Regulation of obese (ob) mRNA and Plasma Leptin Levels in Rhesus Monkeys

EFFECTS OF INSULIN, BODY WEIGHT, AND NON-INSULIN-DEPENDENT DIABETES MELLITUS

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We have cloned the rhesus monkey ob gene cDNA and have analyzed its expression in monkeys with a wide range of body weights (lean to very obese) and with or without non-insulin-dependent diabetes mellitus to examine the relationship of ob gene expression to obesity and non-insulin-dependent diabetes mellitus. The sequence of monkey ob protein, excluding the signal peptide, showed 91% identity with the human protein. We observed a significant correlation between the level of ob mRNA and body weight. We also found a significant relationship between ob mRNA and fasting plasma insulin concentration; however, insulin stimulation during a 100-140-min euglycemic/hyperinsulinemic clamp did not result in any changes in ob mRNA levels. Circulating levels of the ob gene product leptin were also significantly correlated with body weight. These results show that ob gene expression is related to body weight and is not acutely regulated by insulin.

Obesity is the most common nutritional disorder in developed countries and is a major risk factor for hypertension, cardiovascular disease, and non-insulin-dependent diabetes mellitus (NIDDM)1. Recently, several genes responsible for obesity, including the ob and fat genes (2, 3), and the agouti gene (4) have been identified in genetically obese rodent models. The human and rat ob cDNAs and human and mouse ob genes have also been cloned (2, 5, 6). The ob gene encodes the circulating protein termed leptin (2, 12), which may be a helical cytokine (13). Recent experiments utilizing recombinant protein have suggested that leptin modulates appetite, body weight, oxygen consumption, and body temperature (14–19). However, if leptin is truly a satiety hormone, it is not yet clear how humans or rodents become obese in the presence of high levels of leptin (20, 21). The recent identification of a leptin receptor (22) may lead to a better understanding of these discordant findings.

Regulation of the ob gene is an area of intensive investigation. In one study, induction of ob gene expression by corticosterone in normal rats has been shown to reduce body weight and food intake (23). Increased ob mRNA expression has been reported in adipose tissue in ob/ob mice (2) as well as in obese humans, genetically fat rodents (db/db mouse, Zucker fatty rat, Wistar fatty rat, and obese JCR:LA corpulent), ventromedial hypothalamus lesioned obese rodents (6–9, 24–27), and dietary-induced obese rodents (28). There have been some reports suggesting that ob mRNA levels are reduced in fasting normal rodents and streptozotocin-induced diabetic rodents and that these levels are restored to normal after refeeding and partially toward normal with insulin treatment of diabetic animals (29–33). However, this regulation by insulin is still controversial (30, 32, 34).

Many but not all rhesus monkeys (Macaca mulatta) become obese after sexual maturation (35). Obesity in rhesus monkeys, as in humans, is usually associated with insulin resistance and hyperinsulinemia and frequently leads to NIDDM, supporting the rhesus monkey as an excellent model for the study of human obesity and NIDDM (36).

In this paper, we report the cloning of the rhesus monkey ob cDNA. We examine the expression of this gene and its protein in relation to body weight and plasma insulin levels in three groups of monkeys: normal, normoglycemic-hyperinsulinemic, and those that have overt NIDDM. Results showed that the ob mRNA and plasma leptin levels were significantly related to body weight and that ob mRNA was not acutely regulated by insulin.

EXPERIMENTAL PROCEDURES

Animals—Twenty-five rhesus monkeys (Macaca mulatta) were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (37). They were individually housed and provided 8-h/day access to either Ensure® (Ross Laboratories, Columbus, OH), a nutritionally complete liquid diet, or monkey chow® (Purina Mills, Inc., St. Louis, MO) and fresh water ad libitum. The normal monkeys (n = 10) were lean (body weight, 10.2 ± 0.6 kg; body fat, 12.1 ± 3.5%) and had normal fasting plasma glucose (3.4 ± 0.1 mM) and insulin (336 ± 28 pm) concentrations. The hyperinsulinemic monkeys (n = 6) were obese (body weight, 18.2 ± 1.5 kg; body fat, 32.6 ± 2.7%) and were normoglycemic (4.2 ± 0.2 mM) and hyperinsulinemic (1035 ± 217 pm). The NIDDM monkeys (n = 9; body weight, 12.8 ± 1.3 kg; body fat, 35.1 ± 4.0) had hyperglycemia (9.7 ± 1.0 mM) and fasting plasma insulin concentrations of 341 ± 90 pm. Four of the NIDDM monkeys received insulin treatment.

