Downregulation of Leptin by Free Fatty Acids in Rat Adipocytes: Effects of Triacsin C, Palmitate, and 2-Bromopalmitate

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Free fatty acid (FFA) has been reported to decrease leptin mRNA levels in 3T3-L1 adipocytes. When using this cell line, it is difficult to determine the protein levels because a very small amount of leptin is secreted into the medium. The effect of FFA on leptin secretion from adipocytes has not yet been determined. In addition, in vivo studies have failed to demonstrate a FFA-induced decrease in plasma leptin levels. To clarify the effect of FFA on leptin production, we investigated the leptin protein level in the medium and the mRNA level in primary cultured rat adipocytes treated with triacsin C, which is a potent inhibitor of acyl-coenzyme A (CoA) synthetase, palmitate, and 2-bromopalmitate. Triacsin C (0 to 5 × 10^{-5} mol/L) decreased leptin concentrations in the culture medium in a dose-dependent manner. Leptin mRNA levels were decreased to 10% of the control in the presence of triacsin C. The concentration of triacsin C needed to suppress leptin production was similar to the K_i value (≈10^{-5} mol/L) for inhibition of acyl-CoA synthetase. Both palmitate and 2-bromopalmitate decreased leptin concentrations but did not affect the triacsin C–induced decrease in leptin additively. In conclusion, both protein and mRNA levels of leptin were decreased by triacsin C and FFA in primary cultured rat adipocytes. Our findings suggest that FFA is involved in the regulation of leptin production in adipocytes.

OBESITY is a worldwide health problem associated with serious complications, including a high incidence of non–insulin-dependent diabetes mellitus and cardiovascular disease. The obese (*ob*) gene, responsible for obesity in *ob/ob* mice, was recently identified.[1] The product of the *ob* gene, leptin, is produced and secreted from adipocytes and plays a role in the regulation of energy homeostasis. Leptin is thought to regulate food intake through a closed-loop feedback system involving the hypothalamus, and its plasma level has been correlated with the percent body fat (% fat) and body mass index.[2] However, during fasting, a rapid decrease in the plasma leptin concentration was not correlated with changes in % fat in obese rodents and humans, which indicates that leptin levels are regulated by various factors in addition to changes in body fat mass.[3]

With in vitro studies, several substances have been reported to affect leptin production in adipocytes, including thiazolidinediones,[4-8] isoproterenol,[9] glucocorticoids,[9] and insulin.[10,11] 2-Bromopalmitate also has been reported to decrease leptin mRNA expression in 3T3-L1 adipocytes using reverse transcriptase–polymerase chain reaction.[12] Protein levels of leptin are difficult to detect in 3T3-L1 adipocytes because there is a very small amount of leptin in the medium. The effect of free fatty acid (FFA) on leptin secretion from adipocytes has not yet been determined. In addition, in vivo studies have failed to demonstrate a FFA-induced decrease in plasma leptin in humans.[13-15] It is recognized that the control of leptin involves a complex system of interactions through various regulatory systems. The value of an in vitro system is that it allows an investigation of some of the characteristics of different regulatory systems, although in vitro systems do not provide information with regard to which regulatory systems may dominate.

In the present study, to clarify the effect of FFA on leptin production, we examined the leptin protein level in the medium and the mRNA level in primary cultured rat epididymal adipocytes incubated with triacsin C, a potent inhibitor of acyl-coenzyme A (CoA) synthetase[16] and FFA. We chose primary cultured mature adipocytes because it is possible to measure the leptin protein level in the culture medium. Furthermore, the adipocytes are physiologically relevant and are not subject to differentiation.

MATERIALS AND METHODS

Materials

Triacsin C was purchased from Kyowa Medics (Tokyo, Japan), and Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO-BRL (Gaithersburg, MD). 2-Bromopalmitate and all other chemicals were obtained from Sigma (St Louis, MO) unless otherwise noted.

