

Regulation of *ob* gene mRNA levels in cultured adipocytes

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Received 4 December 1995

Abstract mRNA levels of the *ob* gene product, leptin, were investigated by quantitative competitive RT-PCR in a mouse cell line (3T3-L1) which can be induced to differentiate into adipocytes. During conversion to fat cells, the level of leptin mRNA increased several-fold and in parallel to that for typical adipocyte markers like lipoprotein lipase, adipin and glycerophosphate dehydrogenase. Leptin transcription, however, did not correlate with the size of the adipocytes measured as total triglycerides. On the other hand, mRNA levels for leptin in fully differentiated adipocytes were increased 2–3 fold by insulin. In contrast, free fatty acids exerted a concentration-dependent inhibition of leptin transcription while the corticosteroid dexamethasone and an elevation of intracellular cAMP displayed only marginal inhibitory effects on leptin mRNA levels.

Key words: Adipocyte; mRNA; Leptin; *ob* gene; Expression; Obesity

1. Introduction

The *ob* gene has recently been cloned and shown to encode for a 167-amino acid protein, leptin, which is secreted exclusively from the adipocytes of white fat [1–5]. Leptin has been shown to lower body weight by restraining appetite [5–8] and by altering metabolic processes [6]. The hypothalamus seems to be a target tissue for the hormone, since injections of leptin into the intracerebral ventricle lead in mice to a reduction of food intake [7]. A defective *ob* gene promoter or a nonsense mutation within the coding region are believed to be the cause of obesity in the *ob/ob* mouse [1]. Although the mechanism of action of leptin has not been elucidated, this peptide may be the long sought after satiety factor released in the periphery (i.e. the adipocytes) to regulate long-term body weight [9]. In this lipostatic theory of body weight maintenance, as body fat increases more leptin is secreted, thereby bringing the fat mass back to a certain normal 'set point'. In this regard, leptin mRNA levels have been studied in rats with obesity secondary to ventromedial hypothalamic lesions and increase in parallel with body weight [10,11], fulfilling one of the requirement of this regulatory system. Also in humans, it has recently been shown that leptin mRNA levels in white adipocytes increase or decrease in parallel with the body mass index [3,12]. Although believed to be a long-term regulator of body weight, increasing

evidence suggests that leptin may also form part of a short-term regulatory system to maintain body weight since *ob* mRNA levels are rapidly influenced by food intake and cold [13].

Little is known concerning the control of leptin expression. Changes in metabolism during weight loss and gain are associated with alterations in the secretion of many hormones and to changes in the plasma concentration of critical metabolites. To investigate the control of leptin mRNA levels, differentiated mouse adipocytes maintained in culture were studied with the aim of evaluating whether two hormones intimately involved in fat metabolism, insulin and corticosteroids as well as free fatty acids (FFA) contribute to the regulation of leptin mRNA levels in fat cells.

2. Materials and methods

2.1. Differentiation of 3T3-L1 cells and mRNA isolation

Mouse 3T3-L1 cells were maintained as subconfluent cultures in Dulbecco's minimal essential medium (MEM 2052)/5% FCS. Thereafter, the cells were grown to confluency in 6-well plates. To induce adipocyte conversion, the medium was replaced by Dulbecco's modified Eagle's medium (DMEM)/10% FCS supplemented with 10 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.6 μ M dexamethasone. After 2 days, the hormonal cocktail was removed and cells were maintained in DMEM/10% FCS for the indicated time (as outlined in section 3). Overconfluent and time-matched 3T3-L1 preadipocytes did not show cytoplasmic triacylglycerol accumulation over a period of 16 days.

mRNA was isolated using the Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions, except that $\sim 5 \times 10^5$ cells were extracted in 0.6 ml lysis buffer (4 M urea, 100 mM HEPES-KOH pH 7.5, 180 mM NaCl, 1% SDS, 5 mM DTT and 400 μ g/ml proteinase K), homogenized shortly by a Polytron PT1200 tissue homogenizer and incubated at 56°C for 30 min.

