Obese Gene Expression Alters the Ability of 30A5 Preadipocytes to Respond to Lipogenic Hormones

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Leptin, the product of the ob gene, controls food-intake and weight loss in the ob mouse. Although the target(s) of the circulating leptin is presumed to be the brain which then orchestrates food-intake and weight loss, how leptin functions in the process of weight loss is unknown. In this report, we present evidence that ob gene expression in cultured cells suppresses acetyl-CoA carboxylase gene expression and lipid synthesis which are induced by hormone treatment. This is the first example in which leptin has been found to suppress defined biochemical reactions that contribute to lipid accumulation without the participation of the brain.

When the balance between caloric intake and energy expenditure in animals is shifted in the direction of excess caloric intake, obesity occurs. Because obesity in humans may lead to pathological conditions, such as cardiovascular disease, type II diabetes mellitus, and certain forms of cancer, extensive investigations of obesity have been carried out for many years. Earlier studies suggested the presence of a blood-borne factor(s) which might control food-intake and weight in mice (1). Recently, Friedman and his co-workers cloned a gene that is responsible for controlling food-intake and obesity in ob mice (2). In this case, a point mutation in the gene results in the generation of prematurely terminated leptin which is unable to control food-intake in the affected animals (3–5). The accepted hypothesis is that a certain part of the brain responds to the level of ob protein circulating in the blood and uses this information to direct the control of food-intake in animals. However, the lack of leptin, or a mutated leptin might not be perceived by the brain and could result in uncontrolled food-intake which would lead to obesity. Indeed, various experiments in which mice are injected with leptin indicated that leptin curtails food-intake in these experimental animals (3–5). The injected animals quickly lost significant amounts of weight. The interpretation of these observed effects of leptin on obesity that has apparently been accepted recently is that: (a) leptin functions through the brain and results in proper control of food-intake and (b) lipid deposits in fat cells are simply the passive result of excess calories which accumulate in the absence of leptin, at least in the case of the mouse model system. However, neither the target tissue(s) of leptin nor how leptin controls food-intake or weight loss is understood at this time. Indeed, there have not been any reports of biochemical reactions which might be affected by leptin and thus explain the role of leptin in the control of obesity.

In this communication, we report evidence that ob gene expression represses acetyl-CoA carboxylase (ACC) gene expression, fatty acid synthesis, and lipid synthesis in a culture system.

MATERIALS AND METHODS

Cell Culture and Treatment—A preadipocyte cell line, 30A5, was grown in Eagle’s basal medium supplemented with 10% heat-inactivated fetal bovine serum. To induce lipid synthesis and cell differentiation, fresh medium containing 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine were added at confluence (6). After 2 days, the medium was changed to include only insulin, and cells were maintained in this medium for 2 days before ACC was assayed (6). Glycerol phosphate dehydrogenase activity was measured as described (7). The rates of fatty acid and lipid synthesis were determined as described using 14CH3COOH (56 mCi/mmol) (8). To obtain stably transfected 30A5 cells with the ob gene plasmid, at confluence, cells were transfected using the calcium phosphate coprecipitation method (9). Precipitates were formed using 20 μl of plasmid pcR/CVS or pcR/CVS-ob. The precipitate was left on the cells for 5 h at 37°C, after which cells were washed and then shocked with a glycerol solution for 2 min at room temperature. After washing, the cells were incubated at 37°C for an additional 48 h. The stably transfected cells were obtained by using G418.

Assay of ACC, Fatty Acid Synthesis, and Lipid Synthesis—Cells in 100-mm dishes were washed twice with cold phosphate-buffered saline. 400 μl of dialysin buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.25% sucrose, digitonin (0.4 mg/ml), and 1.5 mM phenylmethylsulfon fluoride) were added to the culture dish on ice, and the dish was rocked for 3 min. The diffusate was collected, and, following centrifugation, the supernatant was used for enzyme assay (10).

Northern Blot Analysis of RNA—Total RNA was separated on 1% agarose gel containing 2.2 M formaldehyde, then transferred to nitrocellulose. Hybridization, autoradiography, and washing conditions were as described (9). 32P-Labeled KH-18 ACC cDNA fragment was prepared by the random priming method (Promega), using [α-32P]dCTP (6000 Ci/mmol).