Rat Adipocyte Preparation

Male Sprague-Dawley rats (180 to 200 g; Shimizu Breeding Laboratories, Kyoto, Japan) were maintained with free access to a standard laboratory chow (CLEA Rodent Diet CE-2; Nippon CLEA, Tokyo, Japan) and water. The rats were anesthetized with a gas mixture of 70% CO_2 and 30% O_2 and killed by decapitation. All experiments were approved by the Institutional Animal Care and Use Committee of Kyoto University. The epididymal fat pads were removed, minced, and digested with collagenase (type 1; Cooper Biochemical, Freehold, NJ). Cell preparation was performed at 37°C in Krebs-Ringer bicarbonate-HEPES buffer, pH 7.4, containing 10 mmol/L $NaHCO_3$, 30 mmol/L HEPES, and 1% (wt/vol) bovine serum albumin ([BSA] fraction V; Intergen, Purchase, NY) as described previously.[17] All incubations were performed at 37°C in DMEM supplemented with 5% (wt/vol) BSA, 2 mmol/L L-glutamine, 200 nmol/L adenosine, 50 μg/mL gentamicin, and 100 mg/dL glucose. Isolated adipose cells were distributed equally into plastic dishes to a final incubation vol of 10 mL (10% cytocrit, ≈5 × 10^5

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cells/mL) containing the indicated reagents. Triacsin C was dissolved in dimethyl sulfoxide (DMSO), whereas 2-bromopalmitate and palmitate were dissolved in ethanol. All cells were exposed to a final concentration of 0.1% DMSO and 0.1% ethanol.

Radioimmunoassay for Rat Leptin

Recombinant rat leptin was produced as described previously.[18-20] Antiserum (KN-2-2-96) for rat leptin was raised in Japanese white rabbits immunized with the recombinant rat leptin. ^{125}I-rat leptin was prepared by the chloramine-T method. The specific activity of ^{125}I-rat leptin was 62.5 to 72.5 μCi/μg. The incubation buffer for radioimmunoassay (RIA) was 0.1 mol/L phosphate buffer, pH 7.4, containing 0.5% BSA (fraction V, RIA-grade; Sigma). The RIA incubation mixture consisted of 100 μL standard rat leptin or sample, 100 μL 1:6,000 dilution of the antiserum, and 200 μL incubation buffer and was incubated for 24 hours at 4°C. Then, 100 μL ^{125}I-rat leptin (10,000 cpm) was added, and the mixture was further incubated for 24 hours at 4°C. Bound and free ligands were separated by the double-antibody method.[20] The detection limit was 1 ng/mL, and the 50% binding intercept was 10 ng/mL. The intrassay and interassay coefficient of variation was 5.2% and 5.5%, respectively.

RNA Extraction and Northern Blot Analysis

Total RNA extraction and Northern blot analysis with ^{32}P-labeled rat leptin[19] and lipoprotein lipase (LPL)[21] cDNA probes were performed as previously described. A human β-actin genomic probe (Wako Pure Chemical, Osaka, Japan) was used to confirm the integrity of the RNA samples. The mRNA level (arbitrary units) was expressed relative to the control cells.

Statistical Analysis

Results are expressed as the mean ± SEM. Statistical significance was tested with 1-way ANOVA followed by Duncan's multiple-range test or the Wilcoxon signed-ranks test as appropriate, and differences were regarded as significant at a P level less than .05.

RESULTS

Effect of Triacsin C on Leptin Concentration

The adipocytes were incubated with or without triacsin C for 24 hours, and the leptin concentration in the culture medium was measured using RIA. At 24 hours, triacsin C (5×10^{-5} mol/L) decreased the leptin concentration by 37% (28 ± 4 ng/mL) compared with the controls (44 ± 5 ng/mL, $P < .01$; Fig 1). Figure 2 shows the dose-response relationship between triacsin C and the leptin concentration at 24 hours. Triacsin C decreased leptin dose-dependently (half-maximal effective dose [ED_{50}], $\approx 2 \times 10^{-5}$ mol/L).

