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Transcriptional Regulation of the Leptin Promoter by Insulin-Stimulated Glucose Metabolism in 3T3-L1 Adipocytes

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Insulin-stimulated glucose metabolism plays a key role in the regulation of leptin mRNA expression and protein secretion. However, it is not known whether stimulation of leptin production by glucose metabolism is regulated at the level of promoter activation or at a step distal to the promoter. Therefore, in order to investigate the transcriptional regulation of the leptin promoter by insulin-stimulated glucose metabolism, 3T3-L1 cells were transfected with a plasmid containing the leptin promoter driving a luciferase reporter gene. Leptin promoter activity was increased after 48 hours of treatment by 219 ± 64 (p = 0.028) and 225 ± 69% (p = 0.046) at insulin concentrations of 16 and 160 nM, respectively. The activation of the leptin promoter induced by insulin (16 nM) was markedly inhibited by 2-deoxy-D-glucose (2-DG, 50 mg/dl), a competitive inhibitor of glucose metabolism. The increment of insulin-stimulated leptin promoter activation was reduced by 52 ± 11% (p = 0.028 vs insulin alone). The activity of a control plasmid (pGL2-Control) was unaffected by insulin or 2-DG. These results provide strong evidence that insulin-stimulated glucose metabolism, and not insulin *per se*, mediates the effects of insulin to increase the transcriptional activity of the leptin promoter.

Key Words: **glucose metabolism; insulin; leptin promoter; 2-deoxy-D-glucose.**

The adipocyte hormone leptin is a key regulator of energy balance in animals and in humans (1). Our previous experiments suggest that increased glucose metabolism mediates the effect of insulin to stimulate leptin gene expression and leptin secretion (2). Leptin secretion by cultured adipocytes is more closely related to the amount of glucose metabolized than to the extracellular insulin concentration (2). Inhibitors of glucose uptake and metabolism such as 2-deoxy-D-glucose (2-DG), phloretin and cytochalasin-B, or inhibitors of glycolysis such as sodium fluoride and iodoacetate, inhibit insulin-stimulated leptin secretion (2). Thus, the effect of insulin to increase leptin secretion is likely to be related to its effects to increase adipocyte glucose utilization. A recently published study using cultured human adipose tissue (3) supports our previous findings in rat adipocytes (2). In addition, the results of a recent study conducted in human subjects (4) provides evidence that insulin-stimulated glucose metabolism rather than insulin *per se* is a major determinant of leptin secretion *in vivo.* More recent studies from our laboratory indicate that the anaerobic metabolism of glucose to lactate does not increase leptin secretion (5), and suggest that mitochondrial substrate oxidation is involved in the stimulation of leptin production by glucose metabolism (6).

The mechanism(s) underlying the effects of insulin-stimulated glucose metabolism to increase leptin secretion remain unknown. Our previous studies have shown that insulin increases leptin mRNA expression in isolated rat adipocytes, and that this increase was reversed by blocking glucose utilization with either 2-DG or sodium fluoride. These results suggest that a transcriptional regulatory mechanism is likely to be involved in the regulation of leptin gene expression by insulin-stimulated glucose metabolism. Fukuda *et al.* (7) showed that insulin in the presence of glucose significantly increased leptin promoter activity. This study did not, however, determine whether the stimulation of promoter activity induced by insulin was the result of a direct effect of insulin or if it was mediated by insulin's effects to increase adipocyte glucose utilization. The aim of the present study was, therefore, to examine the effects of glucose metabolism on the transcriptional regulation on the leptin gene. For this purpose, 3T3-L1 adipocytes were transfected with a plasmid containing 762 bp construct of the leptin promoter

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and the promoter activity was evaluated after treatment with glucose and insulin in the absence or presence of 2-DG, an inhibitor of glucose uptake and metabolism.

MATERIALS AND METHODS

Cell culture and differentiation. 3T3-L1 cells (8) were obtained from American Type Culture Collection (Rockville, MD). Passages 3–9 were used in all studies. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose and 10% calf bovine serum. Confluent cells were induced to differentiate by incubating for 72 h with differentiation medium containing 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX) and 10% fetal bovine serum in DMEM. Cells were then maintained in DMEM containing 10% fetal bovine serum, but without dexamethasone or IBMX (post-differentiation medium), for 48 h prior transfection.

