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Effects of inhibiting transcription and protein synthesis on basal and insulin-stimulated leptin gene expression and leptin secretion in cultured rat adipocytes

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Abstract

We have previously reported that glucose metabolism mediates the effects of insulin to increase leptin gene expression and leptin secretion by isolated adipocytes. The aim of the present study was to investigate the role of transcription and translation in the regulation of basal and insulin-stimulated leptin production. The short-term (4 h) and long-term (24–48 h) effects of actinomycin D, a transcriptional inhibitor, and cycloheximide, an inhibitor of protein synthesis, on leptin gene expression and leptin secretion by isolated adipocytes were determined. Actinomycin D (5 μg/ml) increased both basal and insulin-stimulated (1.6 nM) leptin secretion at 4 and 24 h ($193 \pm 14.9\%$ and $153.8 \pm 10.4\%$ of respective controls at 24 h, both $p < 0.001$). Similar effects of actinomycin D were observed on basal and insulin-stimulated leptin mRNA levels. 5,6-Dichlororibofuranosyl benzimidazole (DRB), another inhibitor of transcription, also increased basal ($175.4 \pm 18.2\%$ of control; $p < 0.01$) and insulin-stimulated leptin secretion ($141.0 \pm 11.1\%$ of insulin-treated cells; $p < 0.05$) at 24 h. The effect of actinomycin D and DRB to increase basal leptin secretion observed at 4 and 24 h was not present at 48 h when actinomycin D and DRB both markedly inhibited insulin-stimulated leptin secretion (to $36 \pm 16\%$, $p < 0.05$ and $21.9 \pm 5.6\%$ of control, for actinomycin D and DRB, respectively, both $p < 0.001$). Neither actinomycin D nor DRB had any effect on adipocyte glucose utilization between 24 and 48 h. The observed effects of inhibitors of transcription on leptin gene expression and leptin secretion are consistent with a long-term transcriptional mechanism for insulin-stimulated glucose metabolism to increase leptin production. Cycloheximide treatment (10 μg/ml) abolished the effects of insulin to stimulate leptin secretion ($29 \pm 11\%$ of control, $p < 0.01$) during the first 4 h of treatment and at all later time points, which indicate that de novo protein synthesis is required for insulin-mediated glucose metabolism to increase leptin secretion.

Keywords: Leptin; Insulin-mediated glucose metabolism; Transcription; Actinomycin; Cycloheximide

Leptin, a hormone produced in adipocytes, is involved in the regulation of body weight via its central actions on food intake and energy expenditure [1]. Circulating leptin concentrations decrease, independently of body adiposity, after fasting or caloric restriction in both humans [2,3] and rodents [4] and the decreases of leptin are proportional to changes of plasma insulin and glucose. Leptin gene expression and circulating leptin concentrations are markedly decreased in insulin-deficient diabetic animals [5,6]. The decreases of leptin expression and circulating leptin in insulin-deficient diabetes are reversed by insulin administration in proportion to the degree of glucose lowering [6,7]. These data provide evidence of a role for insulin and glucose to mediate changes of leptin production in vivo [8,9]. Several in vitro studies have also shown that insulin potently stimulates leptin secretion in cultured adipocytes [10,11]. Previously published experiments from our

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laboratory indicated that increased adipocyte glucose metabolism mediates the effect of insulin to stimulate leptin gene expression and leptin secretion [12]. Thus, the effect of insulin to increase adipocyte glucose utilization is likely to contribute to insulin-stimulated leptin production. A recent study in humans also suggests that increased leptin secretion during glucose and insulin infusion is mediated by insulin's actions to increase glucose metabolism rather than to an effect of insulin per se [13].

