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RESEARCH ARTICLE

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The role of leptin in bovine adipocyte fat metabolism *in vitro*

Abstract To identify the effect of leptin on adipocyte, preadipocyte was isolated from male dairy calves and cultured in a monolayer; 5 $\mu g \cdot L^{-1}$ bovine leptin were added in culture liquid when the cell differentiated into adipocytes. The result showed that leptin decreased the deposition of triglyceride (TG) and increased the levels of hormone sensitive lipase (HSL) mRNA in cells. At the same time, leptin suppressed the levels of endogenous leptin mRNA, and the levels of leptin receptor (Ob-R) mRNA were raised significantly. It indicated that leptin may induce more fat metabolism directly.

Keywords leptin, fat metabolism, adipocyte

1 Introduction

Leptin, the product of the *ob* gene, is secreted from adipose tissue and regulates food intake, whole-body energy metabolism, immune reaction, and endocrine by inducing anorexy and increasing energy consumption to a new balance of energy. Its concentration in blood is always correlated with the content of body fat. The discovery of this hormone improved greatly in the domain of obesity, energy homeostasis, and reproductive function. Many researches were done in regulating food intake and whole-body energy metabolism of the human being and murid (Glasow et al., 2001; Peters et al., 2007; Philip et al., 2007). The few reports about livestock leptin were published especially in ruminants.

In the past few years, more research considered that leptin plays a great regulating role on the performance of lactating, body condition, and body fat metabolism in cows. We added leptin in the cell culture fluid of the primarily cultured adipocyte to find out its effect on the cells.

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2 Meterials and methods

Oil red O (Sigma) dissolved in isopropanol was kept overnight at room temperature, filtered, mixed with distilled water, kept overnight in the cold, and finally filtered twice before use (Ramirez et al., 1992). The final staining solution was 0.2% oil red O in 60% isopropanol.

2.1 Bovine adipocyte culture *in vitro* and grouping

New born male dairy calves anesthetized with sodium thiamylal (2 g) via intrajugular injection were taken as the experimental materials. A piece of greater omentum was taken, and the animals were euthanatized immediately by intravenous injection of 10 mL of Beuthanasia-D Special. Adipose cell were dispersed via collagenase digestion described by Xia Cheng and Wang Zhe (Xia et al., 2004; Rodbell, 1964).

Then, immediately, the greater omentum was flushed with 50 mL of D-hanks free Ca^{2+} and Mg^{2+} warmed to 37°C and was sheared to 1 mm^3 tablets. The tablets were rinsed with D-hands free Ca^{2+} and Mg^{2+} and collected in a tube and then suspended with buffer solution containing 0.1% collagenase and D-hanks free Ca^{2+} and Mg^{2+} under 37°C for 90 min. This suspension was filtered through a nylon mesh (grid size: 100 mm), and the filtrate was added to the 1% BSA D-hanks free Ca^{2+} and Mg^{2+} buffer. The cell pellet was collected after centrifugation at 500×g for 5 min and resuspended in 50 mL of DCM. The viability of cells was estimated by trypan blue (0.2% solution), and only preparations in which ⩾90% of the adipocyte excluded dye were used in subsequent experiments. The gained preadipocytes were diluted into a density of 10^6 cells per cm^2 and cultured with DCM under 37°C and 5% CO_2 in six-hole plates (Costar); these were aspirated and refreshed every 24 h. Until the cells differentiated into adipocytes and accumulated lipid droplets in the

cytoplasm, the media were changed to BCM, and then, bovine leptin (final concentration 5 $\mu g \cdot L^{-1}$) was added into the media, respectively. After 24 h, subsequent experiments were done.

2.2 Measurement of TG in cells

TG in cells was quantitated by staining the intracytoplasmic lipids with Oil red O, as described by Ramirez et al. (1992) and Fukumoto and Fujimoto (2002). For staining, the cells were first fixed with 3.7% (v/v) formaldehyde for 10 min and washed twice by PBS buffer. The 1.5 mL staining solution was added to holes and incubated for 15–30 min at room temperature. After that, the cells were washed with PBS buffer twice for 1 min each. Quantitate the extent of adipose conversion by staining the accumulated lipid with Oil red O and determining the amount of extracted dye at 510 nm.

