

Hormonal Regulation of Leptin, Resistin, and Plasminogen Activator Inhibitor-1 Gene Expression in 3T3-L1 Adipocytes

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Abstract

Leptin, resistin and PAI-1 (plasminogen activator inhibitor-1) are synthesized and secreted by rodent fat cells and recently postulated to be an important link to obesity. This study was conducted to characterize the hormonal regulation of leptin, resistin, and PAI-1 gene expression in the 3T3-L1 adipocytes. The cells were treated with 0.5 μ M insulin, 1 μ M dexamethasone (Dex), or 0.05 μ M triiodothyronine (T3) for 72 hours. The mRNA levels of each peptide were measured by semi-quantitative RT-PCR. The mRNA level of the leptin-producing *ob* gene was significantly increased by insulin, Dex, and T3 by 3.2-, 3.1- and 2.7-fold, respectively, compared to the control ($p < 0.05$). The level of resistin mRNA was increased by insulin, Dex, and T3 by 2.7-, 2.5- and 2-fold, respectively, compared to the control ($p < 0.05$). Likewise, the level of PAI-1 mRNA was significantly increased by insulin, Dex, and T3 compared to the control ($p < 0.05$). Taken together, our results suggest that insulin, Dex, and T3 may regulate the gene expression of leptin, resistin, and PAI-1 in 3T3-L1 adipocytes.

Key words: leptin, resistin, plasminogen activator inhibitor-1, 3T3-L1 adipocyte

INTRODUCTION

At one level, obesity can be explained from an energy balance viewpoint, the phenomenon of accumulation of excess energy as fat resulting from a higher intake of energy than the expenditure of energy. Especially as people get older, the frequency of obesity increases because the energy expenditure decreases compared to energy intakes. Obesity belongs to a cluster of metabolic abnormalities, often referred to as "Metabolic Syndrome" or "Syndrome X", characterized by manifestations that include: non-insulin-dependent diabetes, dyslipidemia, hypertension, and cardiovascular diseases (1).

Adipose tissue was previously believed to be largely inert with the simple function of storing excess energy as fat and releasing energy on demand. However, recent research has shown that fat cells secrete substances such as leptin, resistin, plasminogen activator inhibitor-1 (PAI-1), adiponectin, and TNF- α , which means the adipose tissue works as not only fat storage but also as endocrine tissue secreting the controlling substances related to energy balance, insulin resistance, and immune function (1,2). The amount of fat accumulation in the adipose tissue appears to control the level of gene expression of the above proteins, resulting in the energy balance being controlled by the level of exertion.

The discovery of the *ob* gene and 'leptin' protein coded by this gene revealed new possibilities for understanding the physiological mechanisms of obesity (3). Physiologically, leptin serves as a satiety factor that is released by adipose tissue and signals the hypothalamus to decrease food intake and increase energy expenditure (4,5). Expression of the *ob* gene and the consequential release of leptin has been demonstrated in cultured rat and human primary adipocytes (6,7). In the obese mouse (*ob/ob* mouse), a mutant leptin mRNA was produced excessively which resulted in repression of secretion of the normal leptin into the blood. When *ob/ob* mice were treated with leptin, food intake decreased and energy consumption increased, resulting in a decrease in body fat and body weight. This means leptin has an endocrine function of controlling body fat by changing food intake and energy balance (5,8).

Resistin is 12.5-kDa sized cysteine-rich protein which is secreted by adipose tissue (9). Resistin is also called adipocyte-specific secretory factor (ADSF), consistent with its name, it only expressed in adipose tissue. Kim et al. (10) reported that resistin gene expression is greatly increased by the differentiation of fat cells and primary preadipocytes. Also, the amount of resistin mRNA is remarkably decreased in fasting or diabetic animals and remarkably increased by refeeding or insulin treatment

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(10). These results show that the expression of resistin is related to adipogenesis. However, the regulatory mechanism for hormonal regulation of resistin in animals is not clearly understood.

Type 1 plasminogen activator inhibitor (PAI-1) is a primary regulator of the endogenous fibrinolytic system. It decreases fibrinolysis and promotes the progression of thrombosis (1). Plasma PAI-1 levels are related to overall fatness [body mass index (BMI)] and omental fat in particular and are elevated in obese subjects (11). As PAI-1 is a major risk factor for cardiovascular disease, it may be a pathogenetic link between atherothrombosis and obesity (1). In 1991, Sawdey and Loskutoff (12) reported that PAI-1, one of the risk factor in cardiovascular disease, is synthesized and secreted by mammary adipose tissues. Particularly in humans, PAI-1 is synthesized more actively by the visceral adipose tissue than by subcutaneous adipose tissue. Weight loss resulting from energy reduction did not change the PAI-1 concentrations of the subcutaneous adipose tissue but did decrease the PAI-1 of the visceral adipose tissue (12). However, the cellular and molecular basis of this connection is still elusive.

