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Sp1-mediated transcription is involved in the induction of leptin by insulin-stimulated glucose metabolism

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Abstract

We have previously demonstrated that insulin-stimulated glucose metabolism, and not insulin *per se*, mediates the effects of insulin to increase the transcriptional activity of the leptin promoter in adipocytes. Here, we sought to identify the specific *cis*-acting DNA elements required for the upregulation of leptin gene transcription in response to insulin-mediated glucose metabolism. To accomplish this, 3T3-L1 cells and primary rat adipocytes were transfected with a series of luciferase reporter genes containing portions of the mouse leptin promoter. Using this method, we identified an element between −135 and −95 bp (relative to the transcriptional start site) that mediated transcription in response to insulin-stimulated glucose metabolism in adipocytes. This effect was abolished by incubation with 2-deoxy-D-glucose, a competitive inhibitor of glucose metabolism. Gel shift electrophoretic mobility shift assays confirmed that the stimulatory effect of insulin-mediated glucose metabolism on leptin transcription was mediated by a previously identified Sp1 site. Consistent with these findings, incubation of primary rat adipocytes with WP631, a specific inhibitor of specificity protein (Sp)1-dependent transcription, inhibited glucose- and insulin-stimulated, but not basal, leptin secretion. Together, these findings support a key role for Sp1 in the transcriptional activation of the leptin gene promoter by insulin-mediated glucose metabolism.

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Introduction

Leptin is a 167 amino acid-secreted protein produced mainly by adipocytes that maintains energy homeostasis by exerting pleiotropic effects on energy intake and energy expenditure (Havel 2004). Leptin acts primarily as a signal of nutritional deprivation, where decreases of circulating leptin initiate an adaptive response to conserve energy, characterized by hyperphagia, decreased energy expenditure and inhibition of reproductive, and other endocrine systems. Genetic deficiency of either leptin or its receptor in mice and humans results in severe early onset obesity (Farooqi & O'Rahilly 2005).

A more complete understanding of the molecular and biochemical mechanisms regulating leptin secretion in adipocytes may lead to new therapeutic opportunities for managing obesity and related metabolic diseases (Havel 2004, Rosenbaum *et al.* 2005). Several major signaling systems affecting leptin production have been identified. Leptin synthesis and secretion are increased by insulin (Saladin *et al.* 1995, Leroy *et al.* 1996, Bradley & Cheatham 1999) and glucocorticoids (Slieker *et al.* 1996, Kolaczynski *et al.* 1997), and are inhibited by adrenergic agonists (Trayhurn *et al.* 1996, Moreno-Aliaga *et al.* 2002) and activators of peroxisome proliferator activated receptor (PPAR)-γ, such as thiazolidinediones (De Vos *et al.* 1996).

We have demonstrated previously that insulin-stimulated glucose metabolism, rather than a direct effect of insulin *per se*, mediates the actions of insulin to increase the leptin (*ob*) gene expression and leptin secretion in isolated adipocytes (Mueller *et al.* 1998). During incubation of isolated adipocytes with physiological concentrations of insulin (0·16–16 nM), we demonstrated that the increase of leptin secretion was much more closely related to the amount of glucose taken up by the adipocytes than to the concentration of insulin. Consistent with this finding, competitive inhibition of glucose transport and phosphorylation with 2-deoxy-D-glucose (2-DG) caused a concentration-dependent inhibition of leptin release in the presence of 1·6 nM insulin. Other inhibitors of glucose transport (phloretin and cytochalasin B) or metabolism (iodoacetate and fluoride) also inhibited leptin secretion, in direct proportion to glucose uptake (Mueller *et al.* 1998). Additional studies suggested that, not only glucose uptake, but also oxidative metabolism of glucose beyond pyruvate, is required for insulin-mediated leptin secretion (Mueller *et al.* 2000). We have also demonstrated that insulin-mediated glucose metabolism is involved in the activation of the leptin promoter transcription by glucose and insulin in 3T3-L1 cells (Moreno-Aliaga *et al.* 2001).

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While the structure of the promoter region has been well characterized, there is a paucity of data which directly addresses the question of how insulin-stimulated glucose metabolism stimulates leptin synthesis in adipocytes. Fukuda & Iritani (1999) joined two copies of the rat leptin promoter sequence from −101 to −83 (which contains a consensus Sp1-binding site) to a luciferase reporter gene, and found that the activity of this reporter gene in transfected primary rat adipocytes was upregulated by the addition of 0·1 μM insulin compared with incubation in 20 mM glucose alone. Mutation of this Sp1 site in the reporter gene then abolished its responsiveness to glucose and insulin. Other studies have mutated the equivalent Sp1 site in the proximal human (Zhang *et al.* 2002) and murine (Mason *et al.* 1998) promoters and have also observed a reduction in reporter gene activity. Subsequent gel shift experiments by Fukuda & Iritani (1999) confirmed the binding of a protein from adipose tissue nuclear extract to the −101 to −83 sequence. Formation of this DNA–protein complex was inhibited to some degree by competition with a DNA probe containing an Sp1-binding sequence and also by preincubation with an anti-Sp1 antibody. A recent study also observed that mutation in Sp1 motif of the bovine leptin gene decreases the promoter-binding capacity for nuclear proteins and reduces leptin gene expression (Adamowicz *et al.* 2006).

