Obese gene expression: Reduction by fasting and stimulation by insulin and glucose in lean mice, and persistent elevation in acquired (diet-induced) and genetic (yellow agouti) obesity

(leptin/mRNA/hyperinsulinemia/hyperglycemia/diabetes)

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ABSTRACT Mutations in the obese (*ob*) gene lead to obesity. This gene has been recently cloned, but the factors regulating its expression have not been elucidated. To address the regulation of the *ob* gene with regard to body weight and nutritional factors, Northern blot analysis was used to assess *ob* mRNA in adipose tissue from mice [lean, obese due to diet, or genetically (yellow agouti) obese] under different nutritional conditions. *ob* mRNA was elevated in both forms of obesity, compared to lean controls, correlated with elevations in plasma insulin and body weight, but not plasma glucose. In lean C57BL/6J mice, but not in mice with diet-induced obesity, *ob* mRNA decreased after a 48-hr fast. Similarly, in lean C57BL/6J controls, but not in obese yellow mice, i.p. glucose injection significantly increased *ob* mRNA. For up to 30 min after glucose injection, *ob* mRNA in lean mice significantly correlated with plasma glucose, but not with plasma insulin. In a separate study with only lean mice, *ob* mRNA was inhibited >90% by fasting, and elevated $\approx$ 2-fold 30 min after i.p. injection of either glucose or insulin. These results suggest that in lean animals glucose and insulin enhance *ob* gene expression. In contrast to our results in lean mice, in obese animals *ob* mRNA is elevated and relatively insensitive to nutritional state, possibly due to chronic exposure to elevated plasma insulin and/or glucose.

Obesity is a major disorder associated with excessive mortality (1). However, mechanisms leading to different forms of obesity are not well understood. Mutations in the obese (*ob*) gene result in obesity and hyperinsulinemia (2). Parabiosis studies have suggested that the *ob* gene codes for a satiety factor (3). The *ob* gene, recently isolated by positional cloning, codes for a product produced in and secreted by adipose tissue (4). Injection of the *ob* gene product leptin reduced food intake, body weight, plasma glucose, and plasma insulin (5–7). In a recent report, Maffei *et al.* (8) stated that “identification of the molecules that regulate expression of the *ob* gene in adipocytes . . . will greatly enhance our understanding of the physiological mechanisms that regulate body weight”. On the basis of the above observations, we hypothesized that glucose and/or insulin would stimulate expression of the *ob* gene.

Studies demonstrating that leptin injections reduce body weight in obese mice (5–7) have suggested that some forms of obesity, like the *ob/ob* mouse, may be due to insufficient leptin production, and thus, like other hormone-deficient states, may be treatable by replacement therapy. On the other hand, if obesity is generally characterized by overexpression of the *ob* gene, as in *db/db* mice (8), insensitivity to leptin, rather than

