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Changes in Leptin Expression Are Not Associated with Corresponding Changes in CCAAT/Enhancer Binding Protein-α

Kyle W. Sloop, Peggy L. Surface, Mark L. Heiman, and Lawrence J. Slieker[1]
Endocrine Research Division, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

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C/EBP-α binds a C/EBP consensus site in the leptin promoter and activates transcription *in vitro.* We assessed adipose tissue expression of C/EBP-α, leptin and β-actin in Sprague Dawley rats under conditions that modulate leptin mRNA abundance in order to study the relationship between leptin and C/EBP-α expression patterns. During acute fasting, which decreased the level of leptin and β-actin mRNA, C/EBP-α mRNA expression was unaltered. In leptin-treated and pair-fed animals, C/EBP-α mRNA was unaltered compared to *ad libitum* fed controls, while leptin and β-actin mRNA expression was again decreased. These results indicate that changes in the level of leptin gene expression are not directly associated with changes in the level of C/EBP-α abundance.

Since a major role of leptin is to provide information to the hypothalamus concerning adipose tissue energy storage, knowledge of the regulation of its expression is extremely important in understanding energy homeostasis. Leptin expression is regulated by glucocorticoids (1–3), insulin (4,5), β_3-adrenergic agonists (3,6), thiazolidinediones (7–9), adiposity (10,11), and fasting (12–14). Despite the presence of potential transcriptional regulatory elements such as putative cAMP response element-binding protein sites, glucocorticoid response element half sites, and a DR-1 site in the leptin promoter, the mechanisms through which this regulation occurs have not been completely elucidated (15–18).

Four transcription factors functionally implicated in the regulation of leptin gene expression are CCAAT/enhancer binding protein-α (C/EBP-α) (15–18), peroxizomal proliferator activated receptor-γ (PPAR-γ) (7), Sp-1 (18), and a novel factor that binds an LP1 motif (18). Transfection of truncated leptin promoter-luciferase reporter constructs into primary adipocytes and 3T3-L1 adipocytes has identified a C/EBP binding site at nucleotides −58 to −49 as a key regulatory element for adipose tissue specific expression. Reporter activity is completely repressed by C/EBP binding site mutants, and it is enhanced in a dose dependent manner by co-transfection with C/EBP-α (19). The role of PPAR-γ in regulating leptin expression is less clear. Activators of PPAR-γ such as BRL49653 decrease leptin expression *in vivo* and *in vitro* (7), but activity of leptin promoter-luciferase constructs transiently expressed in adipocytes was unaltered by co-transfection with PPAR-γ and RXR-α with or without BRL49653 (18). Thiazolidinediones may antagonize C/EBP-α binding, since the effect of BRL49653 maps to the same region of the promoter which binds C/EBP-α. A canonical DR-1 site at −3951 in the mouse promoter binds BRL49653 and drives a heterologous promoter, but appears to play no functional role in regulating leptin expression (20). Sp-1 has been shown to bind at nucleotides −100 to −95, and an unknown factor has been found to bind an LP1 motif at positions −89 to −82; mutations in these regions abolish transcription factor binding and reduce promoter firing (18). It is not clear whether regulation of the leptin promoter is controlled by the relative expression levels of these transcription factors or by changes in their functional activity.

The goal of these studies was to determine whether changes in the abundance of leptin mRNA in response to fasting or pharmacological treatment could be mediated by concomitant changes in C/EBP-α expression. We analyzed C/EBP-α mRNA under acute pharmacological conditions that have been shown to either increase or decrease leptin mRNA expression, e.g. treatment with glucocorticoids, a β_3 adrenergic receptor agonist, and a thiazolidinedione. In addition, because we have previously demonstrated decreased leptin expression in *ob/ob* mice treated chronically with leptin

[1] To whom correspondence should be addressed at Endocrine Research Division, Drop Code 0545, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285. E-mail: Slieker_Lawrence_J@Lilly.com.

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(3), we also examined adipocyte C/EBP-α mRNA in Sprague Dawley rats treated for two weeks with recombinant human leptin.