Overall, these data indicate that insulin-stimulated glucose metabolism might activate Sp1-mediated transcription of leptin via this Sp1 site in the proximal promoter. However, in the experiments of Fukuda & Iritani (1999), co-transfection of an Sp1 expression vector with their reporter gene decreased promoter activity in adipocytes, suggesting that Sp1 inhibited transcription through this element. This result was clearly inconsistent with the prevailing view that deletion of the Sp1 site reduces promoter activity in adipocytes (Mason *et al.* 1998, Fukuda & Iritani 1999, Zhang *et al.* 2002).

Therefore, the aim of the present study was to investigate the mechanisms underlying the transcriptional regulation of the leptin gene by insulin-stimulated glucose metabolism. First, we carried out deletion mapping studies in adipocytes to identify the *cis*-acting DNA sequences in the proximal promoter mediating the effects of insulin-stimulated glucose metabolism to increase leptin gene transcription. Secondly, we performed electrophoretic mobility shift assays (EMSAs) to test the hypothesis that the transcription factor Sp1 mediates the stimulatory effects of insulin-mediated glucose metabolism. Finally, we incubated primary adipocyte cultures with a specific inhibitor of Sp1-mediated transcription and found that while this intervention had no effect on basal leptin secretion, it abolished insulin-stimulated leptin secretion.

Materials and methods

Cell culture and differentiation

Low-passage (3–9) 3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were used in all studies. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% calf serum, and were maintained in a water-jacketed incubator set to 37 °C and 5% carbon dioxide. 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 to transfection.

Primary culture of adipocytes

Adipocytes were isolated from epididymal fat pads of male Wistar rats, as described by Mueller *et al.* (1998). First, fat pads were minced in Krebs–Ringer HEPES buffer (pH 7·4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2·2 mM $CaCl_2{\cdot}2H_2O$, 1·25 mM $MgSO_4{\cdot}7H_2O$, 0·45 mM KH_2PO_4, 2·17 mM Na_2HPO_4, and 10 mM HEPES). Secondly, tissue fragments were digested in the above buffer supplemented with type I collagenase (1·25 mg/ml per 0·5 g tissue; Worthington, Lakewood, NJ, USA) at 37 °C for 30 min with gentle shaking (60 cycles/min). The resulting cell suspension was diluted in HEPES–phosphate buffer; isolated adipocytes were then separated from the undigested tissue by filtration through a 400 μm nylon mesh and washed thrice. Isolated adipocytes were resuspended in DMEM supplemented with 1% FBS and incubated for 30 min at 37 °C.

Plasmids

Cells were transfected with plasmids containing different constructs of the mouse leptin promoter preceding a luciferase reporter gene (p(−762)lep–luc, p(−135)lep–luc, p(−95)lep–luc, p(−85)lep–luc, m(−140 to −135), and m(−100 to −95)), kindly provided by Dr Marc Reitman (Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health; He *et al.* 1995, Mason *et al.* 1998). Plasmid DNA was transformed into One Shot competent cells (Original TA Cloning Kit, Invitrogen) and all vectors were prepared using an EndoFree Plasmid Maxi kit (Qiagen, Inc.). The concentration of plasmid DNA was determined by both spectrophotometry and restriction enzyme digestion followed by agarose gel electrophoresis and comparison with known DNA standards.

Transient transfection of 3T3-L1 cells

Five days after the induction of differentiation, 3T3-L1 adipocytes were transfected with 6 μg plasmids containing the leptin promoter constructs using a calcium phosphate method (Moreno-Aliaga *et al.* 2001). The pRL-SV40 plasmid (4 ng), encoding *Renilla* luciferase, was used to control for transfection efficiency. After 16–20 h of incubation, the culture medium was removed and cells were washed with Dulbecco's PBS. Cells were then incubated for 48 h in medium containing 25 mM glucose, with or without insulin (16 nM). 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 induces a marked (>90%) suppression of leptin mRNA expression and leptin secretion in primary cultured rat adipocytes, but does not induce cytotoxic effects, as 18S RNA was unaffected and lipoprotein lipase activity was only modestly reduced (Mueller *et al.* 1998).

Transient transfection of primary adipocytes by electroporation

An aliquot of 200 μl adipocyte suspension was placed into 0·4 cm gap electrocuvettes containing 10 μg leptin promoter constructs and 3·5 ng pRL-SV40 plasmid. Cells were electroporated by administering 1 pulse at a voltage of 200 V and a capacitance of 950 μF. After electroporation, adipocytes were transferred to polystyrene tubes containing 2 ml DMEM (5 mM glucose) with 1% fetal bovine serum (FBS) and 1·6 nM insulin with or without 10 mg/dl 2-DG, as reported in the results.

Dual luciferase assay

After 20 h (primary adipocytes) or 48 h (3T3-L1 cells) of treatment, cells were lysed, and firefly and *Renilla* luciferase assays were performed on the lysate using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's standard protocol.