insufficient leptin production, may be a more common impairment, and efficacy of leptin may be limited. The yellow allele (*A^y*) of the agouti gene is, like mutant alleles of the *ob* gene, associated with obesity, hyperinsulinemia, and hyperphagia (9). The agouti gene has also been characterized, and its product appears to inhibit the action of melanocyte-stimulating hormone on its receptor (10). We have therefore examined expression of *ob* mRNA in a model of environmentally acquired obesity, due to a high-calorie diet, and in a model of genetic obesity, the yellow agouti mouse. We have further examined the relationship between *ob* gene expression and plasma glucose and insulin, in lean and obese mice.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice, heterozygous lethal yellow obese mice (C57BL/6J-*A^y*), and CBA mice were purchased at 2 mo of age from The Jackson Laboratory. Mice were housed with free access to feed and water under 12/12 hr light–dark cycle (lights on at 07:00 hr). All studies had been approved by the appropriate Institutional Animal Review Board. To study effects of diet-induced obesity on *ob* mRNA, normal C57BL/6J mice were placed for 14 weeks on a high-fat, high-carbohydrate diet (diet no. F2685, BioServe, Frenchtown, NJ); normal caloric intake was produced by access to mouse chow. After 14 weeks on their respective diets, half of each diet group was sacrificed just before lights out after a 48-hr fast (acutely nutritionally deprived). To produce an equivalent state of acute nutritional stimulation in mice of each diet, food was removed 7 hr before lights out (during which mice normally eat almost nothing even when food is available), then mice were sacrificed 30 min after an i.p. injection of glucose (2 mg/g of body wt) just before lights out. To study effects of genetic obesity and glucose injection on *ob* mRNA, wild-type lean *a/a* or obese yellow *A^y/a* mice (both on the C57BL/6J background) were fasted for 7 hr, then sacrificed (just before lights out) 0, 15, or 30 min after i.p. glucose injection (2 mg/g body wt; *n* = 5 per group). To further define nutritional factors regulating the *ob* gene, lean CBA mice were sacrificed just before lights out after division into four groups (*n* = 4–12 per group): 7-hr daytime fast, sacrificed 30 min after i.p. injection of saline; 7-hr fast, sacrificed 30 min after i.p. injection of glucose (2 mg/g of body wt.); 7-hr fast, sacrificed 30 min after i.p. injection of insulin (33 milliunits/g of body wt.); or 72 hr after food was removed and 30 min after i.p. injection of saline. The nutritional manipulations (prolonged fasting, high calorie-diet, or injection of glucose or insulin) produced no apparent ill effects: there was no mortality, and apart from weight change, the animals appeared healthy. Mice were sacrificed by

exposure to carbon dioxide for ≈ 5 min, followed by cardiac puncture and decapitation between 16:00 and 18:00 hr. Fresh white adipose tissue from the gonadal fat pad was removed, frozen on dry ice, and stored at -70°C until use. Glucose was measured by a Lifescan One-Touch II glucose meter (Johnson & Johnson). Insulin was measured by a species-nonspecific RIA (11) using rat insulin as a standard.

RNA Extraction and Northern Blot Analysis. Total RNA from ≈ 100 mg of adipose tissue (from gonadal fat pad) was extracted in RNazol B (Tel-Test, Friendswood, TX). Pellet was resuspended in 15–20 μl of diethylpyrocarbonate (DEPC)-treated water. The quantity and integrity of RNA isolated was determined for each sample by using both UV absorbance (260/280) as well as by agarose electrophoresis, visualizing the intact ribosomal RNA bands with ethidium bromide. Approximately 7 μg of total RNA from each sample was denatured by incubating with glyoxal and dimethyl sulfoxide for 1 hr at 50°C , loaded into 1.5% agarose gel, and then separated by electrophoresis at 80 V for 2.5 hr. RNA was transferred to Immobilon S (Millipore) by capillary elution in $20\times$ standard saline citrate (SSC) ($1\times$ SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0). After being washed with $6\times$ SSC, membranes were baked at 80°C for 1 hr and exposed to UV light to fix RNA. The membranes were prehybridized in a solution containing 25 mM NaPO_4 (pH 7.0), $4\times$ SSC, 5 mM EDTA, $5\times$ Denhardt's solution, 0.5% (vol/vol) SDS, 50% (vol/vol) deionized formamide, and denatured salmon sperm DNA at 100 $\mu\text{g}/\text{ml}$ (5 Prime $\rightarrow$ 3 Prime, Inc.) at 42°C overnight. Hybridization was done in the same buffer containing 10% (wt/vol) dextran sulfate and ^{32}P -labeled *ob* gene probe at 1.6 $\mu\text{Ci}/\text{ml}$ (1 Ci = 37 GBq; 1.4 ng/ml) at 42°C overnight. The membranes were washed twice in $1\times$ SSC, 0.1% SDS for 20 min at room temperature, followed by two washes in $0.1\times$ SSC/0.1% SDS for 15 min at room temperature. Subsequently, the membranes were washed in $0.1\times$ SSC, 0.1% SDS for 3 hr at 55°C and then exposed to autoradiography film between intensifying screens for 1 to 72 hr at -70°C . To monitor RNA loading, membranes were reprobed and hybridized with ^{32}P -labeled probe encoding 18S ribosomal RNA. The total integrated densities of hybridization signals were determined by computerized densitometric scanning (MCID Systems, St. Catherine's, ON).