2.2. Reverse transcription and quantitative polymerase chain reaction (PCR)

First-strand cDNA synthesis was performed using mRNA derived from $\sim 4 \times 10^5$ cells for each reverse transcription (RT) reaction. 25 pmol of random hexamers were hybridized by incubation for 10 min at 25°C and extended for 30 min at 42°C in the presence of 25 U of *Superscript II* reverse transcriptase (Life Technologies, MD) in PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) containing 5 mM $MgCl_2$, 1 mM each dNTP (deoxynucleotide triphosphate) and supplemented with 8.4 U of RNasin (Promega, Madison, WI) in a total volume of 8.5 μ l. The resulting cDNA was used directly for PCR amplification without further purification. PCR was carried out with *Taq* DNA polymerase (Life Technologies) using 10 pmol of each sense and antisense primer in 45 cycles of 94°C, 65°C, 30 s each and 72°C for 1 min in PCR buffer containing 2 mM $MgCl_2$ in a total volume of 50 μ l. Oligonucleotide primer pairs specific for the following mouse genes were used: β -actin (5'-TGACCCAGATCATGTTTGAGACC-3'/5'-CCATACCC-AAGAAGGAA GGC-3'), glycerophosphate dehydrogenase (GPDH) (5'-GAATTCATCC-GCTGTCTGC-3'/5'-CTTTCCTGTGGCTG-A-AGTGC-3'), lipoprotein lipase (LPL) (5'-ACTCA-TCTCCG-CCAT-GCC-3'/5'-CCAGCTTTCTCCTAGCAAGG-3'), adipin (5'-CTGC-TGGACGAGCAGTGG-3'/5'-GATGACACTCGGGTA-TAGACG-C-3') and leptin (5'-GTGCC-TATCCAGAAAGTCCAG-3'/5'-

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Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; FFA, free fatty acids; LPL lipoprotein lipase; GPDH, glycerophosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; SDS, sodium dodecylsulfate; NPY, neuropeptide-Y.

TCAGCATTCAGGGCTAACA TC-3'). The corresponding heterologous standardcompetitively with the target cDNA, were generated as described previously [14]. The PCR products were separated on agarose gels, stained with ethidium bromide and recorded using the *Cybertech CS* system. The ratio of the signal generated from the defined amount of heterologous template to target cDNA was quantified using the Image Processing program Win Cam 2.1. To validate the method of quantitation, measurements of template dilutions (standard cDNA and target mRNA) were carried out basically as suggested by Raeymaekers [15]. It is possible that a factor present in particular mRNA preparations (i.e. from control or fully differentiated cells) might affect the outcome of the RT-PCR reactions and thus would prevent any meaningful comparison between the samples. Therefore, mRNA preparations from control and fully differentiated adipocytes were mixed at various ratios and the levels of target mRNAs, as determined by competitive RT-PCR, were shown to match exactly the values expected from the individual mRNA preparations.

2.3. Determination of the cellular triglyceride content

Differentiated 3T3-L1 adipocytes were harvested in PBS supplemented with 0.1% SDS and lysed by a short sonication step. Triglyceride measurements were based on the ethoxide transesterification of triglycerides and subsequent color development with acetylacetone as previously described [16]. A Titertek Multiskan MC (Eflab, Helsinki, Finland) was used for photometric readings.

2.4. Data analysis

Results are expressed as mean $\pm$ S.E.M. The value n represents the number of independent cell culture wells. Between group comparison was by two-tailed unpaired Student's t test. The significance was defined as a probability of equal or less than 0.05.

3. Results

The expression of leptin mRNA was studied during the conversion of 3T3-L1 cells to adipocytes. Differentiation of the cells was induced by supplementing the culture medium with IBMX, dexamethasone and insulin for 2 days. The level of leptin mRNA was analysed at various time points by heterologous competitive RT-PCR as shown in Fig. 1 (left panel) and compared with that of other typical adipocyte markers, such as LPL, GPDH and adipsin. Quantitation was obtained by calculating the ratio of the specific signal to that of the corresponding standard. Normalization was achieved after correction for the corresponding levels of β -actin mRNA in the same sample. Expression of LPL, GPDH and adipsin was found to increase gradually, reaching a maximum $\sim$ 7–9 days after induction (Fig. 1, right panel). The increase in adipsin mRNA was slightly delayed, as expected for a late marker of mature adipocytes. The expression of leptin mRNA was also stimulated after conversion of 3T3-L1 cells to adipocytes. Curiously, leptin mRNA levels fell to control levels after removal of the differentiation mixture on the 2nd day after induction (Fig. 1). Thereafter, leptin mRNA increased gradually again to reach maximum levels after about a week, in a manner similar to the level of other adipocyte markers. The heterologous competitive

RT-PCR procedure also allows an estimation of the relative quantities of mRNA encoding for the various genes of interest. Typically, maximal levels of mRNA for GPDH were in the range of those encoding for β -actin whereas those for adipsin and LPL were $\sim 5 \times$ higher. Conversely, maximal levels of leptin mRNA were more than 100 times less abundant.

