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RECOMBINANT OB-GENE PRODUCT REDUCES FOOD INTAKE IN FASTED MICE

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SUMMARY: The ob-gene encodes for a protein of 167 amino acids which is expressed exclusively in white adipose tissue. The ob-gene product is probably released from adipocytes as a soluble hormone of 146 amino acids and has been proposed as a satiety factor. To test this hypothesis, the soluble portion of the ob-gene product devoid of signal sequence was expressed in E. coli and purified. The purified protein, which contains two Cys residues, was recovered from the periplasm in an oxidized form. After a single intravenous injection, the ob-gene product decreased food intake after fasting in normal mice. The results show that recombinant ob-gene product can be obtained in a functionally active conformation and provide direct proof that this protein is a satiety factor.

Obesity is a major risk factor for the development of chronic diseases such as hypertension, coronary artery disease and hyperlipidemia. It is well established that a complex interplay of polygenic and environmental factors contribute to the development of obesity. Although genetic influences may control body mass in approximately one third of the obese human population, the biological events involved in this inheritance, remain to be clarified. The recent cloning of three genes, the fat (1), the agouti (2) and the ob-gene (3), which are responsible for the development of distinct forms of obesity in rodents, will contribute to our understanding of the pathophysiology of this disease.

In each individual a complex regulatory system normally maintains the amount of adipose tissue within a certain approximately constant range. To explain this, the lipostatic hypothesis postulates the existence of a fat-derived satiety factor which exerts a negative feed back on the hypothalamus to suppress appetite (4). Parabiosis and transfusion studies have long predicted the existence of a circulating, long lived satiety

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factor released from adipose tissue (5). The newly discovered ob-gene, encoding for a protein of 167 amino acids, is a good candidate for providing this regulatory role. Transcription of the ob-gene, has been detected only in white adipose tissue (3,6,7,8) where mRNA levels correlate with fat cell size (6,8). The protein product contains a signal sequence of 21 amino acids consistent with a secreted hormone. In addition, genetically obese ob/ob mice either fail to express the ob-gene or express a defective product, due to the presence of a premature stop codon (3). To date, however, the release of the ob-gene product into the blood stream and the presumed appetite suppressing function of the protein remains to be demonstrated. In this study we have produced a recombinant mouse ob-gene product in E. coli and investigated its effect on eating behavior after a 24 hour fast in normal mice.

Materials and Methods

Animals - These studies were conducted with male albino mice [MA01 (Spf)] weighing between 30 and 40 g (CIBA-GEIGY, Sisseln, Switzerland). The mice were maintained within a room with a 12 h light/dark cycle (8.00 a.m. - 8.00 p.m. light) at a temperature of 20-25 ^{0}C. Unless otherwise stated all mice were allowed free access to tap water and a normal diet (NAFAG; Gossau, Switzerland).

Plasmid construct - To clone the translated region of the ob-gene, mRNA of mouse mesenteric fat tissue was applied as a template for first strand cDNA synthesis by reverse transcription (RT) using the antisense PCR-primer 5'-CGCGGATCCTCAGCATTCAGGGCTAACATC-3' (619-599). PCR amplification was performed for 40 cycles with 15 s at 94 ^{0}C, 30 s each at 58 ^{0}C and 72 ^{0}C using the sense oligonucleotide 5'-CGCGGTACCATGGTGCCTATCCAGAAAGTCCAG-3' (179-199). The sense oligonucleotide was designed to allow translation of an ob-gene product in E.coli lacking the putative eukaryotic signal sequence of secreted proteins (3). Both oligonucleotides are extended at their 5' ends (underlined above) containing recognition sequences for endonucleases. The sequence of the amplified PCR fragment was confirmed by the chain-termination DNA sequencing method (9) of double stranded DNA with the Sequence Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, Ohio). The mouse ob-gene fragment was sub-cloned into the *BamHI/NcoI* site of pET-22b (Novagen, Madison, USA) by standard manipulation (Maniatis) to obtain pET-ob22. pET-22b contains the pelB signal sequence leader for potential periplasmic localization.

Protein expression and purification - An overnight culture of the E.coli strain BL21(DE3) harboring pET-ob22 was inoculated (1/100 volume) in fresh medium and grown at 37 ^{0}C according to the manufacturer's guidelines. After induction with isopropyl ß-D thiogalagtopyranoside (IPTG), the cell suspension was incubated at 25 ^{0}C for 3.5 hours. The periplasmic fraction was obtained by cold osmotic shock as previously described (10). Proteins were concentrated by ammonium sulfate precipitation (55% saturation) and resuspended in 20 mM $(NH_4)H_2CO_3$. The protein solution was fractionated by gel filtration on a Sephacryl S-100 (high resolution) column (Pharmacia, Uppsala, Sweden). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (11), pooled and lyophilized. The protein concentration was determined by the Bradford method (12).

