Biochemical Pharmacology, Vol. 55, pp. 1309–1314, 1998.
© 1998 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00638-2

Stimulation of Leptin Release by Actinomycin D in Rat Adipocytes

John N. Fain† and Suleiman W. Bahouth‡*

DEPARTMENTS OF *BIOCHEMISTRY AND ‡PHARMACOLOGY, COLLEGE OF MEDICINE, UNIVERSITY OF TENNESSEE AT MEMPHIS, MEMPHIS, TN 38163, U.S.A.

ABSTRACT. A greater understanding of the factors causing the enhanced release of leptin by adipocytes in obseity is needed. Experiments were designed to determine the effects of actinomycin D on leptin release by isolated rat adipocytes during primary culture for 24 hr. In adipocytes from fed hypothyroid rats, the initial rate of leptin release over the first 6 hr was not maintained over the next 18 hr. The decline in leptin release by adipocytes in primary culture between 6 and 24 hr was reduced markedly by either dexamethasone or actinomycin D. Both actinomycin D and dexamethasone also reduced the loss of leptin mRNA seen over the 24-hr incubation. Maximal effects on leptin release and leptin mRNA accumulation required only 0.1 μM of actinomycin D, a concentration that had no significant effect on the 18S RNA content of adipocytes at the end of a 24-hr incubation. In contrast to the reduced loss of leptin mRNA seen at 24 hr, the loss of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid (GAPDH mRNA) was enhanced in the presence of 0.1 μM of actinomycin D. The effects of dexamethasone could be differentiated from those of actinomycin D by the finding that cycloheximide blocked the reduced loss of leptin mRNA due to dexamethasone while having no effect on that due to actinomycin D. These results point to a unique regulation of leptin release and leptin mRNA levels by actinomycin D. BIOCHEM PHARMACOL **55**;8:1309–1314, 1998.
© 1998 Elsevier Science Inc.

KEY WORDS. leptin; leptin mRNA; dexamethasone; adipocytes; 18S RNA; actinomycin D; cycloheximide; GAPDH mRNA

Leptin is a protein made exclusively by adipocytes. The plasma content of leptin in rats or humans reflects the adipose tissue mass. Leptin is involved in the regulation of appetite and the release of pituitary hormones [1, 2]. Glucocorticoids such as dexamethasone are potent simulators of leptin release and leptin mRNA accumulation [3, 4]. While leptin release appears to be enhanced in the fed state, the role of insulin is unclear. Some investigators found a decrease in leptin mRNA due to insulin [5], others reported no effects [4, 6, 7], but positive effects of insulin on leptin release [8–10] also have been reported.

The factors that are responsible for the enhanced release of leptin by adipocytes in obesity are poorly understood. The present experiments were designed to investigate the effects of actinomycin D on leptin release by isolated rat adipocytes during primary culture for 24 hr.

MATERIALS AND METHODS

Cells

Adipocytes were obtained from hypothyroid male Sprague–Dawley rats (310–390 g) fed a low-iodine diet with 6-*N*-propyl-2-thiouracil in the drinking water (62.5 mg/L) for 3 weeks. We obtained adipocytes from hypothyroid rats because leptin mRNA levels are elevated as compared with those in adipocytes from euthyroid rats [11]. Adipocytes were prepared by dissociation of the epididymal adipose tissue from each rat in 5.5 mL of buffer containing 1 mg/mL of bacterial collagenase (*Clostridium histolyticum* CLS1, 238 U/mg, from Worthington Biochemical) as described by Gokmen–Polar *et al.* [12].

