Production of Leptin in *E. coli* and Its Effect on Glucose and Acetate Transport and Expression of Uncoupling Protein-2 Gene in Adipose Tissues of Korean Cattle (Hanwoo)

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ABSTRACT : Leptin has a major role in the regulation of food intake and energy homeostasis. In addition, leptin participates in many physiological functions including regulation of lipid metabolism. Bovine recombinant leptin protein was produced in *E. coli* cells in order to understand function of leptin in the regulation of lipid metabolism. The leptin expression vector was constructed in pGEX-4T-3 vector and transformed into *E. coli* BL21 cells. Expression of the GST-leptin fusion protein was induced with IPTG. The fusion protein was purified using glutathione sepharose 4B batch method, and the recombinant leptin was eluted after thrombin protease digestion. The effect of leptin on glucose transport was examined in the differentiated adipocytes of 3T3-L1 cells. Leptin had no effect on basal and insulin-stimulated glucose transport in 3T3-L1 cells ($p>0.05$). Effect of recombinant leptin on glucose and acetate transport was examined in adipose tissues of Korean cattle (Hanwoo). Insulin stimulated glucose transport in both intramuscular and subcutaneous adipose tissues ($p<0.05$), but leptin did not affect glucose transport in both adipose tissues ($p>0.05$). Insulin stimulated acetate transport in bovine adipose tissues ($p<0.05$), but leptin did not affect acetate transport ($p>0.05$). Northern and RT-PCR analyses showed that mRNA levels of uncoupling protein-2 were increased by leptin treatment in 3T3-L1 cells without statistical difference ($p>0.05$). In conclusion, bovine recombinant leptin did not affect glucose and acetate transport in both 3T3-L1 adipocytes and bovine adipose tissues, while it stimulates UCP-2 mRNA expression in 3T3-L1 cells. *(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 8 : 1062-1068)*

Key Words : Bovine Leptin, 3T3-L1 Cells, Adipose Tissues, Glucose Transport, Acetate Transport, Uncoupling Protein-2

INTRODUCTION

Lipid metabolism is regulated by many hormones, and leptin is the most recently discovered among them (Zhang et al., 1994). Recently, cDNA microarray method has been employed in order to identify adipose tissue-specific genes (Kim and Moon, 2003). The identification of the obese (ob) gene through positional cloning (Zhang et al., 1994) and the discovery that its encoded protein, leptin, is essential for the normal regulation of body weight, have altered the field of metabolic physiology (Halaas et al., 1995; Pelleymounter et al., 1995; Fruhbeck et al., 2001). Leptin acts on specific regions of the brain to regulate food intake, energy expenditure, and neuroendocrine function (Saladin et al., 1995; Flier and Maratos-Flier, 1998; Houseknecht and Portocarrero, 1998). The profound importance of leptin is demonstrated by the fact that mice which are homozygous for a mutation in the ob gene (Pelleymounter et al., 1995) or in its receptor (Lee et al., 1996), demonstrate massive obesity, a reduced basal metabolic rate, hyperglycemia, hyperinsulinemia, and reduced fertility. Leptin is a 167 amino acid protein whose amino terminal signal sequence is cleaved during secretion and it circulates as a 146 amino acid peptide.

In addition to its role in the regulation of food intake and energy homeostasis, leptin participates in many physiological functions including regulation of neuroendocrine, angiogenesis and lipolysis (Takahashi et al., 1996). In cultured adipocytes, leptin inhibited the insulin and dexamethasone-stimulated synthesis of fatty acids and total lipids (Bai et al., 1996). Muoio et al. (1997) reported that leptin stimulated fatty acid oxidation and inhibited triglyceride synthesis in isolated muscle preparation. Other study reports a leptin-mediated inhibition of insulin action in adipocytes (Muller et al., 1997). Leptin was shown to increase basal glucose transport and glycogen synthesis (Berti et al., 1997). In contrast, leptin had no effect on glucose transport, lipoprotein lipase activity, and insulin action in fat and muscle cells *in vitro* (Ranganathan et al., 1998).

