HOKKAIDO UNIVERSITY

Title	Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes
Author(s)	Soliman, Mohamed; Kimura, Kazuhiro; Ahmed, Mohamed et al.
Citation	Domestic Animal Endocrinology, 33(4), 400-409 https://doi.org/10.1016/j.domaniend.2006.08.005
Issue Date	2007-11
Doc URL	https://hdl.handle.net/2115/30264
Type	journal article
File Information	DAE33-4.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in the cultured bovine adipocytes

Mohamed Soliman[1, 2], Kazuhiro Kimura[1]*, Mohamed Ahmed[1], Daisuke Yamaji[1], Yukiko Matsushita[1] Yuko Okamatsu-Ogura[1], Kennedy Makondo[1], and Masayuki Saito[1]

[1] Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2] Department of Biochemistry and Physiology, Faculty of Veterinary Medicine, Tanta University, Kafr El-Sheikh, Egypt.

RUNNING HEAD: Regulation of bovine leptin by fatty acids

*Corresponding author: Kazuhiro Kimura, Ph.D.

Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Tel: +81-11-706-5206; Fax: +81-11-757-0703

E-mail address: k-kimura@vetmed.hokudai.ac.jp

Abstract

Leptin is an adipose tissue-derived cytokine plays key roles in the regulation of food intake and energy expenditure. However, regulatory mechanisms of leptin gene expression are not fully elucidated in ruminants that utilize short-chain fatty acids (SCFA), known as volatile fatty acids, as principal energy sources. In this study, we determined effects of SCFA and long-chain fatty acids (LCFA) on leptin expression in bovine adipocytes. Bovine stromal vascular cells isolated from subcutaneous adipose tissue of Holstein cows were cultured to confluence and treated sequentially with dexamethasone and isobutylmethylxanthine for 2 days and insulin and troglitazone for 12 days to achieve full differentiation to adipocytes. The cells started to accumulate lipids 4 days after the onset of treatment, with increased mRNAs expression of leptin, as well as aP2, adiponectin, and PPAR-$\tilde{\gamma}$ Removal of fetal calf serum and reduction of glucose in the culture medium of differentiated adipocytes decreased leptin mRNA expression. Subsequent addition of acetate, butyrate, or propionate dose-dependently restored and rather increased leptin expression, while addition of LCFA suppressed it. The stimulatory effect of acetate was abolished by prior treatment of the cells with pertussis toxin and by addition of LCFA. Furthermore, cows fasted for 48h and fed thereafter, elaborate reduced and increased plasma leptin levels, respectively. Thus, these results suggest that plasma leptin levels in cows are inversely controlled at transcription by VFA and LCFA, and that the effects of SCFA possibly act through a G protein-coupled receptor for SCFA.

Key words: adiponectin, G protein-coupled receptor, leptin, MAP kinase, pertussis toxin, volatile fatty acids.

Introduction

Leptin, the product of the *ob* gene, is a plasma protein synthesized and secreted mainly by mature adipocytes, and regulates energy expenditure and food intake through its binding to specific receptors in the hypothalamus [1, 2]. Plasma concentrations of leptin are positively correlated with body fat content in various mammalian species including ruminants [3-11]. Therefore, circulating leptin is a suitable marker for adiposity in livestock animals and pets, and for assessment of long-term nutritional condition in wild animals.

It is, however, noteworthy to note that leptin gene expression in adipocytes and circulating leptin concentrations are subject to short-term regulation, especially by nutritional status [12-14]. For example, food deprivation decreases plasma leptin concentrations with decreased mRNA transcription in adipose tissue. These changes are closely associated with decreased plasma insulin and elevated plasma non-esterified fatty acid (NEFA) consisting of mainly long-chain fatty acids (LCFA). In contrast, food intake and injection of insulin to rodents or human increase leptin expression, probably due to enhancement of glucose utilization.

Similarly, underfeeding and refeeding cows causes a significant decrease and increase in plasma leptin, respectively [15]. Expression of leptin mRNA in subcutaneous adipose tissue from fasted heifer and cows is about half of that of fed control [16, 17]. It is also suggested that plasma leptin is positively related to plasma glucose and negatively related to plasma NEFA [15]. Moreover, hyperinsulinemic-euglycemic clamp study shows a progressive rise in the plasma concentration of leptin that reached maximal levels at 24h in both cows in late pregnancy and early lactation, although there is no apparent increase

in leptin mRNA in the adipose tissue from cows in early lactation [18]. The study also shows that basal NEFA levels are markedly higher in cows in early lactation than cows in late pregnancy and decreased to the levels of late pregnancy by infusion of insulin to cows in early lactation [18]. However, in another study of Holstein cows, jugular infusion of lipid increased plasma leptin concentration, under mechanisms independent of insulin or IGF-1, unlike the stimulatory effect of glucose which related to insulin-mediated glucose metabolism [19].

Recently, it has been shown that short-chain fatty acids (SCFA) such as propionate stimulate leptin production in mouse adipocytes [20], although it is well described that medium-chain fatty acids (MCFA) and LCFA inhibit leptin expression and production in rat adipocytes and differentiated mouse 3T3-L1 cells [21-25]. Similarly, acetate and butyrate stimulate leptin expression in bovine anterior pituitary cells [26]. However, MCFA and LCFA such as caprylate and linoleate, respectively, increase leptin expression in bovine mammary epithelial cells [27, 28]. In addition, acetate and butyrate fail to influence leptin expression in bovine mammary epithelial cells [28]. Thus, these findings indicate that some conflicts in the regulation of leptin expression by fatty acids depending on the cell types.

As well known, ruminants depend mainly on the production of SCFA, known as volatile fatty acids (VFA) by the act of bacterial fermentation in the rumen as principle energy source. Therefore, to establish the role of fatty acids in the regulation of leptin expression in adipocytes from cows, we examined the direct effect of various fatty acids on leptin expression in cultured bovine subcutaneous adipocytes.

Materials and Methods

Materials

Dulbecco's modified Eagles's medium (DMEM, DMEM/F12 and DMEM without glucose), Hank's balanced salt solution, bovine serum albumin (BSA), bovine insulin, isobutylmethylxanthine (IBMX), and various chain fatty acids (sodium form) were bought from Sigma-Aldrich Fine Chemical (St. Louis, MO, USA). Fetal calf serum (FCS) was from Trace Scientific Ltd. (Melbourne, Australia). Collagenase, dexamethasone and troglitazone were purchased from Wako Pure Chemicals Co. (Osaka, Japan), while pertussis toxin (PTX) and a MAPK kinase (MEK) inhibitor, PD 98059, were from List Biological Laboratories (Campell, CA, USA) and Biomol Research Laboratories (Plymouth, PA, USA), respectively. Polyclonal antibodies against phospho-specific p44/p42 MAPK (Thr202/Tyr204) and total p44/p42 MAPK were from Cell Signaling Technology (Beverly, MA, USA).

Fasting and refeeding experiment

The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University. Six Holstein cows weighting 450-550 kg were housed in individual stalls in an animal facility of Hokkaido University with free access to water and trace mineral block, and given a mixture of forage (orchard-grass hay, alfalfa hay cube, corn silage) and concentrates. The feed was completely withdrawn for 48 h (fasting period) and given thereafter (re-feeding). Blood was collected at 0, 24, 48, 49, 50, 51, 52, and 54 h after the onset of food deprivation. Plasma was separated by centrifugation and stored at -20 ^{0}C until

measurement of plasma leptin levels with a multi-species leptin RIA kit (LINCO Research Inc., St. Charles. MO, USA).

Isolation of stromal vascular cells from bovine subcutaneous adipose tissue

Subcutaneous adipose tissue was obtained from three Holstein cows (2-3 years old) and dissected into small pieces in Hank's balanced salt solution containing 2 mg/ml collagenase and 0.1% BSA in sterile 50 ml plastic tube. Following digestion at 37 °C for 90 min with gentle shaking, the solution was filtered through sterile nylon mesh with 80μm pores. The filtrate was centrifuged at 1,000 x g for 5 min at room temperature. The collected cells mainly consisting of stromal vascular cells were treated with an erythrocyte lysis buffer (154 mM NH_4CL, 10 mM $KHCO_3$, and 1 mM EDTA) for 5 min and washed twice with DMEM containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in DMEM (5.5 mM glucose) containing 10% FCS in collagen-coated dishes and media were changed every 2 days. The cells between 3rd and 5th passages were used for the experiments

Differentiation of stromal vascular cells to adipocytes and treatments with various fatty acids

When stromal vascular cells reached confluency (referred to day 0), the cells were further cultured in DMEM/F12 (17.5 mM glucose) containing 1.5% FCS, 0.5 mM IBMX, 1μM dexamethasone, and 10 μg/ml insulin for 2 days, and subsequent in fresh DMEM/F12 containing 1.5% FCS, 10 μM troglitazone and 10 μg/ml insulin every 2 days for 10 or 12 days to achieve cell differentiation to adipocytes. At day 12 or 14, the cells were cultured in DMEM without glucose, containing 0.1% BSA, but not FCS, for 24 h (starvation), and further cultured in DMEM with 2.5 mM glucose containing 0.1% BSA

(control) in the absence or presence of increasing concentrations of acetate, butyrate, propionate, caprylate, stearate and linoleate.