Buffer and Assays

The buffer for dissociation and incubation of cells was Dulbecco's modified Eagle's medium/Ham's F12 (1:1, Sigma No. 2906) containing 17.5 mM of glucose, 121 mM of NaCl, 4 mM of KCl, 1 mM of $CaCl_2$, 25 mM of HEPES, 2.4 mM of sodium bicarbonate, 40 mg/mL of BSA, 5 μg/mL of ethanolamine, 0.1 ng/mL of sodium selenite, 90 μg/mL of penicillin G, and 150 μg/mL of streptomycin sulfate. For incubation of cells, we added ascorbic acid (55 μM), leupeptin (10 μg/mL), and aprotinin (10 μg/mL). The buffer with BSA and other additions was filtered through a 0.2-μm filter. Adipocytes were isolated and incubated under sterile conditions in 50-mL polypropylene tubes incubated on their side and shaken at 11 rpm in a gyratory water bath. Approximately 0.4 to 0.5 g of packed cells (600,000–1,000,000) were incubated in 10 mL of medium

† Corresponding author: Dr. J. M. Fain, University of Tennessee at Memphis, College of Medicine, Department of Biochemistry, Memphis, TN 38163. Tel. (901) 448-4343; FAX (901) 448-7360; E-mail: jfain@utmem1.utmem.edu.

for 24 hr. The adipocytes or cut pieces of epididymal adipose tissue obtained from two rats were divided between 13 or 14 tubes. Lipolysis was based on analysis of glycerol release into the medium and determined on 10-μL aliquots of the medium by the procedure of Boobis and Maughan [13]. Lactate formation was measured using lactate dehydrogenase. The leptin content of 100-μL aliquots of incubation medium was measured using radioimmunoassay kits with antibody raised against rat leptin with rat leptin standards from Linco Research, Inc.

Analysis of mRNA

The analysis for RNA levels was by northern blotting. The leptin cDNA§ probe was a 209-bp fragment of mouse leptin cDNA (nucleotides +101 to +309) that was cloned by reverse transcription and PCR [14]. The rat GAPDH probe was prepared from pTRI-GAPDH vector by religating the linearized plasmid (Ambion Inc.) [11].

Total cellular RNA was extracted from adipocytes by the procedure of Chomczynski and Sacchi [14] and dissolved in 100 μL of water. Twenty microliters of RNA was lyophilized and then dissolved in 20 μL of loading buffer composed of: 50% formamide, 50 mM of boric acid, 5 mM of sodium borate (pH 8.0), 10 mM of sodium sulfate, 0.5 mM of EDTA, 5% formaldehyde, 100 μg/mL of ethidium bromide, 10% glycerol, 0.02% bromophenol blue, and 0.02% xylene cyanol. The RNA was heated to 65° for 20 min and then was electrophoresed at 22 V for 20 hr. The gels were washed extensively with water to remove the formaldehyde and then photographed under UV transillumination (Deltaimager). The digital images were stored as TIFF files and analyzed by the NIH Image program, which calculates the relative intensities of 18S RNA bands. Then the gels were transferred to Nytran membranes in a buffer containing 3 M of NaCl plus 0.3 mM of sodium citrate (pH 7.0), using a Turboblotter (Schleicher & Schuell). After transfer, the blots were crosslinked by UV irradiation in a Stratalinker (Stratagene), and air dried to fix the RNA to the Nytran membrane. After the blots were prehybridized, as described previously [11], they were incubated simultaneously with 2×10^6 cpm/mL of radiolabeled leptin probe and 2.5×10^5 cpm/mL of the GAPDH probe for 18–20 hr at 42° and then washed twice for 5 min in 0.3 M of NaCl plus 30 mM of sodium citrate containing 0.5% SDS at 25° and twice for 15 min in the same buffer at 56°. The washed blot was covered with Saran wrap and subjected to direct counting using an Instantimage analyzer (Packard). The disintegrations per minute of ^{32}P hybridized to the 5.5-kb leptin mRNA and the disintegrations per minute hybridized to an equal area of GAPDH mRNA were determined simultaneously.

§ *Abbreviations:* cDNA, complementary DNA; GAPDH mRNA, glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid; PCR, polymerase chain reaction; and TIFF, tagged image file format.

Received 2 September 1997; accepted 14 October 1997.