Procedures—After a consistent 16-h fast, plasma samples for fasting glucose, insulin, and leptin were obtained under light anesthesia (ketamine hydrochloride (10 mg/kg body weight), with dose repeated as necessary). Plasma was frozen for later assays. Abdominal subcutaneous adipose tissue samples were obtained either under ketamine hydrochloride, as noted above, or immediately after anesthesia by intravenous sodium pentobarbital. Tissue samples were immediately frozen in liquid nitrogen and stored at −165 °C. The euglycemic clamp procedure was carried out in five of the lean young adult monkeys from the

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1. The abbreviation used is: NIDDM, non-insulin-dependent diabetes mellitus.

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normal group described above. The experimental procedure has been described previously (38) and used an insulin infusion rate of 2400 pmol/m² body surface area/min, with glucose maintained at approximately 4.7 mM. Abdominal subcutaneous and omental adipose tissue was separated by 0.8% agarose gel containing 2.2M of formaldehyde. The hybridization was carried out at room temperature and three times at 65°C (41). The 440-base pair BglI fragment of the monkey ob cDNA (47 to 452) was used as a probe. The 440-base pair BglI-PstI DNA fragment of the human HSP83 cDNA was used as an internal control (42). The relative amounts of ob and HSP83 probes bound to the immobilized mRNAs were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Assay of Plasma Leptin—**Plasma leptin levels were measured in a solid-phase sandwich enzyme immunoassay, utilizing an affinity-purified polyclonal antibody immobilized in microtiter wells. Bound leptin was detected with affinity-purified antibody conjugated to horseradish peroxidase and quantitated with a chromogenic substrate. Leptin concentrations were calculated from standard curves generated in the same assay with recombinant leptin.

**Other Assays—**Plasma insulin was assayed using a Glucose Analyzer II (Beckman Instruments). Plasma insulin was determined by radioimmunoassay (43). Body fat was determined by using tritiated water (4 μCi/kg), with dilution determined on plasma samples obtained at 1 h (44).

**Statistical Analysis—**Group differences were compared by one-way analysis of variance. Data are expressed as the mean ± S.E.

**RESULTS AND DISCUSSION**

To identify the monkey ob cDNA, we first screened a monkey adipose tissue cDNA library using a polymerase chain reaction-derived probe as described under “Experimental Procedures.” Twenty-nine clones were identified, and these overlapping clones included the 5'-non-coding region and the poly(A) tail. Sequence analysis of these clones revealed that the predicted monkey ob protein is identical in length (167 amino acids) to the human and mouse proteins. The monkey protein (excluding the signal peptide) shows 91% and 84% identity with human and mouse ob proteins, respectively (Fig. 1). In 1 of 8 clones...
encoding this region, a glutamine at codon 49 was excluded, as has also been reported in the mouse and human ob mRNAs (2, 5). This difference has been postulated to be due to splice site “slippage,” and the functional consequence of this difference is unclear.

We next examined ob mRNA expression in monkey subcutaneous adipose tissue by Northern blot analysis. Northern blot hybridization revealed that the ob mRNA in monkey is much shorter (0.8 kilobase pair) than that of human, mouse, and rat (4.5 kilobase pairs). Monkey ob mRNA was expressed only in adipose tissue, as has also been shown in humans and rodents (2, 5, 6, 8, 9, 25, 27, 29) (Fig. 2). Longer exposures revealed no other bands of other mobilities (data not shown).