These studies utilized "real time" quantitative PCR to determine mRNA expression levels. This new technology utilizes fluorescent oligonucleotide probes that hybridize to a cDNA of interest and allow quantitation by PCR in real time. Although this methodology has been used to measure DNA such as oncogene amplification in human breast tumors, low copy viral targets in human blood, and the Vβ-repertoire of human T cell populations (21–24), few examples exist of its utility in mRNA quantitation through reverse transcriptase coupled PCR (25,26). We feel that this method is more sensitive than Northern or RNAse protection analysis and more accurate and less labor intensive than quantitative competitive RT-PCR.

MATERIALS AND METHODS

Animal Use and Treatments

Acute and chronic studies used male Sprague-Dawley rats from Harlan Sprague-Dawley Inc. (Indianapolis, IN) which were fed Purina Formulab Chow 5001 (Purina Mills, St. Louis, MO) and maintained under 12-h light (6:00 a.m.) and 12-h dark conditions. For the acute study, rats (200 g) were housed 3 per cage and fed *ad libitum* (except fasted group). At 6:00 p.m., rats were injected subcutaneously with 200 μl of 50 mM sodium carbonate (pH 10.0) vehicle (control and fasted rats), dexamethasone (0.74 mg/kg body wt.), CL 316,243 (10 mg/kg body wt), and BRL49653 (1.9 mg/kg body weight). Rats were decapitated at 4 and 12 hrs. following injection, and epididymal adipose tissue was excised, immediately frozen in liquid nitrogen, and stored at −80°C until RNA isolation. For the chronic study, 15 rats (250 g) were housed 1 per cage for 2 weeks. Two groups of animals (5 per group) were injected twice daily with recombinant human leptin (250 μg/injection, Eli Lilly and Co.) or phosphate-buffered saline (PBS, pH 7.8) and fed *ad libitum*. The third group was pair-fed to the leptin treated group and received twice daily injections of PBS. Rats were decapitated and tissue was handled as described above. Serum from trunk blood was collected for leptin analysis and stored at −20°C until assayed (Leptin RIA kit, Linco Research, Inc., St. Charles, MO).

RT Real Time Quantitative PCR

Description of assay. "Real time" quantitative PCR utilizes the ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Applied Biosystems, Foster City, CA) (27). This system exploits the 5′ nuclease activity of Taq polymerase to cleave a fluorogenic probe that has hybridized to a cDNA molecule of interest (28). Separation of the fluorescent probe from an internal quencher increases fluorescent emission which is plotted versus PCR cycle number in order to generate an amplification plot for each sample (28). A threshold based on the variability of the baseline reporter emission is determined, and the cycle number where fluorescent emission increases above baseline emission is defined as "C_T", or threshold cycle (Figure 1a). A standard curve is then generated by plotting C_T versus copy # of a series of target cDNA molecules (Figure 1B) (28).

RT procedure. Total RNA was isolated from rat epididymal fat tissue by the TRI REAGENT method (Molecular Research Center, Cincinnati, OH) as previously described (3). Reverse transcription of total RNA with 3′ primers specific to rat C/EBP-α, leptin, and β-actin was performed using the SuperScript Preamplification System for First Strand cDNA Synthesis (GibcoBRL Life Technologies). Primer extension was performed at 42°C for 50 min with reactions consisting of 3 μg total RNA, 2.5 mM $MgCl_2$, 10 mM DTT, 500 μM each dNTP, 100 nM each primer, 400 units of SuperScript II reverse transcriptase, and 1× PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl) in a final volume of 40 μl. Reactions were then heated to 70°C for 10 min. to denature the reverse transcriptase and incubated with 4 units of RNase H for 20 min at 37°C to remove the mRNA from the cDNA:mRNA hybrids. In addition to the unknowns, reverse transcription was performed on dilutions of RNA ranging from 1 μg to 5 μg in order to verify assay linearity (see below).

