Expression of ob Gene in Adipose Cells

REGULATION BY INSULIN*

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The product of the recently cloned mouse obese (ob) gene is likely to play an important role in a loop regulating the size of the adipose tissue mass. The hormonal regulation of the ob gene could affect adiposity. To investigate this point, the effect of insulin on ob gene expression was examined in cells of the 3T3-F442A preadipocyte clonal line. ob mRNA is absent from exponentially growing, undifferentiated cells as well as from confluent preadipose cells. Terminal differentiation of preadipose to adipose cells leads to the expression of ob mRNA detected by a sensitive and quantitative ribonuclease protection assay. In adipose cells, the level of ob mRNA is sensitive to insulin in the nanomolar range of concentrations with an increase from an average of 1 copy to 5-10 copies/cell. The effect of insulin was fully reversible and takes place primarily at a transcriptional level. The ob mRNA shows a rapid turnover, with a half-life of approximately 2 h in the absence or presence of insulin. The level of secreted Ob protein is also regulated by insulin. These results indicate that the ob gene is expressed in mature fat cells only and support the possibility that insulin is an important regulator of ob gene expression.

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‡ The abbreviations used are: VMH, ventromedial nucleus of the hypothalamus; DME, Dubecco's modified Eagle's medium; bp, base pair(s); 2AZC, a2 chain of type VI collagen; T3, triiodothyronine; GAPDH, glyceraldehyde-phosphate dehydrogenase.

The "lipostat" or "adipostat" theory postulates that the size of body fat stores is regulated by a feedback loop (1). This hypothesis is based upon the recovery of initial body weight following lipectomy (2) and parabiosis experiments between genetically obese and wild-type mice suggesting the existence of putative factors regulating food intake (3). The recently cloned ob gene from mouse, rat, and human encodes a circulating factor of 16 kDa that is secreted from adipocytes from various adipose depots (4–8). The OB protein, named leptin, appears to act at distal sites since injections of the leptin decrease food intake and body weight in ob/ob mice and their lean counterparts (9–12). This phenomenon implicates directly or indirectly the hypothalamus since mice with chemical lesions of the ventromedial nucleus of the hypothalamus (VMH),1 after becoming rapidly hyperinsulinemic, express a dramatic increase in the levels of ob mRNA (5, 13). A substantial fall in ob mRNA in the epididymal fat of lean mice has been observed after fasting; this phenomenon is rapidly reversed on refeeding (13–16). The correlation between insulin level and the levels of ob mRNA and plasma leptin suggests that insulin may have direct effects on ob gene expression (15–18). In this paper, we present data using cultured adipocytes that support this hypothesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Culture conditions of cells of the 3T3-F442A clonal line have been described (19). Cells were plated at 104 cells/cm2 in 60- or 100-mm dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. This medium was defined as standard medium. At confluence, standard medium was supplemented with 2 nm triiodothyronine (T3) and 3 nm insulin, termed Defined Minimum medium, for changed every 2 days. Where indicated, insulin removal from 10-day postconfluent adipose cells was performed by rinsing the cells three times at 37°C (20 min each) with DMEM containing 2% fetal bovine serum and 2 nm T3. Insulin-deprived cells were then maintained for 24 h in standard medium supplemented with 2 nm T3 before adding insulin at the indicated concentrations.

RNA Isolation—Total RNA was prepared as described by Chomczynski and Sacchi (20). At least two dishes were pooled for each condition. Northern blot analyses were performed as described previously (19).