The biochemical and molecular mechanisms underlying the effects of glucose and insulin to increase leptin production have not been identified. Several investigators have reported that leptin gene mRNA expression is increased after insulin treatment both in vivo and in vitro [7,14,15] or in response to glucose administration in rodents [14]. A recent study has also shown that insulin and glucose increase leptin mRNA expression in abdominal subcutaneous tissue in humans [16]. These studies suggest that the regulation of leptin production by insulin and glucose occurs at the level of transcription. Accordingly, it has been shown that insulin increases the transcriptional activity of leptin promoter in isolated adipocytes [17] and that this activation is mediated by increased glucose metabolism [18]. Other studies, however, have suggested that post-transcriptional mechanisms may also be involved in the short-term effects of insulin to stimulate leptin secretion [19,20]. A recent paper described a dual action of insulin on leptin secretion with an early (less than 48 h) inhibitory action followed (48–96 h) by a clear-cut stimulation. In addition, it was reported that these effects were not mediated by changes in leptin gene expression, suggesting that it involved a post-transcriptional mechanism [21].

In the current study, we try to better elucidate the roles of transcriptional and post-transcriptional mechanisms in the actions of insulin-mediated glucose metabolism to increase leptin gene expression and protein secretion. Several previous experiments examining the molecular mechanisms by which glucose and insulin increase leptin production have employed inhibitors of transcription and translation. However, in most experiments, the actions of these agents have been investigated over a short period of time (2 h) [19,20]. In the present studies, we examined and compared both the short-term (4 h) and long-term (24–48 h) effects of two transcriptional inhibitors, (actinomycin D and DRB) and an inhibitor of protein synthesis (cycloheximide), on basal and insulin-stimulated leptin secretion and leptin gene expression.

Materials and methods

Adipocyte isolation and culture. Adipocytes were isolated from epididymal fat pads of nonfasted male Sprague–Dawley rats (3–4 months). The 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). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue; Worthington, Lakewood, NJ) at 37 °C with gentle shaking at 60 cycles/min for 30 min.The resulting cell suspension was diluted in Hepes-phosphate buffer and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400-μm nylon mesh and washed three times. Isolated adipocytes were then resuspended in DMEM supplemented with 1% FBS and incubated for 30 min at 37 °C.

The isolated adipocytes (150 μl of 2:1 ratio of packed cells to medium) were then plated on 500 μl of a collagen matrix (Vitrogen 100, Cohesion Technologies, Palo Alto, CA) in 6-well culture plates. After a 45 min incubation at 37 °C, culture media containing the different treatments were added and the cells were maintained in an incubator at 37 °C in 6% CO_2 for up to 48 h.

Aliquots (300 μl) of the media were collected at 4, 24, and 48 h, and replaced with fresh medium containing the appropriate concentration of insulin (1.6 nM), actinomycin D, DRB, and/or cycloheximide.

Leptin and glucose assay. Leptin concentrations in the medium were determined by a radioimmunoassay for rat leptin as previously described [22] (Linco Research, St. Charles, MO). Glucose was measured with a YSI glucose analyzer (Model 2300, Yellow Springs Instruments, Yellow Springs, OH).

RNA preparation and Northern blot analysis. Leptin mRNA was determined by Northern blotting. The leptin cDNA probe was a 388-bp fragment of mouse leptin cDNA (kindly provided by Dr. Charles Mobbs, Mount Sinai School of Medicine, New York). The 18S ribosomal probe was obtained from Ambion (Ambion, Austin, TX).

RNA was extracted according to the Gibco Life Technologies procedure using Trizol (Life Technologies, Grand Island, NY). UV absorbance and integrity gels were used to estimate RNA. Leptin and 18S cDNA probes were labeled by random priming (Rediprime kit, Amersham, Buckinghamshire, UK) in the presence of [^{32}P]dCTP (3000 Ci/mmol, Amersham). Unincorporated nucleotides were removed using NucTrap probe purification columns (Stratagene, La Jolla, CA). For each tissue sample, 5–7 μg of total RNA was fractionated by electrophoresis on a denaturing 1% agarose gel containing 2.2 M formaldehyde and 1× Mops running buffer. One microliter of a 50 μg/ml ethidium bromide (Gibco BRL, Gaithersburg, MD) stock solution was added in order to check RNA integrity and even loading. After electrophoresis, RNA was transferred to nylon membrane (Duralon-UV, Stratagene, La Jolla, CA) by overnight capillary transfer and UV cross-linked (Stratalinker 1800, Stratagene, La Jolla, CA). Blots were then hybridized for 1 h at 68 °C in presence of the labeled cDNA probe (2×10^6 cpm/ml Express Hyb solution, Clontech, Palo Alto, CA). After washing at high stringency, blots were exposed to X-ray films with an intensifying screen for 1 day at −80 °C. After analysis of leptin mRNA using a single-stranded cDNA probe followed by quantification of bands from film, the blots were re-analyzed using a probe complementary to mouse 18S ribosomal RNA, to allow loading of equal mass of RNA in each well. Leptin mRNA was then normalized with respect to the 18S ribosomal signal.

