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Differentiation Method-Dependent Expression of Leptin in Adipocyte Cell Lines

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Leptin, the product of the *ob* gene, is expressed exclusively in adipose tissue. However, adipocyte cell lines, such as 3T3-L1 adipocytes, have generally been reported to express extremely low levels of leptin mRNA. We compared 3T3-L1's to the closely related line 3T3-F442A, and to another murine adipocyte line, TA1. TA1 cells, when differentiated by indomethacin/insulin treatment, express leptin at levels greater than those of 3T3-L1 adipocytes differentiated by the traditional methylisobutylxanthine/dexamethasone/insulin protocol. However, when 3T3-L1's are differentiated in the presence of indomethacin/insulin their expression levels of leptin increase dramatically. 3T3-F442A preadipocytes also express high levels of leptin when differentiated in the presence of T_3 and insulin, but when differentiated in the presence of indomethacin/insulin, expression levels drop precipitously. These changes in leptin mRNA and protein expression are not reflected by changes in CCAAT/enhancer binding protein-α (c/EBPα), peroxizomal proliferator activated receptor-γ (PPARγ), lipoprotein lipase (LPL), fatty-acid binding protein aP2 or uncoupling protein-2 (UCP2) mRNA levels, and suggest a mechanism unique to the leptin gene.

Leptin is the 17 kDa protein product of the recently cloned *ob* gene (1). Physiologically, leptin serves the role of a satiety factor that is released by adipose tissue and signals the hypothalamus to decrease food intake and increase energy expenditure (2,3). Leptin expression and release by rat and human primary adipocytes in culture have been demonstrated (4,5), but reported expression in continuous adipocyte cell lines such as 3T3-L1 adipocytes has been variable. 3T3-L1 cells have been shown to express the leptin gene and to exhibit normal regulation of its expression by β agonists, but mRNA levels are generally so low as to require RT-PCR for detection (6-8). Primary rat preadipocytes have also been shown to express leptin mRNA upon differentiation, but like 3T3-L1s, RT-PCR was required for detection (9). The adipocyte cell line 3T3-F442A has been demonstrated to express leptin, but apparently at much higher levels than those of 3T3-L1 adipocytes since it was readily detected by Northern analysis (10). Because of this apparent disparity between otherwise similar cell lines, we chose to examine both of these cells in conjunction with another murine adipocyte line, TA1.

MATERIALS AND METHODS

Cell culture. 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). TA1 cells were a gift from Dr. Gordon Ringold (Affymax Research Institute, Palo Alto, CA), and 3T3-F442A cells were supplied by Dr. Howard Green (Harvard University, Cambridge, MA.). TA1 and 3T3-L1 cells were maintained in DMEM containing 25 mM glucose supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD), 100 U/ml streptomycin and 100 μg/ml penicillin (Sigma, St. Louis, MO). 3T3-F442A cells were maintained similarly with the substitution of 10% calf serum. The methylisobutylxanthine/dexamethasone/insulin protocol (MDI) (11) was employed by treating confluent 3T3-L1 preadipocyte monolayers with growth medium supplemented with 0.5 mM methylisobutylxanthine, 0.1 μM dexamethasone and 5 μg/ml human insulin (Eli Lilly, Indianapolis, IN). On day two, and every 2-3 days thereafter, medium was changed to growth medium plus insulin. The indomethacin/insulin protocol (I/I) was performed essentially as described by Ringold (12). Confluent TA1, 3T3-L1 or 3T3-F442A preadipocytes were treated with FBS-containing growth medium supplemented with 125 μM indomethacin and 5 μg/ml insulin. On day 2 and thereafter, medium was changed as described for the MDI protocol. For the TA1 cells, insulin was either maintained throughout the differentiation period (+ ins) or removed at day 8 (− ins). 3T3-F442A cells were also differentiated in FBS-containing growth medium supplemented with 17 nM insulin and 2 nM T_3 throughout the differentiation period according to the method of Leroy et al. (10). On the designated days, medium was removed and stored at −20 C until leptin measurements were performed by radioimmunoassay (RIA) with a murine leptin RIA kit (Linco, St. Charles, MO)