Plasmids. Cells were transfected with a plasmid containing 762 bp construct of the mouse leptin promoter with a luciferase reporter gene [p(−762)lep-luc] (9, 10), which was kindly provided by Dr. Reitman (Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH). This firefly luciferase reporter is derivative of pGL2-Basic. pGL2-Control and pGL2-basic were purchased from Promega (Madison, WI). pRL-SV40, a renilla luciferase control reporter plasmid, was kindly provided from Dr. De Miguel (Department of Biochemistry, University of Navarra, Spain). Plasmid DNA was transformed into One Shot competent cells (Original TA Cloning Kit, Invitrogen, Carlsbad, CA). Milligram quantities of all of these plasmid DNA vectors were prepared using EndoFree Plasmid Maxi kit (Quiagen Inc., Santa Clarita, CA). The concentration of plasmid DNA has been determined by both spectrophotometric assay and by comparison to known DNA markers using ethidium bromide staining of restriction-digested plasmid run on an agarose gel.

Transient transfections. On day 5 after the induction of differentiation, 3T3-L1 adipocytes were transfected with 6 μg of the plasmid p(−762)lep-luc by the calcium phosphate method (11). pRL-SV40 plasmid (4 ng) was transfected at the same time and used as an internal control for transfection efficiency. After 16–20 h of incubation, the culture medium was removed and cells were washed with Dulbecco's phosphate-buffered saline (PBS). Cells were then incubated in the absence or presence of insulin (0.16–1600 nM) for 48 h with 25 mM glucose. The effects of 2-DG (50 mg/dl) on leptin promoter activity in the absence or presence of insulin (16 nM) were also examined. We have previously demonstrated that this concentration of 2-DG induced a marked (>90%) suppression of leptin mRNA expression and leptin secretion in primary cultured rat adipocytes, but did not induce cytotoxic effects since 18S RNA was unaffected and lipoprotein lipase activity was only modestly reduced (2).

To exclude the possibility of a false positive finding in the transfection efficiency, some cells were also transfected with the pGL2-basic plasmid (a promoterless vector that serves as a negative control). A pGL2-control was also transfected and used as a positive control. Cells transfected with this positive control were treated at the same time and under the same conditions as cells transfected with the plasmid containing the leptin promoter.

Dual luciferase assay. After 48 h of treatment, cells were lysed using 1× Passive Lysis Buffer (Promega, Madison, WI). Firefly and renilla luciferase activities were then determined in the cell lysates using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI), which allows sequential measurement of both luciferases in the same sample. Data of firefly luciferase activity were normalized with the data of renilla luciferase activity.

Statistical analysis. Results are presented as mean ± SEM. Data were analyzed with a Wilcoxon signed rank test (Statview for Macintosh, Abacus, Inc., Berkeley, CA).

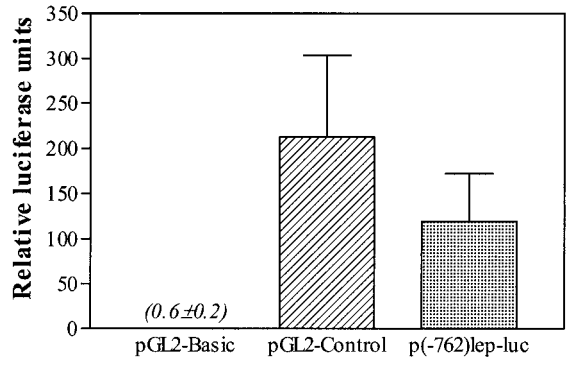

FIG. 1. Activity of the pGL2-Basic (a promoterless vector), pGL2-Control, and p(−762)lep-luc on 3T3-L1 adipocytes. Transient expression was performed as described under Materials and Methods. Data are presented as relative luciferase units. Bars are the means ± SE of at least 3 independent experiments.

RESULTS AND DISCUSSION

3T3-L1 cells are a useful cell culture model system for investigation of adipose differentiation and adipocyte function *in vitro.* For example, 3T3-L1 cells have been used to study the regulation of leptin promoter by glucocorticoids (12) and the regulation of FAS promoter by insulin (13, 14).

The promoter of the mouse *leptin* gene has been cloned and characterized. Four elements in the proximal 109-bp contribute to the leptin promoter activity: the TATA box at −30, a C/EBP motif at −53, a LP1 region at −87, and an Sp1 motif at −97. These sites are conserved among species, including mouse and human. No distant elements with a large effect on adipose expression have been identified, although a placental enhancer was found upstream of the human leptin promoter (9, 10, 15–17). In the present study transient transfection of 3T3-L1 cells with the p(−762)lep-luc construct, containing 762 bp of the leptin promoter sequence, increased reporter activity to a level ~180-fold greater than background. As expected, when cells were transfected with the pGL2-basic construct, a promoterless vector, only background activity was observed. In contrast the level of expression of pGL2-Control, a plasmid with strong luciferase expression in many types of mammalian cells, was increased to ~300-fold over background levels (Fig. 1).