We reported that the expression of the genes encoding PAI-1, resistin, and leptin, which are all secreted by adipocytes are regulated by nutritional status and might vary according to the type of adipose tissue (13). In the present study we investigated the hormonal control of leptin, resistin, and PAI-1 gene expression. We particularly focused on the effects of insulin, dexamethasone (Dex), and triiodothyronine (T3) in 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials

Murine 3T3-L1 adipocytes were purchased from Korean Cell Line Bank (KCLB[®]). Dulbecco's modified eagle's medium (DMEM), glutamine, penicillin/streptomycin, non-essential amino acids, trypsin-EDTA, Hank's balanced bovine serum salt solution, bovine serum albumin, and HEPES were purchased from GIBCO. Insulin, dexamethasone, triiodothyronine (T3), 3-isobutyl-1-methylxanthine and fetal bovine serum (FBS) were obtained from Sigma.

3T3-L1 adipocytes

Murine 3T3-L1 preadipocytes were cultured in DMEM containing 10% FBS, 1% glutamine, 1% non-essential amino acid, 50 U/mL penicillin, and 50 µg/mL streptomycin (standard media). Confluent 3T3-L1 preadipocytes were differentiated into adipocytes by exposing them to 0.5 mmol/L 3-isobutyl-1-methylxanthine and 0.25 µmol/L Dex for 48 h. The cells were then placed in standard media in which they accumulated small lipid

droplets that grew to occupy a large fraction of the cells within 4~5 days. The differentiated cells were then treated further for 72 h in serum free media in the presence or absence of hormone.

Semi-quantitative RT-PCR

The levels of leptin, resistin, and PAI-1 mRNA were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from cultured cells was extracted by the single-step method of Chomczynski and Sacchi (14) using TRIzol reagent (Invitrogen). Reverse transcription was performed using total RNA (4 µg) in a final volume of 20 µL containing buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 50 mM DTT), 1 mM dNTPs (Promega), 200 U M-MLV reverse transcriptase (Promega), and 30 pmole oligo dT₁₉. The reaction mixture was incubated at 37°C for 1 h and then for 15 min at 75°C. cDNA was mixed with 0.2 mM dNTPs, 0.125 U Super Taq polymerase (Super-Bio, Korea), 0.25 µM forward and reverse primers. PCR amplification was carried out using an automated DNA thermal cycler. The sequence of the forward primers was as following: leptin, 5'-TCT ATC AAC AGG TCC TCA CC-3'; resistin, 5'-CTA TTT TCA ACC AGA GGC AC-3'; PAI-1, 5'-ATG GAA GAC CCC TTT CTT AG-3'; β-actin 5'-GGA CCT GAC AGA CTA CCT CA-3'. The sequence of the reverse primers was as following: 5'-ACT GTT GAA GAA TGT CCT GC-3'; 5'-TCC TTC CAC CAT GTA GTT TC-3'; 5'-CCT TCC ATT GTC TGA GT-3'; 5'-GTT GCC AAT AGT GAT GAC CT-3'. The sequence for temperature cycling was as following: 95°C for 5 min, denaturation at 95°C for 15 s, 27 cycles for leptin and resistin, 25 cycles for PAI-1, 20 cycles for β-actin of annealing at 50°C for 30 s, and extension at 72°C for 40 s, and a final extension at 72°C for 10 min. The constitutively expressed β-actin was used as a reference to normalize leptin, resistin, and PAI-1 mRNA levels.

Statistical analysis

Data for the control and individual hormone treatment groups were analyzed by one-way ANOVA: $p \geq 0.05$ was taken as indicating no significant difference. Where ANOVA showed significance, differences among group were evaluated by Duncan's multiple range test.

RESULTS AND DISCUSSION

3T3-L1 adipocytes are a useful cell culture model system for investigation of adipocyte differentiation and function *in vitro*. In the present study, we found that insulin induced a concentration-dependent increase in *ob* gene expression, showing significant effects at 0.05 to

2 μM of insulin in 3T3-L1 adipocytes ($p < 0.05$) (Fig. 1A). Not only was the mRNA level of the *ob* gene was significantly increased by 0.5 μM insulin, but also by 1 μM Dex and 0.05 μM T3 by 3.2-, 3.1- and 2.7-fold, respectively compared to the control ($p < 0.05$) (Fig. 2).