In the time-course experiments (Fig 1), the leptin concentration did not increase linearly in either control or triacsin C–treated cells, and the accumulation rate gradually decreased after 6 hours. However, even at 6 hours, the leptin concentration was lower in triacsin C–treated cells versus the control cells. To examine the possibility of degradation, we investigated the degradation rate of leptin using ^{125}I-labeled leptin and trichloroacetic acid ([TCA] 10%) precipitation. The degradation rate was less than 8% during the time course (data not shown). Moreover, when dexamethasone (10^{-7} mol/L) was added to the adipocytes after incubation for 6 hours, the leptin concentration in the medium increased linearly up to 24 hours (data not shown). In addition, the loss of cells over 24 hours was less than

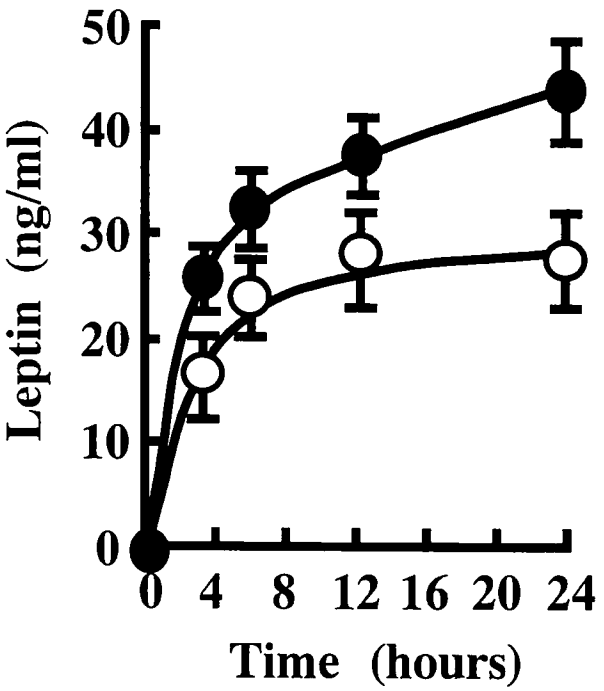

Fig 1. Effect of triacsin C on the time course of leptin concentrations. Rat epididymal adipocytes were incubated with (○) or without (●) triacsin C (5×10^{-5} mol/L) for the indicated time. The leptin concentration in the culture medium was measured in triplicate using RIA. Control cells were incubated with 0.1% DMSO alone in the medium. Values are the mean ± SEM of 3 independent experiments.

10%, and was equivalent to the control and triacsin C–treated cells under these conditions as estimated by cytocrit and lipid weight.[17]

Effect of Triacsin C on Leptin, LPL, and β-Actin mRNA Levels

We next examined the effect of triacsin C on leptin mRNA expression in rat adipocytes. Figure 3 shows the representative Northern blot for leptin mRNA from control cells and triacsin C (5×10^{-5} mol/L)–treated cells at 24 hours. Triacsin C decreased the leptin mRNA level to 10% ± 2% after 24 hours compared with the control ($P < .01$). To determine whether this effect was specific for leptin, we examined the mRNA level of LPL, one of the representative markers of differentiated adipocytes, and β-actin. Triacsin C did not change the mRNA level of LPL or β-actin (98% ± 12% and 101% ± 7%, respectively).

Effect of 2-Bromopalmitate and Palmitate on Leptin Production

To confirm that FFA induces a decrease in leptin production, we studied the effects of 2-bromopalmitate (nonmetabolizable FFA) and palmitate (metabolizable FFA) on leptin production in the adipocytes. 2-Bromopalmitate reduced the leptin concentration in a dose-dependent manner, and at its maximal concentration (3×10^{-4} mol/L), the compound decreased leptin levels to 42% ± 4% (29 ± 3 ng/mL, $P < .01$) compared with the control (Fig 4). Palmitate (3×10^{-4} mol/L) also reduced the leptin concentration to 76% ± 3% (39 ± 4 ng/mL, $P < .01$), although this was less than the reduction for 2-bromopalmitate and