RNA isolation and detection. RNA was isolated on day 11 from differentiated adipocytes by the method of Chomszynski (13) using TRI REAGENT (Molecular Research Centers, Cincinnati, OH). Northern analysis was performed with a ^{32}P labeled full-length murine leptin cDNA probe as described previously (4). A full length probe for rat C/EBPα (14) and a 275 bp fragment of rat PPARγ (15) were kindly provided by Dr. Brad Lowell (Harvard University). A full length cDNA probe of rat aP2 and a 335 bp fragment of rat LPL (bp 153-488) were generated by RT-PCR from rat adipocyte RNA. Quantitation of leptin and UCP2 mRNA was also performed by real-time fluorescence PCR using the Perkin-Elmer ABI 7700 (16). Reverse

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transcription of total RNA with 3′ primers specific to rat leptin, murine β-actin and murine UCP2 (see below for sequences) was performed using the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies). 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 probe. Primer sequences were as follows: leptin primers (forward 5′ ACCAAAACCCTCATCAAGAC 3′ and reverse 5′ CGGGAATGAAGTCCAAACCGG 3′), UCP2 primers (forward 5′ AAGGGACTTCTCCCAATGTT 3′ and reverse 5′ TTGGCTTTCAGGAGAGTATC 3′) and β-actin (forward 5′ CCGTGAAAAGATGACCCAGA 3′ reverse 5′ GTACGACCAGAGGCATACAG 3′). TaqMan probe sequences were as follows: Leptin probe (5′ ATTTCACACACGCAGTCGGTATCCGC 3′), UCP2 probe (5′ AGCTGAGCCGTGAACTGGACACGCT 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 and the reporter dye FAM (6-carboxyfluorescein, emission maximum = 518 nm.) attached to the first base on the 5′ end. 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. Quantitation was accomplished by comparison to standard curves generated from known amounts of full length cDNA for each gene of interest (100 to 10,000,000 copies). All UCP2 and actin primers, probes and cDNA standards were mouse sequence, but the leptin standard cDNA was the rat sequence. The 5′-primer and probes were identical to the mouse sequence, but the 3′-primer had three mismatches: CAGGAATGAAGTCCAAGCCAG.

RESULTS AND DISCUSSION

In preliminary experiments with fully differentiated 3T3-L1 adipocytes using the standard MDI differentiation protocol, we were not able to detect secreted leptin by RIA, or by Northern analysis (results not shown), confirming the very low expression levels reported by others. For this reason, we chose to examine TA1 cells, which are a murine preadipocyte line derived from 10T1/2 cells. The initially described protocol for differentiation of TA1 cells was treatment with dexamethasone/insulin (17), but Knight et al. later reported greater levels of differentiation with indomethacin/insulin (I/I) (12). When we examined TA1 cells during both differentiation protocols for leptin mRNA expression by Northern analysis, we found detectable levels under both conditions, but a greater expression level was observed with the I/I protocol (results not shown). Since 3T3-L1 preadipocytes have been reported to undergo differentiation in response to I/I treatment (18), we compared this method with the standard MDI protocol and examined the expression level of leptin mRNA by Northern analysis and secreted protein by RIA over a 10 day differentiation period. In addition, we compared TA1 and 3T3-F442A cells, the latter being differentiated by both the standard T_3/ins and the I/I protocols.

As shown in Figure 1, secreted leptin increased in a differentiation-dependent manner in the TA1 cells, the

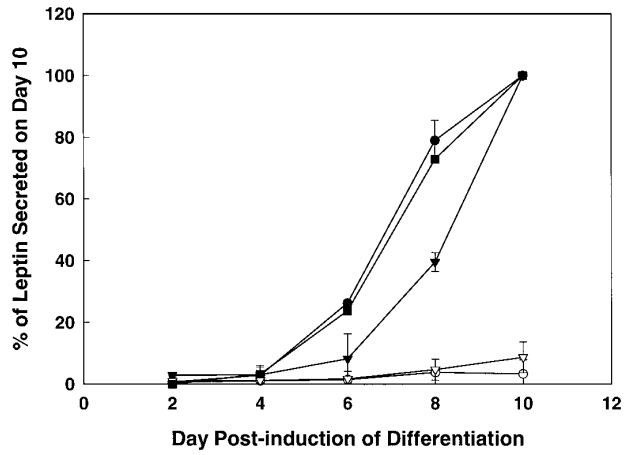

FIG. 1. Effect of different differentiation conditions on the secretion of leptin from TA1 (■), 3T3-L1 I/I (●), 3T3-L1 MDI (○), 3T3-F442A I/T_3 (▼) and 3T3-F442A I/I (▽) adipocytes. Data are normalized to the amount secreted at day 10 for each condition. 3T3-L1 MDI and 3T3-F442A I/I data are normalized to 3T3-L1 I/I and 3T3-F442A I/T_3 results at day 10, respectively. This represents the average of two independent experiments.