Centrally administered leptin has been shown to increase insulin-stimulated glucose utilization and to favor the expression of uncoupling proteins (UCPs). Uncoupling protein-2 (UCP-2) has been proposed to play a prominent role in the regulation of energy balance. UCP-2 is a member of the emerging family of UCP homologues that are activated by β-adrenergic stimulation resulting in an increase in the thermogenesis as observed for UCP-1 in brown adipose tissues. The function of UCP-2 in lipid metabolism has not been clearly described.

Our previous results demonstrate that the expression

level of obese mRNA and its response to fasting are different among several bovine adipose tissues (Kim et al., 2000). In particular, the expression of the obese gene in intramuscular adipose tissues was very low and did not respond to fasting. This result may suggest that increasing intramuscular fat instead of subcutaneous fat would have a favorable effect in that a lower leptin level could result in greater food intake and further marbling. Further studies are required to assess the physiological importance in the regional differences of obese gene expression. Bovine leptin exhibits high homology in mammalian species. We have cloned the bovine leptin cDNA (Kim et al., 2000). The corresponding amino acid sequences show 86% homology with human leptin and 89% homology with mouse leptin (Tellam, 1996).

Although the action of leptin has been extensively studied in rodents and humans, its role in the food intake and energy metabolism is not well known in cattle. In this study, bovine recombinant leptin was produced in *E. coli* and purified, and effects of leptin on glucose or acetate transport were examined in 3T3-L1 adipocytes and in adipose tissues of Korean cattle. The effect of leptin on expression UCP-2 gene was examined by northern and RT-PCR analyses in 3T3-L1 cells.

MATERIALS AND METHODS

Production of leptin protein in *E. coli* and its purification

Leptin expression vector was constructed by using GST fusion system of pGEX vector. The Glutathione S-transferase (GST) gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. The pGEX vector is a *tac* promoter for chemically inducible, high-level expression, and an internal lac Iq gene for use in any *E. coli* host. The pGEX-4T-3 is derived from pGEX-2T and encodes the recognition sequence for site-specific cleavage by thrombin between the GST domain and the multiple cloning sites. The pBluescript II vector containing coding region of obese gene (0.46 kb) and pGEX-4T-3 vector were digested with EcoR I, and the purified obese cDNA and pGEX-4T-3 were ligated using T4 DNA ligase. The ligation product was transformed to JM105 cells. The correct ligation of bovine obese cDNA into pGEX expression vector was confirmed by EcoR I digestion and sequencing of junction region. The leptin expression vector was transformed into *E. coli* BL21 cells in order to express fusion protein.

The BL21 cells containing expression vector were inoculated onto LB/ampicillin (50 μg/ml) plate, and an ampicillin-resistant colony was cultured in 2×YT broth medium containing ampicillin. The 1ml of overnight culture was inoculated in 10 ml 2×YTA broth until cells reach mid-log growth at 30°C for 2 h. and expression of GST-leptin protein was induced with 0.1 mM IPTG. To check expression of GST-leptin protein, 1 ml of cells were collected at 0 h. 2 h. 4 h. and 6 h after incubation, and cell pellet was made by centrifugation. The cell pellet was resuspended in 800 μl of 5×protein loading buffer, and heated at 100°C, and loaded on a 10% SDS polyacrylamide gel electrophoresis. The gel was stained with coomassie brilliant blue to visualize the proteins.

For large culture, cells were cultured in 1 L of 2×YTA medium until cells reach mid-log growth at 30°C for 3 h, then expression of GST-leptin protein was induced with 0.1 mM IPTG for 4 h.

