contains boxes 1-3, which are implicated in signal transduction pathways involving both Janus kinase (JAK) and signal transducers and activators of transcription (Tartaglia, 1997). The presence of Ob-R typical signal transduction, such as phosphorylation of STAT3, were shown in mouse germ cells and phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) and STAT3 in mouse interstitial cells (El-Hefnawy *et al.*, 2000).

In male animals leptin acts at different levels of the hypothalamic-pituitary-testicular system (Tena-Sempere, 2002). It also does participate in functional regulation of the male gonadal axis (Caprio *et al.*, 1999; Tena-Sempere, 2002). The predominant
stimulatory effects of leptin, primarily at the hypothalamus, are observed at its physiological levels above a minimal threshold. In contrast, direct inhibitory actions at the testicular level may take place in the presence of a significantly elevated leptin concentration, as detected in obesity (Tena-Sempere, 2002). Furthermore, leptin has a novel direct negative action on LH/hCG-stimulated androgen production from Leydig cells in culture. It is consistent with the reduced testicular function in obese male rats (Caprio et al., 1999). Leptin has also inhibitory actions on hormonally-stimulated ovarian steroidogenesis in vitro (Spicer and Francisco, 1997).

Leptin treatment rescued the sterility of genetically obese ob/ob males (Mounzih et al., 1997) and observation of testicular histology indicated that leptin stimulate cellular activity in the seminiferous tubules (Barash et al., 1996). The existence of leptin in human testicular tissue and a negative correlation between leptin concentrations in seminal plasma and the motility of human spermatozoa were described (Glander and Kratzsch, 2000).

It has been shown that expression of Ob-Rb in testis is less than liver in cattle (Kawachi et al. 2007). Similarly, the low level of expression of leptin receptor in mouse Leydig cells has been detected but it was highly efficient and functional (Caprio et al., 1999).

Our results showed the presence of leptin and leptin receptor in the bovine testis. These results together with above mentioned findings create the condition for an autoregulative mechanism (autocrine or paracrine leptin loop) at this level, in addition to an endocrine effect. Leptin secretion suggests that the bovine testis has the ability to modulate its desired function such as metabolism, relatively independent of the plasma leptin levels.

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References


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شناسایی بیان mRNA لپتن و گیرنده آن در بیضه گاو

چکیده
لپتن، هورمون حاصل از پایه Ob، مخصوصاً در فراز پرواز و عملکرد آن (به خصوص در حالت محساسی) از ایمنی و عملکرد آن اثرات انسانی با پارکینسونیایی را در عمل می‌کند. لپتن و گیرنده آن در پستانداران نیز در بیضه گاو جلوه‌ی خاصی به دلیل احتمال اصلی به آنها می‌باشد. لپتن و گیرنده آن در بیضه گاو به وسیله‌ی mRNA به وسیله‌ی RT-PCR و با استفاده از زوج پروتئینی از ارتباط مهاجرتی بیش از پاناسوری‌زی بیشتری داشت. اگرچه در مطالعه‌ها هر دوی آنها می‌توانند کنترل‌کننده اثرات پارکینسونیایی را در بیضه گاو ترکیبی از آنها با پارکینسونیایی می‌باشد. لپتن و گیرنده آن در بیضه گاو به وسیله‌ی mRNA به وسیله‌ی RT-PCR، روندهای خاصی به وسیله‌ی mRNA به وسیله‌ی RT-PCR نیز می‌باشد. لپتن و گیرنده آن در بیضه گاو به وسیله‌ی mRNA به وسیله‌ی RT-PCR نیز می‌باشد.