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RESEARCH ARTICLE

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Effects of leptin on the expression of *Ob-Rb* mRNA in the cultured adipocytes of newborn calf

Abstract The effects of additional leptin on the long type receptor (*Ob-Rb* mRNA) for adipocytes of new born calf were tested by means of competitive reverse transcription polymerase chain reaction (RT-PCR). A sample of fine monolayer adipose cells were first obtained and recombination leptins of calf (5 ng/mL·12 h) were added. No additive was adopted as tester in the adipose cell. Total RNA was determined at 4, 12, 24, 36, 48 and 72 h, and duplicated three times in every treatment in the single factor duplicating test. The result, compared with the group of testers, was that the quantity of *Ob-Rb* mRNA in adipose cell cultures was also significantly higher ($P < 0.01$) at the beginning stage. Following this tendency, the quantity was gradually lower with cultured time going on in 12–24 h, and the quantity was in stable level ($P > 0.05$) from 48 to 72 h. It was shown that leptin could improve the level of expression of *Ob-Rb* in cultured adipose cells of new born calf within a definite time.

Keywords recombination leptin, adipocyte, *Ob-Rb* mRNA, in vitro culture

1 Introduction

Leptin, the product of the *Ob* gene (Liu et al., 2000) which is secreted from adipose tissues, regulates food intake, whole-body energy metabolism, immune reaction and endocrine by inducing anorexy and increasing energy consumption to a new balance of energy (Ramsay, 2001; Sun and Huang, 2001; Cettour-Rose et al., 2002; Machinal-Quelin et al., 2002). Leptin plays its role through its receptors. *Ob-Rb*, which has high affinity, was cloned by Tartaglia (Yukio, 1997) from Mus choroid plexus (CP). Leptin receptors can be classified into different groups such as a, b, c, d, e and f. *Ob-Rb*, called a function receptor, belongs to the long configuration receptor which has the function of signal transduction (Hoggard et al., 1997; Lord et al., 1998; Konopleva et al., 1999; Tsuchiya et al., 1999; Martin-Romero et al., 2000; Wu and Zhou, 2000; Zhang et al., 2002). Thus, it is very important to investigate the regular expression of *OB-Rb* for the function of leptin. It is well known that *Ob-Rb* distributes widely at the central and most peripheric tissues (Machinal-Quelin et al., 2002). There are few reports about leptin expression levels that were published. Leptin was added into the cell culture fluid of primary cultured adipocytes to find out its effects on *Ob-Rb* expression levels by means of competitive RT-PCR at 12, 24, 36, 48 and 72 h (12 h each).

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2 Materials and methods

2.1 Recombination bovine leptin

Recombination bovine leptins (Diagnostic Systems Laboratories, INC USA) were dissolved into ddH_2O, underwent filtration sterilization, and stored at –20°C.

2.2 Primers for the competitive reverse transcription polymerase chain reaction (RT-PCR)

The specific oligonucleotide primers of *Ob-Rb* used for PCR reaction were designed according to the bovine mRNA sequence of *Ob-Rb* in Genbank. The forward primer sequence was 5′-tttggccctcttcttttgga-3′; the reverse primer sequence was 5′-tgctcctcctcagtttcacct-3′. The length of the PCR products was 287 bp.

2.3 Cloning and sequencing of the bovine *Ob-Rb*

2.3.1 RNA isolation

Total RNA of the adipose cell was extracted with a commercially available RNA isolation kit (Tripure; Roche).

Total RNA was treated with RNase-free DNase I (Takara) operated according to manufacturer's instructions. Total volume was 50 μL counting total RNA, 10 × DNase I buffer 5 μL, DNase I 4 U, RNase Inhibitor 20 U. Incubation was done at 37°C for 30 min, then RNA was recovered, washed with 70% alcohol, and resuspended with diethylpyrocarbonate (DEPC) water.