Quantitative PCR. Quantitative PCR amplification reactions (50 μl each) included: 2 μl of cDNA synthesis reaction, 1× TaqMan Buffer A, 300 μM dATP, dCTP, dGTP, and 600 μM dUTP, 3.5 mM MgCl2, 1.25 Units AmpliTaq Gold DNA polymerase, 0.5 units of AmpErase uracil N-glycosylase (UNG), 300 nM forward primer and reverse primer, and 200 nM fluorogenic probe. Primer sequences were as follows: C/EBP-α primers (forward 5′ AGTTGACCAGTGACAATGACCG 3′ and reverse 5′ TCAGGCAGCTGGCGGAAGAT 3′ - amplicon = 92 bp), leptin primers (forward 5′ ACCAAAACCCTCATCAAGAC 3′ and reverse 5′ CGGGAATGAAGTCCAAACCGG 3′ - amplicon = 102 bp), and β-actin (forward 5′ CCGTGAAAAGATGACCCAGA 3′ reverse 5′ GTACGACCAGAGGCATACAG 3′ - amplicon = 98 bp). TaqMan probe sequences were as follows: C/EBP-α probe (5′ AGCTGAGCCGTGAACTGGACACGCT 3′), leptin probe (5′ ATTTCACACACGCAGTCGGTATCCGC 3′), and β-actin probe (5′ TTTGAGACCTTCAACACCCCAGCC 3′). Each probe contained the quencher dye TAMRA (6-carboxytetramethyl-rhodamine, emission maximum = 582 nm.) attached to a linker arm nucleotide that is added to the 3′ end. The C/EBP-α and leptin probes contained the reporter dye FAM (6-carboxy-fluorescein, emission maximum = 518 nm.) attached to the first base on the 5′ end, while the β-actin probe contained the reporter dye TET (tetrachloro-6-carboxy-fluorescein, emission maximum = 538 nm). Probes were synthesized and HPLC purified by Perkin-Elmer, Applied Biosystems Division, Foster City, CA. Reaction temperatures and cycling parameters were as follows: 50°C for 2 min, 94°C for 10 min, then 35 cycles at 94°C for 30 sec and 60°C for 1 min.

The slopes (m) of the standard curves generated from cDNA dilutions were equivalent to the slopes of the curves generated from dilutions of RNA converted to cDNA by reverse transcriptase (C/EBP-α mRNA m = −3.13 +/−0.14 vs. C/EBP-α cDNA m = 3.14 +/−0.10, leptin mRNA m = −3.00 +/−0.04 vs. leptin cDNA m = −3.21 +/−0.18, β-actin mRNA m = −3.40 +/−0.15 vs. β-actin cDNA m = −3.66 +/−0.10). This comparison was made in each experiment in order to monitor reverse transcriptase consistency and assay linearity. Utilizing 3′ specific primers in the RT step and amplifying target fragments of less than 100 bp enhances assay reproducibility. These results are similar to those reported that demonstrate colineartiy of slopes generated from serial dilutions of adeno CFTR internal control and total cell RNA containing the adeno CFTR target mRNA (25).

RESULTS AND DISCUSSION

Since C/EBP-α is potentially a significant regulator of leptin expression, we wished to determine if acute changes in leptin mRNA expression correlated with changes in C/EBP-α levels. Figures 2a–d show the expression level of leptin, C/EBP-α, and β-actin mRNA as determined by real-time PCR (normalized to 150 ng of total RNA) for both 4 and 12 hrs. After 4 hrs, there was a 95% increase in the level of leptin mRNA in response to dexamethasone, and a 45 or 70% decrease with fasting or CL 316,243 treatment,