Data analysis. Glucose utilization was determined by subtracting the glucose concentration measured during the incubations from the initial media glucose concentration. Glucose utilization was corrected for the amount of glucose that was removed during each medium sampling and the amount added by the replacement of fresh media (15% of total volume).

The effects of the inhibitors in each adipocyte suspension prepared from a single animal were compared to a control well from the same suspension. Data are expressed as means ± SEM. Data were analyzed by ANOVA followed by a Bonferroni's post hoc test (GraphPad Prism, GraphPad Software, San Diego, CA).

Results

Effects of actinomycin D on basal and insulin-stimulated leptin secretion

Fig. 1A depicts both short (4 h) and long-term (24–48 h) effects of actinomycin D (5 μg/ml) on basal and insulin-stimulated leptin secretion in isolated rat adipocytes. In the absence of insulin, leptin release from the adipocytes (control group) increased between 4 and 48 h. The effects of actinomycin D (5 μg/ml) on basal leptin secretion were time-dependent. At 4 h of incubation, actinomycin D induced a slight but nonsignificant increase in basal leptin secretion (126% of control). This stimulation of basal leptin secretion by actinomycin D was very significant after 24 h of treatment ($193 \pm 14.9\%$ of control, $p < 0.001$). After 48 h of treatment, a significant ($p < 0.001$) but less potent stimulatory effect ($168.7 \pm 11.9\%$) of actinomycin D on cumulative leptin secretion was still observed. However, the amount of leptin secreted between the 24 and 48 h of treatment was similar for control and actinomycin D treated adipocytes (Fig. 1B). Thus, the stimulatory effect of actinomycin D on basal leptin secretion is not present after 24 h.

As expected, insulin (1.6 nM) induced an increase in leptin secretion from 4 to 48 h of treatment (Fig. 1A). Treatment with insulin and actinomycin D for 4 h induced a slight, but nonsignificant increase of leptin secretion. Similar to what was observed with basal leptin secretion, 24 h of actinomycin treatment increased insulin-stimulated leptin secretion ($153.8 \pm 10.4\%$ of insulin-treated cells, $p < 0.001$). However, during 24–48 h of treatment this effect was reversed. As shown in Fig. 1B, from 24 to 48 h, actinomycin D completely prevented the effect of insulin to stimulate leptin secretion, and leptin production was similar to that observed in the absence of insulin.

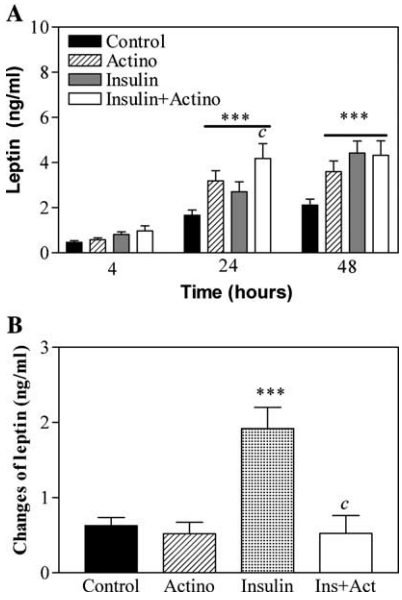

Fig. 1. Effects of actinomycin D on basal and insulin-stimulated leptin secretion. (A) Time-response effects from 4 to 48 h of treatment. (B) Changes in basal and insulin-stimulated leptin secretion between 24 and 48 h of treatment. Cultured adipocytes were incubated for up to 48 h in the absence or presence of insulin (1.6 nM) with actinomycin D (5 μg/ml). $N = 16$ in all groups at 4, 24, and 48 h. ***$p < 0.001$ compared to basal control. *c*: $p < 0.001$ compared with the 1.6 nM insulin-treated control.