2.3 RNA isolation

After the cells were cultured for 24 hours, its total RNA was extracted using commercially available RNA isolation kit (TRIPURE; Roche) and treated with RNase-free DNase I (TAKARA) according to manufacturer's instructions. The reaction volume consisted of total RNA 45 μL, 10×DNase I Buffer 5 μL, DNase I 4U, and RNase Inhibitor 20U. After incubation at 37°C for 30 min, RNA was recovered, washed with 70% alcohol, resuspended with DEPC water, and finally stored at −80°C for use.

2.4 Gene expression analysis

Four pairs of specific primers in Table 1 were designed according to the alignments of the published mRNA sequences of *β-actin*, *Leptin*, *HSL*, and *Ob-R* gene in cells. These four pairs of primers were tested for their specificity by conventional reverse transcription polymerase chain reaction (RT-PCR) before being used for the QRT-PCR studies.

The expression levels of *Leptin*, *HSL*, and *Ob-R* gene were quantified by real-time amplification and the house-keeping gene *β-actin* as control from the mRNA preparation using the ABI7000 system (ABI, USA) and Brilliant® SYBR® Green QRT-PCR Master Mix, 1-Step (Stratagene, USA). The QRT-PCR was performed in 25-μL reaction mixture.

The RT-PCR protocol included reverse transcription at 50°C for 30 min and an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles consisting of a denaturation step at 95°C for 30 s, an annealing step at 60°C for 60 s, and an extension step at 72°C for 30 s. The reaction was then subjected to a melting protocol from 55°C to 95°C with a 0.2°C increment and 1 s holding at each increment to check the specificity of the amplified products. For relative quantification of differential expression between samples, cycle threshold (C_T) values were obtained. The relative expression levels were related to the reference gene. Data analyses of the relative expression levels were carried out according to the following equation (Livak and Schmittgen, 2001):

$$\text{amount of target} = 2^{-\Delta\Delta}C_T$$

$$\Delta\Delta C_T = (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{Time x}} - (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{Time 0}}$$

The representative QRT-PCR products were purified using DNA wizard cleanup kit (Promega, USA) and sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, USA).

2.5 Statistical analysis

The minimum of three separate cell preparations constituted the experiment, and treatments within each preparation were replicated separately in three holes. Results were expressed as the mean±SEM. Comparisons between control and treatments were made using the Student's *t* test for paired data. Values ($P \leqslant 0.05$) were considered significant.

3 Results

3.1 Observation of bovine adipocyte

Freshly isolated bovine preadipocytes were small and round, with a bigger adrationem nuclei and plasm. The

Table 1 Primer pairs for reverse transcriptase-PCR amplification of each target gene and length (bp) of PCR products

target genes	specific oligonucleotide primers	length of PCR products/bp
Leptin	Forward:5′ - ACCAGACATTGGCGATCTACCA-3′	253
	Reverse:5′- TGCCGCAACATGTCCTGTAGT-3′	
HSL	Forward: 5′-GCCCCTCCGTACCCTCAACCATCA AC-3′	299
	Reverse: 5′-AGACGAGTCGTCCAGCATGGG GTCCA-3′	
Ob-R	Forward: 5′-TTTGGCCCTCTTCTTTGGA-3′	287
	Reverse: 5′- TGCTCCTCCTCAGTTTCACCT-3′	
β-actin	Forward: 5′ -GTCATCACCATCGGCAATGAG-3′	403
	Reverse: 5′ -GCTAACAGTCCGCCTAGAAGCA-3′	

attachment of cells to plastic substratum was apparent within 30 min of seeding. Monolayer confluence was achieved within 24 h, and after being proliferated, the cells were turned from rotundity to triangulum or polygon in form (Fig. 1). Then, lipid droplets were accumulated in the cells. The cells that differentiated into adipocytes had multiplicity lipid droplets in intracytoplasm. When intracytoplasmic lipids were stained with Oil red O, the cells appeared as typical rings in form and had a smaller ratio of nuclei and plasm (Fig. 2).