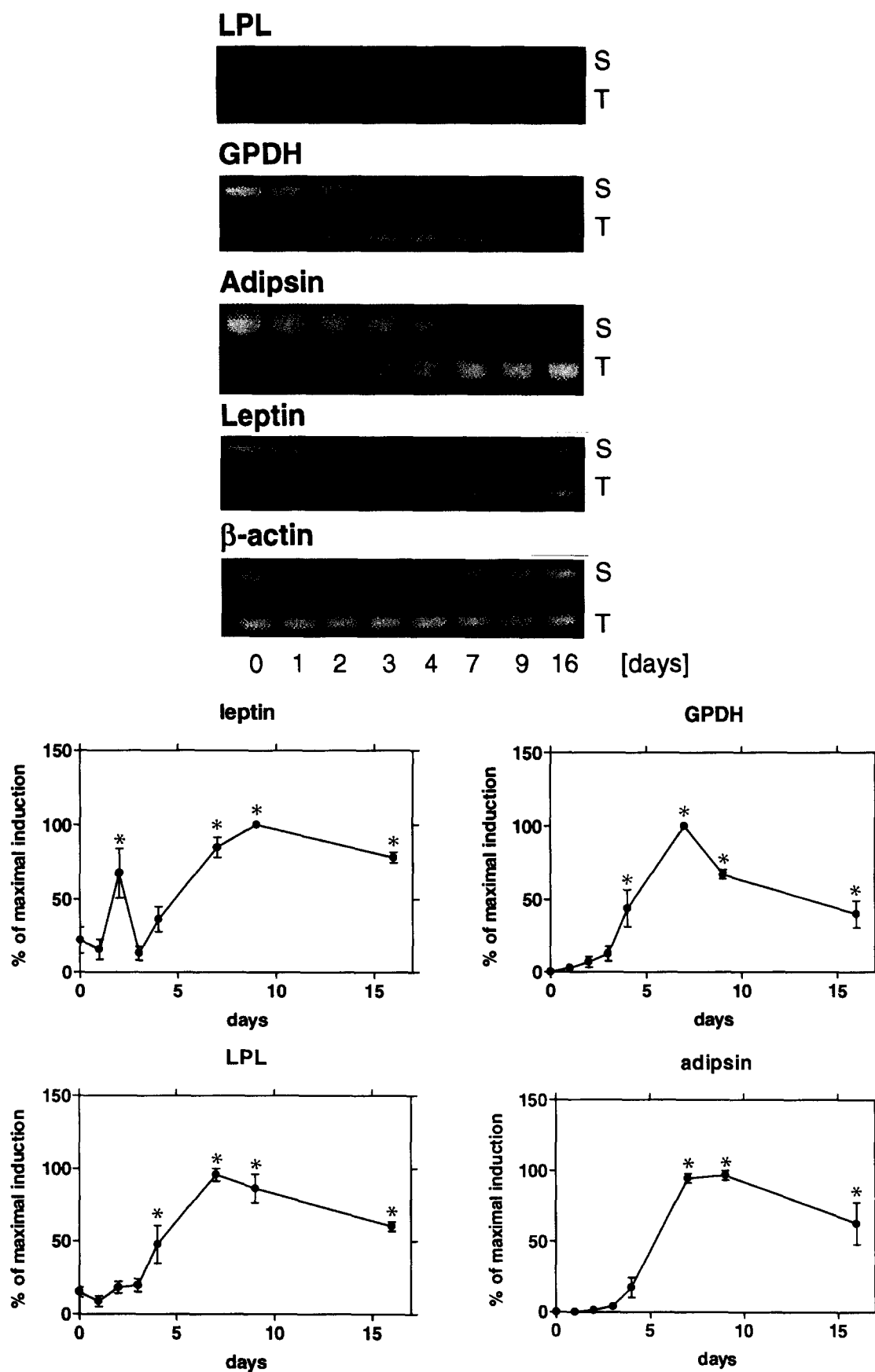
Lipid deposits inside the differentiated adipocytes became visible within the first 3–4 days after induction and were well-developed after 7 days. Thereafter, the size of the cells kept enlarging and at day 16 the cytosol was filled with extremely large fat droplets. As an indication of the size of the fat depots, total triglyceride content is presented in Fig. 2. The quantity of triglycerides at day 16 was 6-fold higher than at day 6. Leptin mRNA, on the other hand, had the tendency to decrease after day 6 (Fig. 2).