The cell pellet was resuspended in 2 ml of 1×PBS per 50 ml cell culture, and 100 μg/ml lysozyme, 1 mM PMSF and 1 mM EDTA were added. The cell suspension was incubated on ice for 30 min. The 0.2% Triton X-100 and 0.1% SDS were added, and cells were disrupted by sonication. The insoluble debris was removed by centrifugation, and supernatant containing soluble protein was collected for purification of the fusion protein. the cell lysate was combined with an 50% slurry of glutathione separose 4B resin in 1×PBS. The mixture was shaken gently for 2 h at 4°C. and the supernatant was removed with centrifugation. Unbounded proteins were removed by washing the resin with 10 bed volumes of 1×PBS and by centrifugation. Bound GST fusion protein was eluted from resin using 10mM reduced glutathione elution buffer with gentle agitation for 3 hours at 4°C. The tube was centrifuged at 3,000 rpm. and the supernatant was saved. To check purification of the fusion protein, aliquots of the supernatant and cell debris were analyzed by SDS-PAGE.

For cleavage of GST-leptin fusion protein, 10 units of thrombin solution were added into the eluate of purified fusion protein. The mixture was incubated with shaking for 12 h at room temperature. Once digestion is complete. GST was removed by extensive dialysis against 2,000 volumes of 1×PBS followed by batch purification on glutathione separose 4B.

Leptin effect on glucose transport in 3T3-L1 cells

Mouse 3T3-L1 preadipocyte cells were obtained from ATCC (CL-173), and the cells were propagated with Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% heat-inactivated bovine calf serum (Gibco BRL) and 50 μg/ml penicillin-50 μg/ml streptomycin (Sigma) at 37°C in 5% CO_2. The medium was changed every 2-3 days. The cells were never allowed to achieve confluence. When cells were arrived at 80% confluent, cells were either subcultured in 6-well polystyrene plates at a density of 4×10^3 cells/cm^3 with 3 ml

of media or stored in liquid nitrogen tank.

3T3-L1 cells were differentiated into adipocytes using DMEM supplemented with 10% FBS, 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-methylxanthine, and 1 µg/ml insulin. Three days later the differentiation media was removed, and replaced with DMEM supplemented with 10% FBS and 1 µg/ml insulin. Three days later cells were fed with DMEM supplemented with 10% FBS for 3 more days. Six days after replacement of differentiation medium, there were lipid droplets visible in the cells.

For glucose transport assay, 100 nM of leptin was treated at 24 h before the day of the experiment. Before glucose transport assay, the cells were preincubated with serum-free DMEM for 4 h. The cells were then washed twice with Krebs-Ringer phosphate buffer (KRB) and incubated in the same buffer for 30 min. To determine insulin-stimulated glucose transport, insulin (10 nM) was added, and the incubation was continued for another 30 min. Leptin was added back to the respective wells during the transport assay. The 1 µl [1-^{14}C] glucose was added and incubated for 10 min at room temperature. The medium was aspirated, and the cells were washed three times with 1×PBS and dissolved in 1.5 ml of 0.5 N NaOH. The cells were resuspended in 10 ml of scintillation cocktail, and radioactivity was measured using β-counter.

Leptin effect on glucose and acetate transport in adipose tissues of Korean cattle

Subcutaneous and intramuscular adipose tissues were isolated from bulls of Korean cattle (Hanwoo). The 0.2 g of subcutaneous adipose tissue were transferred to a 6 well polystylene plates supplied with 3 ml of KRB buffer and then chopped until the slice reached approximately 0.1 mm diameter explants. The intramuscular adipose tissue was collected in petridish. The 0.2 g of intramuscular adipose tissue was transferred to a 6 well polystylene plates supplied with 3 ml of KRB buffer and 0.1 mm diameter explants were prepared with chopping.

To measure glucose uptake, both subcutaneous and intramuscular adipose tissue slices were incubated with 1 µl [U-^{14}C]-glucose in 3 ml KRB buffer, 3 ml KRB buffer containing 2 µg/ml insulin, 3 ml KRB buffer containing 100 nM leptin, or 3 ml KRB buffer containing leptin plus insulin for 2 h at 37°C. Acetate transport assay was performed using [1-^{14}C]-acetic acid with same method as glucose uptake assay. At the end of the incubation, reactions were terminated by addition of 3 ml of 5% trichloroacetic acid (TCA), and explants were rinsed once with KRB buffer and twice with 1×PBS to remove unincorporated radioisotopes. The washed adipocyte slices were dissolved in 1.5 ml of 0.5 N NaOH and resuspended in 10 ml of scintillation cocktail, and radioactivity was measured with

β-counter.