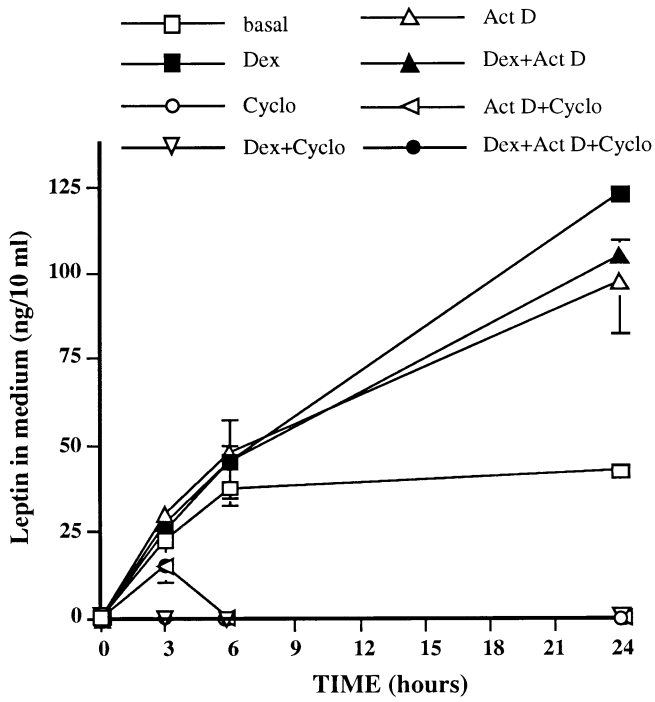

FIG. 1. Effects of dexamethasone and actinomycin D on leptin release in adipocytes. Adipocytes were incubated for 24 hr in 10 mL of incubation buffer, and 0.1-mL aliquots of the medium were removed at 3, 6, and 24 hr for assay of leptin release. The concentration of cycloheximide (Cyclo) was 36 μM, that of actinomycin D (Act D) 0.1 μM, and that of dexamethasone (Dex) 25 nM. Values are the means ± SEM from the six experiments shown in Table 1.

RESULTS

Actinomycin D Stimulation of Leptin Release

Leptin was released from adipocytes in primary culture during the first 3–6 hr of incubation, but there was little further release during the next 18 hr (Fig. 1, open squares). In adipocytes cultured in the presence of 25 nM of dexamethasone, leptin release during the first 3–6 hr was comparable to basal levels, but the rate of leptin release was higher over the next 18 hr (Fig. 1, closed squares). At the end of the 24-hr incubation, leptin release from dexamethasone-treated adipocytes was 3-fold higher than in control adipocytes (Fig. 1). The effects of dexamethasone were mimicked by 0.1 μM of actinomycin D, but the combination of dexamethasone and actinomycin D had no greater effect than actinomycin D alone (Fig. 1). Cycloheximide, an inhibitor of protein synthesis, virtually abolished leptin release at all the time points, indicating the reliance upon new protein synthesis for leptin release. We used a concentration of cycloheximide (36 μM) 10-fold greater than that reported to maximally inhibit protein synthesis in adipocytes [15, 16]. However, in the presence of 3.6 μM of cycloheximide, leptin release was inhibited over 24 hr in the absence of dexamethasone by only 63 ± 11%, and in the presence of 25 nM of dexamethasone by 62 ± 7% (mean ± SEM of 6 paired replications).

TABLE 1. Cycloheximide inhibition of the elevation in leptin mRNA accumulation by dexamethasone but not that by actinomycin D

Additions	Basal	+Dexamethasone (25 nM)
Leptin mRNA at 24 hr (% of zero time)		
None	5 ± 1	27 ± 3
Cycloheximide (36 μM)	7 ± 2	8 ± 2
Actinomycin D (0.1 μM)	25 ± 10	24 ± 2
Cycloheximide + actinomycin D	36 ± 4	26 ± 2
GAPDH mRNA at 24 hr (% of zero time)		
None	62 ± 7	65 ± 12
Cycloheximide	28 ± 7	25 ± 5
Actinomycin D	43 ± 10	45 ± 7
Cycloheximide + actinomycin D	58 ± 2	48 ± 7
Glycerol in medium at 24 hr (μmol/10 mL)		
None	2.8 ± 0.6	2.4 ± 0.6
Cycloheximide	0.3 ± 0.5	1.2 ± 0.4
Actinomycin D	4.7 ± 0.5	5.3 ± 0.2
Cycloheximide + actinomycin D	0.5 ± 0.5	0.9 ± 0.2
Lactate in medium at 24 hr (μmol/10 mL)		
None	2.7 ± 0.6	3.9 ± 0.3
Cycloheximide	4.3 ± 0.4	4.3 ± 0.3
Actinomycin D	4.2 ± 0.6	3.8 ± 0.5
Cycloheximide + actinomycin D	5.5 ± 0.6	5.4 ± 0.7