Next we determined the mRNA levels in monkey subcutaneous adipose tissue and the plasma leptin levels in three groups of monkeys: (a) normal; (b) hyperinsulinemic; and (c) NIDDM. The average ob mRNA levels were highest in hyperinsulinemic monkeys and lowest in NIDDM monkeys, although the differences between the three groups were not statistically significant by analysis of variance ($p = 0.08$; Fig. 3A). There were no significant differences in the levels of the control HSP83 mRNA (Fig. 3B). Among the NIDDM monkeys, four monkeys were treated with insulin. The ob mRNA level of insulin-treated monkeys ($0.3 \pm 0.1 \times 10^4$) was significantly lower than that of the five untreated NIDDM monkeys ($1.1 \pm 0.2 \times 10^4$). The NIDDM monkeys lose weight before insulin treatment and show insulin levels that are reduced relative to the hyperinsulinemic state. The insulin-treated monkeys were lean (9.9 ± 1.2 kg) and had lower levels of fasting plasma insulin ($123 \pm 37$ pm) compared with untreated NIDDM monkeys (15.1 ± 1.5 kg and 516 ± 107 pm). Thus, this difference may be related to the lower body weight and plasma insulin, not to the insulin treatment.

As shown in Fig. 3 (C and D), ob mRNA levels and body weight were correlated ($r = 0.528; p < 0.01$), as were ob mRNA and fasting plasma insulin concentrations ($r = 0.514; p < 0.01$; Fig. 3, C and D). We noted significant variations in the ob mRNA expression levels in six young adult non-obese monkeys that were all of the same age (~7 years old). These monkeys were relatively lean, with body fat less than 22% (body weight, 7.2–11.8 kg; body fat, 5.5–19.3%) and had normal fasting plasma glucose (3.2 ± 0.1 mM) and insulin (354 ± 33 pm) levels. The ob mRNA levels of these young adult monkeys were correlated to body weight ($r =$...
propensity to develop obesity. This relationship was much stronger in these young adult monkeys than in the other monkeys, including older normal, hyperinsulinemic, and NIDDM monkeys (age, 21.5 ± 1.4, 12.7–30.0 years old). The early increased ob expression in some of the lean young monkeys could relate to the later development of obesity or be a marker of the propensity to develop obesity.

In fasting rats or mice and streptozotocin-diabetic rats, ob mRNA levels were reduced, and levels were increased after refeeding or insulin treatment (29–33). However, this insulin effect has been controversial. In fasting rats or streptozotocin-induced diabetic rats, whether lower ob mRNA levels were due to body weight loss or to decreases in insulin levels could not be determined (32). In vitro experiments using primary adipose cells showed no regulation of ob mRNA by insulin (34), however, another group reported ob up-regulation by insulin (30). To address the issue of whether ob mRNA is acutely regulated by insulin in vivo, we performed the euglycemic/hyperinsulinemic clamp using five of the normal lean young adult monkeys to examine the effect of insulin on ob mRNA levels. There were no significant differences in ob mRNA levels in either subcutaneous or omental adipose tissues (Fig. 4, A and B) between basal and maximally insulin-stimulated clamp conditions measured after 100–140 min of insulin infusion. Previously, there have been some reports that in rats, ob mRNA levels increased 3-fold after a 6-h insulin infusion clamp or 2-fold after 2 days (30, 33). Therefore, we cannot exclude the possibility that ob mRNA might increase after a clamp of longer than 2 h. However, the present results together with previously reported studies involving cultured adipose cells (34) suggest that ob mRNA is not acutely regulated by insulin.

We next analyzed the plasma leptin levels of normal, hyperinsulinemic, and NIDDM monkeys. There were no significant differences in plasma leptin levels between these three groups (data not shown), although the hyperinsulinemic group tended to have higher levels. There was a significant correlation between the plasma leptin level and body weight (Fig. 5) in the total group (r = 0.498; p < 0.05). This correlation was greater (r = 0.736; p < 0.001) when the data from one monkey (which was lean and had high leptin levels) was omitted. There were no significant correlations between plasma leptin levels and fasting plasma insulin (r = 0.139) or fasting plasma glucose levels (r = 0.151; data not shown). These results suggest that the expression of the ob gene is related to body weight at both the mRNA and protein levels.

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Fig. 4. The level of ob mRNA before and after a 100–140-min euglycemic/hyperinsulinemic clamp in subcutaneous (A, five monkeys) and omental (B, four monkeys) adipose tissue. Each symbol signifies a different monkey. The amount of ob mRNA was measured by PhosphorImager analysis of slot-blot hybridizations.

Fig. 5. Correlation between plasma leptin level and body weight. O, □, and ■ indicate normal, hyperinsulinemic, and NIDDM monkeys, respectively.

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