Same experiments on glucose and acetate transport assays were done with recombinant human leptin purchased from Research Diagnostics Inc.

RT-PCR and northern analyses for expression of UCP-2 gene in 3T3-L1 adipocytes

Total RNA was prepared from 3T3-L1 cells by acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The first-stranded cDNAs were synthesized using 4 µg of total RNA and reverse transcriptase at 42°C for 1 h. The forward and reverse primers of UCP-2 cDNA were synthesized based on published nucleotides (Fleury et al., 1997): forward primer, 5'CAACAGCCACTGTGAAGTTC3'; reverse primer 5'GG ACCTTTACCACATCTGTAGG3'. The PCR amplification of UCP-2 cDNA was performed for 30 cycles (preheating at 94°C for 5 min; cycling at 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min; final elongation at 72°C for 10 min). Bovine beta-actin cDNA was also amplified by RT-PCR as a control. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide.

For northern analysis, twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the ^{32}P-labeled insert of the cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28 S and 18 S band, and the efficiency of transfer was monitored by ethidium bromide staining.

All data were analyzed by using the General Linear Model Procedures of SAS (1998). Comparison of treatment means was based on Duncan's multiple range test. A significant level of $p < 0.05$ was applied in all case.

RESULTS AND DISCUSSION

Role of bovine leptin in energy and lipid metabolism is not well known in cattle, although many studies on the action of leptin have been done in rodents and humans. In order to understand role of bovine leptin in lipid metabolism, bovine leptin was produced in *E. coli* and purified. Briefly, expression of GST-leptin fusion protein (42 kD) was induced from 2 h after IPTG addition (Figure 1A). The GST-leptin fusion protein was purified using glutathione sepharose 4B batch method (Figure 1B), and digested with thrombin protease. Cleavage of fusion protein (42 kD) into GST (26 kD) and leptin proteins (16 kD) was confirmed by SDS-PAGE (Figure 1C), and the thrombin-digested leptin proteins (16 kD) were purified and concentrated.

Role of leptin in glucose or acetate transport were
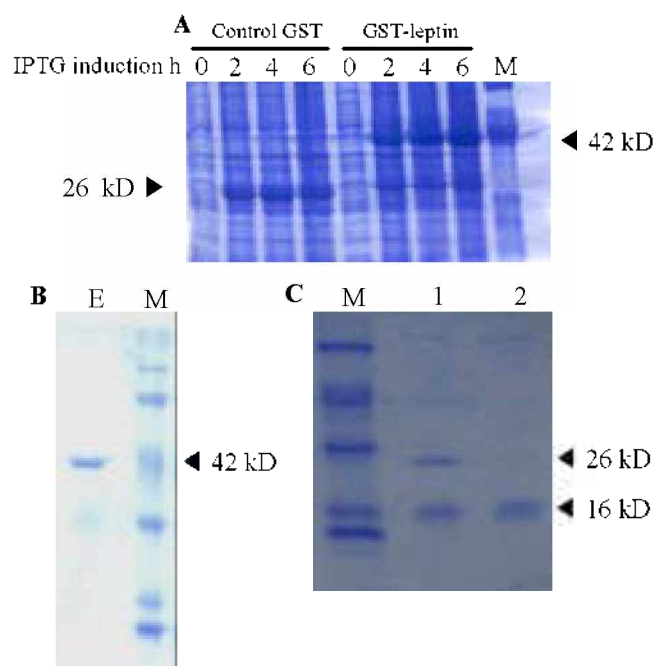
Figure 1. Expression and purification bovine leptin protein in *E. coli* cells. A. BL21 cells containing either control vector or GST-leptin expression vector were collected at 0, 2, 4 and 6 h after 0.1 mM IPTG induction. Cell lysates by boiling method were subjected to 10% SDS-PAGE. B. BL21 cells containing GST-leptin expression vector were collected after IPTG induction. GST-leptin fusion protein was purified with glutathione sepharose 4B resin. The eluate (E) was subjected to 10% SDS-PAGE. C. Eluted GST-leptin fusion protein was digested with thrombin protease and subjected to SDS-PAGE. Lane M; marker, lane 1; the thrombin-treatment protein, lane 2; Eluted protein after thrombin digestion.