Several studies have previously reported that leptin expression in mature 3T3-L1 adipocytes is increased by insulin (18). In the present study, we found that insulin induced a concentration-dependent increase of leptin promoter activity. Thus, no increase in promoter activity was found with 0.16 nM insulin (103 ± 38% of control). Treatment with 1.6 nM insulin, a physiological concentration of insulin, may have caused a slight increase (126 ± 27%) of promoter activity, while significant stimulatory effects (220 ± 64, 225 ± 69, 240 ±

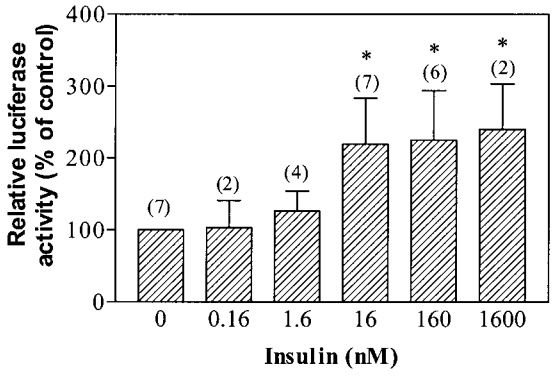

FIG. 2. Dose-response effects of insulin on the transcriptional activity of leptin promoter. A plasmid containing 762 bp construct of the leptin promoter with a luciferase reporter gene p(−762)lep-luc was introduced into 3T3-L1 adipocytes. Cells were then cultured for 48 h in DMEM containing 25 mM glucose without or with insulin ranging from 0.16–1600 nM. A pRL-SV40 plasmid was also cotransfected and used to normalize the leptin promoter activity data. Data are expressed as percentage of the activity in the absence of insulin. Bars are the mean ± SE of several independent experiments performed in triplicates. The number above each bar is the number of independent experiments. * $p < 0.05$ compared with control group.

63%) were observed at 16, 160 and 1600 nM insulin (Fig. 2). Although, the differences in promoter activation were small between 16 and 1600 nM, overall, the percent increase of leptin promoter activity induced by insulin was concentration-dependent ($p < 0.02$). Kim *et al.* (18) have reported also a similar degree of stimulation of leptin expression by insulin in 3T3-L1 adipocytes at concentrations between 10 and 1000 nM, suggesting that in this cell line both transcriptional activation of the promoter and leptin gene expression are maximal at moderate and at high insulin concentrations. In previous studies in primary rat adipocytes, which are substantially more sensitive to insulin than 3T3-L1 adipocytes, we have observed concentration-dependent increases of leptin secretion in presence of 0.16 to 1.6 nM insulin. In one previous study, Fukuda *et al.* (7) reported that insulin at a high concentration of 100 nM increased leptin promoter activity by 1.5-fold in primary rat adipocytes.

In the experiment by Fukuda *et al.* (7), the culture media contained both glucose and insulin. Therefore, it was not possible to differentiate between the direct effects of insulin *per se* and insulin's actions to stimulate cellular glucose metabolism in the stimulation of the transcriptional activity of the leptin promoter in that study. Our previous published results provided evidence that insulin-stimulated glucose metabolism mediates the effects of insulin to increase leptin mRNA expression and secretion in isolated rat adipocytes (2). In order to determine if glucose metabolism could regulate leptin production at the level of promoter activation, we incubated 3T3-L1 adipocytes with glucose and insulin in the absence and the presence of 2-DG, an inhibitor of glucose uptake and metabolism, and assessed the activity of a transfected construct of the leptin promoter. As shown in Fig. 3, the increase in the leptin promoter activity induced by insulin (16 nM) was completely abolished in the presence of 2-DG (50 mg/dL) indicating that activation of the insulin signal transduction pathway *per se*, in the absence of insulin's effects to stimulate glucose utilization, does not activate the promoter. However, 2-DG did not induce any significant change on leptin promoter activity in the absence of insulin (data not shown). In addition, the activity of the pGL2-control plasmid was unaffected either by insulin or 2-DG treatment, suggesting that insulin-mediated glucose metabolism specifically activates the transcriptional activity of this promoter, and that the metabolic block produced by 2-DG does not induce a generalized inhibition of gene transcription (Fig. 3).