Electrophoretic mobility shift assays (EMSAs)

Nuclei were isolated from primary adipocytes, treated without or with insulin (1·6 nM) in the absence or presence of 2-DG, according to the method of Dignam *et al.* (1983) with minor modifications. Nuclear extracts were then prepared from these nuclei by a slight modification of the method of Lavery & Schibler (1993). EMSA studies were performed as described by Moreno-Aliaga & Matsumura (1999). The sequences of double-stranded oligonucleotides for the Sp1-binding assays were as follows: wt−112/−83 (5′-GCCCGCTGGGTGGGGCGGGAGTTGGCGCTC-3′), mut−112/−83 (5′-GCCCGCTGGGTGaaGCttGAGTTGGCGCTC-3′). Sp1 consensus oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Oligonucleotides were end-labeled using [γ-^{32}P]ATP (Amersham Life Sciences) and T4 polynucleotide kinase (Promega) according to the standard methods.

Nuclear extracts (10 μg) were incubated in a buffer containing 25 mM HEPES (pH 7·9), 10% glycerol, and 0·5 mM dithiothreitol, with 3 μg poly (d(I−C)) (Boehringer Mannheim, Indianapolis, IN, USA), and 5 μg acetylated BSA for 30 min at 4 °C. A 100-fold excess of specific competitor was added to some samples. Lastly, the different radiolabeled double-strand oligonucleotides (50 000 c.p.m.) were added and incubated for an additional 20 min at room temperature. For supershift assays, a specific antibody against Sp1 (Santa Cruz Biotechnology) was added. Oligonucleotide–nuclear factor complexes were determined by electrophoresis in a non-denaturing 4% polyacrylamide gel at 150–200 V for 2–3 h. Gels were dried and exposed to film with an intensifying screen at −80 °C.

Measurement of leptin secretion, glucose utilization, and lactate production in cultured primary rat adipocytes

Epididymal rat adipocytes (150 μl of 2:1 ratio of packed cells to medium), isolated as previously described, were plated on 500 μl collagen matrix (Vitrogen 100, Cohesion Technologies, Palo Alto, CA, USA) in six-well culture plates. After a 45-min incubation at 37 °C, culture media (5 mM glucose DMEM) containing the different treatments (1·6 nM insulin and/or 0·1 μM WP631) were added, and the cells were maintained in an incubator at 37 °C in 5% CO_2 for up to 48 h. Leptin concentrations in the media were determined with an RIA for rat leptin (Linco Research, St Charles, MO, USA). Glucose utilization and lactate production were assessed by measuring their concentration in the media with an Autoanalyzer (Cobas Roche Diagnostic). The amount of carbon released as lactate per amount of carbon taken up as glucose over 48 h was calculated as Δ(lactate)/Δ(glucose), where Δ is the change and expressed as a percentage.

Real-time PCR analysis

Total RNA was extracted using TRIZOL reagent (GIBCO-Life Technologies, Inc.) according to the manufacturer's instructions and incubated with RNase-free kit DNase (Ambion, Austin, TX, USA) for 30 min at 37 °C. RNA concentrations were measured spectrophotometrically and its quality was verified by ethidium bromide staining after agarose gel electrophoresis.

For each sample, 1·5 μg RNA were reverse transcribed to cDNA using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 26·6 μl. Reverse transcription was performed under the following conditions: 60 min at 37 °C and 5 min at 95 °C. cDNA samples were then frozen at −20 °C in several aliquots until gene expression assays were performed.

For real-time PCR analysis, 9 μl cDNA per reaction were used. Reagents for real-time PCR analysis of leptin, Sp1, and 18S (Assays-on-Demand, TaqMan Universal PCR Master mix) were purchased from Applied Biosystems and the conditions were used according to manufacturer's protocol. Amplification and detection of specific products were performed with the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems). A standard curve was plotted for each primer–probe set with a decimal serial dilution of several cDNA samples to ensure that the end of the reaction for control and different treatment samples was in the middle of the exponential curve of amplification.

18S ribosomal primer–probe was used to normalize the expression levels between samples allowing data to be expressed relative to 18S rRNA, therefore, compensating any difference in reverse transcriptase efficiency. All standards and samples were analyzed as duplicates. Data were obtained as Ct values (the cycle where the emitted fluorescence signal is significantly above the background levels and is inversely proportional to the initial template copy number) according to manufacturer's guidelines, and used to determine ΔCt values (ΔCt = Ct of the target gene – Ct of the housekeeping gene 18S) of each sample. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Statistical analysis

For the transfection studies, the means were compared by one-way ANOVA followed by a Bonferroni's *post hoc* test (GraphPad Prism, GraphPad Software, San Diego, CA, USA). The statistical analysis of the effects of WP631 on leptin secretion, glucose utilization and the percentage of glucose converted to lactate were performed by a repeated-measures ANOVA followed by a Bonferroni's *post hoc* test, because of the high variability between adipocytes cultures from different rats; for this reason, the experimental results from each adipocyte suspension prepared from a single animal were analyzed in relation to a control well from the same suspension (Perez-Matute *et al.* 2005). Differences were considered as statistically significant at $P<0·05$.