Ribonuclease Protection Assay—The EcoRI-PstI ob probe consisting of a 297-bp fragment starting 9 bp upstream of the first methionine codon was derived from mgt2b plasmid (4). The template for in vitro transcription, which consists of the partial cDNA flanked with plasmid sequences, was prepared by gel purification of the fragment released by digestion with BssHII restriction enzyme. RNA probe was produced by T3 RNA polymerase in the presence of [32P]CTP (800 Ci/mmol, ICN France). The size of the probe was 365 nucleotides, and the size of the protected fragment was 297 bp. Used as an internal control, the GAPDH probe was synthesized from a construct provided by Dr. A. G. Smith (Edinburgh, UK). The RNA probe was produced by SP6 transcription. [32P]CTP was diluted with unlabeled CTP in order to obtain a GAPDH probe of specific activity approximately 20-fold lower than that of the ob probe. The size of the probe was 235 bp and that of the protected fragment was 220 bp (21). Ribonuclease protection assays were performed with 20–40 µg of RNA essentially as described previously (22), except that hybridizations were done at 65°C overnight. Protected species were resolved on a 6% acrylamide/urea gel and visualized by autoradiographic exposure on x-ray film (Kodak) at ~70°C for 5–7 days.

Estimation of the Number of ob Transcripts per Cell—The number of ob RNA/cell was determined by comparison with varying amounts of ob RNA synthesized in vitro. Briefly, ob sense RNA was produced by T7 transcription of the 297-bp fragment. The integrity and quantification of the product were confirmed by agarose gel electrophoresis and ethidium bromide staining. Quantification of the hybridization signal was performed using a PhosphorImager apparatus (Molecular Dynamics). As little as 0.1 pg of in vitro synthesized RNA could be detected under the conditions described above. This RNA has a molecular weight of approximately 105,000. Therefore, 0.1 pg corresponds to 6 × 105 molecules. Assuming a yield of 20 µg of RNA/106 cells, this corresponds, in a sample of 20 µg of total RNA, to the detection of one copy of ob RNA/cell.

Detection of Leptin in Conditioned Media—Differentiated 3T3-F442A cells were washed as described above and then maintained under serum-free conditions in H-16F-12 medium (1:1, v/v) supplemented with 0.2 nm T3, 20 nm sodium selenite, 10 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin, in the absence or presence of insulin. Media were then collected and analyzed for leptin as described previously (10).
RESULTS AND DISCUSSION

Expression of ob Gene during Differentiation of 3T3-F442A Cells—We have previously reported that ob mRNA is detected in the adipocyte fraction of mouse adipose tissues only (5). The major, if not exclusive, expression of ob gene in the adipocyte was reminiscent of the expression of adipin gene (19) and suggested that ob gene was a marker of terminally differentiated cells. This has been studied more directly during the differentiation of cells from the 3T3-F442A preadipocyte clonal line. Induction of ob gene expression was compared with that of adipin gene and also with that of the n2 chain of type V1 collagen (A2COL6) gene, a very early marker expressed before triacylglycerol deposition (23, 24). The amount of A2COL6 and adipin mRNA transcripts was determined by Northern blot analysis whereas the relatively low level of ob mRNA in cultured adipocytes necessitated the use of a highly sensitive ribonuclease protection assay. No expression of A2COL6, adipin, and ob genes was observed in exponentially growing, undifferentiated cells (Fig. 1). A2COL6 gene was expressed at confluence, and a maximal level of A2COL6 mRNA was attained 2 days later at a time at which no lipid droplets could yet be seen by microscopy examination. The kinetics of induction of adipin and ob genes showed a similar time course. This relatively late expression followed the accumulation of small lipid droplets in a majority of cells between day 2 and day 4. Ten days after confluence, when the lipid accumulation was near maximal, the ob mRNA level reached 5–10 copies/cell (assuming it is evenly expressed in all cells) in the continuous presence of insulin. The removal of insulin led to a lower ob mRNA content (1 copy/cell, not shown). Under these conditions, as previously reported (19), the content in β-actin mRNA did not vary by more than ±5% whereas that in GAPDH mRNA did not vary by more than ±8%. Since an appropriate GAPDH riboprobe was readily available, the GAPDH mRNA content was used as an internal control. These results indicated that ob mRNA accumulated as a function of differentiation and suggested that insulin was involved in the regulation of ob gene expression.