Isolation of cDNA Encoding ob Protein—Total RNA was isolated from differentiated 30A5 adipocytes, using the guanidinium thiocyanate method (11). Total RNA (2 to 4 μg) was heated for 10 min at 70°C and cooled on ice for 2 min. The RNA was mixed in a final volume of 20 μl with 0.5 μg of ob cDNA specific antisense primer 430H (GATCCAAACCTTTCCCTTCAGGCTTCA), 500 μM each dNTP, and 200 units of SuperScriptII RNase H Reverse Transcriptase (Life Technologies, Inc.) in 1× reverse transcription buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol). The mixture was incubated for 30 min at 42°C, then for 5 min at 55°C. Two units of RNase H was added and incubated for 10 min at 55°C. Target cDNA derived from reverse transcription was amplified by PCR using two sets of primers. The primers for the first round of PCR were 600H with a HindIII site (5′-CCCAAGCTTGAAGAAGATCCCAGGGAGGAAAATGTG-3′) and 430H. The primers for the second round of PCR were 600H and 601H with a Xba site (GCTCTAGACAAGCATTGTGGAGTCAT). Target cDNA (5 μl) was combined with 15 pmol of each PCR primer, 200 μl dNTP, and 2 units of deep vent DNA polymerase in 1× PCR buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgCl2, 0.1% Triton X-100) in final volume of 100 μl. Cycling conditions for the PCR were denaturation at 94°C for 5 min,
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**Table 1**

| Sample | Culture condition | ob medium | Hormone treatment | ACC activity (microunits/mg protein) |
|--------|-------------------|-----------|------------------|-------------------------------------|
|        | ml                |           |                  |                                     |
| 1      | 5                 | 5         |                 | 62.2                                |
| 2      | 5                 | 5         | +               | 163.8                               |
| 3      | 5                 | 2.5       | 2.5             | 58.2                                |
| 3      | 5                 | 2.5       | +               | 73.0                                |

**Figure 1.** Ob gene expression in 30A5 cells. Total RNA was extracted from 30A5 cells. Reverse transcription reactions were performed using 4 µg of total RNA. The first-strand cDNA synthesis and PCR amplification conditions have been described under "Materials and Methods." PCR products were resolved in a 1.2% agarose gel run in 1× TAE buffer. The bands shown in the gel are the 0.68-kb ob fragments. Lane 1, PCR products on RNA from control 30A5 cells with pRc/RSV; lane 2, PCR products on RNA from 30A5 cells containing pRc/RSV-ob; lane 3, molecular marker; lane 4, molecular marker

**Figure 2.** Effect of ob gene expression on ACC. 30A5 cells containing pRc/RSV (lanes 1 and 3) and stable clones containing the ob gene, pRc/RSV-ob (lanes 2 and 4), were treated with hormones as described. After incubation for 4 days, cell extracts were prepared and ACC activity was assayed. Lanes 1 and 2, cells without hormone treatment; lanes 3 and 4, cells treated with dexamethasone and insulin. Northern analysis was carried out using total RNAs that were prepared from cells that had been treated with the same procedure; in each lane, 15 µg of RNA was used. Lane 1, control RNA; lane 2, RNA from cells expressing ob gene; lane 3, control treated with hormones; lane 4, RNA from ob gene-expressing cells treated with hormones.

annealing at 55 °C for 2 min, and extension at 72 °C for 3 min for one cycle, followed by denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min for 29 cycles, finally followed by an extension step at 72 °C for 10 min.

**Construction of ob Expression Vector—** The ob expression vector pRc/RSV-ob (5.9 kb) was constructed by inserting the ob gene obtained as a 0.68-kb HindIII-XhoI fragment, which includes 501-bp coding for ob protein, into plasmid pRc/RSV, and the construct was confirmed by DNA sequencing (12). For the expression of ob gene in Escherichia coli, the coding region was amplified by PCR to have an Ncol site at the 5'-end and a XhoI site at the 3'-end using pRc/RSV-ob as template. 440 bp of Ncol/XhoI fragment was then inserted into the plasmid pET22b (+), E. coli BL21(DE3) was transformed with the vector, and overexpression was made with the use of isopropyl-1-thio-

**Figure 3.** Effect of leptin on ACC activity. 30A5 cells were grown in 100-mm culture plates containing 10 ml of medium. At confluence, cells were treated in differentiation medium in the absence (x—x) or presence (O—O) of 2, 4, or 6 µg of leptin, respectively. After 2 days of incubation with dexamethasone and insulin, the medium was changed to include only insulin and leptin and the cells were incubated for 2 additional days. ACC activities of cell extracts were assayed as described under "Materials and Methods." RESULTS

To examine the effect of ob gene expression on lipid and fatty acid synthesis, 30A5 cells which express the ob gene were constructed by stably transfecting with the expression plasmid containing the ob gene, pRc/RSV-ob. Stably transfected cells with pRc/RSV-ob were isolated, and the expression of the ob gene was examined by reverse transcription-PCR to detect ob mRNA in the total RNA preparations (Fig. 1). In lane 1, amplified ob DNA, generated on ob cDNA from 30A5 cells containing pRc/RSV-ob plasmid, is shown. PCR-amplified DNA on ob mRNA is 680 bases long. This 680-base sequence contains 501 base pairs encoding ob protein. Lane 2 shows PCR-amplified

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DNA on the total RNA preparation from 30A5 cells containing empty vector pRc/RSV. This shows the presence of ob mRNA in the total RNA preparation from 30A5 cells containing pRc/RSV-ob plasmid. Lane 3 shows the migration pattern of molecular marker. This ob gene-expressing cell line was used to examine the effect of ob gene expression on lipid synthesis.