Insulin-stimulated glucose metabolism has been previously demonstrated to regulate the expression of a number of genes involved in the storage of energy, such as lipogenic and glycolytic enzymes in liver and adipose tissue, at the level of transcription (19, 20). Rutter *et al.* (21) has referred to these genes as GIR (Glucose and Insulin Responsive) genes because of their responsiveness to both glucose and insulin, with the aim to differentiate them from the "purely insulin-responsive" (pIR) genes. These GIR genes include fatty acid synthase (FAS), the liver isoform of pyruvate kinase (L-PK) and acetyl-CoAcarboxylase (ACC) and Spot-14 (S14). It has been suggested that in order for glucose metabolism to exert its effect on the expression of these genes, insulin is required to stimulate the glucose uptake and/or metabolism (21). Our previous data demonstrated that insulin-stimulated glucose metabolism

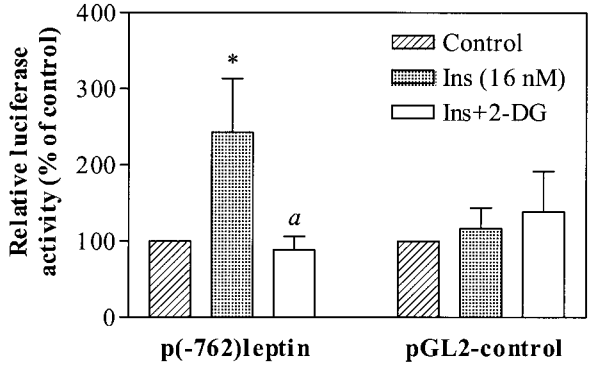

FIG. 3. Effects of 2-DG on the action of insulin on leptin and SV40 (pGL2-Control) promoter activity. Cells were transfected with the p(−762)lep-luc or pGL2-Control plasmids and treated for 48 h without or with insulin (16 nM) in the absence or presence of 2-DG (50 mg/dl). A pRL-SV40 plasmid was also cotransfected and used to normalize the data. Data are expressed as percentage of the activity in the absence of insulin. Bars are the mean ± SE of six independent experiments. * $p < 0.05$ compared with the control cells. *a*, $p < 0.05$ compared with the insulin-treated group.

and not insulin *per se* regulates leptin expression and secretion (2). Wellhoener *et al.* (4) provided *in vivo* support that glucose metabolism rather than insulin is a major determinant of leptin production in humans. These studies, along with our present data, suggest that leptin gene may be considered as a GIR gene.

Nutrient regulation of gene expression is likely to represent an important mechanism by which organisms adapt to their nutritional environment (20). We have reported that the consumption of high carbohydrate, low fat meals increases circulating leptin concentrations over 24 h in women compared to a high fat, low carbohydrate meal (22). A similar pattern in the regulation of some of the lipogenic GIR genes by dietary macronutrient content has been described (23).

The mechanisms linking glucose metabolism to the glucose-responsive transcription complex are largely unknown. Glucose response elements (GlREs), which have a common sequence 5′-CACGTG-3′ termed E box, have been identified for the L-PK, the S14 and the FAS genes (24–26). Our present data have shown the responsiveness of leptin promoter to insulin-mediated glucose metabolism, which raises the possibility, that leptin promoter may have a cis-acting DNA element involved in the response to glucose metabolism. Two E box motifs (CannTG) overlapping the C/EBP site has been identified in the leptin promoter. However, mutations of these E boxes (m47 and m59) did not affect promoter activity (10). The studies of Fukuda *et al.* (7) have suggested that the region from −101 to −83 of the leptin gene is responsible for glucose/insulin stimulation of transcription, and that the transcription factor Sp1 is somehow involved in this regulation.

In conclusion, the results of the present study suggest that it is insulin-stimulated glucose metabolism and not insulin *per se* which regulates the transcriptional activity of the leptin gene. These data along with previous *in vitro* and *in vivo* studies (2, 4, 5, 22) suggest that leptin is likely to be a GIR gene. Further experiments are necessary to more fully characterize the molecular mechanisms by which insulin-stimulated glucose metabolism regulates transcription of leptin gene. This will include the more precise identification of *cis*-acting DNA sequences as well as the *trans*-acting factors involved in the regulation of leptin gene by glucose metabolism.

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