Template Production and Probe Labeling. A template for a single-stranded complementary DNA (cDNA) probe for the *ob* gene was produced from RNA of lean mouse adipose tissue by using a reverse transcriptase-polymerase chain reaction (RT-PCR). A mixture of 1 μg of total adipose RNA, $1\times$ PCR buffer, 5 mM MgCl_2 , 1 mM dNTPs, 20 units of RNase inhibitor, 50 units of reverse transcriptase, and 2.5 μM random hexamers in a total volume of 20 μl was incubated at room temperature for 10 min to allow for the hybridization of the hexameric primer. Subsequently, reverse transcription was carried out using the following sequence of conditions: 42°C (15 min), 99.9°C (5 min), and 4°C (5 min). The product of the reverse transcription was mixed with $1\times$ PCR buffer, 2 mM MgCl_2 , 2.5 units of Taq polymerase, 50 pmol of *ob* gene N-terminal primer: 5'-CTGCAAGGTGCAAGAAGAAG-3' (Oligos Etc., Portland, OR), and 50 pmol of *ob* gene C-terminal primer: 5'-TCGGAGATTCTCCAGGTCAT-3', in a final volume of 100 μl . Amplification conditions were as follows: 94°C (1 min), 58°C (2 min), and 72°C (3 min) for 30 cycles. The amplified fragments were separated on a low-melting-point agarose gel, and the appropriate band (352 bp) was cut out, purified, diluted to 50 ng/ μl , and stored at -20°C for labeling as described below. The identity of the purified template of *ob* gene was confirmed by direct sequencing, and the sequence was almost completely complementary to mouse *ob* gene bases 78–430, as numbered in ref. 4. The 18S ribosomal RNA probe was generated in an analogous fashion.

Single-stranded internally labeled DNA probes were produced by amplified primer extension labeling (12), which

produces single-stranded cDNA probes with high specific activity analogous to RNA probes, but which produces less nonspecific binding than RNA probes. The reaction mixture contained 50 ng of template cDNA, $1\times$ PCR buffer, 1.5 mM MgCl_2 , deoxy G, A, and T (0.2 mM each), 150 μCi of [^{32}P]dCTP (800 Ci/mmol, DuPont), 50 pmol of C-terminal primer, and 2.5 units of Taq polymerase in a total 50- μl vol. Amplification was done in a thermal cycler by using the following sequence: 94°C (30 sec), 60°C (30 sec), and 72°C (30 sec) for 50 cycles. Labeled probes were purified from unincorporated nucleotides and excess primers by G-50 spin columns (5 Prime $\rightarrow$ 3 Prime) and stored at -70°C until use.

Statistical Analysis. Statistical analysis was performed using JMP software (SAS), entailing a two-way analysis of variance (ANOVA) followed by Tukey–Kramer honestly significant difference (HSD) pair-wise comparisons, or linear regression, unless otherwise specified.

RESULTS

Using a single-stranded cDNA probe generated by RT-PCR, Northern blot analysis revealed an expected 4.5-kb band of *ob* gene mRNA from lean mouse adipose tissue (Fig. 1*A* and 4*A*). Yield of total RNA per mg of wet weight of adipose tissue was similar in lean and obese mice. The *ob* mRNA per μg of total RNA (Fig. 1*A* and *B*), was persistently elevated in mice on a high-calorie diet for 14 weeks, compared to *ob* mRNA from mice on a normal diet ($P < 0.05$, two-way ANOVA, Fig. 1*C* and *D*). The obesity-related elevation of *ob* mRNA was significant compared with lean animals, whether mice were fasted for 48 hr or fasted for 7 hr and injected with glucose (Tukey–Kramer; Fig. 1*C* and *D*). In lean mice *ob* mRNA was significantly elevated in 7-hr fasted, glucose-injected mice, compared to mice fasted for 48 hr ($P < 0.05$, Tukey–Kramer), but this effect of acute nutritional status was not significant in obese mice. Body weight and insulin were also significantly elevated in mice with diet-induced obesity ($P < 0.05$, ANOVA followed by Tukey–Kramer test, Fig. 1*C* and *D*). When lean and obese mice were analyzed together, analysis by linear regression indicated that *ob* mRNA correlated significantly with body weight ($r = 0.79$, $P < 0.05$), and insulin ($r = 0.54$, $P < 0.05$), but the correlation between *ob* mRNA and glucose did not achieve statistical significance ($r = 0.4$, $P = 0.08$).