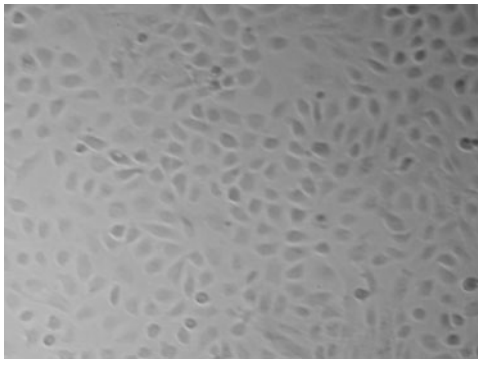

Fig. 1 Preadipocyte of bovine (×100)

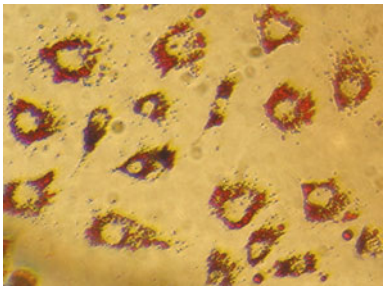

Fig. 2 Adipocytes of bovine (×250)

3.2 Effect of leptin on the triglyceride content of bovine adipocyte *in vitro* culture (Figs. 3 and 4)

Effects of leptin assessed with cell triglyceride were tested using staining intracytoplasmic lipids with Oil red O. Twelve hours after adding leptin, the cells and lipid droplets obviously diminished. The content of the triglyceride in cells was significantly decreased ($P \leqslant 0.05$) by 42% than the control group. Therefore, it is considered that bovine leptin can promote the decomposition of triglyceride and enhance fat metabolism in mature fat cells.

3.3 Effect of leptin on *Leptin*, *HSL*, and *Ob-R* expression (Fig. 5)

The abundances of *Leptin*, *HSL*, and *Ob-R* mRNA were detected by quantity RT-PCR. The result showed that the

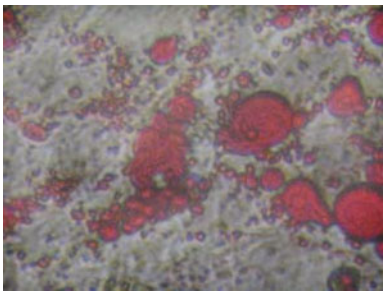

Fig. 3 Control group (×250)

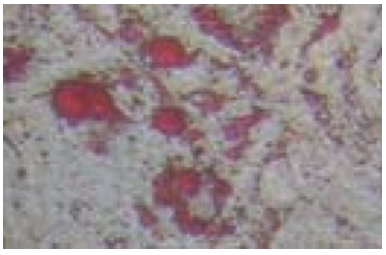

Fig. 4 Leptin-added group (×250)

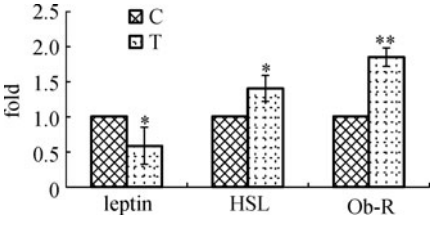

Fig. 5 Effect of leptin on *leptin, HSL*, and *Ob-R* expression
Note: * and ** represent $P \leqslant 0.05$ and $P \leqslant 0.01$, respectively.

expression of *HSL* and *Ob-R* was increased by 42% and 0.84%, respectively ($P \leqslant 0.05$ or $P \leqslant 0.01$), while the expression of *leptin* was decreased by 40% ($P \leqslant 0.05$).

4 Discussion

It was systemically described by Van et al. (1976) that Stromal vascular fractions (SVF) were the classic preadipocytes. In brief, adipose tissue was treated by collagenase, and then, the adipocyte suspensions were centrifuged. The sediments were SVF, which can differentiate into adipocyte. In our experiments, SVF was separated from greater omentum and differentiated into adipocyte in DCM, which was according with the research on *Homo sapiens*, mice, and swine (Casanueva and Dieguez, 1999; Ramsay, 2001; Spiegelman and Flier, 2001; Van et al., 2004).