2.3.2 Reverse transcription (RT) reaction

The first strand cDNA was synthesized with reagant purchased from TAKARA Company and operated according to manufacturer's instructions. Briefly, total volume was 20 μL counting total RNA 1 μg for each samples, 5 × reverse transcriptase buffer 4 μL, dNTP mixture (10 mmol each) 2 μL, RNase inhibitor 20 U, Oligo(dT)18 primer 100 pmol, avian myeloblastosis virus (AMV) reverse transcriptase 10 U and DEPC water. Mixture was agitated slightly for 10 min at room temperature, incubated at 42°C for 90 min, and cooled for 2 min in ice. The reaction liquid was then used in the PCR reaction.

2.3.3 Cloning and sequencing of the bovine *Ob-Rb*

Aliquots of 2 μL of the first strand cDNA reaction were amplified in a 50 μL reactive volume containing a final concentration of 5 μL of 10 × PCR buffer, 0.2 mmol dNTP mix, 0.4 μmol of each primer, 2 U of recombinant Ex *Taq* DNA polymerase, and sterile distilled water.

The RT-PCR was carried out in a DNA thermocycler (Tpersonal 2000, Biometra, Germany). Following an initial denaturation at 94°C for 4 min, PCR was performed for 30 cycles of denaturation at 94°C for 45 sec, specific annealing at 57°C for 45 sec, extension at 72°C for 1 min and a final extension of 72°C for 10 min in the last cycle. The PCR products were electrophoresed on a 2% agarose gel in Tris-borate triethylenediaminetetraacetic acid (EDTA) buffer, stained with ethidium bromide, and linked with vector of PMD18-T, named PMD18-T Lept in recepter (PMD-LR), and then sequenced.

2.4 Competitive template of *Ob-Rb*

A 470-bp-long fragment digested from the vector of pFast-BacI by incision enzyme *Bgl*II was inserted into *Ob-Rb*. As the competitive template of *Ob-Rb*, the new fragment was 757 bp.

2.5 Bovine adipose cell culture in vitro and grouping

Adipose cells were dispersed via collagenase digestion described by Xia et al. (2005), Rodbell (1964). A single factor duplicate test using competitive-PCR was designed to investigate whether additional leptin (5 ng/mL · 12 h) in adipose cell cultured medium at 4, 12, 24, 36, 48 and 72 h (12 h each) had a role in *Ob-Rb* mRNA expression. The monolayer adipose cells were the control group.

2.6 Quantification of expression of *Ob-Rb* mRNA by polymerase chain reaction (PCR)

Aliquots of 2 μL of the first strand cDNA reaction were amplified in a 50 μL reactive volume containing a final concentration of 5 μL of 10 × PCR buffer, 0.2 mmol dNTP mix, 0.4 μmol of each primer, 2 U of recombinant Ex *Taq* DNA polymerase, competitive template of *Ob-Rb* 2 μL, and sterile distilled water. The RT-PCR was carried out in a DNA thermocycler (Tpersonal 2000, Biometra, Germany). Following an initial denaturation at 94°C for 4 min, PCR was performed for 30 cycles of denaturation at 94°C for 45 sec, with specific annealing at 57°C for 45 sec, extension at 72°C for 1 min, and a final extension of 72°C for 10 min in the last cycle. The cell abundance of *Ob-Rb* mRNA in the six experimental stages was compared by competitive RT-PCR (Zhang et al., 2005). The PCR products were electrophoresed on a 2% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide, the images captured and the PCR product density analyzed with a Tanneng Gel Imaging system (Tanneng Instrument, Shanghai, China).

2.7 Statistical analysis

A minimum of three separate cell preparations constituted an experiment, and treatments within each preparation were replicated in three holes. Results that were expressed as the mean ± standard deviation (SD) comparisons between control and treatment were made using the Student's *t* test for paired data. When $P \leqslant 0.05$, the results were considered significant.