The *ob* mRNA was also significantly elevated in genetically obese yellow mice, compared to *ob* mRNA from lean wild-type C57BL/6J mice ($P < 0.05$, two-way ANOVA, followed by Tukey–Kramer; Fig. 2). Furthermore, *ob* mRNA was significantly induced within 30 min after glucose injection in lean mice ($P < 0.05$, Tukey–Kramer; Fig. 2), but not in obese mice ($P > 0.05$, Tukey–Kramer; Fig. 2). Body weight and plasma insulin were significantly increased in obese mice ($P < 0.05$, ANOVA). Plasma glucose was significantly influenced by glucose injection ($P < 0.05$, two-way ANOVA), but not genotype ($P > 0.05$). Pooling across all groups, analysis by linear regression demonstrated that *ob* mRNA correlated significantly with body weight ($r = 0.71$, $P < 0.05$) and insulin ($r = 0.45$, $P < 0.05$). Within wild-type (lean) mice, *ob* mRNA was highly and significantly correlated with glucose ($r = 0.81$, $P < 0.05$; Fig. 3), but not body weight or insulin. Within genetically obese mice, *ob* mRNA did not correlate with glucose, body weight, or insulin ($P > 0.05$). These statistical relationships suggest that the differences in *ob* mRNA between lean mice and obese mice are more strongly related to group differences in body weight and insulin than to acute differences in plasma glucose. However, the individual differences in *ob* gene expression among lean mice (due to acute effects of glucose injection) may be related more to acute differences in plasma glucose than to acute differences in plasma insulin. Furthermore, genetically obese mice appear to be relatively insensitive to these acute effects of glucose.

FIG. 1. Chronic and acute nutritional stimulation of *ob* gene expression. (A) Representative Northern blot of *ob* gene mRNA (4.5 kb) isolated from adipose tissue. Seven micrograms of total RNA was applied to each lane. (B) Same blot as in A but reprobed with a cDNA for 18S ribosomal RNA. 48h Fast + Chow, food removed 48 hr before sacrifice (just before lights out) of male C57BL/6J mice on a standard Purina mouse chow diet; 48h Fast + Hi Cal, food removed 48 hr before sacrifice of mice maintained for 14 weeks on a high-calorie (high-fat, high-carbohydrate) diet; 7h Fast + Chow, food removed 7 hr, then mice sacrificed 30 min after i.p. injection with glucose (2 mg/g body wt); mice maintained on a chow diet. 7h Fast + Hi Cal, food removed 7 hr, then mice sacrificed 30 min after i.p. injection with glucose (2

FIG. 2. Effect of genetic obesity (yellow agouti) and glucose injection on *ob* mRNA. Lean, male C57BL/6J (a/a); Obese, yellow agouti heterozygous (A^y/a on C57BL/6J background) obese. Values are means ± SEM ($n = 5$ per group). Food was removed 7 hr before sacrifice; then mice were sacrificed 0, 15, and 30 min after i.p. injection with glucose (2 mg/g of body wt). *ob* mRNA was significantly influenced by both genotype and glucose injection ($P < 0.05$, two-way ANOVA). *, Significantly higher than lean mice at corresponding time-point ($P < 0.05$, Tukey-Kramer). §, Significantly higher than group of same genotype at 0 min ($P < 0.05$, Tukey-Kramer test).