3T3-L1 cells using the I/I protocol and in the 3T3-F442A cells using the T_3/ins protocol. Although the absolute amounts of leptin varied somewhat from experiment to experiment, when normalized to the amount secreted at day 10, the results were quite reproducible. The time course for TA1 and 3T3-L1 were quite similar, while leptin secretion from the 3T3-F442A cells was delayed by approximately 48 hours. However, when the 3T3-L1 cells were differentiated with the standard MDI protocol, leptin levels were barely detectable, as was the case with 3T3-F442A adipocytes differentiated with the I/I protocol. These results indicate that all three of the adipocyte cell lines are capable of expressing leptin at comparable levels, but that it is extremely dependent on the differentiation conditions employed.

Leptin expression has been shown to be regulated by glucocorticoids and β-agonists *in vivo* (19,20) and in isolated primary adipocytes *in vitro* (4,21). In order to examine this regulation under the conditions reported here, 3T3-L1 adipocytes differentiated under the I/I protocol and 3T3-F442A cells differentiated under the T_3/ins protocol were treated at day 10 with 50 nM dexamethasone or 1 μM BRL 37344 (rodent specific β_3 agonist) for 24 hrs. As shown in Figure 2, dexamethasone increased leptin secretion from 60 to 90%, while β_3 adrenergic receptor stimulation decreased leptin expression 50-70%. These results are qualitatively similar to those obtained both *in vivo* and *in vitro,* and suggest that under these differentiation conditions leptin expression is regulated normally with respect to glucocorticoids and β-agonists.

In order to verify that leptin mRNA levels correlated with leptin protein secretion, total RNA was isolated

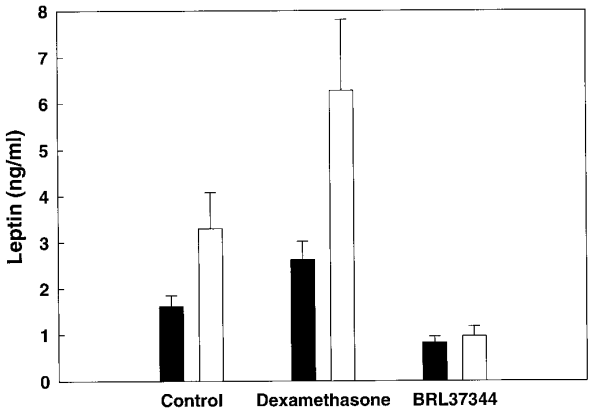

FIG. 2. Effect of dexamethasone (50 nM) and BRL37344 (1 μM) on leptin secretion from 3T3-L1 I/I (■) and 3T3-F442A I/T_3 (□) adipocytes. Cells were differentiated in T75 flasks as described in Methods and Materials. Compounds were added on day 10, and medium was removed 24 hrs later for RIA analysis.

from TA1, 3T3-L1 (MDI and I/I) and 3T3-F442A (T_3/ins and I/I) adipocytes on day 11, after 24 hour treatment ± 50 nM dexamethasone (as described in Methods, TA1(− ins) cells had insulin removed at day 8). Secreted leptin was measured in 24 hour conditioned medium by RIA. As expected, Figure 3A shows that leptin protein secretion was essentially undetectable in 3T3-L1 adipocytes under MDI conditions and only slightly higher in 3T3-F442A adipocytes under I/I conditions, but was secreted at high levels under 3T3-L1 I/I and 3T3-F442A T_3/ins conditions. As shown previously, dexamethasone increased leptin levels in the latter case, but interestingly not in the TA1 cells where it actually decreased leptin expression in the TA1 (− ins) cells. Northern analysis (Figure 3B) clearly showed that leptin mRNA levels correlated very well with measured protein.