In some experiments, cells were treated with either 100 ng/ml PTX or 10 μM PD98059 from 2 h and 30 min, respectively, before the addition of acetate.

Oil red O staining

Cultured stromal vascular cells before and after induction of adipogenic differentiation were fixed with 10% formalin in isotonic phosphate buffer for 1 h, stained with 0.5% oil red O in 60% isopropyl alcohol for 1 h and rinsed extensively with water. After visualization, oil red O stained lipid droplets were extracted by 1 ml of absolute isopropyl alcohol and quantified by measuring absorbance at 540 nm.

RT-PCR

Total cellular RNA was isolated from cultured adipocytes and bovine subcutaneous adipose tissue by the guanidine-isothiocyanate method using TRIzol reagent (Gibco BRL, Rochville, MD, USA).

RNA (1 μg) was treated at 72 ^{0}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 ^{0}C for 1 h. After heating at 94 ^{0}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 specific for respective genes in a total volume of 50 μl. The primer pairs and PCR condition of bovine peroxisome proliferator-activated receptor-γ (PPAR-γ), bovine glucose transporter 4 (GLUT4) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) are summarized in Table 1. After electrophoresis in 1.5% agarose gel, the PCR

products were stained with ethidium bromide.

The mRNA of bovine leptin, bovine adiponectin, bovine adipocyte-type fatty acid binding protein (aP2) and bovine G3PDH were also amplified from total RNA of bovine subcutaneous adipose tissue by RT-PCR as described above (see Table 1), and subcloned into pGEM®-T Easy vector (Promega; Madison, WI, U.S.A). The nucleotide sequence of each cDNA was confirmed and the cDNAs were used as probes for Northern bolt.

Northern blot

Total RNA (18-20 μg) was resolved on 1% agarose-formaldehyde gel, transferred onto a nylon membrane (Hybond-N^+; Amersham Pharmaceutical Biotec. Buckinghamshire, UK) and cross−linked under UV light for 2 min. Both prehybridization and hybridization were performed at 65°C for 2 h and overnight, respectively, in a buffer containing 7% SDS, 0.5 M Church's phosphate buffer, pH 7.2, 1 mM EDTA and 0.5 mg/ml salmon sperm DNA. After prehybridization, the membrane was sequentially hybridized with a cDNA probe encoding bovine leptin, bovine adiponectin, bovine aP2 and/or bovine G3PDH as the internal control for loading. The probe was labeled with [α-^{32}P]dCTP using the Megaprime™ DNA labeling systems (Amersham) according to the instructions provided. After hybridization, the membrane was stringently washed for 20 min twice with 2x SSC and 0.1% SDS, and once with 0.1 x SSC and 0.1% SDS at 65°C before exposure onto a phospho-imaging plate overnight. Detection and quantification of the hybridization signals were carried out using a phosphoimage analyzer (BAS 2500, FUJIFILM, Tokyo, Japan).

Western blot

Following the experimental treatments, cells were washed with ice-cold PBS and scraped in ice-cold lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 5mM EDTA, 20 mM sodium fluoride, 10 mM sodium pyrophosphate, 2mM sodium vanadate, 1% Nonidet 40 and protease inhibitor cocktail (Complete^{TM}; Boehringer Mannheim, Mannheim, Germany)]. Harvested cells were incubated on ice for 30 min followed by centrifugation at 12,000 g for 20 min at 4 ^{0}C to obtain cell lysate. Aliquot of cell lysate (20 µg protein) was resolved by SDS-PAGE (10% gel)under reducing conditions and proteins were electroblotted onto PVDF membrane (Immobilon^{TM}, Millipore, Bedford, MA, USA). The membrane was blocked for 2 h at room temperature in 5% (w/v) skimmed milk in 20mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20, followed by incubation with primary antibody overnight at 4 ^{0}C. The membrane was washed 3 times with 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20, and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (Zymed laboratories, Inc. South San Francisco, CA, USA) for 1 h at room temperature. Visualization was performed using enhanced chemiluminescence detection system (Amersham) according to the manufactures' instruction. Intensities of immunoreactive bands were analyzed densitometrically using NIH Image program (http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Results are expressed as means ± S.E. of independent experiments. Statistical analysis was done using ANOVA and Fischer's post-hoc test, with $p < 0.05$ being considered as statistically significant.

Results

To confirm alteration of circulating leptin concentrations by changes in nutritional and physiological conditions such as feeding-fasting cycles in ruminants, we measured the plasma leptin obtained from cows whose food was withdrawn for 48 h and given thereafter. As shown in Fig. 1, plasma leptin levels were significantly decreased during the food deprivation, increased at 3 h after the onset of re-feeding and returned to basal levels at 6 h.

To test the effect of nutritional factors on leptin expression in bovine adipocytes, we first established primary culture of bovine adipocytes. Stromal vascular cells were isolated from bovine subcutaneous adipose tissue and cultured to confluence. The cells were then treated sequentially with dexamethasone and IBMX for 2 days and with insulin and troglitazone for 10 or 12 days to achieve differentiation to adipocytes. The cells started to accumulate lipids 4 days after the onset of treatments and the lipid content increased daily (data not shown). In proportion to the increase of lipid accumulation, expression of adipogenesis-related genes such as adipocyte-type fatty acid binding protein (aP2) and peroxisome proliferator-activated receptor-γ (PPAR-γ) increased (Fig. 2). Expression of insulin-regulated glucose transporter (GLUT4) mRNA also increased, a rather retarded time-point than aP2. Similarly, expression of two adipocytokines, leptin and adiponectin, started to increase from day 6 after the onset of treatments and further increased thereafter. There were three adiponectin transcripts, consisting of approximately 2.5 kb predominant transcript and two minor transcripts of approximately 1.8 kb and 1.2 kb.

To examine the effect of glucose, SCFA and MCFA on leptin expression in bovine

adipocytes, stromal vascular cells were differentiated to adipocytes, followed by withdrawal of glucose and FCS and subsequent refeeding glucose in combination with SCFA or caprylate. Removal of glucose (17.5 mM) and FCS clearly decreased leptin mRNA expression (Fig.3). Re-feeding of glucose (2.5 mM), which is within physiological range in cows, partially but significantly restored leptin expression. Addition of any one of SCFA（acetate, butyrate and propionate）significantly enhanced leptin expression. However, addition of caprylate suppressed leptin expression.

To determine possible signaling mechanism(s) of the SCFA action on leptin expression, bovine adipocytes were treated with pertussis toxin (PTX) to inactivate Gi/o proteins before addition of acetate. As shown in Fig.4, the treatment with PTX reduced basal and acetate-induced leptin expression. Moreover, addition of acetate time-dependently increased phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) (Fig. 5A) that was also abrogated by prior treatment with PTX (Fig. 5B). To test the involvement of MAPK pathway in the acetate-induced upregulation of leptin expression, the cells were also treated with PD98059, a MAPK kinase inhibitor. However, it failed to inhibit the acetate-induced increase in leptin expression (data not shown).

To examine the effect of LCFA on leptin expression in bovine adipocytes, the cells were treated with increasing concentrations of stearate (saturated fatty acid) and linoleate (unsaturated fatty acid). Addition of either fatty acid dose-dependently reduced leptin expression (Fig. 6A). Similarly, treatments with palmitate, oleate, linolenate, or arachidonate (0.3mM each) suppressed leptin expression (data not shown). Furthermore, addition of stearate or linoleate to the cells cultured with acetate abrogated the stimulatory effects of acetate (Fig. 6B).

When cells were treated with insulin, leptin expression was increased as much as acetate alone and there was no synergistic effect between them (Fig.7). Addition of stearate abrogated the increase in leptin expression by insulin and acetate (Fig.7), while linoleate was less effective (Fig.7) as seen in Fig. 6B.

Discussion

The present data shows that stromal vascular cells from subcutaneous adipose tissue of Holstein cows can be differentiated to adipocytes with concomitant increase in expression of adipogenesis-related genes (aP2, PPAR-γ and GLUT4) and adipocytokine genes (leptin and adiponectin). Of these, PPAR-γ was constitutively expressed as reported previously in bovine perirenal adipocytes [29] and possibly enhanced the transcription of a number of genes that regulates the conversion of preadipocytes into mature adipocytes following the treatment with PPAR-γ agonist, troglitazone. Abundant mRNA expression of aP2 related to lipid was also the feature of mature bovine perirenal adipocytes [30], while that of insulin-regulated glucose transporter (GLUT4) was not found in bovine intramuscular adipocytes [31].