Effects of actinomycin D on leptin gene expression

In order to determine if the effects of actinomycin D on leptin secretion were mediated at the transcriptional level on leptin gene expression, we measured the expression of both leptin mRNA and 18S RNA by Northern blot in control and actinomycin D treated cells. Treatment with actinomycin D for 24 h increased both basal ($223.6 \pm 65.2\%$ of control) and insulin-stimulated ($128.9 \pm 6.9\%$ of insulin-treated cells) leptin mRNA (Fig. 2A). Treatment with actinomycin D for 48 h totally abolished the effect of insulin to increase leptin gene expression (Fig. 2B). These findings are consistent with the observed effects on leptin secretion. Actinomycin D did not affect the expression of the 18S RNA in the presence or absence of insulin.

Effects of actinomycin D on basal and insulin-stimulated glucose utilization

Results from our previous studies indicated that insulin-stimulated glucose metabolism has an important role in the regulation of both leptin gene expression and secretion. Treatment with actinomycin D for 24 h induced a significant increase ($169.4 \pm 16.4\%$, $p < 0.01$) of basal glucose utilization compared to control. Insulin induced a significant stimulation ($349.1 \pm 33.6\%$, $p < 0.001$) in the amount of glucose utilized by the adipocytes, and actinomycin D further stimulated glucose utilization compared to insulin treatment alone during the first 24 h ($124.7 \pm 4.3\%$ of insulin-treated cells; $p < 0.01$) (Fig. 3A). The amount of glucose utilized by adipocytes between 24 and 48 h was not significantly affected by actinomycin D in the presence or absence of insulin (Fig. 3B).

Effects of DRB on basal and insulin-stimulated leptin secretion

In order to determine if the early stimulatory effect of actinomycin D on leptin expression and secretion was due to its transcriptional inhibitory properties, we examined the effect of 100 μM DRB, an inhibitor of transcription that acts through a different mechanism than that of actinomycin D, on leptin secretion at 24 and

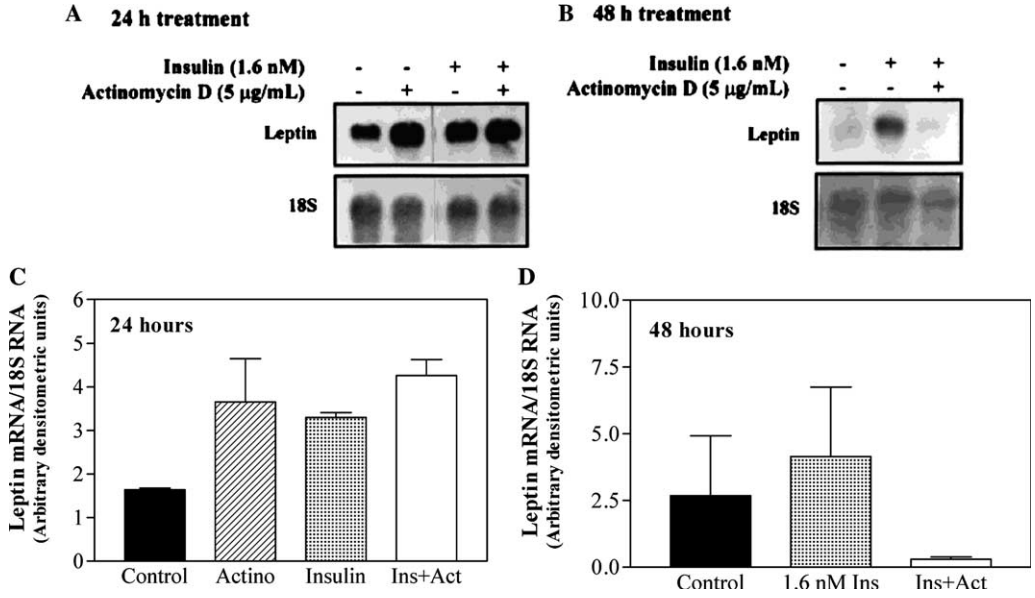

Fig. 2. Effects of actinomycin D on basal and insulin-stimulated leptin mRNA, as assessed by Northern blots (A and B) after 24 ($n = 3$) and 48 h ($n = 2$) of treatment. The expression level of 18S ribosomal RNA was determined and used as an internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of leptin mRNA and 18 S RNA (C and D).