3 Result

3.1 RNA isolation

Total RNA of the adipose cells has three visible straps of 28 S RNA, 18 S RNA and 5 S RNA. The value of A260/A280 was 1.8. Therefore, the total RNA could be used in this experiment.

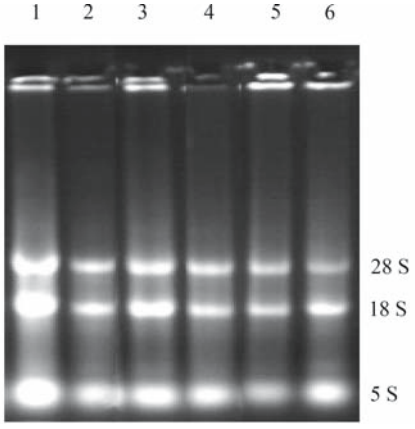
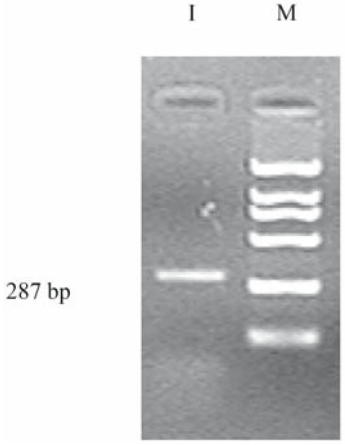


Fig. 1 The electrophoresis result of total RNA from adipose cells

3.2 Cloning and sequencing of the bovine *Ob-Rb* (Fig. 2)

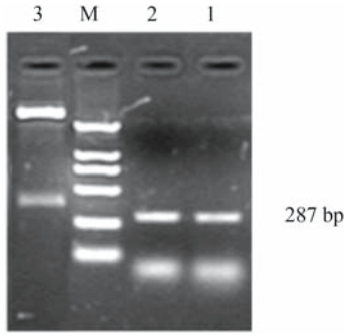
The PCR products were as long as the expected fragments, and their sequence was 99% matched with the *Ob-Rb* sequence in Genbank, the production was *Ob-Rb*.



Notes: M stands for DL-2000 DNA Marker and 1 for PCR products of *Ob-R* gene.

Fig. 2 PCR products of *Ob-R* gene

3.3 Identification of PMD-LR plasmids by PCR and enzyme digestion (Fig. 3)



Notes: M stands for DL-2000 DNA marker; 1 for PCR products of PMD-LR plasmids; 2 for PCR products of PMD-LR plasmids and 3 for Digested PMD-LR plasmids by *Eco*R I and *Hin*dIII.

Fig. 3 Identification of PMD-LR plasmids by PCR and enzyme digestion

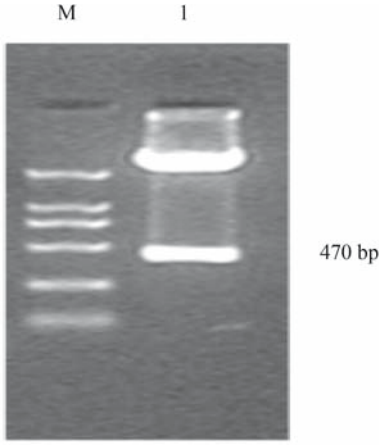
3.4 Competitive template of *Ob-Rb* (Figs. 4 and 5)

Therefore, it is evident that a 470-bp fragment was digested by *Bgl*II from pFastBacI vector.

And, it is also evident that a new fragment was 757-bp long.

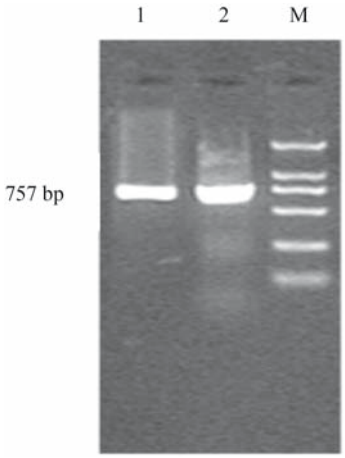
3.5 Competitive PCR products of *OB-Rb* (Fig. 6)

It can be seen that the competitive template and *Ob-Rb* can be amplified in the same reaction system.