Bovine adiponectin has been isolated from serum and cloned from adipose tissue [32]. This study is the first to show adiponectin expression during bovine adipocytes differentiation. The major adiponectin transcript in bovine adipocytes is approximately 2.5kb and there are at least two additional transcripts, like swine and rat adiponectin genes [33-35], whereas mouse adipose tissue expresses a single transcript [33].

The linkage analyses between dominant character and gene polymorphism or biochemical markers are useful methods for selective breeding of dairy cows and beef cattle. For example, the *obese* gene (leptin) polymorphism is highly associated with milk and protein yield in dairy cows [36]. Moreover, plasma concentration of leptin is significantly associated with carcass composition (marbling, back fat depth, and kidney, pelvic and heart fat) in beef cattle [37]. Since genetic variation of the adiponectin gene is reported to predispose human to insulin resistance and type 2 diabetes [38, 39] and

bovine adiponectin purified from serum exhibited comparable biological activities to that of other species [40], analysis on polymorphism(s) of bovine adiponectin gene may be of interest in elucidating its relation with fat-related traits.

The present results demonstrate that expression of leptin gene in bovine adipocytes was greatly influenced by nutritional factors including glucose, fatty acids and/or insulin. Among these, short chain volatile fatty acids, a principle energy source in ruminants, within their physiological levels (total ~1mM) [41], stimulated leptin expression. Since the stimulatory effect of acetate and its activation of p44/p42 MPPK were abolished by prior treatment of the cells with pertussis toxin (PTX), an inhibitor of Gi/Go proteins [42], it is suggested that SCFA might act on the cells through a G protein-coupled receptor for SCFA (GPR41), a counterpart of which in ruminants has not been cloned yet. Indeed, effective doses of SCFA in bovine adipocytes are comparable with those of mouse adipocytes and GPR41 transfected cells [20]. Further, there is a cell type specific difference in the leptin expression by addition of SCFA [26, 28]. The difference between bovine mammary epithelial cells and anterior pituitary cells may be explained possibly by different expression of GPR41-like receptor.

In contrast to SCFA, MCFA and LCFA either saturated or unsaturated inhibited leptin expression in bovine adipocytes, in line with those reported in rat and mouse adipocytes [21-25], although they increase leptin expression in bovine mammary epithelial cells [27, 28]. The mechanism of the inhibitory effect of LCFA in bovine adipocytes is still obscure, but one plausible explanation is that LCFA acts as a PPAR-γ agonist that has been shown to inhibit leptin expression and secretion in adipocytes [43]. MCFA such as caprylate might also suppress leptin expression at transcription, because it

inhibits adipogenesis through the interference with transcription factors such as SREBP-1c [22].

Expression of leptin gene in bovine adipocytes was increased by treatment of insulin to the similar extent with that of acetate. However, there is no synergistic effect between acetate and insulin, while it is reported that in mouse adipocytes propionate and insulin synergistically enhanced leptin production [20]. This difference is interesting in considering the fact that the rise of plasma insulin in cows did not influence plasma leptin levels [44], unless hyperinsulinemic-euglycemic clamp tests were performed [18]. Actually, single injection of insulin did not affect plasma leptin levels in cows (Soliman et al. unpublished results) and a postprandial increase in plasma leptin is usually not seen in ruminants [45].

In the presence of LCFA, especially stearate, the stimulatory effects of acetate either in the presence or absence of insulin was abrogated, suggesting that LCFA predominantly influences leptin expression. This notion supports the findings that fasted cows being expected to have raised LCFA as NEFA in plasma, have reduced plasma leptin levels, whereas after refeeding, being expected to increased SCFA as VFA concentrations increased leptin, in line with previous reports in heifers and cattle [15, 16]. Moreover, plasma leptin concentration is reduced by ~50% after parturition and remains depressed during lactation, but doubles after elimination of the negative energy balance of early lactation [45].

In conclusion, leptin expression in bovine adipocytes is upregulated by SCFA through the stimulation of G-protein coupled receptors and downregulated by LCFA.

Acknowledgements

This work was supported in part by a Grant-in-Aid of the investigation on the etiology and prevention of bovine mastitis (No. 2210) by the Ministry of Agriculture, Forestry and Fisheries of Japan, and by grants from the Ministry of Education, Science and Culture of Japan, and from the Japanese Society for Animal Cytokine Research. The work was also supported in part by JSPS postdoctoral fellowships for foreign researchers to M.S. and K.M. and for young scientist to D.Y.

References

1. Friedman JM, Halaas JL. Leptin and the regulation of body weight in animals. Nature 1998; 395: 763-770.

2. Houseknecht KL, Baile CA, Matteri RL, Spurlock ME. The biology of leptin: a review. J Anim Sci 1998; 76: 1405-1420.

3. Ehrhardt RA,Slepetis RM, Siegal-Willott J, Van Amburgh ME, Bell AW, Boisclar YR. Development of a specific radioimmunoassay to measure physiological changes of circulating leptin in cattle, and sheep. J Endocrinol 2000; 166, 519-528.

4. Maffei M, Halaas J, Ravussin E, Pratler RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM. Leptin levels in human and rodent – measurement of blood leptin and *ob* RNA in obese and weight-reduced subjects. Nat Med 1995; 1: 1155-1161.

5. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. New Engl J Med 1996; 334: 292-295.

6. Appleton DJ, Rand JS, Sunvold GD. Blood leptin concentrations in cats: reference range, effect of weight gain and relationship with adiposity as measured by dual energy X-ray absorptiometry. J Feline Med Surg 2000; 2: 191-199.

7. Backus RC, Havel PJ, Gingerich RL, Rogers QR. Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats. Am J Vet Res 2000; 61: 796-801.

8. Ishioka K, Soliman MM, Sagawa M, Nakadomo F, Shibata H, Honjoh T, Hashimoto A,

Kimura K, Saito, M. Experimental and clinical studies on blood leptin in obese dogs. J Vet Med Sci 2002; 64: 349-353.

9. Sagawa MM, Nakadomo F, Honjoh T, Ishioka K, Saito M. Correlation between blood leptin concentration and body fat content in dogs. Am J Vet Res 2002; 63: 7-10.

10. Shibata H, Sasaki N, Honjoh T, Ohishi I, Takiguchi M, Ishioka K, Ahmed M, Soliman M, Kimura K, Saito M. Feline leptin: Immunogenic and biological activities of the recombinant protein, and its measurement by ELISA. J Vet Med Sci 2003; 65: 1207-1211.

11. Shibata H, Akahane R, Honjoh T, Asano M, Mominoki K, Fujii K, Suzuki M. Ohtaishi N, Ishioka K, Ahmed M, Soliman M, Kimura K, Saito M. Seasonal changes in serum leptin of the feral Raccoon (*Procyon lotor*) determined by canine leptin-specific ELISA. J Exp Zool 2005; 303A, 527-533.

12. Coleman RA, Hermann TS. Nutritional regulation of leptin in humans. Diabetologia 1999; 42 639-646.

13. Rayner DV, Trayhurn P. Regulation of leptin production: sympathetic nervous system interactions. J Mol Med 2001; 79: 8-20.

14. Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J. Transient increase in obese gene expression after food intake or insulin administration. Nature 1995; 377: 527-529.

15. Amstalden M, Garcia MR, Williams SW, Stanko RL, Nizielski SE, Morrison CD, Keisler DH, Williams GL. Leptin gene expression, circulating leptin, and luteinizing hormone pulsatility are acutely responsive to short-term fasting in prepubertal heifers: relationship to circulating insulin and insulin-like growth factor I. Biol Reprod 2000; 63:

127-133.

16. Tsuchiya T, Nagano Y, Ozawa A, Matsumoto M, Sugahara K, Kubo T, Kato H. Decrease of the obese gene expression in bovine subcutaneous adipose tissue by fasting. Biosci Biotechnol Biochem 1998; 62: 2068-2069.

17. Delavaud C, Ferlay A, Faulconnier Y, Bocquier F, Kann G, Chilliard Y. Plasma leptin concentration in adult cattle: effects of breed, adiposity, feeding level, and meal intake. J Anim Sci 2002; 80, 1317-1328.

18. Leury BJ, Baumgard LH, Block SS, Segoale N, Ehrhardt RA, Rhoads RP, Bauman DE, Bell AW, Boisclar YR. Effect of insulin and growth hormone on plasma leptin in periparturient dairy cows. Am J Physiol Regul Integr Comp Physiol 2003; 285, R1107-1115.

19. Chelikani PK, Keisler DH, Kennelly JJ. Response of plasma leptin concentration to jugular infusion of glucose or lipid is dependent on the stage of lactation of Holstein cows. J Nutr 2003; 133, 4163-4171.

20. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci USA 2004; 101:1045-1050.

21. Cammisotto PG, Gelinas Y, Deshaies Y, Bukowiecki LJ. Regulation of leptin secretion from white adipocytes by free fatty acids. Am J Physiol Endocrinol Metab 2003; 285: E521-E526.