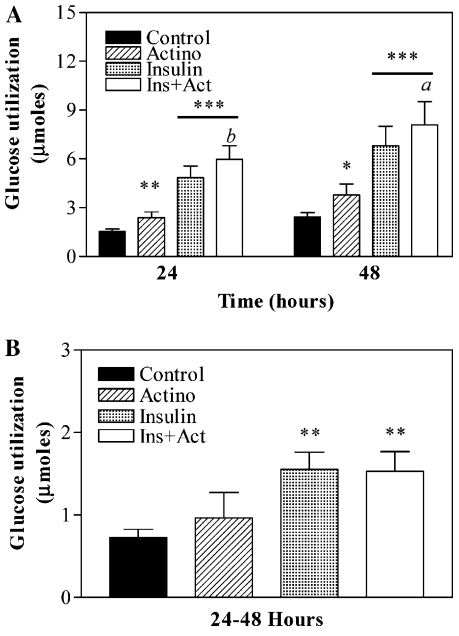

Fig. 3. Effects of actinomycin D on basal and insulin-stimulated glucose utilization. (A) Glucose utilization after 24 and 48 h of treatment. (B) Glucose utilization between 24 and 48 h of treatment. Isolated adipocytes were incubated for up to 48 h in the absence or presence of insulin (1.6 nM) with actinomycin D (5 μg/ml). $N = 13$ for 24 h and $N = 10$ for 48 h. $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to basal control. *a* and *b*: $p < 0.05$, $p < 0.01$ compared with the 1.6 nM insulin-treated control.

48 h. This concentration of DRB has been shown to inhibit the transcription of several genes in adipocytes during even shorter time periods [23,24].

The effect of DRB on both basal and insulin-stimulated leptin secretion was similar to what was observed for actinomycin D at both 24 and 48 h. Treatment for 24 h with DRB (100 μM) significantly increased basal ($175.4 \pm 18.2\%$ of control, $p < 0.01$) and insulin-stimulated leptin secretion ($141.0 \pm 11.1\%$ of insulin-treated cells, $p < 0.05$). Basal leptin secretion was unaffected by DRB treatment between 24 and 48 h. Insulin-stimulated increase of leptin secretion was completely blocked by DRB treatment ($21.9 \pm 5.6\%$ of insulin-treated cells, $p < 0.001$) (Fig. 4) and the 24–48 h leptin production was similar to that observed in the absence of insulin.

Effects of cycloheximide on basal and insulin-stimulated leptin secretion

The effects of cycloheximide, an inhibitor of protein synthesis, on both basal and insulin-stimulated leptin secretion were studied at different periods of treatment (4–48 h). Cycloheximide significantly decreased both basal ($39.0 \pm 10.5\%$ of basal, $p < 0.05$) and insulin-stimulated ($29.1 \pm 11.0\%$ of control, $p < 0.001$) leptin secretion during the first 4 h of treatment (Fig. 5). The inhibition of leptin secretion by cycloheximide was even greater during 24 h and longer periods of treatment (data not shown). These results indicate that de novo protein synthesis is required for both basal and insulin-stimulated leptin secretion.