Adipocytes (1.2×10^6) were incubated in 10 mL of medium for 24 hr and all agents were added at the start of the incubation. Values are the means ± SEM of six paired replications except for those in the presence of actinomycin D plus cycloheximide which are from three experiments. The zero-time value for leptin mRNA was 74,000 counts and for GAPDH mRNA was 26,000 counts.

Prevention of Loss of Leptin mRNA over 24 Hr by actinomycin D

The level of leptin mRNA in adipocytes cultured for 24 hr was 5% of the initial value (Table 1). Dexamethasone at 25 nM reduced, but did not prevent, the fall in leptin mRNA seen at the end of the 24-hr incubation period (Table 1). Actinomycin D produced similar effects on leptin mRNA levels in the absence or presence of dexamethasone. The presence of cycloheximide did not affect the basal leptin mRNA content at 24 hr, but abolished the effect of dexamethasone on leptin mRNA content (Table 1). In contrast, the increase in leptin mRNA due to actinomycin D was unaffected by the concurrent presence of cycloheximide (Table 1).

The increase due to dexamethasone in leptin mRNA was specific, because there was no change in GAPDH mRNA levels at 24 hr (Table 1). GAPDH mRNA levels in actinomycin D-treated adipocytes were 30% lower than those in control or dexamethasone-treated adipocytes (Table 1). In contrast, the levels of leptin mRNA were 5-fold higher in actinomycin D-treated versus control adipocytes.

The effects of cycloheximide and actinomycin D on lipolysis (measured by the release of glycerol) and glucose metabolism (measured by lactate formation) over the 24-hr incubation also are shown in Table 1. Basal lipolysis was

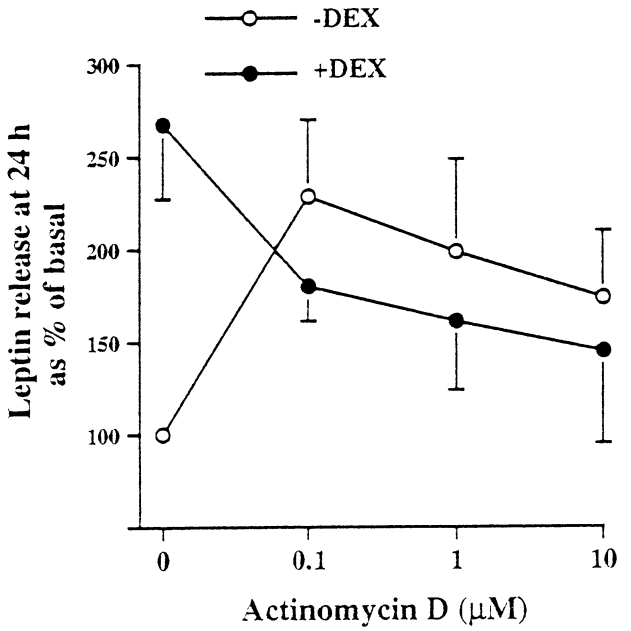

FIG. 2. Maximal stimulation of leptin release by 0.1 μM of actinomycin D. Adipocytes (1×10^6) were incubated in 10 mL of medium for 24 hr. Values are the means ± SEM of four paired replications. The 100% value for leptin release represented that seen in the absence of added agents and was 36 ng of leptin over 24 hr.

enhanced by actinomycin D, and this effect was abolished by cycloheximide, which also inhibited basal lipolysis. In contrast, lactate formation was enhanced slightly by either cycloheximide or actinomycin, while the combination had additive effects. Dexamethasone did not affect either lipolysis or lactate formation under the conditions of these experiments.

The concentration–response relationships for actinomycin D are shown in Fig. 2 and demonstrate that the maximal stimulatory effect on leptin release was seen with 0.1 μM of actinomycin D. This concentration of actinomycin D produced a 95% inhibition of uridine incorporation into total RNA by rat adipocytes over a 4-hr incubation [15].