The peculiarity of the pattern of leptin expression in the first phase of cellular conversion to adipocytes prompted the investigation of the effect of some of the individual components of the differentiation cocktail on fully differentiated 3T3-L1 adipocytes (i.e. 1 week after induction). Addition of the phosphodiesterase inhibitor IBMX (0.5 mM) to the cells caused a minor decrease of leptin mRNA level, which, however, did not reach statistical significance (Fig. 3). Dexamethasone induced a marginal inhibition of leptin expression. The inhibition was neither concentration-dependent nor selective for leptin mRNA. The expression of adipsin, the other marker for mature adipocytes, was inhibited similarly by the corticosteroid (Fig. 3). Insulin, on the other hand, increased leptin mRNA levels 2–3 fold above baseline even at the lowest concentration tested (10 nM). Adipsin mRNA levels were only marginally affected by insulin even at the highest concentration of 1 μ M. FFA are known to regulate the expression level of various enzymes involved in fatty acid metabolism. The effect of 2-bromopalmitate, a non-metabolizable fatty acid, on the expression of leptin is also presented in Fig. 3. This compound was found to strongly reduce the mRNA levels of leptin while adipsin expression was minimally affected.

4. Discussion

The lipostatic hypothesis postulates that appetite is controlled by a negative feedback mechanism, e.g. a satiety factor released from adipose tissue [9]. To fulfill this role, circulating leptin levels should be proportional to the total fat mass. Several studies have indeed found that the expression of leptin mRNA in various animal models of obesity [4,7,10,11] and also in obese humans [3,12] correlates well with body mass index. Since in obesity, the increased size of the fat mass is partially due to an enlargement of existing adipocytes rather than to proliferation or to the recruitment of new fat cells, a direct link between the size of adipocytes and the expression of leptin has

Fig. 1. mRNA levels of adipocyte markers during differentiation. 3T3-L1 cells were cultured until confluent (day 0) and then differentiation was induced by adding a cocktail supplemented with IBMX, insulin and dexamethasone for 2 days. After differentiation, adipocytes were kept in culture in DMEM medium supplemented with 10% calf serum. Poly mRNA was isolated from cell samples at various times during culture. Semiquantification of the mRNA levels of the various adipocyte markers (LPL, GPDH, adipsin and leptin) and of β -actin was obtained by competitive RT-PCR as described in section 2. The cDNAs were amplified with specific primers in the presence of known amounts of the respective standards. Upper panel: the PCR products obtained in a typical experiment were separated on 0.8% agarose gel. S and T correspond to the bands of the standard and of the target, respectively. Lower panel: semiquantification of the various mRNAs was carried out for each cell sample and normalized to the amount of β -actin mRNA. Maximal levels of expression were set to 100%. The figure shows the mean $\pm$ S.E.M., $n = 3$. A significant change over $t = 0$ is indicated by *.