22. Han J, Farmer SR, Kirkland JL, Corkey BE, Yoon R, Pirtskhalava T, Ido Y, Guo W. Octanoate attenuates adipogenesis in 3T3-L1 preadipocytes. J Nutr 2002; 132:904-910.

23. Han J, Hamilton JA, Kirkland JL, Corkey BE, Guo W. Medium-chain oil reduces fat

mass and down-regulates expression of adipogenic genes in rats. Obes Res 2003; 11: 734-744.

24. Rentsch J, Chiesi M. Regulation of *ob* gene mRNA levels in cultured adipocytes. FEBS Lett 1996; 379: 55-59.

25. Shintani M, Nishimura H, Yonemitsu S, Masuzaki H, Ogawa Y, Hosoda K, Inoue G, Yoshimasa Y, Nakao K. Downregulation of leptin by free fatty acids in rat adipocytes: effects of triacsin C, palmitate, and 2-bromopalmitate. Metabolism 2000; 49: 326-330.

26. Yonekura S, Senoo T, Kobayashi Y, Yonezawa T, Katoh K, Obara Y. Effect of acetate and butyrate on the expression of leptin and short-form leptin receptor in bovine and rat anterior pituitary cells. Gen Comp Endocrinol 2003; 133: 165-172.

27. Yonezawa T, Yonekura S, Kobayashi Y, Hagino A, Katoh K, Obara Y. Effects of long-chain fatty acids on cytosolic triacylglycerol accumulation and lipid droplet formation in primary cultured bovine mammary epithelial cells. J Dairy Sci 2004; 87:2527-2534.

28. Yonezawa T, Yonekura S, Sanosaka M, Hagino A, Katoh K, Obara Y. Octanoate stimulates cytosolic triacylglycerol accumulation and CD36 mRNA expression but inhibits acetyl coenzyme A carboxylase activity in primary cultured bovine mammary epithelial cells. J Dairy Res 2004; 71:398-404.

29. Ohyama M, Matsuda K, Torii S, Matsui T, Yano H, Kawada T, Ishihara T. The interaction between vitamin A and thiazolidinedion on bovine adipocyte differentiation in primary culture. J Anim Sci 1998; 76: 61-65.

30. Oishi M, Taniguchi Y, Nishimura K, Yamada T, Sasaki Y. Characterization of gene expression in bovine adipose tissue before and after fattening. Anim. Genet. 2000; 31:

166-170.

31. Aso H, Abe H, Nakajima I, Ozutsumi K, Yamaguchi T, Takamori Y, Kodama A, Hoshino FB, Takano S. A preadipocyte clonal line from bovine intramuscular adipose tissue: nonexpression of GLUT-4 protein during adipocyte differentiation. Biochem Biophys Res Commun 1995; 213: 369-375.

32. Sato C, Yasukawa Z, Honda N, Matsuda T, Kitajima K. Identification and adipocyte differentiation-dependent expression of the unique disialic acid residue in an adipose tissue-specific glycoprotein, adipo Q. J Biol Chem 2001; 276: 28849-28856.

33. Hu E, Liang P, Spiegelman BM. Adipo Q is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 1996; 271: 10697-10703.

34. Jacobi SK, Ajuwon KM, Weber TE, Kuske JL, Dyer CJ, Spurlock ME. Cloning and expression of porcine adiponectin, and its relationship to adiposity, lipogenesis and the acute phase response. J Endocrinol 2004; 182: 133-144.

35. Bensaid M, Gary-Bobo M, Esclangon A, Maffrand JP, Le Fur G, Oury-Donat F, Soubrie P. The cannabinoid CB1 receptor antagonist SR141716 increases Acrp30 mRNA expression in adipose tissue of obese *fa/fa* rats and in cultured adipocyte cells. Mol Pharmacol. 2003; 63: 908-914.

36. Buchanan FC, van Kessel AG, Waldner C, Christensen DA, Laarveld B, Shumutz SM. An association between a leptin single nucleotide polymorphism and milk and protein yield. J Dairy Sci 2003; 86: 3164-3166.

37. Geary TW, McFadin EL, MacNeil MD, Grings EE, Short RE, Funston RN, Keisler DH. Leptin as a predictor of carcass composition in beef cattle. J Anim Sci 2003; 81: 1-8.

38. Hara K, Boutin P, Mori Y, Tobe K, Dina C, Yasuda K, Yamauchi T, Otabe S, Okada

T, Eto K, Kadowaki H, Hagura R, Akanuma Y, Yazaki Y, Nagai R, Taniyama M, Matsubara K, Yoda M, Nakano Y, Tomita M, Kimura S, Ito C, Froguel P, Kadowaki T. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes 2002; 51: 536-540.

39. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 2000; 20: 1595-1599.

40. Wang Y, Lu G, Wong WPS, Vliegenthart JFG, Gerwig GJ, Lam KSL, Cooper GJS, Xu A. Proteomic and functional characterization of endogenous adiponectin purified from fetal bovine serum. Proteomics 2004; 4: 3933-3942.

41. Quigley III JD, Smith ZP, Heitmann RN. Changes in plasma volatile fatty acids in response to weaning and feed intake in young calves. J Dairy Sci 1991; 74: 258-263.

42. Blaukat A, Barac A, Cross MJ, Offermanns S, Dikic I. G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Gαq and Gαi signals. Mol Cell Biol 2000; 20: 6837-6848.

43. Considine RV. Regulation of leptin production. Rev Endocrinol Metab Disord 2001; 2: 357-363.

44. Soliman M, Ishioka K, Kushibiki S, Kimura K, Saito M. Plasma leptin responses to lipopolysaccharide and tumor necrosis factor-α in cows. Jpn. J. Vet. Res. 2002; 50: 107-114.

45. Block SS, Butler WR, Ehrhardt RA, Bell AW, Van Amburgh ME, Boisclar YR.

Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. J Endocrinol 2001; 171: 339-348.

Table 1. PCR primers

PPAR-γ (Y12420, +1388 to + 1601, 214bp) [55 ^{0}C, 60 sec, 30 cycles]
Forward primer: 5'-CGCACTGGAATTAGATGACAGC-3'
Reverse primer: 5'-CACAATCTGTCTGAGGTCTGTC-3'

GLUT4 (AY458600, +14 to +341, 328 bp) [60 ^{0}C, 90 sec, 30 cycles]
Forward primer: 5'-CCGTCGGGCTTCCAACAGAT-3'
Reverse primer: 5'-CCTCTTCCTTCCCAGCCACT-3'

G3PDH (U85042, +490 to +934 and additional nucleotides underlined, 453bp) [59 ^{0}C, 30 sec, 25 cycles]
Forward primer: 5'-ACCACTGTCCACGCCATCAC-3'
Reverse primer: 5'-TCCACCACCCTGTTTGCTGTA-3'

Leptin (AB003143, +80 to +365, 286bp) [61 ^{0}C, 70 sec, 30 cycles]
Forward primer: 5'-CGCAGTCCGTCTCCTCCAAACAG -3'
Reverse primer: 5'-TCGGTGGAGTAGAGGGAAGC-3'

Adiponectin (NM_174742, +372 to +731, 360 bp) [60 ^{0}C, 75 sec, 30 cycles]
Forward primer: 5'-GCCGCTTATGTGTATCGCTCAG-3'
Reverse primer: 5'-TGCATAGACCCCATTGTGATTT-3'

aP2 (X89244, +78 to +525, 448 bp) [58 ^{0}C, 60 sec, 30 cycles]
Forward primer: 5'-AGGTACCTGGAAACTTGTCT-3'
Reverse primer: 5'-GACAACGTATCCAGCAGAA-3'

In the first parenthesis, GenBank accession number of bovine genes, corresponding region of PCR amplification and size of PCR product are shown. In the second parenthesis, annealing temperature, time and number of PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94 $^{\circ}$C, 30 sec and 72 $^{\circ}$C, 60 sec, respectively.

Figure legends

Fig. 1. Effect of fasting and re-feeding on plasma leptin levels in Holstein cows.

Cows were deprived of food for 48h and fed thereafter. Blood samples were collected at 0, 24, 48, 49(1), 50(2), 51(3), 52(4) 54(6) h after the onset of food deprivation. Numbers in the parenthesis indicate the time after re-feeding. Plasma leptin levels were measured using a RIA kit. Values are means ± SE for 6 cows. * $p< 0.05$ vs. 0 h.

Fig. 2. Expression of adipogenesis-related genes during differentiation of stromal vascular cells.

Stromal vascular cells were isolated from bovine subcutaneous adipose tissue and cultured as described in the Materials and Methods. Total RNA was extracted from cells at different time points. (A) mRNA expression of leptin, adiponectin, aP2 and G3PDH were analyzed by Northern blot and (B) those of GLUT4, PPAR-γ and G3PDH by RT-PCR. Shown are the representative results of three separate experiments.

Fig. 3. Effect of short and medium chain fatty acids on leptin mRNA expression in differentiated adipocytes.