Results

Identification of *cis*-acting DNA sequences involved in the regulation of leptin gene by glucose metabolism

To define the *cis*-acting DNA sequences required for the upregulation of leptin gene by insulin-mediated glucose metabolism, we performed a deletion analysis of the leptin promoter in differentiated 3T3-L1 adipocytes and primary rat adipocytes. 3T3-L1 cells were transiently transfected with a series of reporter constructs containing various portions of the leptin promoter sequence. Previous studies have shown that the proximal promoter (up to −161 bp from the transcriptional start site) is sufficient for leptin expression in adipocytes (He *et al.* 1995).

As shown in Fig. 1, insulin and glucose-stimulated reporter gene activity of the −762 and −135 constructs at least twofold in 3T3-L1 adipocytes. This increase was abolished by incubation with 2-DG (data not shown). In contrast, a marked reduction in insulin-stimulated luciferase activity was observed when reporter genes of −95 and −85 bp were transfected. Similar results were observed in primary adipocytes transfected with the −135 and −95 constructs (Fig. 2). The transcriptional activity of the leptin promoter, as assessed by increased luciferase activity, was increased threefold in the presence of a physiological concentration of insulin (1·6 nM) in cells transfected with the p(−135) construct but was unaffected in the p(−95) construct. Furthermore, the increase of transcriptional activity induced by insulin was abolished by co-incubation with the inhibitor of glucose metabolism, 2-DG. Thus, these data indicate that the *cis*-acting DNA sequence which mediates the activation of the leptin promoter in response to insulin-stimulated glucose metabolism is located between positions −135 and −95 of the leptin gene.

The sequence centered at −97 is an exact match to Sp1 core motif sequence (Mason *et al.* 1998). To study the contribution of this region of the promoter to the insulin and glucose responsiveness, primary adipocytes were transfected with a plasmid m(−100/−95) containing four point mutations between bases −100 and −95. These mutations resulted in a loss of promoter activation (Fig. 3). As a control, we also transfected a mutated −140/−135 construct, which contains an intact Sp1 sequence centered on −97. In primary cells transfected with this construct, insulin induced a twofold increase of transcriptional activity that was abolished by 2-DG. Together, the results demonstrate that mutation of the Sp1 site, but not an upstream site, results in a loss of responsiveness to insulin-stimulated glucose metabolism.

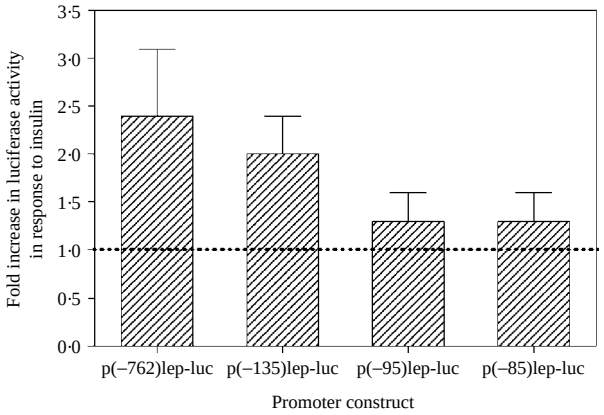

Figure 1 Locations of insulin-mediated glucose metabolism regulatory sequences in the leptin promoter in 3T3-L1 adipocytes. Cells were transfected with the −762, −135, −95, and −85 bp plasmid constructs and treated for 48 h without or with insulin (16 nM). A pRL-SV40 plasmid was co-transfected to control for transfection efficiency. The mean ± S.E.M. of at least four independent experiments is shown.

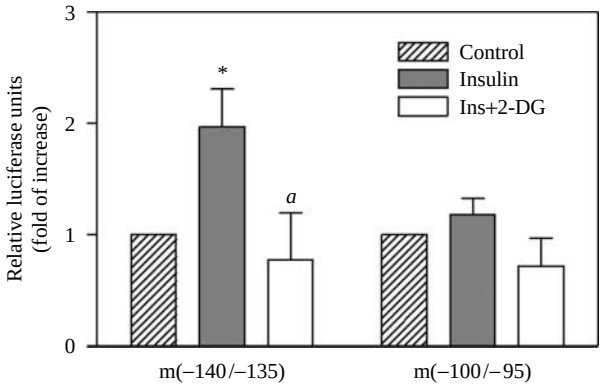

Figure 3 Effects of insulin and 2-DG in cells transfected with the mutant plasmids m(−140/−135) and m(−100/−95) of leptin promoter in primary rat adipocytes. Cells were treated for 20 h without or with insulin (1·6 nM) in the absence or presence of 2-DG (10 mg/dl). A pRL-SV40 plasmid was co-transfected to control for transfection efficiency. Data are expressed as fold of increase of the activity in the absence of insulin. The mean ± S.E.M. of six independent experiments is shown. $^{*}P<0{\cdot}05$ compared with the control cells. $^{a}P<0{\cdot}05$ compared with the insulin-treated group.

Identification of trans-acting factor involved in the regulation of the leptin gene by glucose metabolism

The data from the previous experiments suggest that the transcription factor, Sp1, mediates the activation of the leptin promoter in response to insulin-stimulated glucose metabolism. While previous studies have reported that Sp1 binds to this site (or to its equivalent in the rat gene; Mason *et al.* 1998, Fukuda & Iritani 1999), there are currently no available data addressing whether this Sp1-mediated transcription is regulated by insulin-stimulated glucose metabolism. To test this hypothesis, EMSAs were performed with adipocyte nuclear extracts from control and insulin-treated cells (in the absence or presence of 2-DG).