Effects of Insulin on ob Gene Expression and Leptin Secretion—In order to ascertain whether insulin had direct effects on ob gene expression, the levels of ob mRNA and leptin were measured in 10-day postconfluent, differentiated 3T3-F442A adipose cells. Insulin deprivation of differentiated cells for 24 h resulted in a dramatic decrease in ob mRNA content (Fig. 2). After insulin removal, the average number of ob transcripts was estimated to be one copy/cell and was similar to that determined in 10-day postconfluent cells never exposed to the hormone. The addition of insulin to insulin-deprived cells restored the steady-state level of ob mRNA to the starting level within 48 h. As shown previously (19), insulin had an opposite effect on adipin gene expression. The time course of insulin action on the steady-state level of ob mRNA in 10-day postconfluent, differentiated 3T3-F442A cells has also been determined. As shown in Fig. 3, the effect of insulin on the accumulation of ob mRNA was not immediate. Insulin treatment led to a 3-fold increase in the ob mRNA content within 10 h whereas a maximal 5–10-fold increase was obtained within 24 h. During the same period of time, insulin treatment led to a significant decrease of adipin mRNA. The half-maximal effective concentration (EC50) was estimated to be 0.3–1 nM (Fig. 4), in good agreement with the affinity of the insulin receptor for insulin in these cells and the EC50 values determined for the insulin effects on glucose and amino acid uptake (25). Altogether, these results indicate that insulin has a reversible effect and is a potent modulator of ob gene expression in differentiated adipose cells. These results are also in agreement with the high level of ob mRNA observed in vivo in the hyperinsulinemic state in refed or VMH-lesioned mice (5). In order to see whether insulin effects could also be detected at the protein level, analysis of leptin was performed from conditioned media of differentiated cells exposed or not to the hormone. As shown in Fig. 5, the amount of secreted leptin was greatly decreased when insulin was removed for 24 or 48 h (lanes 2 and 4 compared with lane 1). As anticipated, readdition of insulin to the insulin-deprived cells led to an increase of secreted leptin (lanes 3 and
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5). No protein could be detected in the conditioned medium of undifferentiated cells exposed to insulin (lane 7) as well as from fresh serum-free (lane 6) or serum-containing medium (lane 8).

Half-life of ob mRNA and Transcriptional Activation of ob Gene by Insulin—Previous data suggested that insulin resulted in transcriptional and post-transcriptional regulation of the expression of adipocyte-specific genes in 3T3-F442A cells (19). In order to delineate the mechanisms by which insulin regulates ob gene expression in differentiated adipose cells, the stability of ob mRNA was compared in the absence or the presence of insulin. The rapid decay of ob mRNA content after insulin removal (see Fig. 2) suggested a rapid turnover of ob mRNA. As shown in Fig. 6 (upper panel), within 2 h following addition of actinomycin D, an inhibitor of transcription, the level of ob mRNA content decreased by approximately 50%. This decrease was similar in the absence or the presence of insulin. Therefore, ob mRNA has a half-life of approximately 2 h whereas insulin does not have any significant stabilizing effect. The lack of ob mRNA accumulation after adding insulin in cells preincubated with actinomycin D for 15 min indicated that insulin regulates the expression of the ob gene by means of transcriptional activation (Fig. 6, lower panel). The characterization of trans-acting factors recognizing insulin response element(s) of ob promoter has not yet been identified.

In conclusion, ob gene is expressed in adipose cells only, and its regulation is reversibly and exquisitely sensitive to insulin at physiological concentrations. Secreted leptin can be detected in conditioned media of differentiated cells exposed to the hormone, and its concentration is also modulated by insulin in a reversible manner. The low expression of ob gene in vitro compared with in vivo suggests that other factors are required for maximal expression. The identification of additional factors that regulate ob gene expression is under investigation. It also appears that glucocorticoids regulate ob gene expression in vivo (26), but the pharmacological doses used in this study prevent any definite conclusion. Adipogenic factors other than insulin, which have been reported to trigger terminal differentiation of adipocyte precursor cells to adipose cells (27), are likely to play a regulatory role in the expression of ob gene.

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