Confluent stromal vascular cells were cultured under the condition of adipogenic differentiation for 12 days (Dif: differentiated) and further cultured in DMEM without glucose and FCS for 24 h (Sta: starvation). The cells were then cultured in DMEM with 2.5 mM glucose containing 0.1% BSA in the absence (Co: control) or presence of increasing concentrations of acetate (Ac), butyrate (Bu), propionate (Pr), or caprylate (Cap) for additional 24 h. Total RNA was extracted from the cells and Northern blot (20μg/lane) was performed to determine expression of leptin and G3PDH genes. Representative blots and densitometric analyses of bands expressed relative to control are

shown. Values are means ± SE of 5 independent experiments. * $p<0.05$ vs. control.

Fig. 4. Effect of pertussis toxin on acetate-induced leptin mRNA expression in differentiated adipocytes.

Differentiated adipocytes were starved as described in Fig. 3 legend and then cultured in the absence (-) or presence (+) of acetate (1mM) for 24h. In some preparations, cells were treated with PTX (100 ng/ml) 2 h before addition of acetate. Total RNA was extracted and Northern blot (20μg/lane) was performed to determine expression of leptin and G3PDH genes. Representative blots and densitometric analyses of bands are shown. Values are means ± SE for four independent experiments. * $p<0.05$ vs. control. $^{\#}p<0.05$ vs. acetate alone.

Fig. 5. Effect of acetate on MAPK kinase activation in differentiated adipocytes.

(A) Differentiated adipocytes were starved as described in Fig. 3 legend and then cultured in the presence of acetate (1mM) for indicated period. Cell lysate (20 μg/lane) was prepared, followed by SDS-PAGE (10% gel) and Western blot analysis to detect phosphorylated and total p44/p42 MAPK. (B) Cell lysate (20 μg/lane) was prepared 30min after addition of acetate in the absence (-) or presence (+) of PTX (100 ng/ml) and analyzed. Representative blots and densitometric analyses of bands are shown. Values are means ± SE for four and three independent experiments, respectively. * $p<0.05$ vs. control. $^{\#}p<0.05$ vs. acetate alone.

Fig. 6. Effect of long chain fatty acids on basal and acetate-induced leptin mRNA expression in differentiated adipocytes.

(A) Differentiated adipocytes were starved as described in Fig. 3 legend and then cultured in the absence (-) or presence of increasing concentrations of stearate or linoleate for 24h.

(B) the cells were cultured in the absence (-) or presence (+) of acetate (1 mM), in combination with stearate (0.3 mM) and/or linoleate (0.3 mM) for 24h. Total RNA was extracted and Northern blots (18μg/lane) were performed to determine expression of leptin and G3PDH genes. Representative blots and densitometric analyses of bands are shown. Values are means ± SE for four and three independent experiments, respectively. * $p<0.05$ vs. no addition. # $p<0.05$ vs. acetate alone.

Fig. 7. Effect of acetate and long chain fatty acids on insulin-induced leptin mRNA expression in differentiated adipocytes.

Differentiated adipocytes were starved as described in Fig. 3 legend and then cultured in the absence (-) or presence (+) of insulin (1□M), in combination with acetate (1mM), stearate (0.3 mM) and/or linoleate (0.3 mM), for 24h. Total RNA was extracted and Northern blots (18μg/lane) were performed to determine expression of leptin and G3PDH genes. Representative blots and densitometric analyses of bands are shown. Values are means ± SE for four independent experiments, respectively. * $p<0.05$ vs. no addition. # $p<0.05$ vs. insulin alone.

Fig. 1

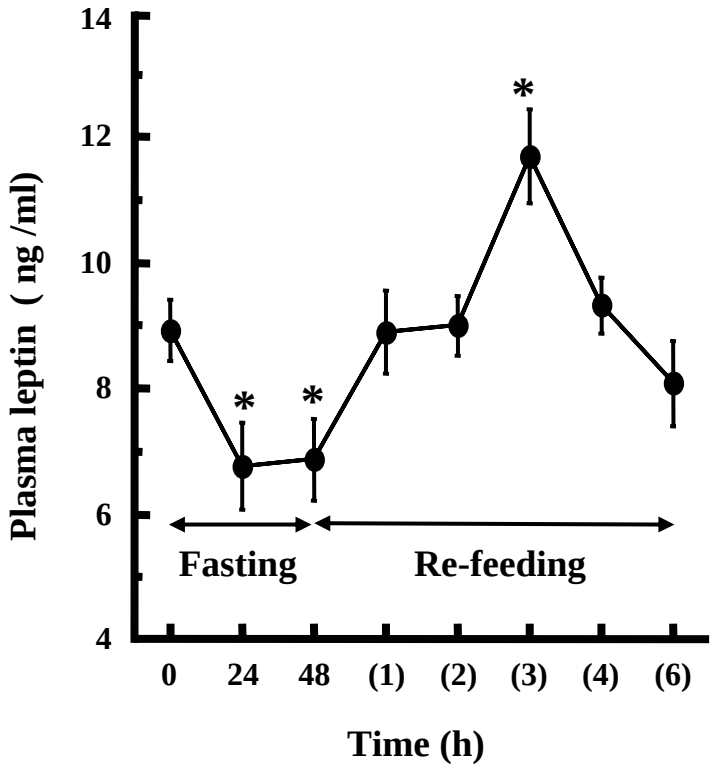

14
12
10
8
6
4
Plasma leptin (ng /ml)
*
*
*
Fasting
Re-feeding
0
24
48
(1)
(2)
(3)
(4)
(6)
Time (h)

Fig. 2

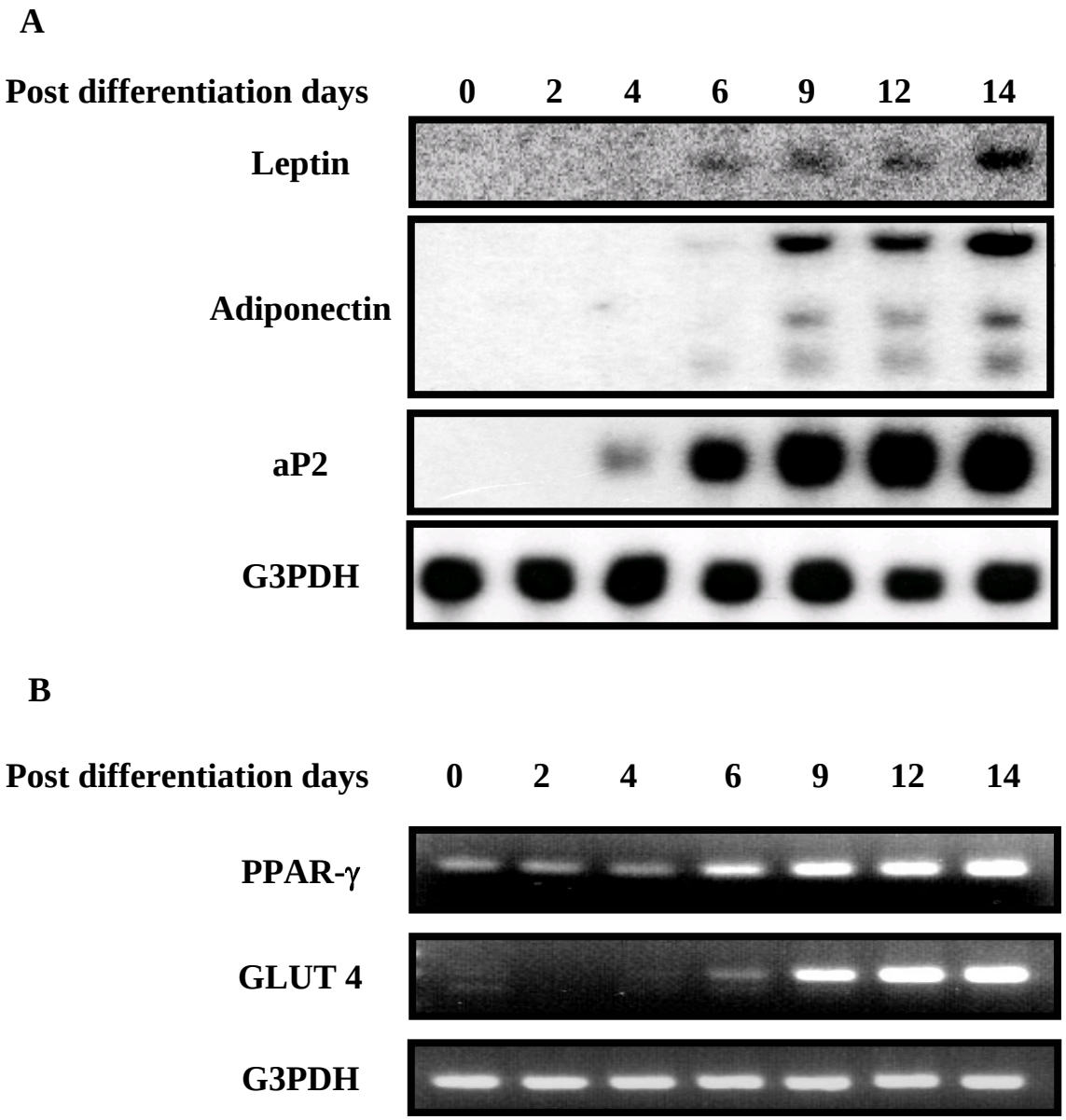

A
Post differentiation days
0
2
4
6
9
12
14
Leptin
Adiponectin
aP2
G3PDH
B
Post differentiation days
0
2
4
6
9
12
14
PPAR-γ
GLUT 4
G3PDH