Controlling for effects of the injection procedure, mice were sacrificed 30 minutes after an i.p. injection of saline, glucose, or insulin, after a 7-hr fast, or 30 min after an i.p. injection of saline after a 72-hr fast. In normal lean mice, *ob* mRNA was significantly influenced by these acute nutritional manipulations ($P < 0.05$, ANOVA). The *ob* mRNA in mice fasted for 7 hr was ≈ 10 -fold higher than *ob* mRNA in mice fasted for 72 hr ($P < 0.05$, ANOVA followed by Tukey-Kramer test; Fig. 4). Furthermore, in mice fasted for 7 hr, i.p. injection of either glucose or insulin resulted in ≈ 2 -fold increase in *ob* mRNA 30 min after injection ($P < 0.05$, Tukey-Kramer, Fig. 4).

DISCUSSION

The product of the obese gene, leptin, is hypothesized to constitute a satiety factor, because absence of a functional obese gene in *ob/ob* mice leads to hyperphagia and obesity (2), and injection of leptin reduces feeding and body weight in *ob/ob* mice (5–7). It was of particular interest that at the highest doses of leptin, mice with diet-induced obesity also lost weight (7). These results raised the possibility that, regardless of etiology, obesity might entail impaired production of leptin and might be reversible by injection of leptin. However, in the present study, we report that both diet-induced obesity and obesity due to the yellow agouti mutation were associated with dramatically elevated levels of *ob* mRNA. Because the expression of *ob* mRNA was normalized per mg of total RNA, the actual amount of total (presumably secreted) *ob* gene product in obese mice is even greater because obese animals also have a greater mass of adipose tissue, and the yield of total RNA per mg of wet weight adipose tissue in the present study was at least as great in obese animals as in lean animals. Therefore,

mg/g of body wt); mice maintained on a high-calorie (high-fat, high-carbohydrate) diet. (C and D) quantification of *ob* gene mRNA (arbitrary OD units) in adipose tissue, of mice fasted for 48 hr, or fasted 7 hr and then sacrificed 30 min after glucose injection. Values are means ± SEM. B.W., body weight in g at time of sacrifice. *ob* mRNA, body weight, and insulin were significantly influenced by both diet and acute nutritional manipulation (fasting/glucose injection) ($P < 0.05$, two-way ANOVA). *Group on high-calorie diet higher than corresponding (48-hr or 7-hr fast) chow diet group ($P < 0.05$, Tukey-Kramer test).

FIG. 3. Correlation between *ob* mRNA and blood glucose in lean mice from Fig. 2, pooling 0, 15, and 30 min after i.p. injection of glucose. *ob* mRNA was highly correlated with glucose in lean animals ($r = 0.81$, $P < 0.05$), but not in genetically obese A^y/y animals (data not shown).

consistent with other recent reports (8, 13), obesity may be generally associated with elevated expression of the obese gene.

Elevated expression of the leptin gene in association with many forms of obesity suggests that obesity may generally be characterized by a relative insensitivity to leptin, rather than insufficient leptin production. Consistent with this hypothesis, 5-fold to 10-fold greater doses of leptin were required to produce an equivalent weight loss in mice with diet-induced obesity, compared to *ob/ob* mice (7). Thus, simply consuming a high-fat, high-carbohydrate diet appears to be associated with a remarkable loss of leptin sensitivity. Similarly, leptin insensitivity appears to be one of the effects of the yellow agouti mutation, whose obesity appears to be due to ectopic expression of an antagonist to melanocyte-stimulating hormone (10), the relationship of which to leptin action, if any, remains undefined. The general association of obesity with insensitivity to leptin may therefore limit the clinical efficacy of leptin in the treatment of human obesity.