The maximal inhibition of the loss in leptin mRNA accumulation seen over 24 hr required only 0.1 μM of actinomycin D. This concentration of actinomycin D reduced the accumulation of GAPDH mRNA by 45% (Fig. 3). However, increasing the concentration of actinomycin D to 1 μM of reduced GAPDH mRNA by 68–86% without further increasing leptin mRNA or decreasing 18S RNA levels (Fig. 3). A higher concentration of actinomycin D (10 μM) reduced 18S RNA by 30% without having any additional effects on leptin or GAPDH content.

Enhancement by actinomycin D of Leptin Release in Intact Adipose Tissue

The data in Fig. 4 indicate that increases in leptin release and leptin mRNA accumulation were also seen in intact pieces of epididymal adipose tissue incubated for 24 hr with

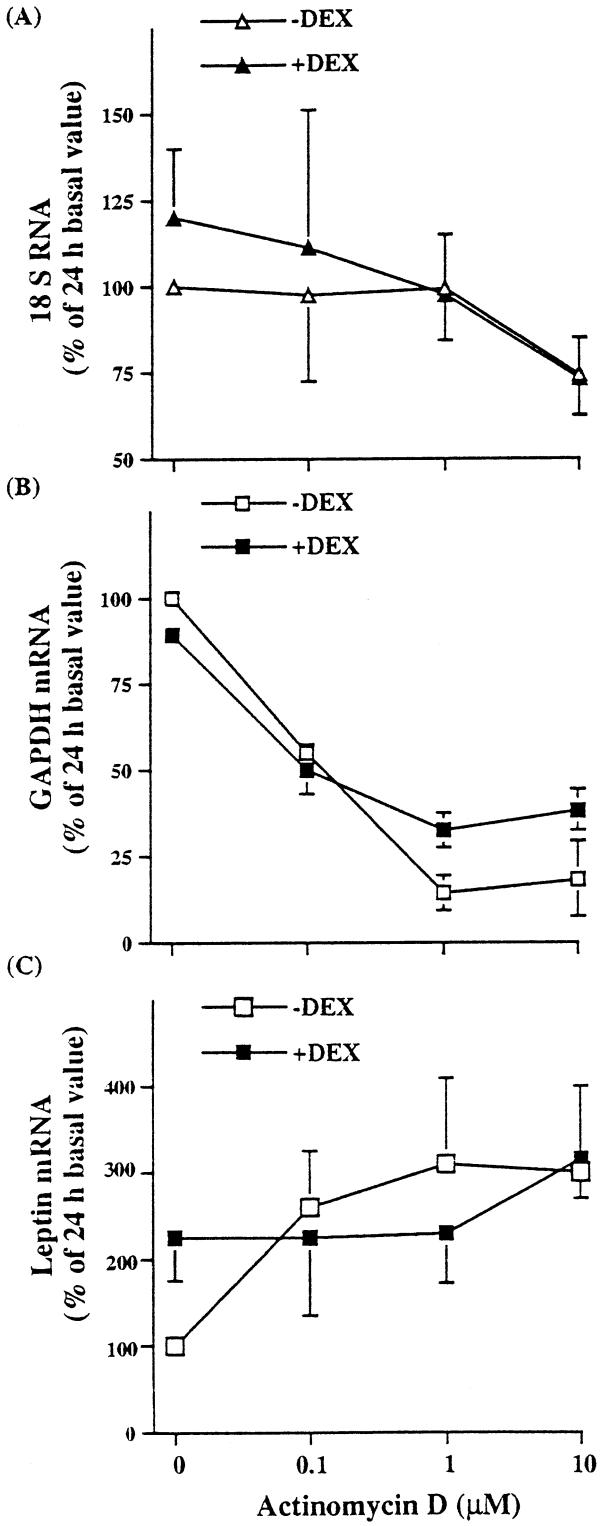

FIG. 3. Enhancement of leptin mRNA accumulation at 24 hr by actinomycin D accompanied by inhibition of GAPDH mRNA accumulation. The values are from the same experiments shown in Fig. 2. The 24-hr value for leptin mRNA was 840 counts and for GAPDH mRNA was 1460 counts. The 24-hr value for 18S RNA in arbitrary units was 1500.