examined in 3T3-L1 adipocyte cells and in adipose tissues of Korean cattle. The effect of leptin on expression UCP-2 gene was examined by northern and RT-PCR analyses in 3T3-L1 cells.

Leptin effect on glucose and acetate transport in 3T3-L1 cells

Effect of bovine recombinant leptin on glucose transport was examined in 3T3-L1 adipocyte cells. The 3T3-L1 adipocyte cells are known to be maintained in a preadipose state until 80% confluency. And the cells undergo a preadipose state to adipose-like conversion as they progress from rapidly diving to a confluent and contact inhibited state in high serum with dexamethasone, isobutyl dimethyl-xanthine and insulin. As shown in Figure 2, 70% confluent-preadipocytes were converted into differentiated adipocytes with lipid droplets visible when cells were cultured in the medium containing dexamethasone, isobutyl dimethyl-xanthine and insulin.

The differentiated cells were treated with either 10 nM insulin or 100 nM leptin, and glucose transport was

Preadipocyte   Differentiated adipocyte
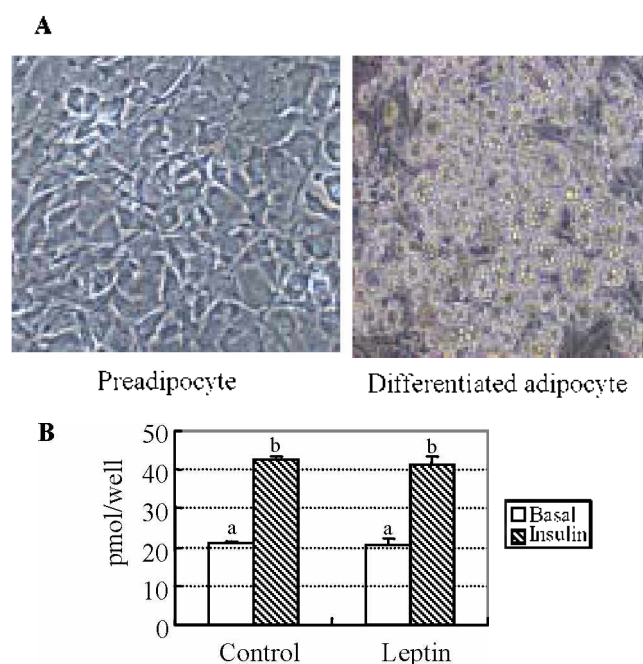
Figure 2. Leptin effect on glucose transport in 3T3-L1 adipocytes. A. 3T3-L1 preadipocytes (70% confluency) were grown. Two days after confluency, differentiation was induced with dexamethasone, isobutyl dimethylxanthine and insulin for two days. B. The differentiated cells were treated with either 10 nM insulin or 100 nM leptin, and glucose transport was determined using ^{14}C-glucose. Bar indicates SD (n=3). Different letters on the bars denote significant differences (p<0.05).

determined using [U-^{14}C]-glucose. Insulin stimulated glucose transport (p<0.05), but leptin had no effect on basal and insulin-stimulated glucose transport in 3T3-L1 adipocytes (Figure 2B).