The hypothesis that leptin constitutes a physiological satiety factor suggests that leptin might be induced by nutritional factors that produce satiety. The present studies indicate that *ob* gene expression is highly sensitive to nutritional status. Both chronic (high-calorie diet) and acute (i.p. injection of glucose) nutritional stimulation lead to elevated *ob* mRNA, whereas acute nutritional deprivation (48-hr or 72-hr fast) dramatically reduced *ob* mRNA. When comparing across groups with different body weights, *ob* mRNA (per mg of adipose RNA) was highly and positively correlated with body weight. On the other hand, it may be revealing that fasting and i.p. glucose injection did not significantly influence *ob* mRNA in obese mice, suggesting that in obesity sensitivity of *ob* gene expression to nutritional status may be attenuated. These data suggest that an insensitivity of the *ob* gene to nutritional status may contribute to the maintenance of the obese state.

Although *ob* mRNA was highly correlated with body weight, the mechanism that links *ob* mRNA with total adiposity is unclear. However, the observation that nutritional stimulation enhances expression of the *ob* gene suggests that the anabolic processes that lead to increased body weight (hyperphagia, hyperinsulinemia, and hyperglycemia) may concomitantly enhance expression of the *ob* gene. Thus, *ob* gene expression and body weight would be positively correlated because of a third factor that stimulates both body weight and *ob* mRNA. The present studies addressed the hypothesis that nutritional stimulation of *ob* mRNA may be mediated by elevations in glucose

FIG. 4. Effect of fasting or injection of insulin or glucose on *ob* mRNA. (A) Representative Northern blot of *ob* gene mRNA (4.5 kb) isolated from adipose tissue. Seven micrograms of total RNA was applied to each lane. (B) Same blot as in A, but reprobed with a cDNA complementary to 18S ribosomal RNA. 7h-Fast + Saline, food removed 7 hr before sacrifice (during lights on), then sacrificed (just before lights out) 30 min after i.p. injection with saline; 72-h Fast + Saline, food removed 72 hr before sacrifice and then sacrificed 30 min after i.p. injection of saline; 7h-Fast + Insulin, food removed 7 hr before sacrifice and then sacrificed 30 min after i.p. with insulin (33 mU/g body wt); 7h-Fast + Glucose, food removed 7 hr before sacrifice and then sacrificed 30 min after i.p. injection with glucose (2 mg/g of body wt.). (C) Quantification of *ob* gene mRNA (arbitrary OD units) in adipose tissue. Values are means $\pm$ SEM ($n = 4-12$). Groups with different letters are statistically different ($P < 0.05$, ANOVA followed by Tukey-Kramer).

or insulin. When analyzed across groups of animals with different body weights (due to obesity or fasting), *ob* mRNA was significantly correlated with both body weight and insulin, but not with glucose. Furthermore, acute injection of insulin significantly increased *ob* mRNA. Therefore hyperinsulinemia could plausibly constitute a common signal that causes both increased body weight and increased *ob* mRNA during the development of obesity.

Nevertheless, in normal lean mice, glucose may be at least as important as insulin in mediating acute effects of nutrition on *ob* mRNA. As described in the present report, in two different studies with lean mice, *ob* mRNA was induced within 30 min after i.p. injection of glucose. Furthermore, after i.p. glucose injection in normal lean mice, *ob* mRNA highly correlated with plasma glucose but not with plasma insulin. Furthermore, the levels of insulin after injection of the pharmacological doses used in these studies were far higher than the physiological levels produced by glucose injection. We therefore suggest that glucose and insulin may each have

independent effects on *ob* mRNA, similar to the independent effects of insulin and glucose on liver function (14) and gene expression (15). The statistical relationships in the present study suggest that insulin is more important for chronic regulation of *ob* gene expression and glucose is more important for acute regulation. The inhibition of *ob* mRNA in fasted mice may be due to the decreased plasma glucose as well as decreased plasma insulin, which are characteristic of the fasted state. A common glucose response element (GIRE), responsible for the transcriptional response to carbohydrates and hormones, has been characterized in several genes that are regulated by insulin and glucose (15). It will be of interest to examine the promoter of the *ob* gene for these elements.