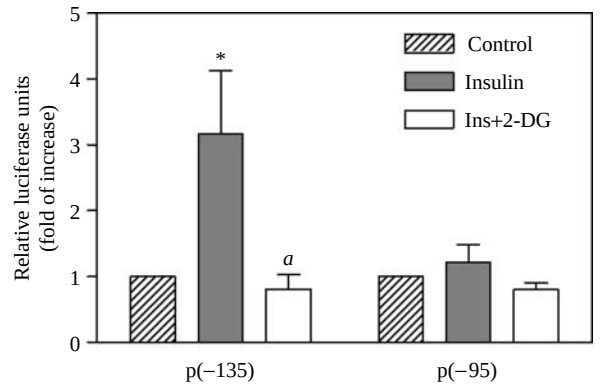

Figure 2 Effects of 2-DG on the action of insulin on leptin promoter activity in primary rat adipocytes. Cells were transfected with the p(−135)lep–luc or p(−95)lep–luc plasmids and treated for 20 h without or with insulin (1·6 nM) in the absence or presence of 2-DG (10 mg/dl). A pRL-SV40 plasmid was co-transfected to control for transfection efficiency. Data are expressed as fold of increase of the activity in the absence of insulin. The mean ± S.E.M. of six independent experiments is shown. $^{*}P<0{\cdot}05$ compared with the control cells. $^{a}P<0{\cdot}05$ compared with the insulin-treated group.

Using nuclear extracts from cells treated in the absence of insulin, DNA–protein complexes of the same mobility were observed for the wild-type sequence of the leptin promoter from bases −112 to −83 (wt(−112/−83), Fig. 4, lane 1) and for an Sp1 consensus oligonucleotide (lane 8). Subsequently, treatment with insulin (1·6 nM) increased the abundance of the Sp1–DNA complex (lane 1 versus lane 2 and lane 8 versus lane 9), and this increase was abolished by the addition of 50 mg/dl 2-DG (lanes 3 and 10). The intensity of the Sp1–DNA complexes was also markedly decreased upon preincubation with an antibody directed against Sp1, indicating that Sp1 is present in the complex (lanes 4–6 and 11). The specific binding of Sp1 to the wt−112/−83 probe was eliminated by competition with an excess of homologous unlabeled probe (lane 7). Also, mutation of four bases in the Sp1 site (in oligonucleotide m(−112/−83)) prevented the formation of a DNA–Sp1 protein complex under these conditions (lanes 12–14).

Inhibition of Sp1-mediated transcription prevents the stimulatory effects of insulin-stimulated glucose metabolism on leptin secretion

To verify that increased leptin secretion in response to insulin-stimulated glucose metabolism involves Sp1-mediated transcription, primary adipocytes were incubated with WP631, a bisintercalating anthracycline drug, which specifically inhibits Sp1-dependent transcription (Botella *et al.* 2001, Mansilla *et al.* 2004).

Probe	wt(–112/–83)							Spl				m(–112/–83)		
Competition							#							
Spl-Ab	–	–	–	+	+	+	–	–	–	–	+	–	–	–
2-DG	–	–	+	–	–	+	–	–	–	+	–	–	–	+
Insulin	–	+	+	–	+	+	–	–	+	+	+	–	+	+
Lane no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14