Leptin effect on glucose and acetate transport in Hanwoo adipose tissues

Leptin effect on glucose and acetate transport was examined in Hanwoo adipose tissues. Subcutaneous and intramuscular adipose tissues were isolated from Hanwoo bull. The 0.2 g of explant slices were treated with either 2 μg/ml insulin or 100 nM leptin, and glucose transport was determined using [U-^{14}C]-glucose. Insulin stimulated glucose transport in both subcutaneous and intramuscular adipose tissues (p<0.05), but leptin did not affect glucose transport in both adipose tissues (Figure 3). Leptin effect on acetate transport was similar to results of glucose transport: insulin stimulated acetate transport in both subcutaneous and intramuscular adipose tissues (p<0.05), but leptin did not affect acetate transport in both adipose tissues (Figure 4). Intramuscular adipose exhibited lower rates of lipogenesis than subcutaneous adipose tissue (May et al., 1994). This trend was similar in this study. The present studies indicate that leptin does not affect glucose and acetate transport in both 3T3-L1 adipocytes and bovine
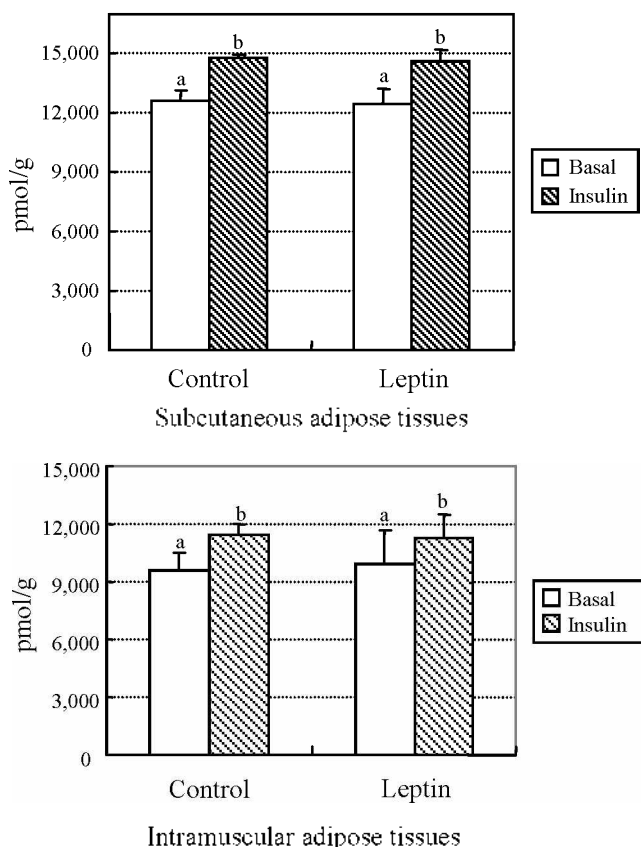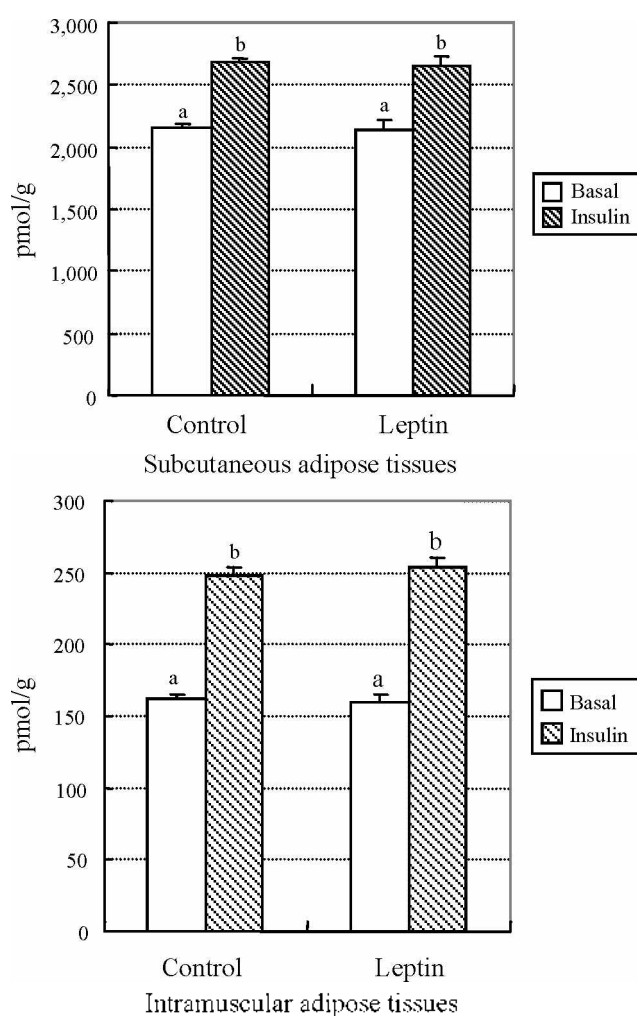
Figure 3. Leptin effect on glucose transport in Hanwoo adipose tissues. Subcutaneous (top) and intramuscular (bottom) adipose tissues were isolated from Hanwoo bull. The 200 mg of explant slices were treated with either 2 ug/ml insulin or 100nM leptin, and glucose transport was determined using ^{14}C-glucose. Bar indicates SD (n=3). Different letters on the bars denote significant differences (p<0.05).