0.1 μM actinomycin D. Cycloheximide abolished the effect of dexamethasone on leptin mRNA in intact adipose tissue, but had no effect on leptin mRNA levels in actinomycin D-treated tissue. Therefore, the effects of cycloheximide on intact adipose tissue are comparable to those observed in adipocyte suspensions (Fig. 4 vs Table 1).

DISCUSSION

There was a marked loss of leptin mRNA in adipocytes during the first 24 hr of primary culture. The decline in leptin mRNA correlated with attenuation of leptin release from these cells. Dexamethasone markedly reduced the loss of leptin mRNA content and elevated leptin release over the 24-hr culture period. Actinomycin D produced effects that were essentially similar to those of dexamethasone on both leptin mRNA and leptin release from adipocytes in primary culture. These effects were produced by low (0.1 μM) and high concentrations of actinomycin D (10 μM). In contrast to these findings, Hardie *et al.* [9] reported that 8 μM actinomycin D blocks leptin release by rat adipocytes incubated for 24 hr in the presence of dexamethasone or insulin plus dexamethasone. There are numerous differences between our culture conditions and those used by Hardie *et al.* [9]. First, Hardie *et al.* incubated about one-tenth as many cells per milliliter of medium. Second, these cells were cultured in Medium 199 containing 10% heat-inactivated fetal bovine serum, while our medium did not contain serum but instead had 4% albumin. The use of high concentrations of actinomycin D and the low concentration of cells by Hardie *et al.* [9] probably resulted in the toxic effects of actinomycin D, since there were no such effects in our study even in the presence of 10 μM actinomycin D.

The mechanism for the increase in leptin release and the prevention of the loss of leptin mRNA due to actinomycin D remains to be elucidated, but appears to be specific for leptin mRNA because actinomycin D had just the opposite effect on GAPDH mRNA. The degradation of leptin mRNA in adipocytes over the 24-hr incubation period in the absence of dexamethasone was considerably larger than the degradation of GAPDH mRNA. The loss of leptin mRNA was about 95% at the end of the 24-hr culture period, while GAPDH mRNA loss amounted to ~36%. Therefore, there appear to be specific factors that destabilize leptin mRNA in adipocytes or adipose tissue. The specificity of the response to actinomycin D rules out inactivation of a ribonuclease that degrades all mRNA and suggests that stabilization of leptin mRNA does not involve protein synthesis. In contrast, stabilization of leptin mRNA by dexamethasone requires active protein synthesis. Actinomycin D appears to block the formation of RNA molecules that destabilize leptin mRNA and reduce leptin formation, while dexamethasone may stimulate the synthesis of proteins that stabilize leptin mRNA. There is very little known about the type of mechanism involved in