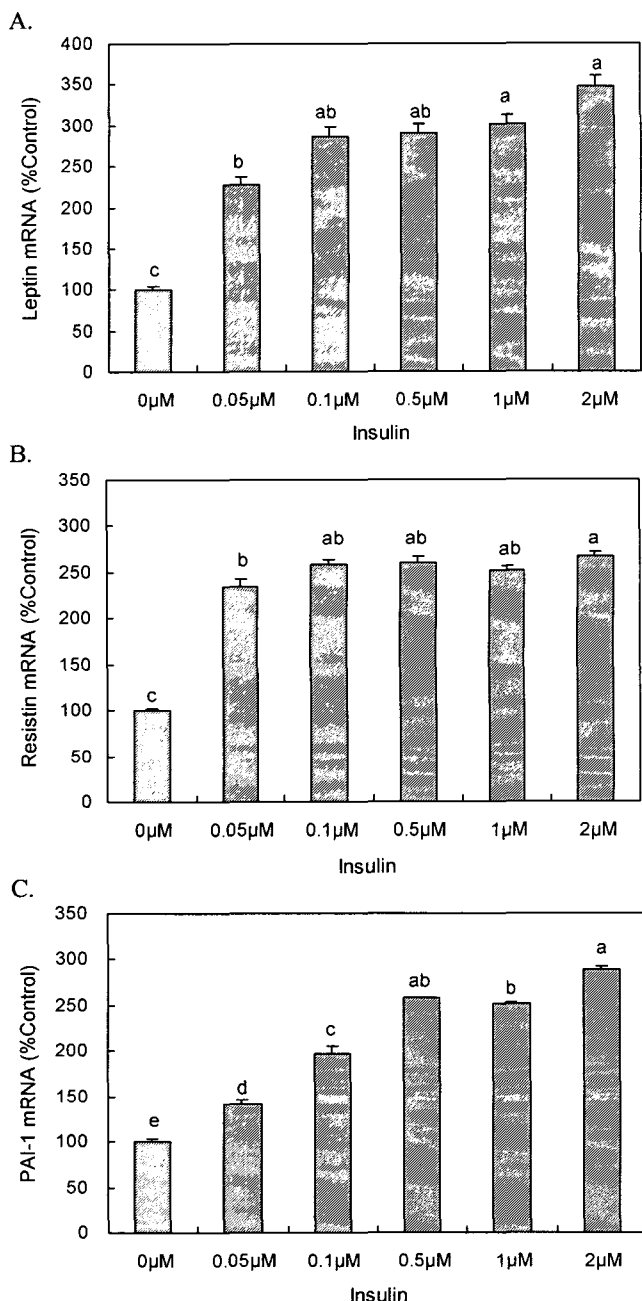


Fig. 1. Effect of insulin on leptin, resistin, and PAI-1 gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 72 hours in serum free media in the presence of varied concentrations of insulin. A, leptin mRNA; B, resistin mRNA; C, PAI-1 mRNA. Values are expressed as means \pm SD, and are shown as percent of control (no insulin); different superscript letters in the upper part of the graph indicate significant differences at $p < 0.05$. The experiments were repeated three times with triplicate samples for each point and showed similar response patterns.

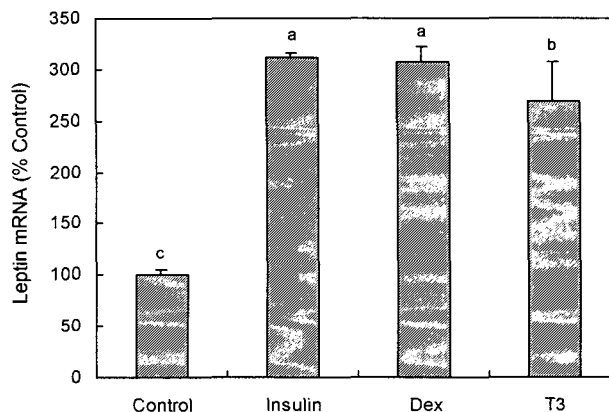
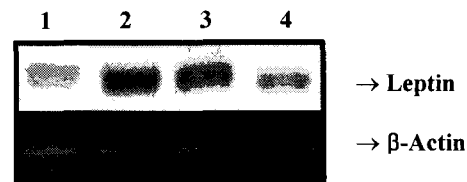


Fig. 2. Effects of insulin, Dex, and T3 on leptin gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 72 hours in serum free media with or without hormones at the concentrations listed below. 1, Control (no treatment); 2, 0.5 μM insulin; 3, 1 μM Dex; 4, 0.05 μM T3. The leptin mRNA was determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The constitutively expressed β -actin was used as a reference to normalize leptin mRNA level. Values are expressed as means \pm SD, and are shown as percent of control (no treatment); different superscript letters in the upper part of the graph indicate significant differences at $p < 0.05$. The experiments were repeated three times with triplicate samples for each point and showed similar response patterns.