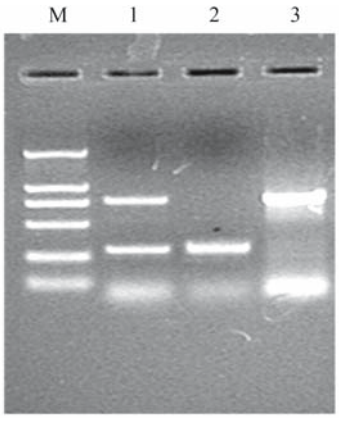
Notes: M stands for DL-2000 DNA marker and 1 for pFastBacI vector digested by *Bgl*II.

Fig. 4 Map of pFastBacI vector enzyme digestion by *Bgl*II



Notes: M stands for DL-2000 DNA marker; 1 for PCR product of *Ob-Rb* competitive plasmid and 2 for PCR product of *Ob-Rb* competitive plasmid.

Fig. 5 PCR product of *Ob-Rb* competitive plasmid



Notes: M represents DL-2000 DNA marker; 1, 2 and 3 stand for competitive PCR products of *Ob-Rb*, PCR products of *Ob-Rb* and PCR products of competitive template, respectively.

Fig. 6 Competitive PCR products of *Ob-Rb*

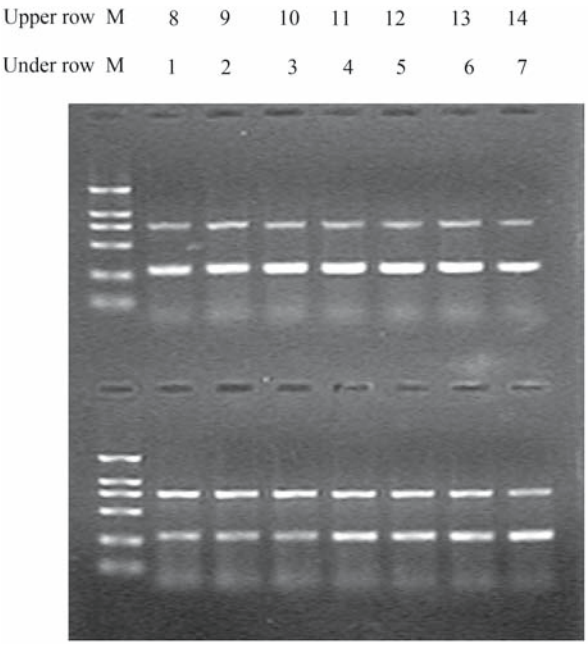
3.6 Effect of leptin on *Ob-Rb* mRNA abundance of bovine adipocyte in vitro culture

The effects of leptin on *Ob-Rb* mRNA abundance of bovine adipocyte are shown in Table 1 and Figs. 7 and 8.

Table 1 Area rate of *Ob-Rb* mRNA to competitive template by competitive Q-PCR ($\overline{X} \pm SD$, $n = 4$)

Time/h	*Ob-Rb*/ competitive template
0	0.4525 ± 0.2657^{ABab}
4	0.5247 ± 0.4180^{Bb}
12	0.8333 ± 0.2669^{Cc}
24	1.0805 ± 0.4133^{Dd}
36	0.4258 ± 0.093^{ABa}
48	0.3839 ± 0.060^{Aa}
72	0.3732 ± 0.1220^{Aa}

Notes: values in one line with different superscripts are significantly different at $P < 0.05$. The result was that the quantity of *Ob-Rb* mRNA in adipose cell cultured for 12–24 h was significantly higher in the treated groups than that of the control group ($P < 0.01$). Following a gradual decrease, the expression of *Ob-Rb* kept a stable level from 48 to 72 h ($P > 0.05$).