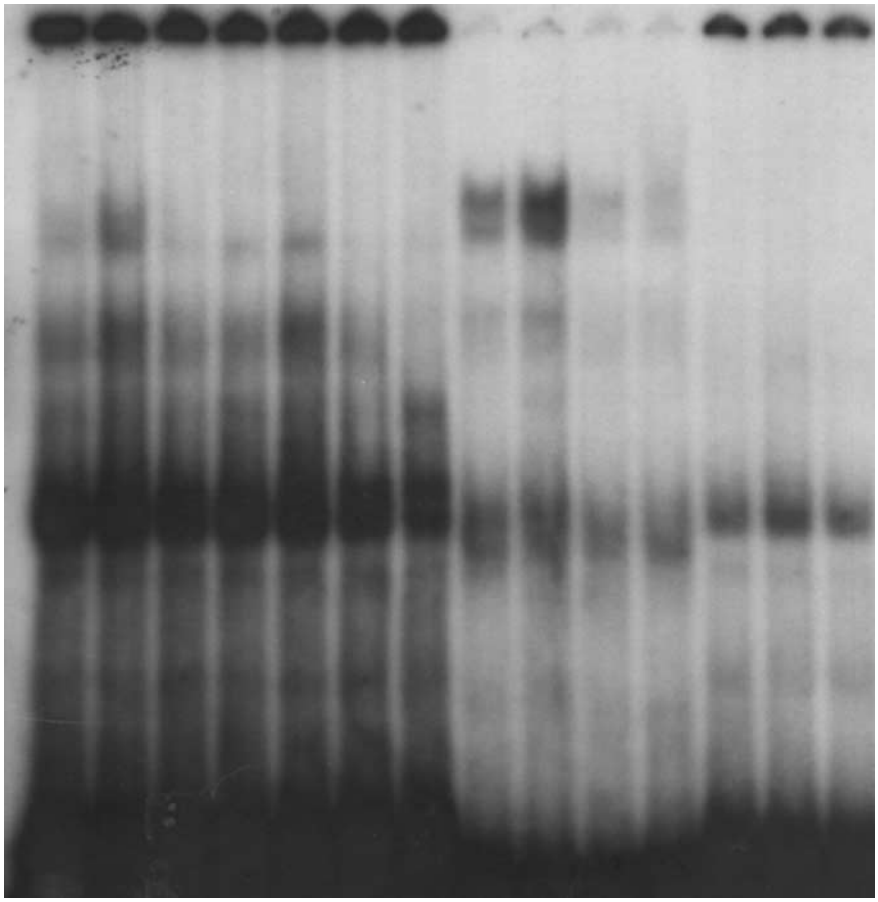

Figure 4 Effects of 2-DG on insulin-stimulated binding of Sp1 to its consensus sequence in the leptin promoter. Gel shift analysis was performed in nuclear extracts prepared from primary rat adipocytes treated for 24 h without or with insulin (1·6 nM) in the absence or presence of 2-DG (50 mg/dl). Sp1 consensus oligonucleotide and wild-type (wt−112/−83) and mutant (m−112/−83) oligonucleotide sequences of the leptin promoter were used as probes. For supershift complexes, nuclear extracts were also incubated with a specific anti-Sp1 antibody. #A competition assay using a 100-fold excess of corresponding unlabeled oligonucleotide was performed to obtain non-specific-binding activity. Each shift is representative of three independent experiments.

Treatment with WP631 (0·1 μM) for 48 h had no effect on basal leptin secretion in cultured primary rat adipocytes, but completely suppressed insulin-stimulated leptin secretion (Fig. 5A). These results were confirmed by RT-PCR, where the insulin-stimulated increase in leptin mRNA expression was blocked by WP631 (Fig. 5B). The effects of WP631 on glucose utilization and lactate production were also determined. Figure 5C and D shows that WP631 treatment did not significantly modify either glucose utilization or the percentage of glucose carbon released as lactate, suggesting that the compound does not exert non-specific effects on cellular metabolism which could affect insulin stimulation of leptin secretion. Therefore, the suppression of insulin-stimulated leptin secretion caused by WP631 (0·1 μM) is likely to be due to its inhibitory properties of Sp1-dependent transcription.

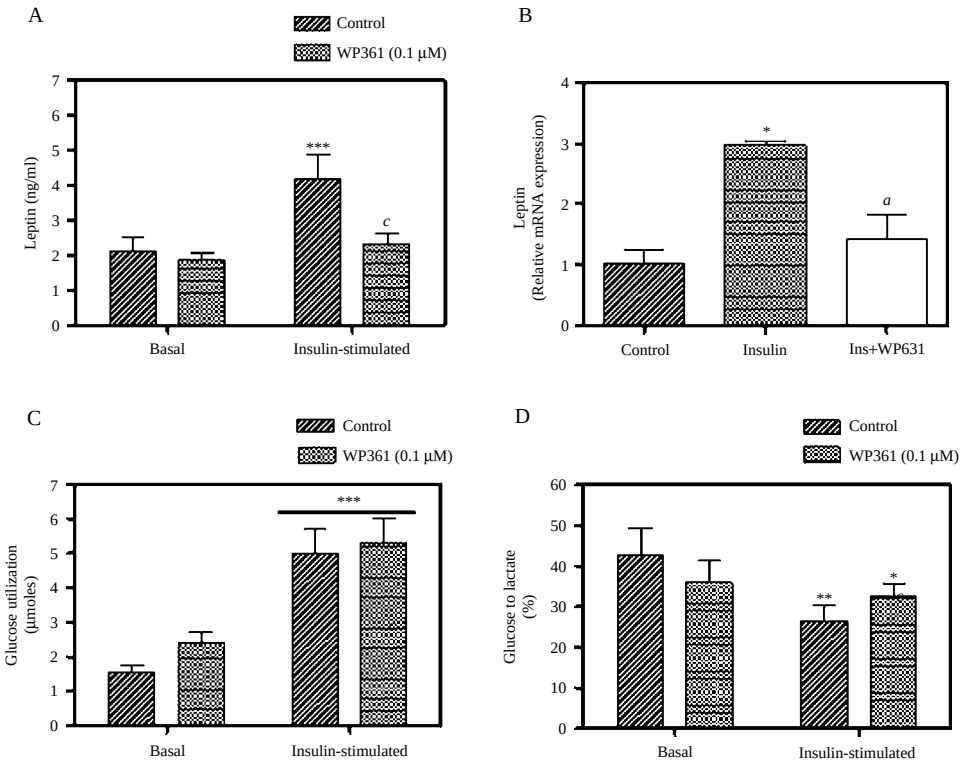

Figure 5 Effects of WP631 on basal and insulin-stimulated leptin secretion (A), leptin gene expression (B), glucose utilization (C), and the percentage of glucose carbon released as lactate (D) after 48 h of culture. Isolated adipocytes were incubated in the absence or presence of insulin (1·6 nM) with WP631 (0·1 μM). $N=8$. *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ compared with control (basal group). $^{a}P<0{\cdot}05$, $^{c}P<0{\cdot}001$ compared with the insulin-stimulated group.