ACC is the rate-limiting enzyme in long-chain fatty acid synthesis, and the activity of ACC is reflected in the biogenesis of lipids (10). In addition, the functioning of ACC is essential for the conversion of carbohydrate into fatty acids and lipids. We have also previously shown that the differentiation of 30A5 preadipocytes into lipid-laden adipocytes which is induced by hormone treatment accompanies ACC induction because the hormones increase the rate of transcription of the ACC gene (13). In order to examine the effect of ob gene expression on ACC induction, the induction of ACC by hormone treatment in the control and the ob gene-expressing cells was examined. As shown in Fig. 2, the cells expressing ob gene did not respond to hormone treatment in the induction of ACC at all, while in control cells containing the empty vector, the activities increased about 2-fold. The same effect of ob gene expression was observed at the level of ACC mRNA accumulation (inset, Fig. 2). The cells with the empty vector showed a 2- to 3-fold increase in mRNA upon hormone treatment (lane 1 versus lane 3 of the inset, Fig. 2), whereas those cells containing the ob gene did not respond to hormone treatment (lane 2 versus lane 4). In a separate experiment using Western analysis, we confirmed that the amounts of ACC in different samples reflected the level of activity.

To eliminate any possibility that the G418 resistant cells that express the ob gene did not constitute a population of cells that are resistant to hormone induction of ACC gene expression, we have examined the presence of ob gene product in the culture medium. The culture medium was then added to the medium in which control cells were treated with hormone. For the control we used the culture medium in which the control 30A5 cells were grown. The effect of the culture medium from the ob gene-expressing cells on ACC gene expression by normal cells is shown in Table I. The 30A5 cells in the control medium showed 62 microunits of ACC/mg of protein; this increased to 163 microunits/mg upon hormone treatment. However, the addition of 2.5 ml of the culture medium from the ob gene-expressing cells completely repressed ACC induction by hormone treatment. The amount of leptin in the culture medium was too low to be detected by Western analysis.

To show that leptin itself affects the gene expression, we have overexpressed leptin in E. coli, and partially purified leptin was used to examine the repression of ACC gene expression (Fig. 3). Although leptin repressed ACC induction in a concentration-dependent manner, leptin added to the culture medium was less effective than the culture medium. The leptin that we overexpressed in E. coli did not contain the 21 amino acids at the N terminus which constitute the signal peptide sequence. Whether or not this lack of signal peptide and/or a low level of leptin receptors in fat cells (14) is the cause of its low efficiency has yet to be determined. However, the data in Fig. 3 do show that leptin represses ACC gene expression. Following the establishment of the suppressive effect of ob gene expression on ACC gene induction, we have examined the effect of ob gene expression on overall fatty acid synthesis (Fig. 4A) and lipid synthesis (Fig. 4B), in addition to glycerol phos-