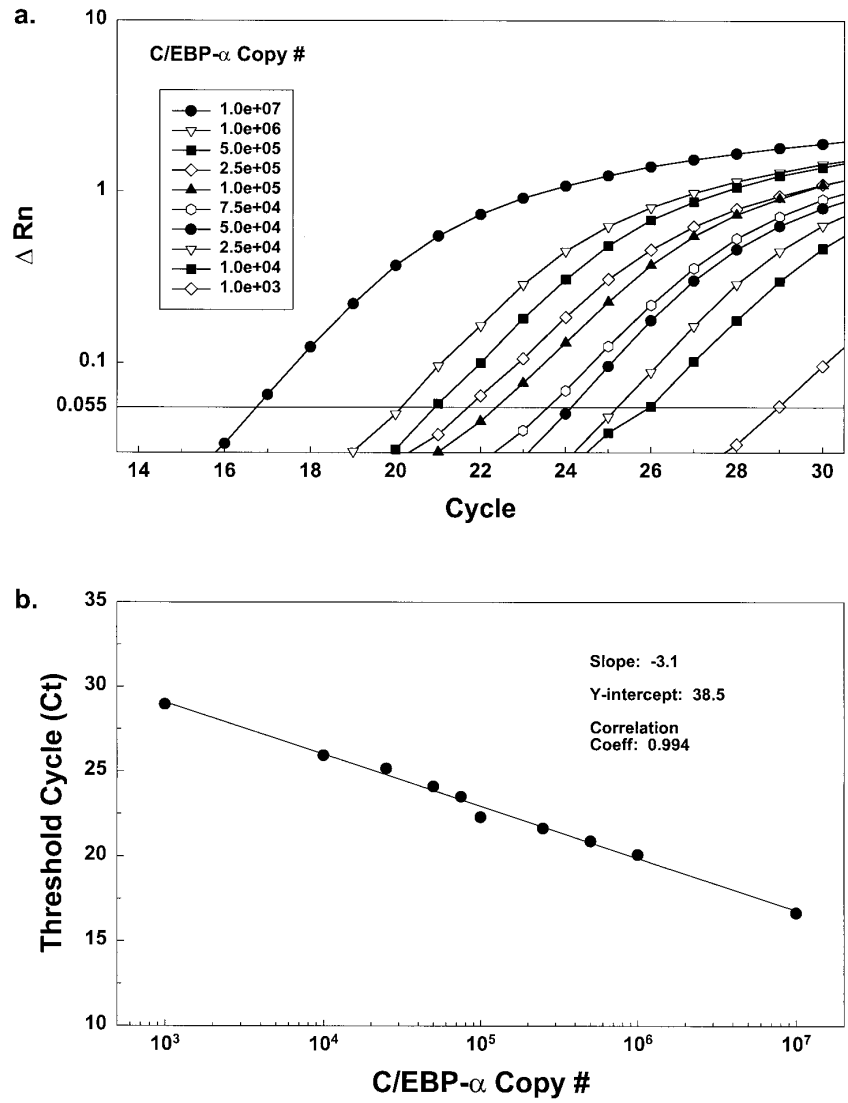

FIG. 1. (a and b) C/EBP-α amplification plot and standard curve. ΔRn, representing normalized fluorescent emission resulting from probe cleavage, was plotted against PCR cycle number for dilutions of C/EBP-α cDNA in order to generate an amplification plot (Fig. 1a) (28). A threshold at 0.055 ΔRn was determined based on the variability of the baseline reporter emission, and the point where the amplification plot crosses it is defined as "C_T", or threshold cycle. The standard curve (Fig. 1b) generated by plotting C_T versus C/EBP-α copy # was used to determine the abundance of C/EBP-α expression in rat epididymal adipose tissue samples (see assay description in text).

respectively. These results were closely mirrored by the level of circulating leptin (Figure 2e). After 12 hrs, the leptin expression pattern was similar to that observed at 4 hrs, but the response to fasting was more pronounced, and the effects of dexamethasone and CL 316,243 were reduced. β-actin expression was unchanged with all pharmacological treatments, but was decreased in the fasted group. C/EBP-α levels were not significantly different under any conditions, although there was a trend towards decreased expression with dexamethasone after 4 hrs followed by increased expression after 12 hrs. This profile is similar to the pattern of C/EBP-α expression previously observed in 3T3-L1 adipocytes treated with dexamethasone for 24 hrs. which showed an acute decrease in C/EBP-α expression followed by a recovery (29).

There was a trend toward reduced leptin expression with BRL49653 treatment, but this was also not significant. One week oral treatment of rats with 2–5 mg/kg BRL49653 (8) and 4 week treatment of *db/db* mice with the same doses of AD-5075 (8) decreased leptin mRNA, but we are unaware of any acute studies demonstrating an effect of thiazolidinediones on leptin expression *in vivo.* Since we would have expected to see a direct transcriptional effect after 12 hrs., it is possible that our method and level of dosing did not achieve high enough blood levels.