adipose tissues. Same experiments were done with recombinant human leptin purchased from Research Diagnostics Inc. Human recombinant leptin also did not affect glucose and acetate transport in both 3T3-L1 cells and bovine adipose tissues. Leptin was shown to increase basal glucose transport and glycogen synthesis (Berti et al., 1997). In contrast, leptin had no effect on glucose transport, lipoprotein lipase activity, and insulin action in fat and muscle cells *in vitro* (Ranganathan et al., 1998). Other study demonstrates that glucose metabolism regulates leptin secretion in rat adipocytes (Mueller et al., 1998). Further research is needed to examine whether leptin affects lipid metabolism other than glucose and acetate transport in bovine adipose tissues.

Effect of leptin on expression of UCP-2 gene in adipocytes

UCP-2 belongs to a family of inner mitochondrial membrane proteins that, in general, function as carriers. The function(s) of UCP-2 have not yet been definitively
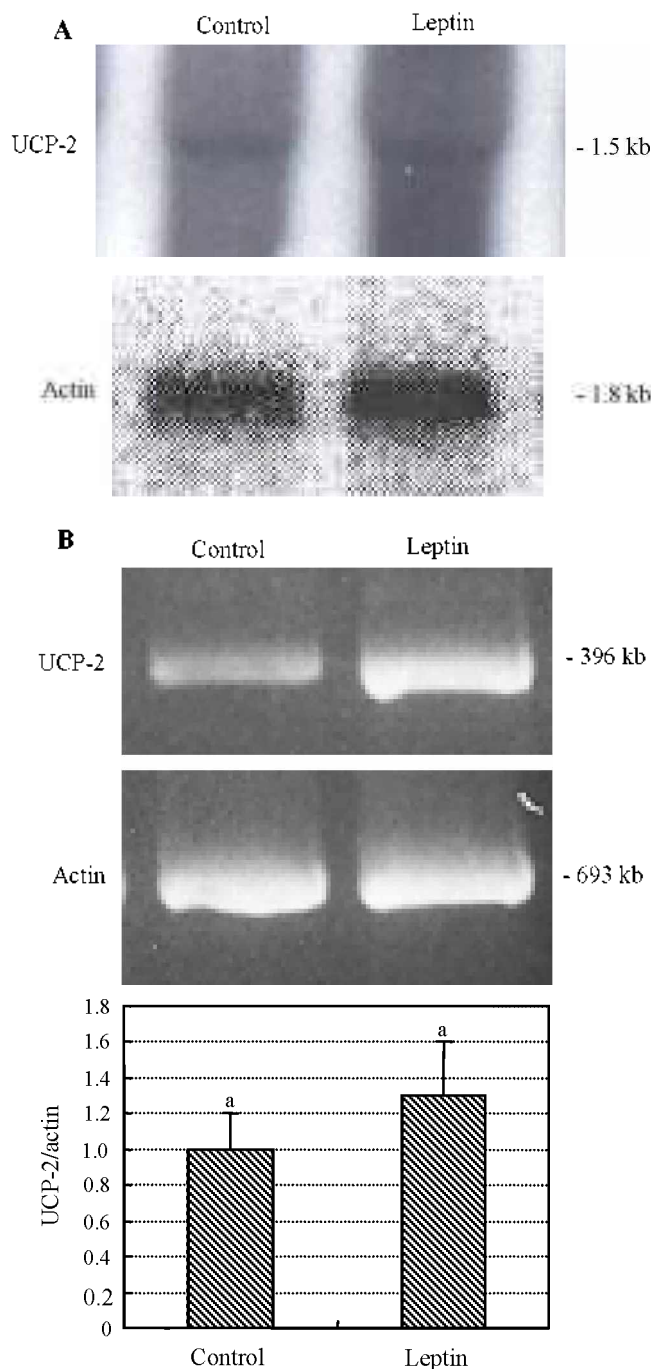