These data demonstrate that insulin, glucocorticoids and thyroid hormone are regulators of leptin mRNA expression in 3T3-L1 adipocytes.

Several studies have previously reported that *ob* gene expression in mature 3T3-L1 adipocytes was increased by insulin (7,15). Kim et al. (15) reported a similar degree of stimulation of *ob* gene expression by insulin in 3T3-L1 adipocytes at concentrations between 10 and 1000 nM. Mouse primary adipocytes treated with insulin caused an increase in leptin mRNA expression levels and Dex also induced an elevation of leptin mRNA (7). The level of leptin protein in cultured media of mouse primary adipocytes treated with insulin or Dex was also higher. Lee et al. (7) postulated that gene expression in adipocytes may be regulated in response to insulin concentration in adipocytes and that leptin may alleviate the hyperglycemia in diabetes mellitus with high insulin resistance.

There was a controversy on the effects of T3 on the leptin synthesis. Yoshida et al. (16) reported that T3 at 10~1000 nmol/L significantly increased the expression of the *ob* gene (237~337%), suggesting that thyroid

hormone is a novel regulator of *ob* gene expression in 3T3-L1 adipocytes. However, when rat primary cultures of white and brown adipocytes were treated with increasing concentrations of T3, the mRNA levels and leptin secretion were inhibited (17). We found that 0.05 μM T3 increased the leptin mRNA by 2.7-fold. While the explanation for this difference is unclear, the differences between primary and 3T3-L1 adipocytes might be one possible clue.

Treatment with 0.1 μM insulin, increased the resistin mRNA, showing stimulatory effects between 0.05 to 2 μM insulin ($p < 0.05$) (Fig. 1B), with 0.5 μM insulin significantly increasing the level of resistin mRNA by 2.7-fold compared to the control ($p < 0.05$) (Fig. 3). However, Shojima et al. (18) reported that resistin expression in 3T3-L1 adipocytes was significantly up-regulated by high glucose concentrations and was suppressed by 0.1 μM insulin. They treated cells with insulin for 24 h, but we incubated for 72 h, showing that the different incubation time can cause opposite results. In the present study, 1 μM Dex significantly increased the level of resistin mRNA by 2.5-fold compared to the control ($p < 0.05$) (Fig. 3), indicating that glucocorticoids

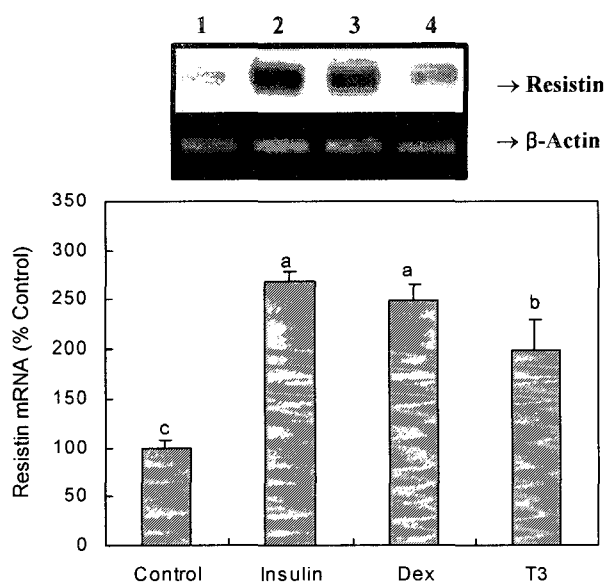


Fig. 3. Effects of insulin, Dex, and T3 on resistin gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes incubated for 72 hours in serum free media with or without hormones at concentrations listed below. 1, Control (no treatment); 2, 0.5 μM insulin; 3, 1 μM Dex; 4, 0.05 μM T3. The resistin mRNA was determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The constitutively expressed β -actin was used as a reference to normalize resistin mRNA level. Values are expressed as means \pm SD, and are shown as percent of control (no treatment); different superscript letters in the upper part of the graph indicate significant differences at $p < 0.05$. The experiments were repeated three times with triplicate samples for each point and showed similar response patterns.

stimulate the expression of the resistin gene in a manner similar to its effect on *ob* gene expression. Also, the resistin gene expression was significantly increased in the presence of 0.05 μM T3 by 2-fold compared to the control ($p < 0.05$) (Fig. 3). In the present study of 3T3-L1 adipocytes, resistin mRNA expression was markedly increased by insulin, Dex and T3. Resistin was recently identified as a hormone secreted by adipocytes that is under both hormonal and nutritional control. This hormone has been suggested to be the link between obesity and type 2 diabetes. Nogueiras et al. (19) reported that resistin was severely decreased in hyperthyroid rats and that chronic food restriction (30% of *ad libitum* food intake) led to a decrease in adipose tissue resistin mRNA levels in normal rats. Sometimes different model systems can show different results; they used *in vivo* animal model, but we used *in vitro* cell culture system.