Two week administration of recombinant human leptin (250 μg/injection, twice daily) decreased food

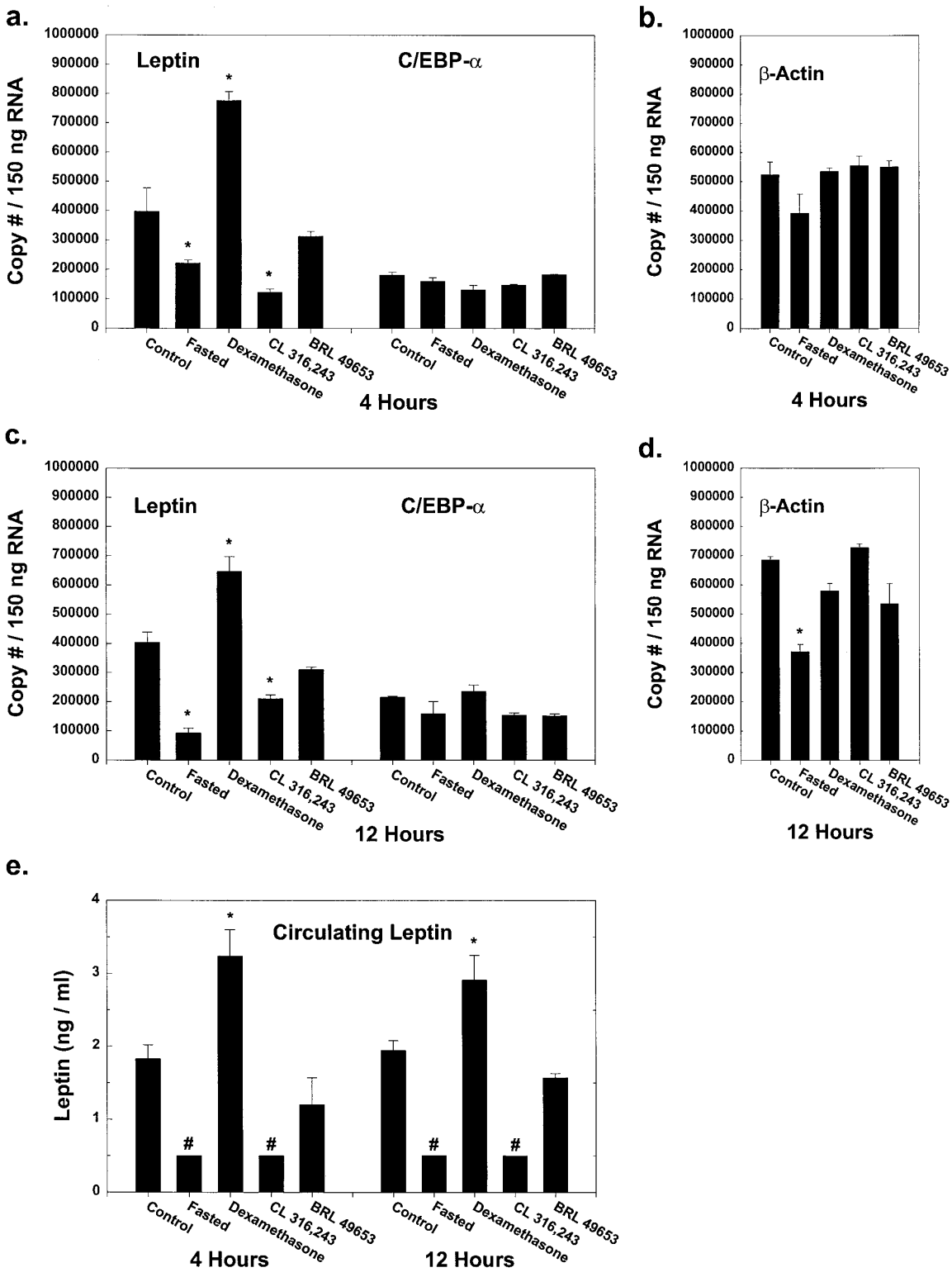

FIG. 2. (a–e) Acute study. Epididymal adipose tissue leptin, C/EBP-α, and β-actin mRNA levels in Sprague Dawley rats. Expression levels were assayed after 4 and 12 hrs. in rats fasted, treated with dexamethasone, CL 316,243, or BRL 49653 and compared to *ad libitum* controls (Fig. 2a–d). Serum leptin levels were determined at 4 and 12 hrs. for each treatment (Fig. 2e). * $p < 0.05$ compared to control, ANOVA. # represents < 0.5 ng/ml.