Effects of cycloheximide on the actions of actinomycin D on leptin secretion

To further investigate the mechanisms underlying the paradoxical short-term stimulation of leptin secretion induced by actinomycin D, we examined the effects of

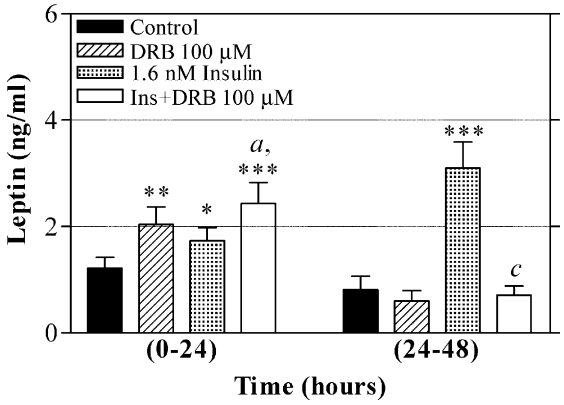

Fig. 4. Effects of DRB on basal and insulin-stimulated leptin secretion. Isolated adipocytes were incubated for up to 48 h in the absence or presence of insulin (1.6 nM) with DRB (100 μM). $N = 6$ in all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to basal control. *a* and *c*: $p < 0.05$, $p < 0.001$ compared with the 1.6 nM insulin-treated control.

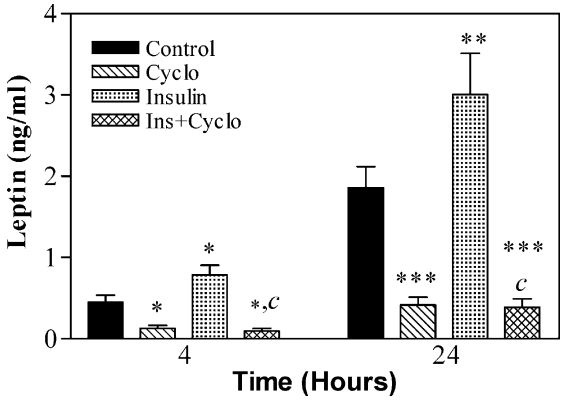

Fig. 5. Effects of cycloheximide on both basal and insulin-stimulated leptin secretion. Isolated adipocytes were incubated for up to 24 h in the absence or presence of insulin (1.6 nM) with cycloheximide (10 μg/ml). $N = 12$ in all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control. *c*: $p < 0.001$ compared with the 1.6 nM insulin-treated group, respectively.

co-treatment with cycloheximide. Cycloheximide treatment completely prevented the effect of actinomycin D to increase either basal or insulin-stimulated leptin secretion at 24 h of treatment (Fig. 6). These data suggest that the early stimulation of leptin secretion induced by actinomycin D requires de novo protein synthesis.

Effects of cycloheximide on the actions of actinomycin D on glucose utilization

Cycloheximide did not have any significant effect on the amount of glucose utilized at 24 h. However, cycloheximide prevented the short-term increase of glucose utilization induced by actinomycin D (Fig. 7).

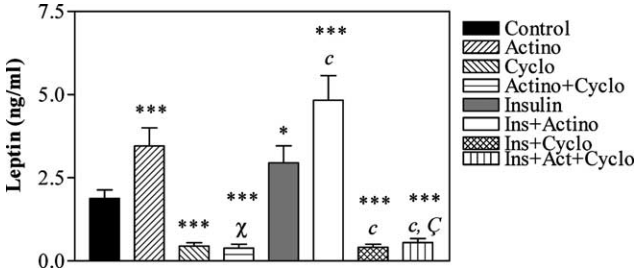

Fig. 6. Effects of cycloheximide on the actions of actinomycin D on basal and insulin-stimulated leptin secretion. Isolated adipocytes were incubated for 24 h in the absence or presence of insulin (1.6 nM) with or without actinomycin D (5 μg/ml) and/or cycloheximide (10 μg/ml). $N = 12$ in all groups. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to basal control. *c*: $p < 0.001$ compared with the 1.6 nM insulin-treated control. χ: $p < 0.001$ compared with the actinomycin treatment. ς: $p < 0.001$ compared to the actinomycin + insulin treatment.