In the present study, we found that PAI-1 gene expression was significantly increased 1.4-fold by 0.05 μM insulin ($p < 0.05$) (Fig. 1C). The PAI-1 gene expression was induced in a concentration-dependent manner in 3T3-L1 adipocytes (Fig. 1C). Crandall et al. (20) reported that human preadipocyte migration is regulated through the endogenous expression of PAI-1, which affords a novel autocrine mechanism for potentially regulating cell cluster formation in adipogenesis. PAI-1 was reported to have an effect on weight gain and adipose tissue cellularity in the induction of obesity in mice (21). Furthermore, PAI-1 produced by the mature adipocytes may be present in the latent form, which is observed to be dominant in cultured cells (22). Plasma PAI-1 is augmented by the intravenous injection of insulin *in vivo* (23). Insulin is also known to elevate PAI-1 production in human hepatocytes in primary culture (24). Samad and Loskutoff (25) reported that PAI-1 synthesis was increased 5- to 6-fold by insulin in the mature 3T3-L1 adipocytes.

In our study, the level of PAI-1 mRNA was significantly increased by 0.5 μM insulin, 1 μM Dex and 0.05 μM T3 by 2.5-, 3- and 2-fold, respectively, compared to the control ($p < 0.05$) (Fig. 4). We found the PAI-1 gene expression in 3T3-L1 adipocytes was significantly up-regulated by insulin, Dex and T3. In a study of PAI-1 expression in non-obese and obese individuals (26), adipocyte PAI-1 mRNA levels were 2-fold in the obese group, suggesting that PAI-1 secretion is related to the lipid content and volume of fat cells. Dex, which down-regulates PA activity and PA mRNA in several cells (27), increased PAI-1 mRNA and activity in mature hepatocytes. Wahrenberg et al. (28) reported that plasma PAI-1 activity was increased in Graves' hyperthyroidism before as compared with during anti-thyroid treatment.

In summary, our present data suggest that insulin, Dex,

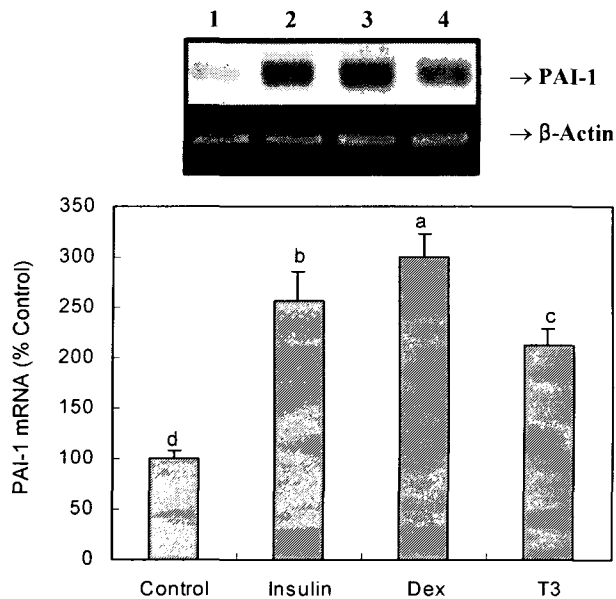


Fig. 4. Effects of insulin, Dex, and T3 on PAI-1 gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 72 hours in serum free media with or without hormones at concentrations listed below. 1, Control (no treatment); 2, 0.5 μ M insulin; 3, 1 μ M Dex; 4, 0.05 μ M T3. The PAI-1 mRNA was determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The constitutively expressed β -actin was used as a reference to normalize PAI-1 mRNA level. Values are expressed as means \pm SD, and are shown as percent of control (no treatment); different superscript letters in the upper part of the graph indicate significant differences at $p < 0.05$. The experiments were repeated three times with triplicate samples for each point and showed similar response patterns.

and T3 may up-regulate the gene expression of leptin, resistin, and PAI-1 in 3T3-L1 adipocytes.

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