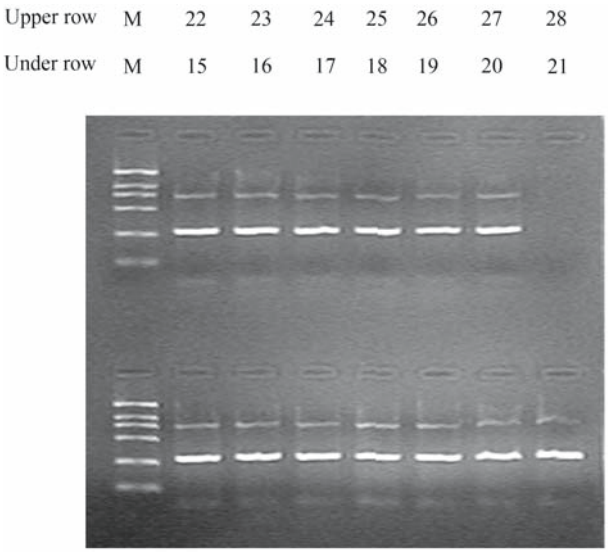


Notes: M represents DL-2000 DNA marker; 1–4 are control group; 5–8 are added 4 h group; 9–12 are added 12 h group and 13–14 are added 24 h group.

Fig. 7 Competitive PCR products of *Ob-Rb* mRNA

4 Discussion

Compared with the traditional RNA analytical method, quantitative RT-PCR has more sensitivity, more particularity and more samples can be quantified at the same time. The accuracy of quantitative RT-PCR depends on the choice of standard substance which obeys external standard or internal standard (Sun and Huang, 2001). External standard is divided



Notes: M represents DL-2000 DNA Marker: 15–16, 17–20, 21–24 and 25–27 stand for added 24 h group, added 36 h group, added 48 h group and added 72 h group, respectively.

Fig. 8 Competitive Q-PCR products of *Ob-Rb* mRNA

into two types. One is endogenous gene, normally choosing home gene which is invariably expressed in cyto relatively, by double PCR amplification. The disadvantage of this method is that the accuracy of quantity is affected by the variable influencing factors, so we called them semi-quantity or relative quantity. In absolute quantity of mRNA, the choice of standard substance should be in substantial agreement with the sequence of the model. At least, the sequences of the premier bounding pad are all the same, insuring that "fair competition" for the bond of two competitive models, then deal with quantity error from different amplification effect. As a quantity detecting method of *Ob-Rb* gene expression, competitive RT-PCR has the advantage of sensitivity, credibility and nicety.

An *Ob-Rb* competitive template was constructed in this experiment. The result of the competitive RT-PCR showed that the quantity of *Ob-Rb* mRNA in adipose cell cultured for 12–24 h was significantly higher in the treated groups than that of the control group. Following a gradual decrease, the expression of *Ob-Rb* was kept at a stable level from 48 to 72 h. That is to say, leptin has different effects in different cell growth periods (Lin et al., 2000; Lin et al., 2001). This result is consistent with those of other researches (Ramsay, 2001; Cettour-Rose et al., 2002). *Ob-Rb* was presented widespread on various kinds of tissues including adipose tissue. That leptin produced a marked effect must be binding with *Ob-Rb*. In simulation, *Ob-Rb* was adjusted by leptin. CHO cells, which stably expressed *Ob-Rb*, were treated with leptin. After 30 min, the binding ability of *Ob-Rb* with leptin was degraded (Mccowen et al., 1998). Leptin has a negative accommodation expression as to the other hormone's feature. However, whether the role of leptin is direct is still not clear. The added recombination human or mouse leptin in adipocyte

has no significant effects on the endogenous leptin mRNA, and injected leptin in vivo decreased endogenous leptin expression. This research may help in the investigation of leptins.