Figure 4. Leptin effect on acetate transport in Hanwoo adipose tissues. Subcutaneous (top) and intramuscular (bottom) adipose tissues were isolated from Hanwoo bull. The 200 mg of explant slices were treated with either 2 ug/ml insulin or 100 nM leptin, and acetate transport was determined using ^{14}C-acetic acid. Bar indicates SD (n=3). Different letters on the bars denote significant differences (p<0.05).

described. However, mounting evidence suggests that UCP-2 could act in multiple tissues as a regulator of lipid metabolism. UCP-2 is widely expressed in many tissues, especially in white adipose tissues, where it is postulated to play an important role in energy balance, body weight regulation, and thermoregulation (Fleury et al., 1997).

The effect of leptin on expression of uncoupling protein-2 (UCP-2) gene was examined by northern and RT-PCR analyses in adipocytes. Northern analysis showed slight increase in UCP-2 mRNA expression (Figure 5A). RT-PCR analysis showed about 30% increase of UCP-2 by leptin treatment (Figure 5B), although there was no statistical difference (p>0.05). High blood levels of leptin resulted in a greater than 10 fold increase in UCP-2 mRNA in white adipose tissues (Zhou et al., 1997). Other studies also report that leptin stimulated UCP-2 mRNA expression

A Control   Leptin

UCP-2   - 1.5 kb

Actin   - 1.8 kb

B Control   Leptin

UCP-2   - 396 kb

Actin   - 693 kb

Figure 5. Leptin effect on expression of uncoupling protein-2 gene (UCP-2) in adipocytes. A. The differentiated 3T3-L1 cells were treated with 100 nM leptin as described in Figure 2, and total RNA was extracted. The 20 µg of total RNA were analyzed by northern method using the ^{32}P-labeled UCP-2 cDNA probe. B. The first-stranded cDNA was synthesized by using RNA template and reverse transcriptase. UCP-2 cDNA was amplified by PCR with specific primers, and beta-actin was also amplified as a control. The band intensities of PCR products were quantitated by image analyzer. The ratio of UCP-2/actin mRNA was normalized to 1.0. The values are shown as mean and standard deviation of three experiments. There was no statistical difference between control and leptin treatment (p>0.05).

and Krebs cycle activity and inhibits lipid synthesis in

isolated rat white adipocytes (Ceddia et al., 2000). Centrally administered leptin has been shown to increase insulin-stimulated glucose utilization and to favor the expression of UCPs. Our data suggest that UCP-2 plays a role in the increased energy dissipation caused by leptin. However, in other study, no changes in UCP-2 mRNA were observed in white adipose tissues when circulating leptin concentrations are increased threefold by inhibiting the sympathetic nervous system over 18 h (Sivitz et al., 1999). This null effect is similar to studies in which feeding increases leptin but not UCP-2 in subcutaneous abdominal fat (Pinkney et al., 2000). Further study is required to understand function of UCP-2 in regulator of lipid metabolism.

In conclusion, bovine recombinant leptin did not affect glucose and acetate transport in both 3T3-L1 adipocytes and bovine adipose tissues, while it stimulates UCP-2 mRNA expression in 3T3-L1 cells.

ACKNOWLEDGMENT

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