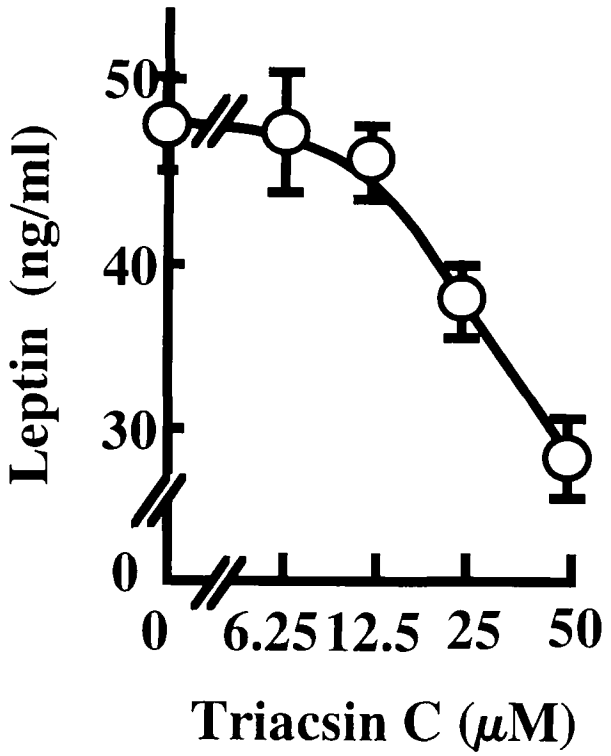

Fig 2. Dose-response curve between triacsin C and leptin concentration. Rat epididymal adipocytes were incubated with various concentrations of triacsin C (0-5 × 10^{-5} mol/L) for 24 hours. The leptin concentration in the culture medium was measured in triplicate using RIA. Control cells were incubated with 0.1% DMSO alone in the medium. Values are the mean ± SEM of 3 independent experiments.

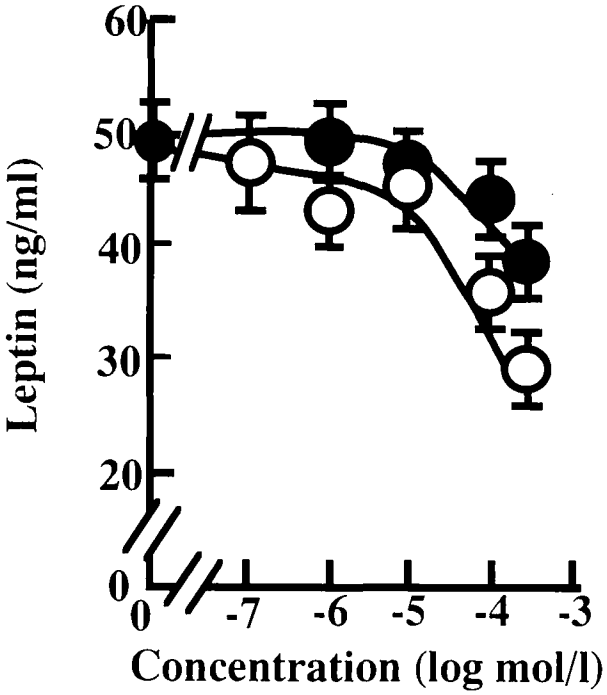

Fig 4. Dose-response curve between palmitate or 2-bromopalmitate and the leptin concentration in the medium. Adipocytes were incubated with various concentrations (0-3 × 10^{-4} mol/L) of palmitate (●) or 2-bromopalmitate (○) for 24 hours. The leptin concentration in the medium was measured in triplicate using RIA. Values are the mean ± SEM of 3 independent experiments.

triacsin C ($P < .05$). We further examined whether these FFAs affected the triacsin C–induced decrease in leptin levels in adipocytes. The triacsin C–induced decrease in leptin was not affected by 2-bromopalmitate or palmitate additively (Fig 5).

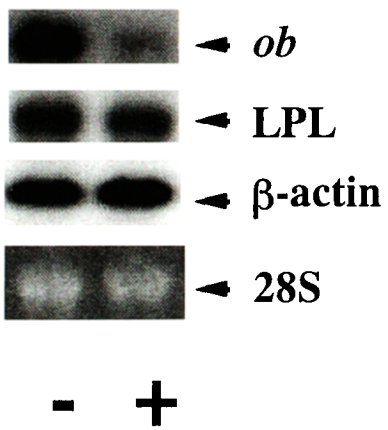

Fig 3. Effect of triacsin C on mRNA levels of leptin, LPL, and β-actin. Adipocytes were incubated with or without triacsin C (5 × 10^{-5} mol/L) for 24 hours. Total RNA (20 μg/lane) was prepared, and leptin, LPL, and β-actin mRNA levels were detected by Northern blot analysis. Panels show representative blots from 3 experiments. Bottom panel shows 28S rRNA detected by ethidium bromide.