Effects of okadaic acid (OA) on insulin-stimulated leptin expression and secretion

Several studies have demonstrated that changes in the phosphorylation status of Sp1 are controlling the ability of this transcription factor to bind to DNA (Samson & Wong 2002). The effects of okadaic acid (OA), a potent phosphatase inhibitor, on leptin gene expression and protein secretion were also examined. The results showed that both leptin secretion (Fig. 6) and leptin mRNA levels (data not shown) were inhibited by OA from the first 4 h of culture in primary adipocytes.

In addition, our data showed that the expression levels of Sp1 were not changed as a result of insulin treatment of adipocytes, suggesting that the overall quantity of Sp1 does not seem to be increased (data not shown).

Together, these data suggest a potential role for dephosphorylation, likely involving Sp1 in the transcriptional regulation of leptin by insulin-stimulated glucose metabolism.

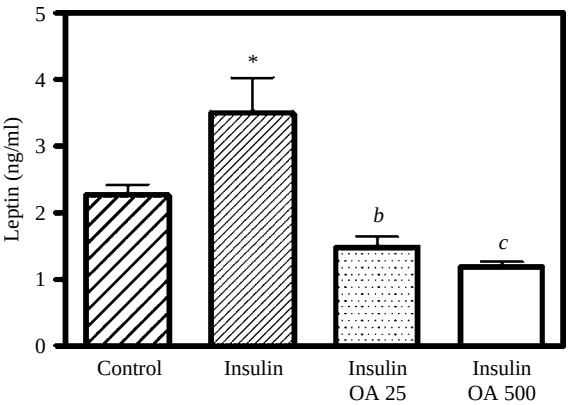

Figure 6 Effects of okadaic acid (OA 25 and 500 nM) on insulin-stimulated leptin secretion in cultured adipocytes. $N=6$ in all groups. *$P<0{\cdot}05$ compared with control group. $^{b}P<0{\cdot}01$ and $^{c}P<0{\cdot}001$ compared with the insulin-stimulated group.

Discussion

The present study was conducted to investigate the mechanisms by which insulin-stimulated glucose metabolism stimulates leptin secretion in adipocytes. Leptin is a major determinant of body weight/adiposity in mammals via its actions to regulate both food intake and energy expenditure (Havel 2004). A thorough understanding of the mechanisms regulating leptin synthesis and secretion may yield new therapeutic strategies to prevent recidivism following weight loss (Rosenbaum *et al.* 2005). We have previously reported that insulin-stimulated glucose metabolism, rather than a direct action of insulin itself, is a major determinant of leptin production in adipocytes (Mueller *et al.* 1998, 2000, Moreno-Aliaga *et al.* 2001), and our findings have been corroborated by *in vivo* data from human subjects (Wellhoener *et al.* 2000). In the present study, we first identified a region of the leptin promoter (−135 to −95 bp, relative to the transcription start site) required to increase the transcriptional activity of the leptin promoter in response to insulin-stimulated glucose metabolism in 3T3-L1 cells and in primary cultured rat adipocytes. This sequence contains a consensus Sp1 site, centered at −97 bp, and a mutation of four nucleotides at this site, but not at an upstream site, prevents the increase of transcription induced by insulin. Again, this was demonstrated to be mediated by insulin-stimulated glucose metabolism as it was abolished by co-incubation with 2-DG, a competitive inhibitor of glucose uptake and phosphorylation. This inhibitory effect of 2-DG is unlikely to be due to a depletion of adipocyte energy stores, as it is known that adipocytes can generate energy (ATP) by oxidizing fatty acids via mitochondrial β-oxidation (Mayers 1993, Moore *et al.* 1996). Using gel shift assays, we showed that insulin increased the binding of Sp1 to this site, and that formation of the Sp1–DNA complex in response to insulin was prevented by treatment with 2-DG, demonstrating a requirement for glucose metabolism. Consistent with these results, we also found that incubating primary adipocyte cultures with a specific inhibitor of Sp1-mediated transcription had no effect on basal leptin secretion, but completely prevented the increase of leptin secretion induced by insulin.