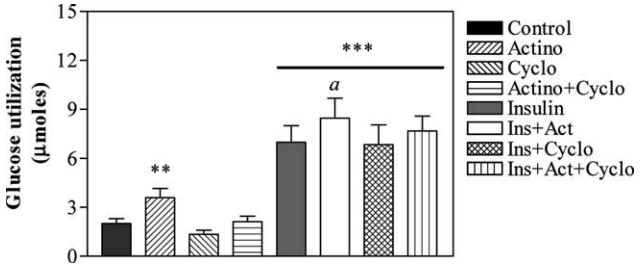

Fig. 7. Effects of cycloheximide on the actions of actinomycin D on basal and insulin-stimulated glucose utilization. Isolated adipocytes were incubated for 24 h in the absence or presence of insulin (1.6 nM) with or without actinomycin D (5 μg/ml) and/or cycloheximide (10 μg/ml). $N = 12$ in all groups. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to basal control. *a*: $p < 0.05$ compared with the 1.6 nM insulin-treated control.

Discussion

Our previous results demonstrated that increased glucose metabolism mediates the long-term effects of insulin to stimulate leptin secretion in rat cultured adipocytes. This stimulation of leptin secretion was accompanied by a glucose-metabolism dependent increase of leptin mRNA expression [12]. Other studies examining the effects of insulin and glucose on leptin gene expression and secretion have reported similar results [7,14,15,25]. Together, these data suggest that a transcriptional mechanism mediates the effect of insulin-stimulated glucose metabolism to increase leptin production. This is further supported by studies showing that the activity of the leptin promoter in transfected cells is increased by insulin and glucose [17] and this increase is dependent on increased glucose metabolism [18]. However, it is possible that glucose and insulin also induce a post-transcriptional stabilization of the leptin mRNA which could contribute to increased leptin production.

To investigate the role of transcription in the regulation of leptin secretion, we examined the short- and long-term effects of actinomycin D, an inhibitor of transcription, on leptin expression and secretion. The concentration of actinomycin D used in this study (5 μg/ml) has been previously employed in several studies to determine the role of transcription in the regulation of leptin or other adipocyte genes [26,27]. Short-term treatments (4 and 24 h) with actinomycin D increased both leptin expression and secretion. A similar effect of actinomycin D on leptin has been previously reported by Fain and Bahouth [28], and more recently by Bradley and Cheatham [19]. Hardie et al. [10] observed a significant decrease in leptin after actinomycin treatment from the first hours of treatment. This result may be due to the higher concentration of actinomycin (10 μg/ml) employed in that study.

Actinomycin D has been previously reported to inhibit the transcription of several adipocytes genes, including fatty acid synthase and pref-1 [29,30]. In fact, the studies of Fain and Bahouth [28] and Bradley and Cheatham [19] confirmed that actinomycin D inhibited the transcription of GAPDH and C/EBPδ, respectively, at the same time when leptin mRNA expression was increased. This suggests that the increase of both leptin mRNA and secretion results from a specific effect of actinomycin on the leptin gene.

Bradley and Cheatham [19] suggest that actinomycin D may inhibit a short-lived regulatory protein that either represses transcription of the leptin gene or destabilizes the message. Fain and Bahouth [28] suggest that actinomycin D stabilizes leptin mRNA and therefore increases leptin release. Alterations of mRNA stability have been described as a universal post-transcriptional mechanism for modulating gene expression [31]. In support of this idea, several studies have shown that actinomycin D can prevent the destabilization of neurofilament mRNA in primary sensory neurons [32] as well as stabilize the urokinase mRNA in macrophages [33]. DRB may have a similar effect. The mechanisms underlying the alteration of mRNA stability remain yet unclear. Regarding the possibility that actinomycin D may interfere with a factor that represses transcription, Wang et al. [34] have identified a negative *cis*-acting element located in the mouse leptin promoter that is repressed by insulin and glucose. It is possible that actinomycin D and DRB induce a short-term stimulation of leptin expression by repressing the binding of a transcription factor to this element.