**Fig. 4. Effect of ob gene expression on the synthetic rates of fatty acids (A), neutral lipids (B), and glycerol phosphate dehydrogenase activity (C).** The synthesis of lipids and fatty acids in intact cells was assayed by measuring [14C]acetate incorporation into lipids (7). Cells were incubated in fresh medium containing [14C]acetate (56 mCi/mmol) for 1 h. After saponification of the samples, neutral lipids were extracted with petroleum ether. After acidification of the samples, the free fatty acids were similarly extracted and counted. 1, control cells were not treated with hormones; 2, cells were treated with dexamethasone and insulin; 3, cells were treated only with dexamethasone; 4, cells were treated only with insulin. 30A5 cells containing pRc/RSV (lanes 1 and 3) and stable clones containing the ob gene, pRcRSV-ob (lanes 2 and 4) were treated with hormones as described (13). After incubation for 4 days, cell extracts were prepared and glycerol phosphate dehydrogenase activity was assayed at 25°C and pH 7.85 in a buffer containing 50 mM Tris-HCl, 10 mM dithiothreitol, 0.2 mM NADH, and 2 mM dihydroxyacetone phosphate. The reaction volume is 1 ml. Enzyme activity was monitored by decrease in absorbance at 340 nm. The data shown are the mean value ± S.E. of three determinations from three separate experiments.
phate dehydrogenase (Fig. 4C). The rate of fatty acid synthesis in the control cells increased about 3-fold, reflecting the increased activity of ACC following hormone treatment (Figs. 2 and 3). However, fatty acid synthesis (Fig. 4A), lipid synthesis (Fig. 4B), and glycerol phosphate dehydrogenase (Fig. 4C), induced by hormones in ob gene-expressing cells, were almost completely curtailed. The effects of ob gene expression on these three parameters (Figs. 2 and 4, A and B) were apparent even in the cells which had not been treated with hormones. For example, those cells expressing the ob gene supported a rate of lipid synthesis less than 30% that of the control cells (control column, Fig. 4B). The differences in the ACC activities and fatty acid synthesis between the control cells and the ob gene-expressing cells were not, however, as dramatic as lipid synthesis. In the control cells, the hormone induced lipid synthesis about 4-fold, and this rate of lipid synthesis was reduced even less than that in the control cells. These studies clearly show that ob gene expression affects the synthesis of fatty acids and lipids, particularly those which are induced by hormones. Lipid synthesis and fatty acid synthesis are also induced by dexamethasone or insulin treatment alone (Fig. 4, A and B), and these increases were also repressed by the expression of ob gene. These observations suggest that hormone-induced increases in the rates of synthesis of fatty acids and lipids are independent of 30A5 cell differentiation which requires sequential treatment with two hormones (6). The observation that the basal levels of ACC and fatty acid synthesis are not grossly affected in the cells that express the ob gene, although induced synthesis by hormones was almost completely suppressed, suggests that ob gene expression primarily affects the increase in rates of synthesis of those compounds.

**DISCUSSION**

Ever since the ob gene was cloned and the effects of leptin on food-intake and weight loss in mice were reported, it was clear that intensive efforts would have to be made to find leptin’s target tissues. These studies are directed toward identifying the presence of leptin receptors in different tissues (14, 15). A complex series of receptors have been identified (14, 15). Leptin is apparently made in adipose tissues and is carried to different parts of the animal. One of the targets appears to be the brain (5, 8), which is thought to orchestrate the appetite and food-intake of the experimental subjects. Recent experiments demonstrated that ob protein had the same effect on food-intake and weight loss when it was injected directly into the brain (5) as when it was injected intravenously (3, 4). These experiments supported the idea that, at least where food-intake problems are involved, the brain may be involved in the final manifestations of leptin action. On the other hand, the leptin receptors are found in various peripheral tissues (14, 15). An immediate question arising from such findings is what are the biochemical consequences of the interaction between leptin and the receptors.

Our experiments provide evidence that the ob protein targets ACC, the rate-limiting enzyme in the biosynthesis of long chain fatty acids, and, therefore, affects the subsequent processes of lipid synthesis in the fat cells without involving the brain, or any signal from the brain. Leptin inhibits lipogenesis, and we have observed that leptin suppresses the accumulation of lipid droplets that occurs in these 30A5 adipocytes (data not shown).

In living cells, the amounts of all cellular components, proteins, and energy stores of carbohydrate (glycogen) and fat or lipids are in a dynamic state and are controlled by the rates of both synthesis and degradation. Thus, the steady state level of, for example, the storage lipids, which is the basis of obesity, is determined by these two parameters. Therefore, our observation that ob protein inhibits the synthesis of lipids is very interesting. Indeed, this observation explains why the injection of leptin in normal individuals also causes weight loss without apparent changes in “metabolic parameters” (5). Under normal conditions, the ob gene product affects the basal rate of synthesis of lipid, and, therefore, the effect of leptin is not dramatic until lipid synthesis is stimulated by the hormone treatment. However, leptin does affect the basal level of the synthetic rates of ACC, fatty acids, and lipids, suggesting that leptin affects these parameters independently of 30A5 cell differentiation. Furthermore, increased fatty acid synthesis and lipid synthesis that occur in the presence of insulin or dexamethasone alone are affected by leptin although the cells are not differentiated under such conditions.

In whole animals, the extent of lipid storage is not a simple passive process transferring excess foods into lipid molecules. Components of food elicit a variety of changes in hormonal systems which in turn activate and inactivate key enzyme systems in such a way as to change metabolite flow in favor of one storage compound or another. The present studies suggest that leptin more efficiently suppresses newly induced fatty acid and lipid synthesis, which occurs as a result of changes in hormonal status, i.e., following food-intake. Using our cell system, we have clearly shown how ob gene expression suppresses hormone-induced lipid storage.

In summary, the general belief that leptin levels in the blood are perceived by some brain cells, and that this results in the release of appetite-controlling substances, may still be true. However, we have shown here that leptin has an effect on the machinery of lipid biosynthesis.

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