Due to the large difference in expression levels of leptin under the different differentiation conditions, we used Northern analysis to examine several other differentially expressed adipocyte genes: aP2, LPL, PPARγ and C/EBPα. The latter two are of particular interest since a C/EBPα site in the leptin promoter at −51 has been shown to be a potent enhancer of leptin expression (22,23), and PPARγ activators such as BRL 49653 may antagonize this regulation (14). Figure 3B shows that the expression level of each of these genes is essentially unchanged as a function of differentiation protocol within each cell type, despite dramatic differences in leptin expression.

UCP2 is another recently identified gene shown to be expressed in a number of tissues including adipose tissue (24,25). Due to its possible role in thermogenesis, as well as its apparent regulation of expression in adipose tissue by high fat feeding (24), we were particularly interested in determining if its expression were altered under these conditions. In this case, both leptin and UCP2 mRNA was determined by real time fluorescence quantitative PCR using the Perkin Elmer 7700 PCR machine combined with TaqMan technology. Figure 4 shows that the quantitation of leptin mRNA by RT-PCR agrees very

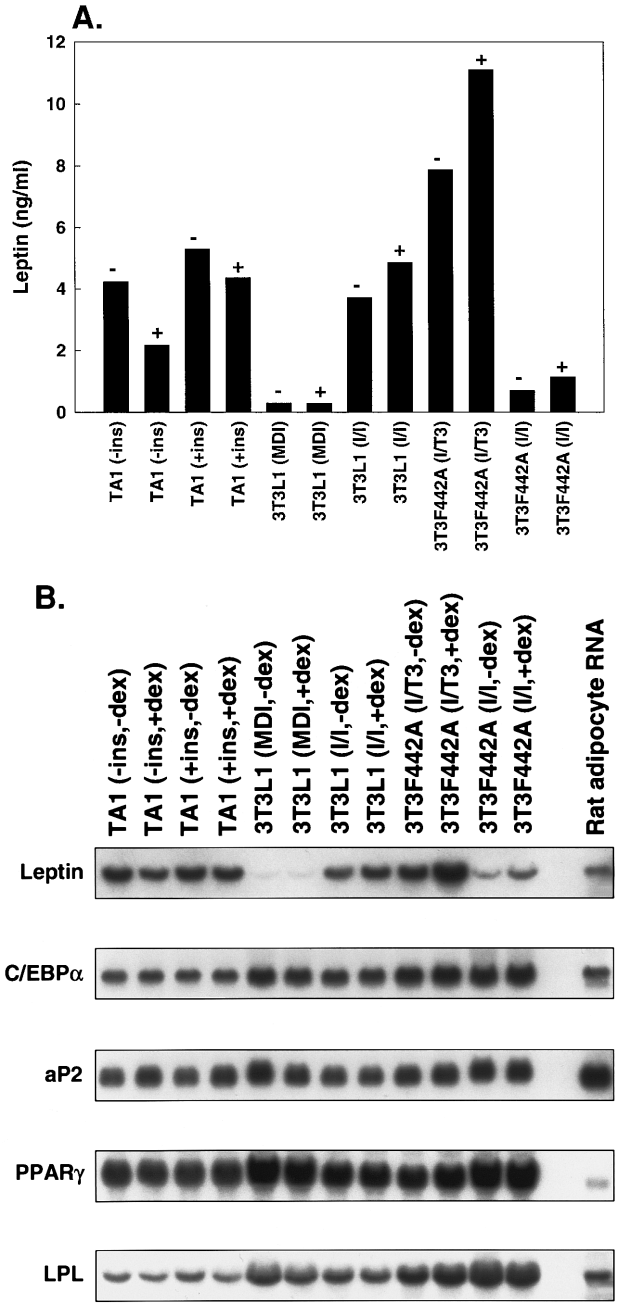

FIG. 3. Effect of dexamethasone on leptin expression in TA1, 3T3-L1 and 3T3-F442A adipocytes under varying differentiation conditions. Cells were treated with vehicle (−) or with 50 nM dexamethasone (+) on day 11 and assayed after 24 hrs. A. RIA analysis of leptin secretion. B. Northern analysis of leptin, C/EBPα, aP2, PPARγ and LPL RNA. See Materials and Methods for details. Twenty μg of total RNA was run per lane. Four μg of rat adipocyte RNA was included for comparison.