Sp1 is a ubiquitously expressed transcription factor that recognizes GC-rich sequences and may function as a cellular glucose sensor (Vaulont *et al.* 2000). Low glucose levels have been shown to attenuate the DNA-binding activity of Sp1 via hypo-GlcNAcylation (Kang *et al.* 2003), a post-translational modification that involves the covalent linkage of the monosaccharide *O*-GlcNAc to serine and threonine residues. In this case, this is followed by proteolytic degradation (Han & Kudlow 1997). Interestingly, Kang *et al.* (2003) have reported that 2-DG downregulates Sp1 activity, although via hyper-GlcNAcylation, which does not attenuate Sp1's DNA-binding activity but rather appears to interfere with its activation domain. In our previous study (Moreno-Aliaga *et al.* 2001), we did not detect significant changes in leptin promoter activity in response to 2-DG in the absence of insulin, suggesting that its effects on the leptin promoter are specific for insulin-mediated glucose metabolism. Other genes regulated by Sp1 in response to glucose include ACC (Daniel & Kim 1996), aldolase A and pyruvate kinase M2 (Schafer *et al.* 1997), and plasminogen activator inhibitor-1 (Chen *et al.* 1998). Sp1 and the closely related Sp3 transcription factors also appear to be involved in glucose- and insulin-dependent expression of fatty acid synthase and ATP citrate-lyase (Fukuda *et al.* 1999). A growing body of evidence suggests that the DNA-binding and transcription activity of Sp1 may increase or decrease in response to changes in phosphorylation (Samson & Wong 2002, Lam *et al.* 2003). Thus, dephosphorylation of Sp1 by protein phosphatase 1 has been involved in the glucose-mediated activation of the acetyl-CoA carboxylase, aldolase, and pyruvate kinase (Daniel *et al.* 1996, Schafer *et al.* 1997). Our data also suggest a potential role for dephosphorylation, likely involving Sp1 in the transcriptional regulation of leptin by insulin-stimulated glucose metabolism.

Although the Sp1 site in the leptin promoter has been identified in prior studies (Mason *et al.* 1998, Fukuda & Iritani 1999), the present work is significant and novel in that we identified this site by determining which regions of the promoter were required to stimulate leptin transcription in response to insulin-stimulated glucose metabolism. Moreover, our finding that inhibition of Sp1-mediated transcription (with WP631) prevents the increase in leptin secretion from adipocytes cultured in the presence of glucose and insulin has not been previously reported.

The present results conflict with those previously reported in some other studies, however, Fukuda & Iritani (1999) also found that Sp1 bound to the equivalent site in the rat leptin promoter, but their data suggested that Sp1 inhibited leptin transcription in adipocytes. The authors constructed a reporter gene containing two copies of the −101 to −83 bp leptin promoter sequence, which was then transfected into adipocytes cultured in 20 mM glucose and 0·1 μM insulin. When an Sp1 expression vector was co-transfected, a significant inhibition of transcription was observed after 48 h. Mutation of the Sp1 site in their reporter gene also resulted in a loss of responsiveness to glucose and insulin, leading the authors to conclude that Sp1 might inactivate leptin transcription by binding to this sequence. The discrepancies between the present study and the findings of Fukuda & Iritani (1999) may be resolved by considering that native leptin

mRNA or protein levels in response to Sp1 transfection were not assessed in their study, and that the reporter gene used to perform the experiments was relatively simple (they used two repeats of a 19 bp sequence compared with the larger 135 bp promoter sequence in the present study). Moreover, it is plausible that binding sites for additional transcription factors, such as C/enhancer binding protein (EBP)-α (He *et al.* 1995), may be required to accurately assess the full response to Sp1 co-transfection. Finally, the concentrations of glucose and insulin in those previous studies were much higher than we employed in the present study (5 mM and 1·6 nM), which are within the physiological range. However, their data concerning the binding of Sp1 to this sequence and concomitant increase in transcriptional activity in response to glucose and insulin treatment are entirely consistent with the present study.

Other investigators (Wang *et al.* 2000) have identified a glucose- and insulin-responsive element in the sequence between −1698 and −1692 bp of the mouse leptin promoter. Glucose and insulin prevented the binding of a nuclear protein to this promoter element. The authors did not detect any glucose/insulin-responsive region in the mouse proximal promoter after transfection of 3T3-L1 adipocytes with constructs with serial deletions of the mouse leptin promoter. We do not dispute their results; however, we maintain that the elements controlling expression of leptin in adipocytes are likely to be proximal, as others have found that deletion of bases −762 to −135 of the leptin promoter does not compromise its expression in adipocytes (He *et al.* 1995, Mason *et al.* 1998). Our view is further supported by the results of Zhang *et al.* (2002), who found evidence for a glucose-responsive element is located within the proximal portion of the human leptin promoter (−219 to +29 bp). It must also be mentioned that the proximal ∼150 bp of the leptin promoter is highly conserved (60–80%) between mice, rats, and humans (as represented on the VISTA Genome Browser, available at www.pipeline.lbl.gov (Couronne *et al.* 2003)).

In summary, the results of this study reveal a role for the ubiquitous transcription factor Sp1 in the regulation of leptin synthesis and secretion in response to insulin-stimulated glucose metabolism in adipocytes. First, we identified the region (−135 to −95 bp) of the proximal promoter that mediates the increase in leptin transcription in response to glucose and insulin, and found that mutation of a consensus Sp1-binding site abolished this response. Next, we demonstrated that the binding of Sp1 to this site was increased in response to insulin treatment, and was inhibited when glucose metabolism is blocked by 2-DG treatment. To subsequently confirm the involvement of Sp1 in this process, we found that inhibition of Sp1-mediated transcription prevented insulin-stimulated leptin secretion in adipocytes. These novel findings are consistent with the known role of Sp1 as a mediator of transcriptional events in response to glucose (Vaulont *et al.* 2000) and significantly enhance our understanding of how leptin synthesis and secretion by adipocytes are linked to changes in glucose metabolism and nutritional status in mammals.

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