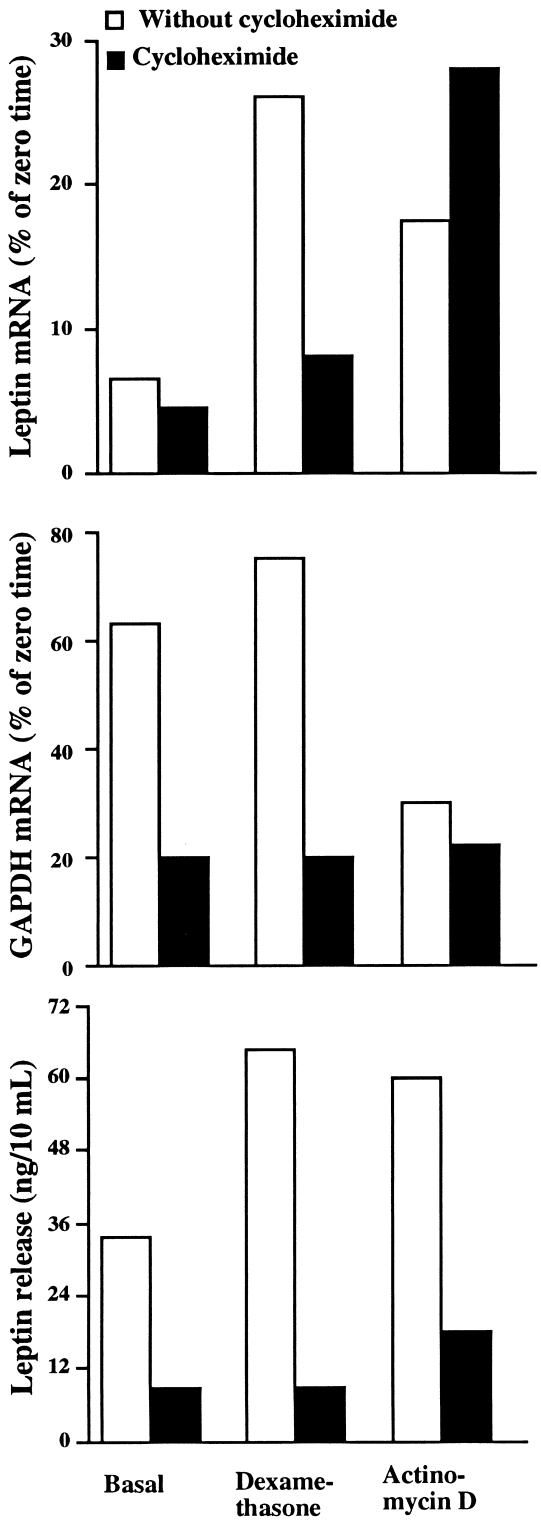

FIG. 4. Effects of dexamethasone and actinomycin D on epididymal adipose tissue incubated for 24 hr. Intact pieces of epididymal adipose tissue weighing approximately 600 mg were incubated for 24 hr in 10 mL of incubation buffer either in the absence (open bars) or presence (filled bars) of 36 μM of cycloheximide. Actinomycin D (0.1 μM) and dexamethasone (25 nM) were present as indicated on the figure. Values are the means of two paired replications. The zero-time value for leptin mRNA was 54,000 counts and for GAPDH mRNA was 36,000 counts.

actinomycin D-mediated stabilization of specific mRNAs [17].

There are a number of studies reporting intriguing effects of actinomycin D. Stabilization of urokinase mRNA has been observed in macrophages in the presence of actinomycin D [18] and in renal epithelial cells in the presence of cycloheximide [19]. Similar results have been seen in primary sensory neurons where actinomycin D prevented destabilization of neurofilament mRNA, and this effect was mimicked, to a lesser extent, by cycloheximide [20]. Knutsen *et al.* [21] reported that nuclear degradation of the mRNA for the regulatory subunit of protein kinase A was blocked by actinomycin D but not by inhibitors of protein synthesis. These investigators found a marked increase of a large mRNA species in the nuclear compartment of cells treated with inhibitors of RNA synthesis. They suggested that this was probably pre-mRNA containing unexcised introns and that this RNA stabilizes the message without involving labile proteins.

Actinomycin D is commonly used to determine the half-life of a particular mRNA on the assumption that it blocks transcriptional activity. The present results indicate at least one situation where the half-life of a mRNA, namely that for leptin, is greatly extended by actinomycin D. In rat mesangial cells, the half-life for prostaglandin G/H synthase mRNA induced by stimuli was less that 2 hr, but the half-life was about 5 hr in the presence of actinomycin D [22]. Apparently some mRNAs are regulated through labile factors involved in destabilization of mRNA, whose formation is blocked by actinomycin D. While dexamethasone also stabilizes leptin mRNA either by stimulating transcription or blocking degradation or both, it has been reported that cytokine-induced cyclooxygenase-2 mRNA is actually destabilized by dexamethasone in human lung and kidney cells [23]. Our data suggest that there is a novel mechanism for the regulation of leptin mRNA involving synthesis of an RNA molecule via a process blocked by actinomycin D that actually destabilizes leptin mRNA.