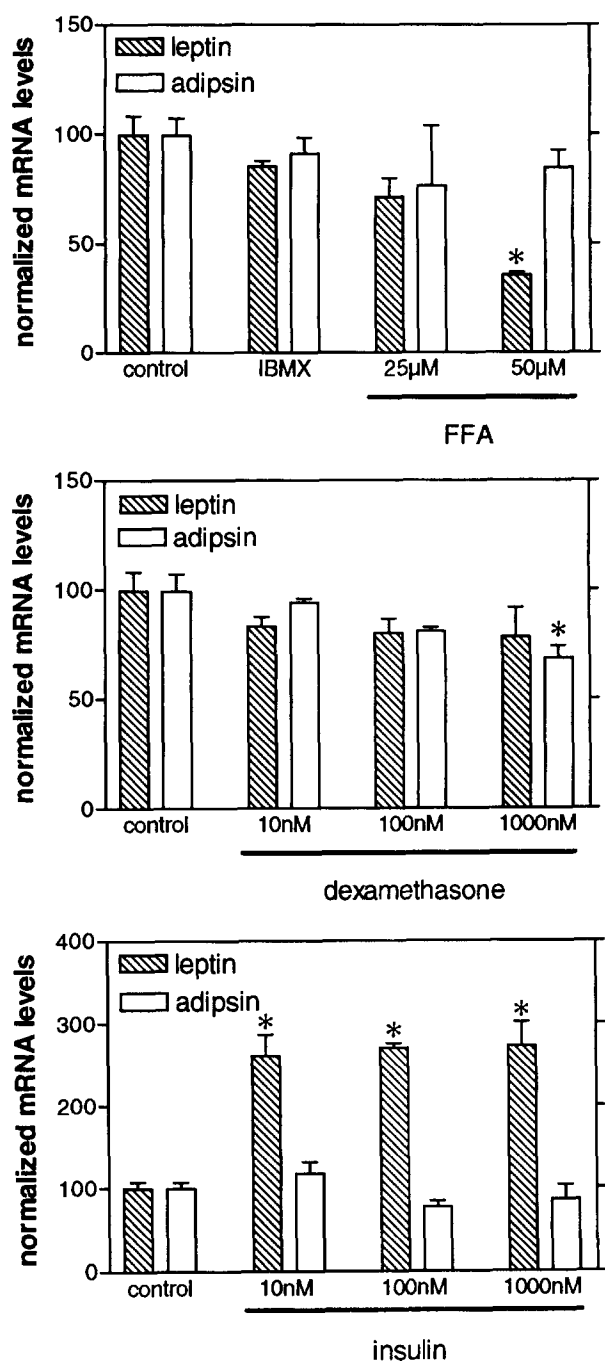


Fig. 2. Determination of total triglycerides and of leptin mRNA in cultured 3T3-L1 cells after differentiation. Fully differentiated 3T3-L1 adipocytes were harvested at various times and split for the colorimetric determination of triglycerides and for the isolation of mRNA. Leptin mRNA was quantified by heterologous competitive RT-PCR (see section 2) and normalized to the corresponding β -actin levels. Data are given as the mean $\pm$ S.E.M., $n = 3$. A significant change over control value is indicated by *.

been postulated. Indeed, within individuals preliminary evidence suggests that the level of leptin mRNA is higher in larger than in smaller adipocytes [12]. Alternatively, leptin production by white fat could be regulated by changes in humoral factors or substrate flux rather than by the actual size of the adipocytes. Using differentiated mouse adipocytes in culture, the present

study has investigated the role of cell size and of selected hormones and metabolites, whose concentration is known to be altered in obese animals, on the regulation of leptin expression.

It remains a controversial issue whether preadipocytes can express leptin. For example, preadipocytes isolated from human fat pads express leptin mRNA whereas mouse preadipocytes do not, even after partial differentiation [12]. In 3T3-F442A cells, a mouse cell line which can also be induced to store lipid, leptin mRNA levels are undetectable before full differentiation into adipocytes [11]. In contrast, the present studies demonstrate that in another mouse cell line (3T3-L1) leptin mRNA is detectable before differentiation. These observations suggest that expression of leptin by cells other than mature adipocytes depends on various factors, which include the cell system and the animal species under investigation.

There is substantial but indirect evidence that insulin regulates leptin expression in white adipose tissue. In situations where insulin secretion is increased, e.g. following a meal or after chronic central neuro peptide-Y (NPY) infusion, adipocyte leptin mRNA levels increase in parallel [13,17]. The stimulatory effect of NPY on leptin mRNA persists even when NPY-induced hyperphagia is compensated for by pair-feeding [17]. These observations strongly suggest that it is not hyperphagia or the consumption of a meal which correlates with increased leptin expression but rather the resulting elevation of circulating insulin. In support of this hypothesis, it has been shown that fasting reduces both the circulating insulin and leptin mRNA levels [13]. The present studies extend these observations to provide direct evidence that the level of leptin mRNA in fully differentiated 3T3-L1 adipocytes is under the control of insulin. The stimulatory effect of insulin is quite pronounced and selective for leptin, since the mRNA for adipsin, another substance secreted by the adipocytes, is barely affected (Fig. 3). Our experiments, therefore, support the contention that rapid changes in circulating insulin might be responsible, at least in part, for the changes in leptin mRNA levels observed during the fasting–eating cycle. Moreover, the elevated leptin expression observed in various obesity models