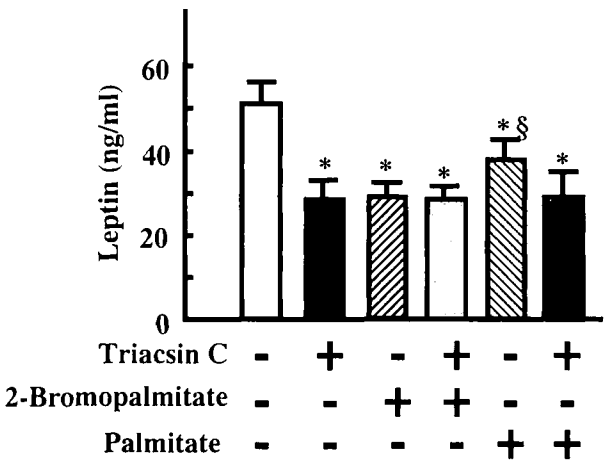

Fig 5. Effect of triacsin C, 2-bromopalmitate, or palmitate on leptin concentrations. Adipocytes were incubated with or without the agents for 24 hours: lane 1, control; 2, triacsin C (5 × 10^{-5} mol/L); 3, 2-bromopalmitate (3 × 10^{-4} mol/L); 4, triacsin C and 2-bromopalmitate; 5, palmitate (3 × 10^{-4} mol/L); and 6, triacsin C and palmitate. The leptin concentration in the culture medium was measured by RIA in duplicate. Values are the mean ± SEM of 3 independent experiments. *$P < .01$ *v* control, §$P < .05$ *v* triacsin C or 2-bromopalmitate alone.

DISCUSSION

The present study demonstrates that triacsin C, 2-bromopalmitate, and palmitate decrease both the protein and mRNA levels of leptin in primary cultured rat adipocytes. Triacsin C is a potent inhibitor of acyl-CoA synthetase, with a K_i value of about 10^{-5} mol/L.[16] The concentration of triacsin C (1 to 5×10^{-5} mol/L) required to reduce the leptin concentration and leptin mRNA expression found in this study was similar to the K_i value for the inhibition of acyl-CoA synthetase. Therefore, it is suggested that the inhibitory effects of triacsin C are directly caused by the inhibition of acyl-CoA synthetase.

Isolated adipocytes present spontaneous hydrolysis of triglycerides with FFA production and reesterification. Triacsin C inhibits the conversion of FFA to acyl-CoA by inhibiting acyl-coA synthetase. Triacsin C is supposed to inhibit lipid synthesis and reuse of postlipolysis fatty acid but not to decrease FFA produced via spontaneous hydrolysis of adipocytes. The decrease in leptin production by triacsin C was not enhanced by addition of 2-bromopalmitate or palmitate. It is thus speculated that triacsin C decreased leptin production through the same mechanism as 2-bromopalmitate and palmitate. In this study, intracellular FFA was not determined. It has been reported that intracellular FFA was increased by triacsin C in 3T3-L1 adipocytes using [1-^{14}C]-labeled palmitate and thin-layer chromatography.[22] Therefore, we speculate that intracellular FFA may have affected leptin production in this study. The recent finding that FFA may affect gene expression by directly binding to the nuclear receptor, peroxisome proliferator–activated receptor (PPAR)[23] or fatty acid–activated receptor,[24] might help to explain our results. PPARγ, a member of the PPAR family, is abundant in adipocytes and is reported to be activated by 2-bromopalmitate at a concentration of 10^{-5} mol/L in PPARs-overexpressing cells.[25] Thiazolidinediones, which are PPARγ agonists, have been reported to reduce leptin levels in in vivo[7,8] and in vitro[4-6] studies. FFA may involve the PPARγ-mediated mechanism in its reduction of leptin production. Further study is necessary to clarify the regulatory mechanism of leptin production by FFA.