References

1. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chair RT, Rabinowitz D, Lallone RL, Burley SK and Friedman JM, Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* **269:** 543–546, 1995.
2. Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E and Flier JS, Role of leptin in the neuroendocrine response to fasting. *Nature* **382:** 250–252, 1996.
3. Murakami T, Iida M and Shima K, Dexamethasone regulates *obese* expression in isolated rat adipocytes. *Biochem Biophys Res Commun* **214:** 1260–1267, 1995.
4. Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, LaQuier F, Manetta J, Bue-Valleskey J and Stephens TW, Regulation of expression of *ob* mRNA and protein by glucocorticoids and cAMP. *J Biol Chem* **271:** 5301–5304, 1996.
5. Reul BA, Ongemba LN, Pottier A-M, Henquin J-C and Brichard SM, Insulin and insulin-like growth factor 1 antagonize the stimulation of *ob* gene expression by dexamethasone in cultured rat adipose tissue. *Biochem J* **324:** 605–610, 1997.
6. Becker DJ, Ongemba LN, Brichard V, Henquin J-C and

Brichard SM, Diet- and diabetes-induced changes of *ob* gene expression in rat adipose tissue. *FEBS Lett* **371:** 324–328, 1995.
7. Vidal H, Auboeuf D, De Vos P, Staels B, Riou JP, Auwerx J and Laville M, The expression of *ob* gene is not acutely regulated by insulin and fasting in human abdominal subcutaneous adipose tissue. *J Clin Invest* **98:** 251–255, 1996.
8. Gettys TW, Harkness PJ and Watson PM, The β_3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology* **137:** 4054–4057, 1996.
9. Hardie LJ, Guilhot N and Trayhurn P, Regulation of leptin production in cultured mature white adipocytes. *Horm Metab Res* **28:** 685–689, 1996.
10. Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B and Auwerx J, Transient increase in *obese* gene expression after food intake or insulin administration. *Nature* **377:** 527–529, 1995.
11. Fain JN, Coronel EC, Beauchamp MJ and Bahouth SW, Expression of leptin and β_3-adrenergic receptors in rat adipose tissue in altered thyroid states. *Biochem J* **322:** 145–150, 1997.
12. Gokmen-Polar Y, Coronel EC, Bahouth SW and Fain JN, Insulin sensitizes β-agonist and forskolin-stimulated lipolysis to inhibition by 2′5′-dideoxyadenosine. *Am J Physiol* **270:** C562–C569, 1996.
13. Boobis LH and Maughan RL, A simple one-step enzymatic flourometric method for the determination of glycerol in 20 microliters of plasma. *Clin Chim Acta* **132:** 173–179, 1983.
14. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
15. Fain JN, Studies on the role of RNA and protein synthesis in the lipolytic action of growth hormone in isolated fat cells. *Adv Enzyme Regul* **5:** 39–51, 1967.
16. Marshall S, Kinetics of insulin action on protein synthesis in isolated adipocytes. *J Biol Chem* **264:** 2029–2036, 1989.
17. Ross J, Control of messenger RNA stability in higher eukaryotes. *Trends Genet* **12:** 171–175, 1996.
18. Stacey KJ, Nagamine Y and Hume DA, RNA synthesis inhibition stabilises urokinase mRNA in macrophages. *FEBS Lett* **356:** 311–313, 1994.
19. Altus MS and Nagamine Y, Protein synthesis inhibition stabilizes urokinase-type plasminogen activator mRNA. *J Biol Chem* **266:** 21190–21196, 1991.
20. Schwartz ML, Shneidman PS, Bruce J and Schlaepfer WW, Actinomycin prevents the destabilization of neurofilament mRNA in primary sensory neurons. *J Biol Chem* **267:** 24596–24600, 1992.
21. Knutsen HK, Taskén KA, Eskild W and Hansson V, Inhibitors of RNA and protein synthesis stabilize messenger RNA for the R11β subunit of protein kinase A in different cellular compartments. *Biochem Biophys Res Commun* **183:** 632–639, 1992.
22. Stroebel M and Goppelt-Struebe M, Signal transduction pathways responsible for serotonin-mediated prostaglandin G/H synthase expression in rat mesangial cells. *J Biol Chem* **269:** 22952–22957, 1994.
23. Ristimaki A, Narko K and Hla T, Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: Evidence for post-transcriptional regulation. *Biochem J* **318:** 325–331, 1996.