Fig. 3

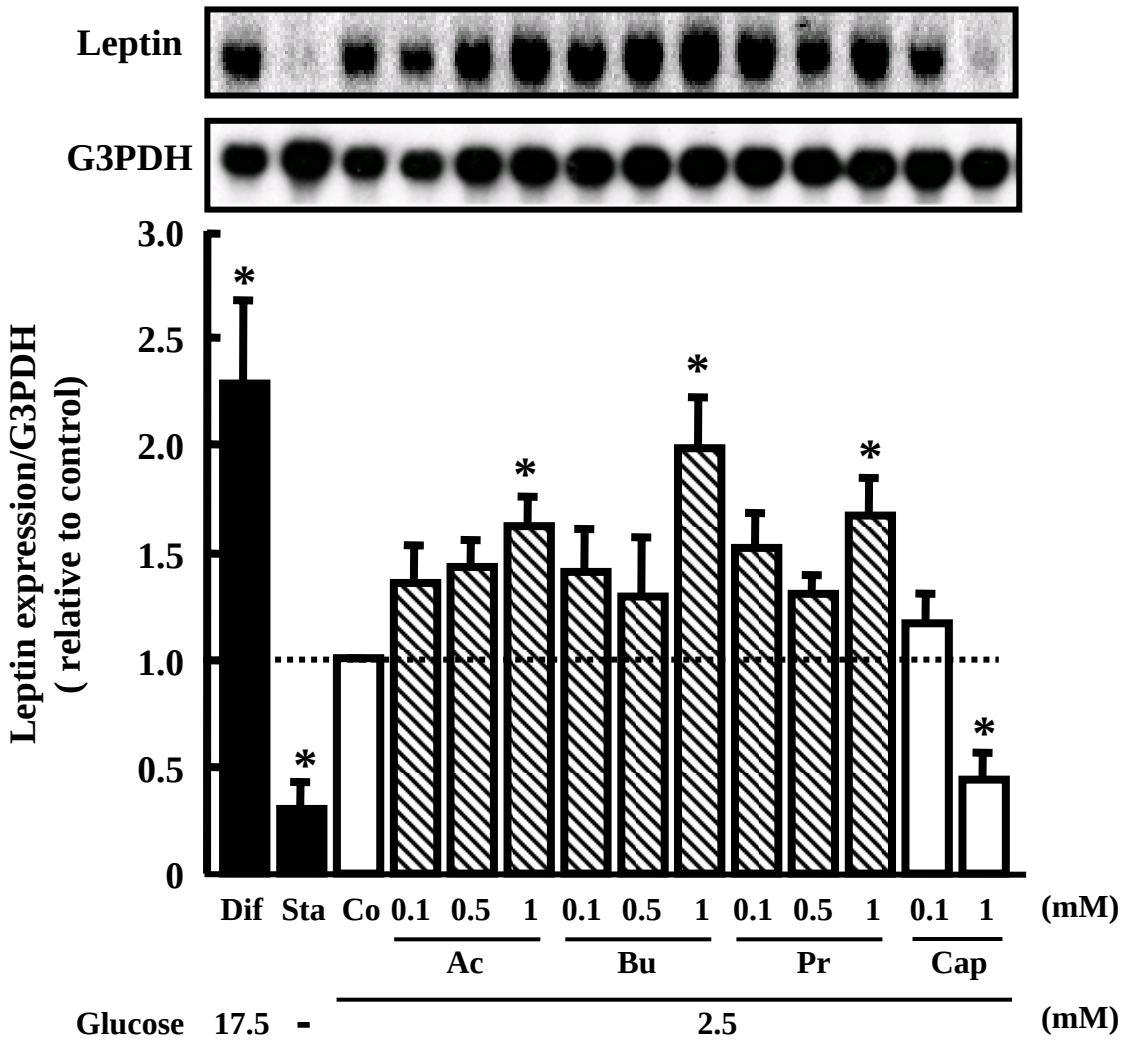

Leptin
G3PDH
Leptin expression/G3PDH
(relative to control)
3.0
2.5
2.0
1.5
1.0
0.5
0
Dif
Sta
Co
0.1
0.5
1
0.1
0.5
1
0.1
0.5
1
0.1
1
(mM)
Ac
Bu
Pr
Cap
Glucose
17.5
-
2.5
(mM)

Fig . 4

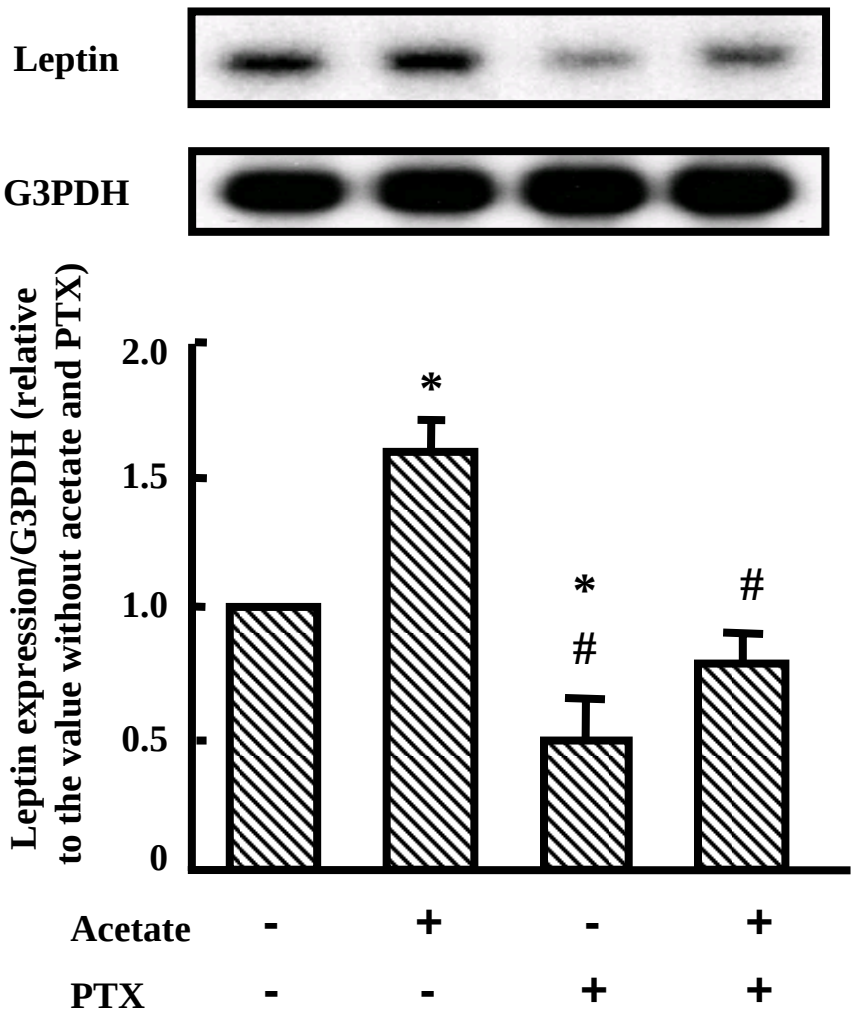

Leptin
G3PDH
Leptin expression/G3PDH (relative to the value without acetate and PTX)
2.0
1.5
1.0
0.5
0
*
*
#
#
Acetate - + - +
PTX - - + +