The leptin concentration in the medium did not increase linearly in the control cells, and the accumulation rate gradually decreased after 6 hours (Fig 1). The expression of leptin mRNA was decreased after 24 hours' incubation compared with the initial level (data not shown). There are several possible explanations for the spontaneous changes in the accumulation rate and mRNA level of leptin. The most likely explanation may be the isolation of adipocytes away from the blood supply, innervation, and stromal support. Isolated adipocytes are not supplied with various factors that increase leptin, including insulin[10,11] and glucocorticoids,[9] in in vitro conditions. The possibility of a decrease in cell number and cell viability may be unlikely. The loss of cells over 24 hours was less than 10% as estimated by cytocrit and lipid weight.[17] We also examined cell viability using fluorescein diacetate (FDA).[26] FDA, the nonfluorescent aliphatic acid ester of fluorescein, is hydrolyzed to produce a fluorescent product that accumulates in living cells. Injury to the cells results in a loss of the ability to accumulate fluorescein. Using FDA, we found that after incubation for 24 hours, greater than 90% of the cells maintained a bright-green fluorescence. Moreover, when dexamethasone was added to the adipocytes after incubation for 6 hours, the leptin concentration in the medium increased linearly (data not shown). Thus, a decrease of cell viability may not be involved. Another possibility is the exhaustion of stored leptin. Although leptin has been reported to be secreted as it is produced,[27] the presence of a preexisting intracellular pool of leptin has also been suggested.[28,29] Further study is necessary to clarify the secretion mechanism of leptin. The possibility of leptin degradation is considered unlikely, because the degradation rate estimated using ^{125}I-labeled leptin and TCA (10%) precipitation was less than 8% during the time course (data not shown). The presence of feedback regulation of leptin in in vitro conditions may not be involved. It has been reported that treatment of isolated rat adipocytes with leptin in vitro has no effect on endogenous leptin mRNA levels.[9] We also confirmed that the addition of leptin to the medium did not change the endogenous leptin mRNA expression in rat adipocytes (data not shown).

It is unlikely that the results of this study are due to unexpected cytotoxic effects or cell damage by triacsin C. To eliminate these possibilities, we measured the level of LPL mRNA as a typical adipocyte marker. The LPL mRNA level was not changed by triacsin C in the present study. Furthermore, triacsin C did not decrease the viability of cells as determined using FDA[26] or increase the loss of cells as estimated by cytocrit and lipid weight (data not shown) compared with the control. Therefore, we conclude that the reduction in leptin production was induced specifically by triacsin C. Leptin mRNA spontaneously decreased over 24 hours as described previously; however, triacsin C specifically decreased leptin mRNA levels more than the spontaneous decrease.

It has been reported that a rapid decrease in plasma leptin is not correlated with changes in % fat during fasting in obese rodents and humans.[3] Short-term fasting in humans results in many metabolic changes, including glucose, FFA, and ketones.[30-32] These alterations are caused or followed by several hormonal changes, including a reduction in insulin and an increase in glucagon, catecholamine, cortisol, and growth hormone.[30-32] The reduction in leptin levels under fasting conditions might be regulated coordinately by those various factors including FFA. In obese subjects, plasma leptin and FFA are both elevated. If FFA also reduces plasma leptin levels in humans as in this in vitro study, it is speculated that the leptin-reduction mechanism of FFA may be disturbed in obesity. Alternatively, leptin resistance, which has been considered to be present in obesity,[33,34] might overcome the FFA-induced decrease in leptin. It is possible that leptin levels may be regulated not only by FFA but also by other factors that increase leptin, including insulin and glucocorticoids in obesity. Insulin has been reported to increase plasma leptin in humans.[10,35] An elevation of plasma FFA levels by lipid-heparin infusion has not been reported to alter plasma leptin levels in humans.[13-15] A possible explanation for the discrepancy between the reduction in leptin production in this study and in vivo studies may be that the plasma leptin level is regulated coordinately with various hormones and metabolic factors in addition to FFA in vivo. It is

also possible that the effects of FFA on plasma leptin in human subjects are slow in onset and undetectable in the time course of such studies.

Triacsin C decreased the leptin concentration in the medium and the leptin mRNA level in the adipocytes. Both palmitate and 2-bromopalmitate decreased the leptin concentration, but did not affect the triacsin C–induced decrease in leptin additively. We speculate that FFA is involved in the regulation of leptin production in adipocytes.

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