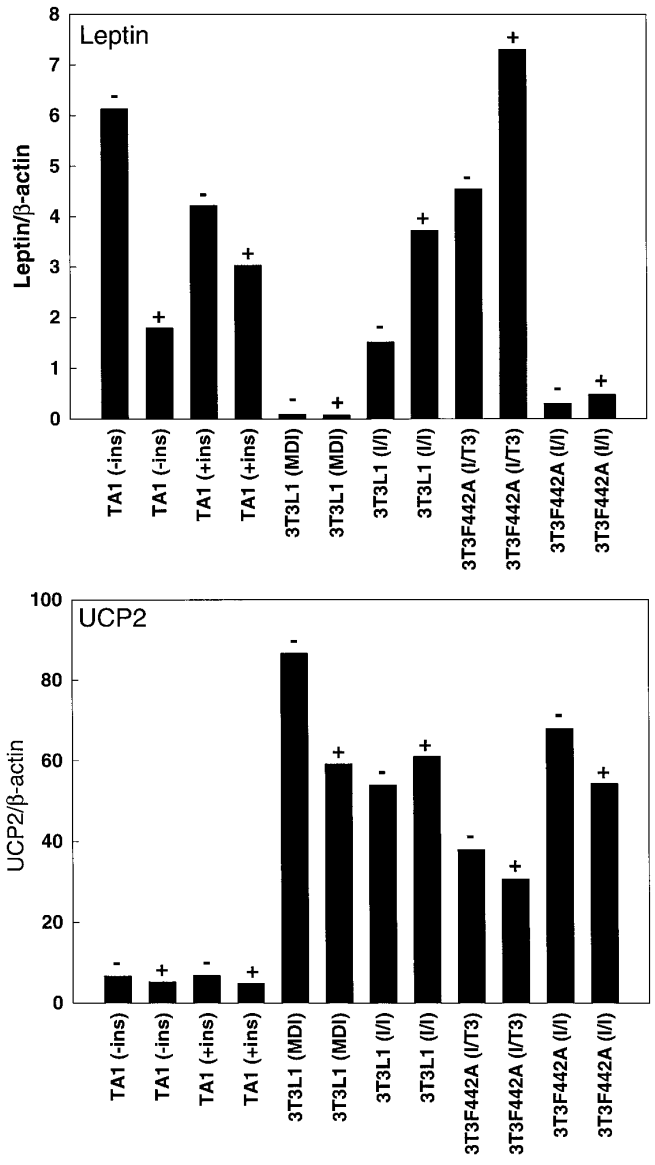

FIG. 4. TaqMan quantitative RT-PCR analysis of leptin and UCP2. RNA samples depicted in Fig. 3 were assayed for leptin and UCP2 expression by combining reverse transcriptase cDNA synthesis and TaqMan fluorescense-based PCR. Quantitation was assessed relative to a series of cDNA standards for each gene and normalized to β-actin.

closely with both the Northern analysis and the secreted leptin protein. UCP2 mRNA did not vary appreciably between differentiation conditions, but was interestingly expressed at approximately a 10 fold higher level in 3T3-L1 and -F442A adipocytes compared to the TA1 cells. The absolute quantitation of leptin in this analysis may be low due to the mismatched downstream primer (see Methods), which could have contributed to decreased efficiency of both the RT and PCR amplification steps. However, the measurement of relative abundance would not be expected to be effected, as is born out by comparison to the Northern and RIA analysis (Figure 3).

These results indicate that the expression of leptin mRNA and protein in adipocyte cell lines is very dependent on specific differentiation conditions. This is not mirrored by several other adipocyte expressed genes, including aP2, C/EBPα, PPARγ, LPL and UCP2. Whether this is due to specific transcriptional events early in the differentiation process (induced by insulin plus indomethacin), or is a result of differences in the final differentiated adipocyte (eg, variation in lipid droplet size or amount of triglyceride per cell) is unclear. The fact that indomethacin/insulin treatment increased leptin in 3T3-L1 adipocytes, but had the exact opposite effect in 3T3-F442A cells (which do not normally require pharmacologic induction of differentiation) might suggest the latter.

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