Fig. 5A

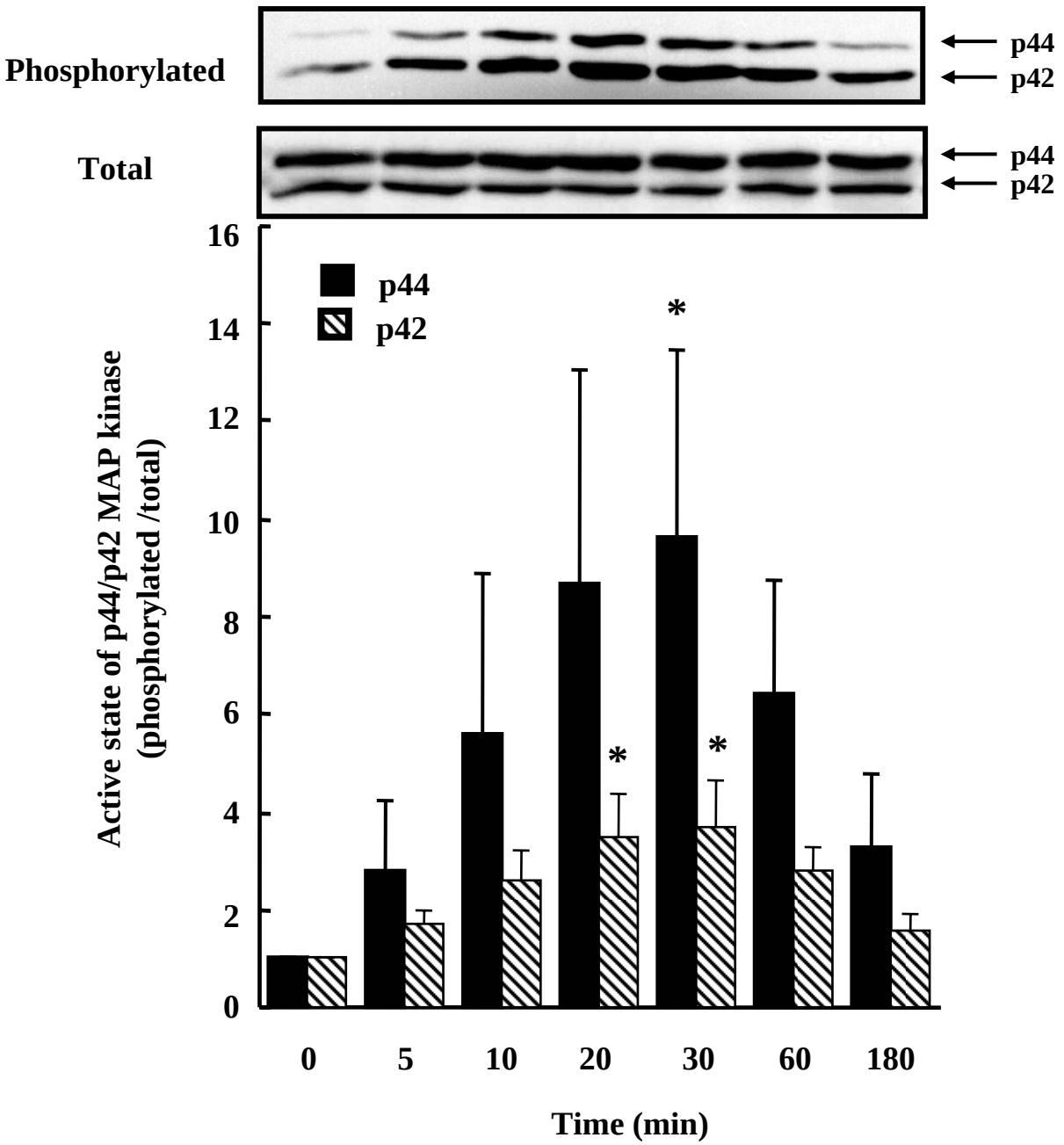

Phosphorylated
p44
p42
Total
p44
p42
p44
p42
Active state of p44/p42 MAP kinase
(phosphorylated /total)
16
14
12
10
8
6
4
2
0
*
*
*
0
5
10
20
30
60
180
Time (min)

Fig. 5B

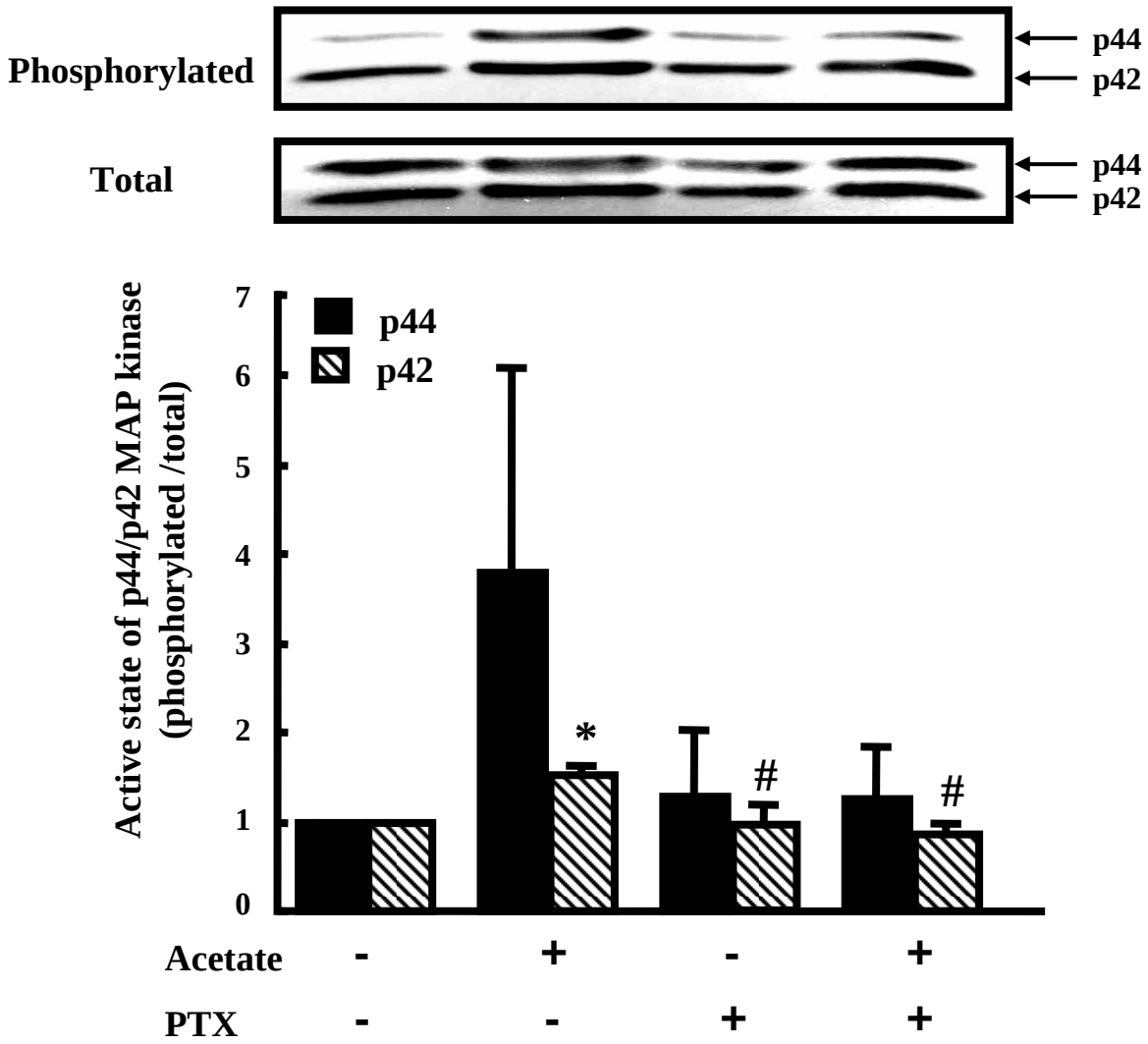

Phosphorylated
p44
p42
Total
p44
p42
Active state of p44/p42 MAP kinase
(phosphorylated /total)
p44
p42
7
6
5
4
3
2
1
0
*
#
#
Acetate - + - +
PTX - - + +