Our previous studies [12] have shown that insulin-mediated glucose metabolism plays a key role in regulating both leptin gene expression and secretion in isolated rat adipocytes. Wellhoener et al. [13] have also provided evidence that glucose metabolism is an important determinant of insulin-mediated leptin secretion in humans. We observed that actinomycin D induced a significant increase in both basal and insulin-stimulated glucose utilization after 24 h. In addition, between 24 and 48 h of treatment when the stimulatory effects of actinomycin D on glucose utilization are no longer present, the stimulatory effect on leptin secretion disappears. In addition, treatment with cycloheximide, which blocks the stimulatory effects of actinomycin D on glucose utilization at 24 h, also prevents its stimulatory action on leptin secretion. These results suggest that the mechanism by which actinomycin D increases leptin secretion and expression may be mediated by glucose metabolism. However, DRB, which also induces a short-term stimulation of leptin secretion, did not affect glucose uptake (data not shown). Therefore, it is unclear whether the stimulatory effect of actinomycin involves a mechanism related to glucose metabolism or is related to its properties to inhibit transcription.

Between 24 and 48 h of treatment, the stimulatory effect of actinomycin D and DRB on basal leptin secretion disappeared and leptin secretion was similar in the control and actinomycin-treated cells. Between 24 and 48 h, actinomycin D and DRB totally prevented the increases of leptin mRNA expression and leptin secretion induced by insulin-mediated glucose metabolism. Neither actinomycin D nor DRB affected basal or insulin-stimulated glucose utilization during this time period. These results, along with the work of Fukuda and Iritani [17] and Moreno-Aliaga et al. [18], strongly support a transcriptional mechanism by which glucose and insulin increase long-term leptin production. Fukuda and Iritani [17] reported that insulin and glucose activate the leptin promoter in rat adipocytes. Wang et al. [34] suggested that insulin and glucose stimulate mouse leptin gene expression by repressing the binding of a transcription factor to its negative *cis*-acting element of the leptin promoter. In addition, our previous experiments in 3T3-L1 adipocytes [18], and our recent data in primary cultured rat adipocytes [35], demonstrate that the activation of the promoter is mediated by the effects of insulin to increase cellular glucose utilization.

While the available data strongly support a transcriptional regulatory mechanism for insulin-mediated glucose metabolism to increase leptin production, it remains to be determined whether activation of transcription by insulin-mediated glucose metabolism is due to stimulation of transcriptional co-activators or due to the inhibition of transcriptional co-repressor. Further experiments are required to more precisely define the transcriptional mechanism underlying the stimulatory effect of glucose and insulin on leptin expression.

With the present data, a possible post-transcriptional mechanism by which increased leptin mRNA stability increases leptin production during the first 24 h of treatment cannot be ruled out.

Inhibition of protein synthesis with cycloheximide blocked leptin secretion within the first 4 h of treatment,

indicating that de novo protein synthesis is required for insulin and glucose to stimulate leptin production. A previous study [19] also showed that cycloheximide inhibits leptin secretion. However, these authors also reported that insulin could increase leptin secretion in the presence of cycloheximide, possibly from a preexisting intracellular pool. Another study [20] has identified a leptin-containing intracellular compartment in rat adipose cells and showed that cycloheximide did not prevent serum-induced acute increase in leptin secretion. However, a more recent study by Levy and Stevens [36] provides additional evidence against the release of leptin from a preformed intracellular pool in response to insulin and glucose. These investigators examined both leptin secretion and intracellular leptin and observed that cycloheximide completely blocks leptin synthesis and leptin secretion in response to the metabolic secretagogues (glucose, insulin, and pyruvate). They concluded that the secretagogues increased leptin secretion by increasing leptin synthesis rather than enhancing the release of a preexisting cytosolic pool [36]. These authors attributed the conflicting results to differences in the method of incubation with cycloheximide. For the two earlier studies [19,20], cells were incubated with cycloheximide for only 20 min, washed, and then re-incubated with fresh media without cycloheximide. For both the Levy and Stevens [36] and present study, the cells were incubated continuously in the presence of cycloheximide.

In summary, the results of these experiments provide evidence that glucose metabolism regulates leptin expression and secretion at the level of transcription. Further experiments are required to determine the mechanisms and transcription factors underlying the effects of insulin-stimulated glucose metabolism on leptin gene expression. The possibility of a post-translational regulatory mechanism during the first 24 h cannot be excluded. De novo protein synthesis is required for insulin and insulin-mediated glucose metabolism to increase leptin production.

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