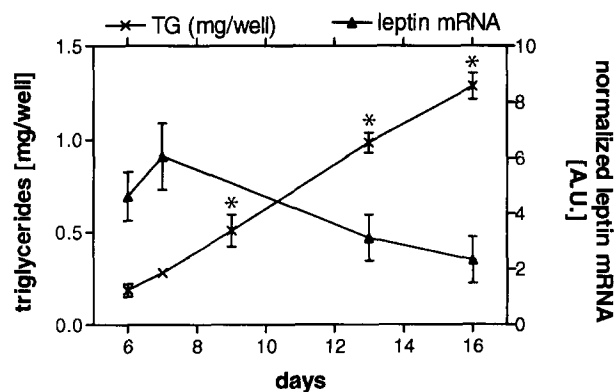


Fig. 3. Regulation of leptin and adipsin expression in fully differentiated 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated for 24 h in the presence of the given concentrations of dexamethasone, 2-bromopalmitate (FFA), IBMX and insulin. mRNA levels of leptin and adipsin were determined by quantitative RT-PCR, normalized for the β -actin amounts in the same samples and presented as percentage of the mRNA amount of untreated control cells. Data are given as the mean $\pm$ S.E.M., $n = 3$. A significant change over day 6 is indicated by a *.

could simply reflect the fact that these animals are hyperinsulinemic. In a recent publication, De Vos et al. [18] provided convincing evidence that the inhibitory effect of pharmacological concentrations of corticosteroids on food intake and the consequent loss of body weight are mediated by an increase of adipocyte leptin mRNA. The present experiments using isolated adipocytes in culture, however, do not demonstrate a direct stimulatory effect of the corticosteroid dexamethasone on leptin mRNA levels (Fig. 3). It is likely, therefore, that the rapid elevation of insulin induced by the high doses of corticosteroid, could have accounted for the stimulatory effect on leptin mRNA observed in vivo [13].

FFA are also obvious candidates for a possible regulatory signal controlling adipocyte leptin production. Prior studies conducted using Ob1771 preadipocytes have shown FFA to upregulate the expression of terminal differentiation-related genes and genes involved in fatty acid metabolism [19]. The effect of FFA on transcription is normally mediated by the interaction with peroxisome proliferator-activated receptors (PPAR), which are nuclear receptors of the steroid/thyroid hormone superfamily. Our studies indicate that the level of intracellular FFA could play a crucial role also in the downregulation of leptin expression. In support of this contention are prior studies showing that most conditions known to inhibit leptin synthesis, such as starvation and cold exposure [13], are accompanied by increased lipolysis and elevated concentrations of intracellular FFA. Similarly, a reduction of cytosolic FFA induced by the antilipolytic action of insulin could mediate the stimulatory effect of the hormone on leptin transcription in adipocytes.

It has been suggested that the fat cell size may control, via a hitherto unknown mechanism, the quantities of leptin produced [1,10]. However, the results of the present study do not support this contention since the amount of leptin mRNA did not correlate with the size of fully differentiated 3T3-L1 adipocytes (Fig. 2). The possibility should be considered, however, that the isolated cell system we used as a model might not be truly representative of fat cell hypertrophy in the intact animal.

In conclusion, the observations presented in this study suggest that insulin acts directly on adipocytes to increase the mRNA levels while FFA, on the other hand, are inhibitory to leptin expression. Extending these observations to the whole animal may mean that the plasma concentration of insulin and FFA, which reflect the metabolic state of the adipocytes, could play a central role in leptin expression. Finally, the extent of

leptin mRNA expression in fat tissue reflects rather the number than the size composition of fat cells.

Acknowledgements: Many thanks are due to Dr. N. Levens for kindly reviewing the manuscript, to Mr. S. Grueninger for the preparation of the standards and to Mrs. H. Thomann for helping in the triglyceride determination.

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