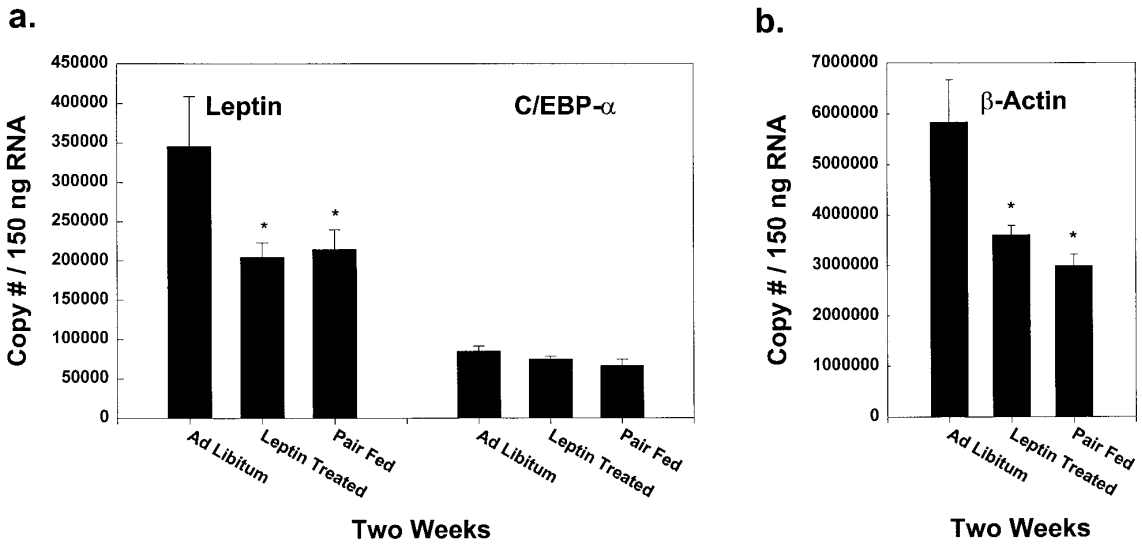

FIG. 3. (a and b) Chronic study. Epididymal adipose tissue leptin, C/EBP-α, and β-actin mRNA levels in Sprague Dawley rats. Expression levels were assayed after two weeks in rats treated with recombinant human leptin, pair fed controls, and *ad libitum* controls. * $p < 0.05$ compared to control, ANOVA.

consumption and body weight in male Sprague-Dawley rats compared to *ad libitum* fed controls (data not shown). In addition, a similar decrease in body weight was observed in animals that were pair fed to the leptin treated group. Both of these treatments also decreased the level of epididymal adipose tissue leptin mRNA but not the level of C/EBP-α mRNA (Figure 3a). Similarly, a previous report has shown that lean 2-week-old Zucker rats (+/*fa*) exhibit the same level of C/EBP-α mRNA expression compared to (*fa/fa*) rats that have 2.3 fold more inguinal subcutaneous fat (30). The observation that C/EBP-α levels were maintained in the leptin treated and pair fed animals suggests that the reduced expression of leptin is not secondary to reduced expression of C/EBP-α.

β-actin, which is typically used as a "housekeeping" gene to normalize RNA loading, was also significantly decreased in both leptin-treated and pair-fed animals (Figure 3b), similar to the effect of fasting observed in the acute experiments (Figure 2b,d). The use of equal amounts of RNA in the RT step was confirmed by agarose gel electrophoresis and ethidium bromide staining (data not shown), so RT-PCR data were normalized to total RNA, not to β-actin. In addition to leptin and β-actin, several genes expressed in adipose tissue are reduced during fasting, e.g. adipsin, GLUT4, tubulin, glycerophosphate dehydrogenase, and PPAR-γ (31–33). Since PPAR-γ and C/EBP-α both play important roles in regulating the adipose tissue differentiation pathway, it is interesting that we were unable to demonstrate any effect of acute or chronic food restriction on adipose tissue C/EBP-α expression.

In summary, our results do not demonstrate a correlation between the level of C/EBP-α and leptin mRNA. However, C/EBP-α may modulate leptin gene expression by mechanisms that do not require changes in its expression level. For example, dephosphorylation of C/EBP-α has been implicated in the insulin-stimulated repression of GLUT4 gene expression in 3T3-L1 adipocytes in the absence of changes in C/EBP-α mRNA abundance (34,35). Our results do not rule out the possibility that the function of C/EBP-α may be modulated during fasting or conditions that alter leptin gene expression, but do suggest that the level of its expression is not altered.

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