References

Cettour-Rose P, Burger A G, Meier C A, Rohner-Jeanrenaud F (2002). Central stimulatory effect of leptin on T3 production is mediated by brown adipose tissue type II deiodinase. Am J Physiol Endocrinol Metab, 283(5): E980–E987

Hoggard N, Mercer J G, Rayner D V, Moar K, Trayhurn P, Williams L M (1997). Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. Biochem Biophys Res Commun, 232(2): 383–387

Konopleva M, Mikhail A, Estrov Z, Zhao S, Harris D, Sanchez-Williams G, Kornblau S M, Dong J, Kliche K O, Jiang S, Snodgrass H R, Estey E H, Andreeff M (1999). Expression and function of leptin receptor isoforms in myeloid leukemia and myelodysplastic syndromes: Proliferative and anti-apoptotic activities. Blood, 93(5): 1668–1676

Lin J, Barb C R, Matteri R L, Kraeling R R (2000). Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig. Domest Anim Endocrinol, 19(1): 53–61

Lin J, Richard Barb C, Kraeling R R, Rampacek G B (2001). Developmental changes in the long form leptin receptor and related neuropeptide gene expression in the pig brain. Biol Reprod, 64(6): 1614–1618

Liu J Z, Li N, Xiong Y Z, Deng C Y (2000). Recent progress in the studies of obese gene and its encoding product. Journal of Agricultural Biotechnology, 8(4): 396–402 (in Chinese)

Lord G M, Matarese G, Howard J K, Baker R J, Bloom S R, Lechler R I (1998). Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature, 394(6696): 897–901

Machinal-Quelin F, Dieudonne M N, Leneveu M C, Pecquery R, Giudicelli Y (2002). Proadipogenic effect of leptin on rat preadipocytes in vitro: Activation of MAPK and STAT3 signaling pathways. Am J Physiol Cell Physiol, 282(4): C853–C863

Martin-Romero C, Santos-Alvarez J, Goberna R, Sánchez-Margalet V (2000). Human leptin enhances activation and proliferation of human circulating T lymphocytes. Cell Immunol, 199(1): 15–24

Mccowen K C, Chow J C, Smith R J (1998). Leptin signaling in the hypothalamus of normal rats in vivo. Endocrinology, 139(11): 4441–4447

Ramsay T G (2001). Porcine leptin alters insulin inhibition of lipolysis in porcine adipocytes in vitro. J Anim Sci, 79(3): 653–657

Rodbell M (1964). Metabolism of isolated fat cells. J Biol Chem, 239: 375–380

Sun Z J, Huang Z Y (2001). Progress in the study of obesity. Journal of Physiology Science Progress, 32 (1): 41–44 (in Chinese)

Tsuchiya T, Shimizu H, Horie T, Mori M (1999). Expression of Leptin receptor in lung: Leptin as a growth factor. Eur J Pharmacol, 365 (2–3): 273–279

Wu J, Zhou D J (2000). Progress in Leptin receptor. Journal of Physiology Science Progress, 31(2): 143–146 (in Chinese)

Xia C, Wang Z, Niu S L, Zhang C, Zhang H Y (2005). Primary culture of calf preadipocyte. Chinese Journal of Cell Biology, 27: 89–92 (in Chinese)

Yukio T (1997). Radioimmunoassay of Leptin in human plasma. Endocrinology, 138: 4513–4516

Zhang C, Xia C, Niu S, Wang Z, Xu C (2005). Constructing RT—PCR inter-reference of Leptin long form receptor mRNA in dairy cows. Jilin Animal Science and Veterinary Medicine, 4: 9–10, 21 (in Chinese)

Zhang X R, Liu G Q, Shen G M (2002). Expression of the long form Leptin receptor mRNA in rat digestive tract. Journal of Anhui Agricultural University, 29(3): 276–278 (in Chinese)