Adjusting fat metabolism and cutting down fatty deposition *in vivo* were considered as the primary function

of leptin (Fukumoto and Fujimoto, 2002). Leptin can adjust fat metabolism directly and change the fat degradation of adipose cells. However, the effects of leptin were completely different on the adipocyte in different developmental stages. On one hand, leptin stimulated cell differentiation and multiplication on proadipocyte that made the cells differentiated into lipocytes. Leptin increased the growth rate, cell volume, and fat drop in mouse subcutaneous proadipocyte *in vitro*. On the other hand, glucose uptake, lipogenesis, glycogen synthase activity, and protein synthesis stimulated by insulin were inhibited (Casanueva and Dieguez, 1999), and thereafter, the efficiency of fat-splitting was raised by 10 times (Spiegelman and Flier, 2001). The analogical results were received on the leptin of swine (Ramsay, 2001). In our research, 5 $ng \cdot mL^{-1}$ leptin was added into the adipose cell differentiated from preadipocyte to test its effect on adipose cell. The result showed that leptin inhibited the utilization of glucose in culture fluid and decreased the deposition of TG in cells; however, it increased the levels of *HSL* mRNA that made TG decompose greatly. Therefore, the content of TG in the cells was decreased.

In addition, leptin has a negative accommodation expression of *leptin*, just like the other hormones. However, whether or not the role of Leptin is direct is still unclear. The added recombination of human being or mouse leptin in adipocyte had no significant effects on endogenous *leptin* mRNA. Injecting leptin *in vivo* decreased endogenous *leptin* expression. Leptin had effects through binding with *Ob-R*, and it regulated *Ob-R* at the same time. CHO cells that stably expressed *Ob-R* were treated by leptin. After 30 min binding, the ability of *Ob-R* with *leptin* was degraded (Glasow et al., 2001; Ramsay, 2001). In our experiment, the leptin suppressed the levels of endogenous *leptin* mRNA, which was different from that of other researches. It indicated that the *leptin* expression was negatively accommodated by itself directly and possibly caused by differentia of species or by different added times and concentrations. The level of *Ob-R* mRNA was raised significantly, and it was possibly the action to redeem the degression of the binding ability of *Ob-R* with *leptin*.

References

Casanueva F F, Dieguez C (1999). Neuroendocrine regulation and actions of leptin. Front Neuroendocrinol, 20(4): 317–363

Fukumoto S, Fujimoto T (2002). Deformation of lipid droplets infixed samples. Histochemistry and Cell Biology, 118(5):423–428

Glasow A, Kiess W, Anderegg U, Berthold A, Bottner A, Kratzsch J (2001). Expression of leptin (Ob) and leptin receptor (Ob-R) in human fibroblasts: regulation of leptin secretion by insulin. J Clin Endocrinol Metab, 86(9): 4472–4479

Livak K J, Schmittgen T D (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4): 402–408

Peters J H, Simasko S M, Ritter R C (2007). Leptin analog antagonizes leptin effects on food intake and body weight but mimics leptin-induced vagal afferent activation. Endocrinology, 148(6): 2878–2885

Philip J, Scarpace, Michael M, Zhang Y, Cheng Kit-Yan, Nihal T (2007). Leptin antagonist reveals an uncoupling between leptin receptor signal transducer and activator of transcription 3 signaling and metabolic responses with central leptin resistance. J Pharmacol Exp Ther, 320: 706–712

Ramirez Z J L, Castro M F, Kuri H W (1992). Quantitation of adipose conversion and triglycerides by staining in intracyctoplasmic lipids with oil red O. Histochemistry, 97(6): 493–497

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

Spiegelman B M, Flier J S (2001). Obesity and the regulation of energy balance. Cell, 104(4): 531–543

Van Harmelen V, Rohrig K, Hauner H (2004). Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. Metabolism, Clinical and Experimental, 53 (5): 632–637

Van R L, Bayliss C E, Roncari D A (1976). Cytological and enzymological characterization of adult human adipocyte precursors in culture. J Clin Invest, 58(3): 699–704

Xia C, Wang Z, Niu S L, Zhang C, Zhang H Y (2004). Culture of calf preadipocyte and establishment of its proliferation and differentiation model. Chinese Journal of Veterinary Science and Technology, 34(5): 26–29 (in Chinese)