Fig. 6A

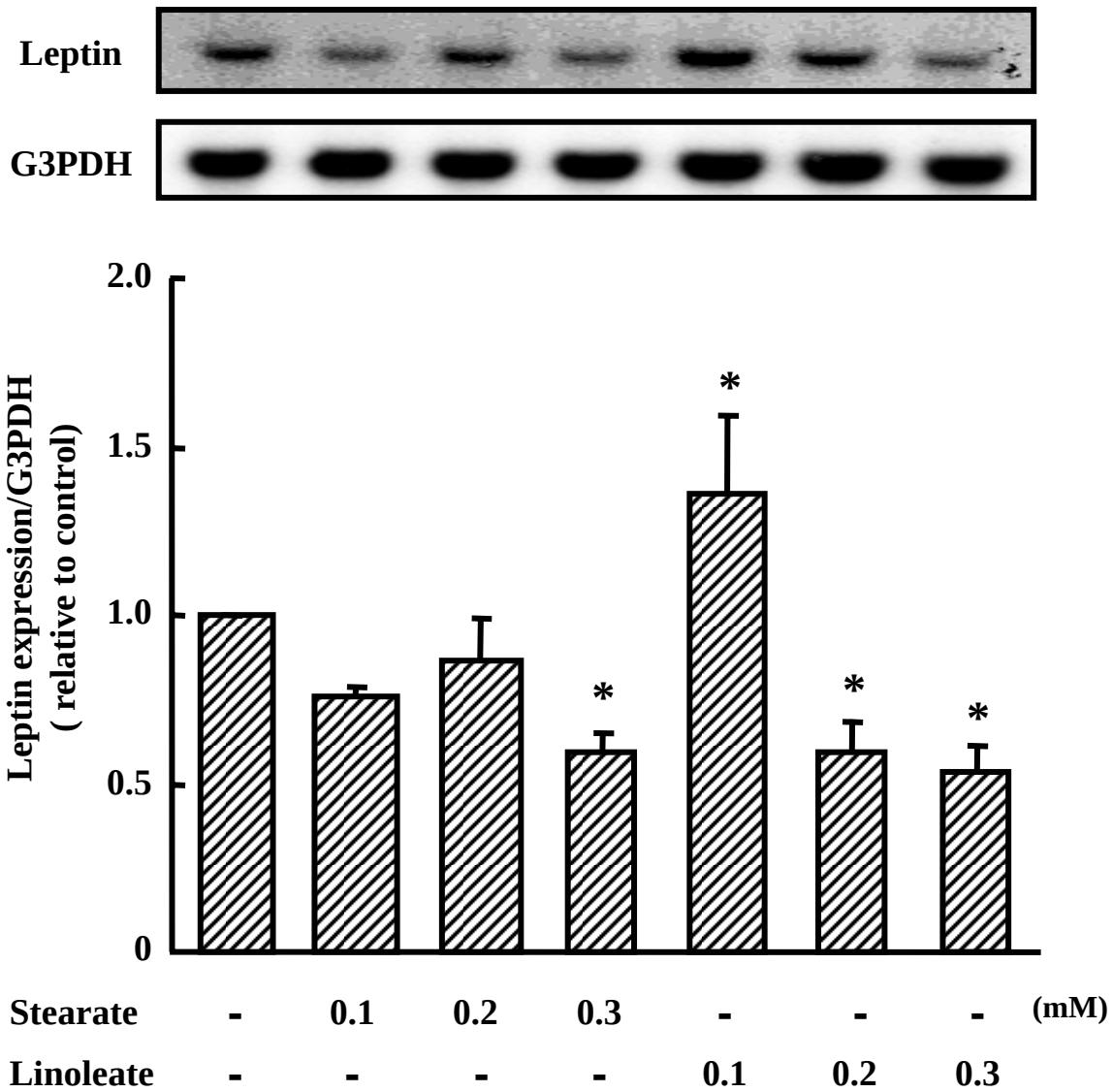

Leptin
G3PDH
Leptin expression/G3PDH
(relative to control)
2.0
1.5
1.0
0.5
0
*
*
*
*
Stearate - 0.1 0.2 0.3 - - - (mM)
Linoleate - - - - 0.1 0.2 0.3

Fig. 6B

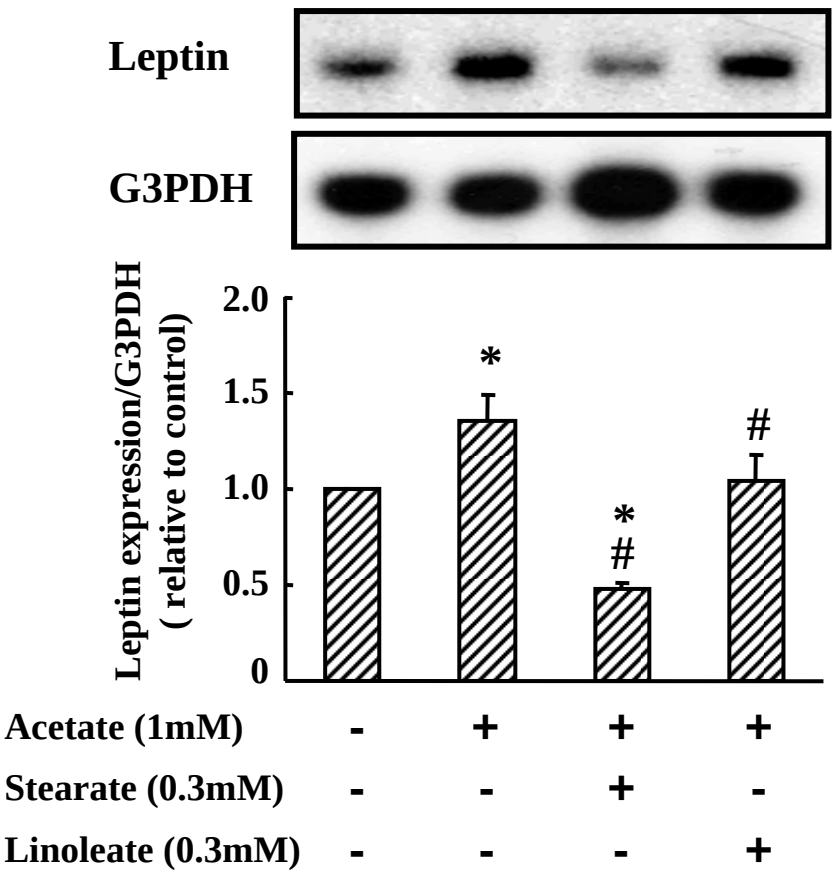
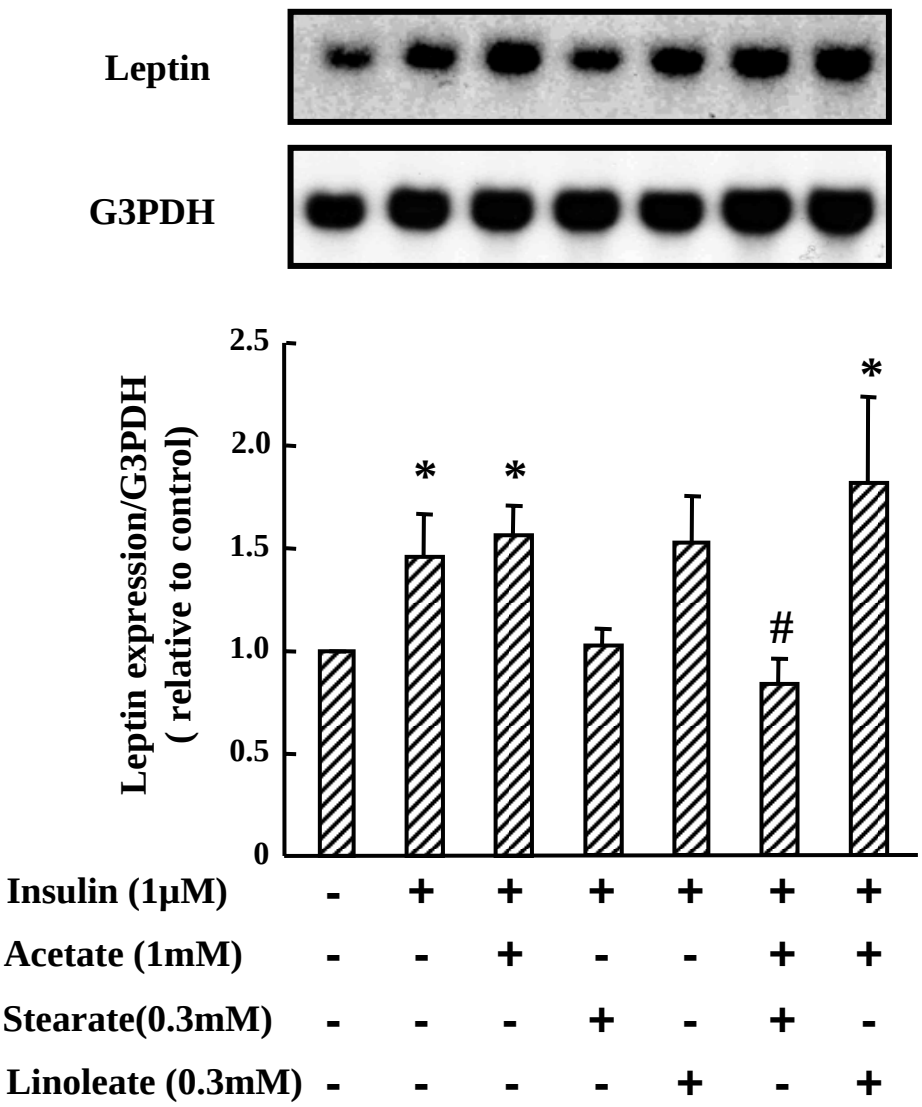

Leptin
G3PDH
Leptin expression/G3PDH
(relative to control)
2.0
1.5
1.0
0.5
0
*
*
#
#
Acetate (1mM) - + + +
Stearate (0.3mM) - - + -
Linoleate (0.3mM) - - - +

Fig. 7


Leptin
G3PDH
Leptin expression/G3PDH
(relative to control)
2.5
2.0
1.5
1.0
0.5
0
*
*
*
#
Insulin (1μM) - + + + + + +
Acetate (1mM) - - + - - + +
Stearate(0.3mM) - - - + - + -
Linoleate (0.3mM) - - - - + - +