The wild-type allele of the obese gene is, of course, more properly thought of as an anti-obese gene because the absence of this gene product produces obesity. (In contrast, the [ectopic] expression of the yellow allele of the agouti gene produces obesity). A critical question is, therefore, what mediates the anti-obesity effect of the wild-type obese gene allele? One of the earliest detectable differences between *ob/ob* mice (which lack functional *ob* gene product) and wild-type controls (which produce the *ob* gene product) is an elevation of plasma insulin in *ob/ob* mice before the development of obesity. Fourteen days after birth, plasma insulin is elevated in *ob/ob* mice, compared to wild-type controls (16), whereas at this age neither food intake nor body weight differs between *ob/ob* and wild-type mice (17). Such results suggest that an early effect of leptin is to inhibit insulin secretion, independent of its effects on feeding. The present studies suggest that integrated or chronic insulin levels are positively correlated with *ob* mRNA and that insulin injection can stimulate *ob* mRNA. Thus the elevation of *ob* mRNA in both acquired and genetic obesity (refs. 8, 13; present studies) is plausibly related to the hyperinsulinemia that is associated with obesity. Therefore we hypothesize that insulin and leptin constitute a negative feedback loop, in which insulin (with glucose) stimulates leptin, and leptin inhibits insulin secretion, at least partly independent of its effects on feeding. The hypothesis that "*ob* mRNA was increased [in *ob/ob* mice] as part of a possible feedback loop" (4) could thus be explained as a failure in *ob/ob* mice to inhibit insulin secretion, in the absence of *ob* gene product. Conversely, the anti-obesity effect of the wild-type obese gene allele could be understood as being mediated, at least in part, by its effects in inhibiting insulin secretion. Further studies will be

needed to address whether leptin acts at the pancreas to inhibit insulin secretion and whether blocking hyperinsulinemia in obesity will prevent the elevation of *ob* mRNA associated with obesity.

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1. Consensus Conference Statement (1985) *Ann. Int. Med.* **103**, 1073–1077.
2. Coleman, D. L. (1982) *Diabetes* **31**, Suppl., 1–6.
3. Coleman, D. L. (1973) *Diabetologia* **9**, 294–298.
4. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J. (1994) *Nature (London)* **372**, 425–431.
5. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K. & Friedman, J. M. (1995) *Science* **269**, 543–546.
6. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. & Collins, F. (1995) *Science* **269**, 540–543.
7. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R. & Burn, P. (1995) *Science* **269**, 546–549.
8. Maffei, M., Fei, H., Lee, G.-H., Dani, C., Leroy, P., Zhang, Y., Proenca, R., Negrel, R., Ailhaud, G. & Friedman, J. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6957–6960.
9. Yen, T. T., Gill, A. M., Frigeri, L. G., Barsch, G. S. & Wolff, G. L. (1994) *FASEB J.* **8**, 479–488.
10. Lu, D., Willard, D., Patel, I. R., Kaddwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O. & Cone, R. D. (1994) *Nature (London)* **371**, 799–801.
11. Yalow, R. S. & Berson, S. A. (1973) in *Methods in Investigative and Diagnostic Endocrinology*, eds. Berson, S. A. & Yalow, R. S. (North-Holland, Amsterdam, The Netherlands), Vol. 2, pp. 864–870.
12. Brooks, P. J., Kaplitt, M. G., Kleopoulos, S. P., Funabashi, T., Mobbs, C. V. & Pfaff, D. W. (1993) *J. Histochem. Cytochem.* **41**, 1761–1766.
13. Considine, R. V., Considine, E. L., Williams, C. J., Nyce, M. R., Manosin, S. A., Bauer, T. L., Roato, E. L., Colberg, J. & Caro, J. F. (1995) *J. Clin. Invest.* **95**, 2986–2988.
14. Liu, Z., Gardner, L. B. & Barrett, E. J. (1993) *Metabolism* **42**, 1546–1551.
15. Vaulont, S. & Kahn, A. (1994) *FASEB J.* **8**, 28–35.
16. Chen, N.-G. & Romsos, E. R. (1995) *Endocrinology* **136**, 505–511.
17. Rath, E. & Thenen, S. W. (1979) *J. Nutr.* **109**, 840–847.