Feeding studies - After acclimatization, the mice were placed individually into metabolism cages with free access to water and the normal diet in powdered form. After a 3 day habituation period, food intake over 2 consecutive 24-hour periods (8.0 a.m. to 8.00 a.m.), was measured to establish baseline values. Food was withheld from the animals over the next 24 hour period and, at the end of the fast, the mice were removed from the cages, weighed and injected via the tail vein with either the ob-gene product (6, 18, 60, 180 & 600 nmol/kg) or the isotonic saline vehicle (0.2 ml) in random order. Immediately after injection, the animals were replaced in their home cages and 0-30 min later food was returned to the animals. Food intake during the following 24 h period was measured at various times after returning food to the animals.

Statistical analysis of the feeding studies -Data from each treatment group were added together and expressed as mean + S.E. The data were analyzed using multivariate analysis of variance for repeated measures. Post hoc test was the least significant difference procedure.

Results and Discussion

Expression and isolation of the ob-gene product - The translated region of the postulated secreted product of the ob-gene was cloned into a pET22b expression vector. This vector is designed for the periplasmic translocation of heterologous proteins in E. coli since it allows the N-terminal fusion to the bacterial pelB leader sequence. For expression, E.coli BL21(DE3) was transformed with the construct and, after induction with IPTG, was allowed to synthesize the ob-gene product for 3.5 hours at room temperature. Even though at this temperature the amount of protein produced was quite modest (compare bacterial protein before and after induction in Figure 1A), most of it was exported to the periplasm from which the product could be obtained in a fairly enriched form (see Figure 1A, lane 3). Considerably more recombinant protein could be produced at higher temperatures but it was mostly recovered in inclusion bodies. Subsequent differential precipitation (Figure 1A, lane 4) and fractionation on a molecular seaving column were sufficient to isolate the product to purity (Figure 1A, lane 5). The Sephacryl S-100 column was calibrated with proteins of various molecular weights. The retention volume of the ob-gene product corresponded to that of a protein with a molecular weight between 10000 and 20000 Daltons thus showing that the product was monomeric under non-denaturing conditions. Typically, 0.5 to 1 mg recombinant protein could be isolated from each liter of solution containing the recombinant bacteria. The molecular weight of the product was estimated to be about 16700 Daltons by SDS-PAGE analysis. This molecular weight corresponds to that of the postulated mature product of the ob-protein, leaving an additional N-terminal methionine after removal of the signal sequence (3). This indicates that the bacterial pelB leader sequence is processed endogenously during the translocation of the ob-gene product into the periplasm.

From the amino acid sequence (as derived from the cDNA) it is known that the ob-gene product contains 2 cysteine residues which could form one possible disulfide bridge. At

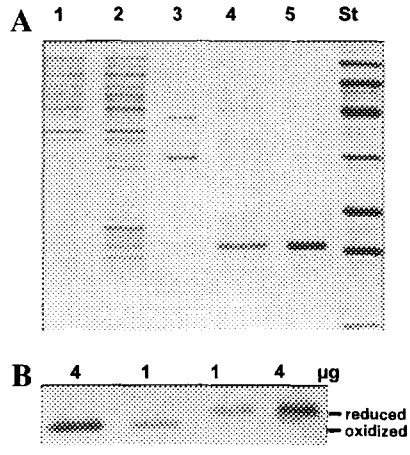

Figure 1. A: Expression and isolation of the ob-protein from E. coli.
Bacteria were transformed with the pET-ob22 expression vector. Recombinant protein synthesis was then induced for 3.5 hours at room temperature. The periplasmic fraction was isolated and the ob-gene product concentrated by ammonium sulfate precipitation (55% sat.). Final purification was achieved by gel permeation through a Sephacryl S-100 high resolution column. Various steps of the purification of the recombinant ob-protein were analyzed by SDS-PAGE. Experimental details are given in the Methods section. Lane1: bacteria before induction. Lane 2: bacteria after induction. Lane 3: isolated periplasmic fraction. Lane 4: the pellet obtained at 55% sat. ammonium sulfate. Lane 5: the pooled peak of pure ob-protein after gel permeation.
B: Analysis of the oxidation state of the recombinant ob-gene product.
A portion of purified ob-protein was treated with 5 mM dithiothritol for 1 hour at room temperature (reduced) and then lyophilized similarly to the untreated protein (oxidized). Both proteins were then analyzed by SDS-PAGE but in the absence of reducing agents in the sample buffer.

present, it is not known whether the putative hormone requires the disulfide bridge to be functional. Proteins targeted to the periplasmic environment have a good chance to assume the active physiological conformation and to form the appropriate disulfide bridges when expressed in a bacterial system. The oxidation state of our product was checked by analyzing its migration characteristics after separation by SDS-PAGE. A portion of the isolated ob-gene product was treated for 1 hour at room temperature with 5 mM dithiothreitol before lyophilization, and then the proteins were analyzed by SDS-PAGE in the absence of reducing agents. Figure 1B shows a clear increase of the migration velocity of the recombinant ob-protein which was not treated with dithiothreitol. This observation suggests that the ob-protein was recovered from the bacteria in an oxidized form.

Feeding studies - Immediately after restoring food, the fasted control animals began to eat and continued to do so at varying rates over the subsequent 24 h period (Fig. 2.). Except for an increase in food intake following injection at 6 nmol/kg during the first 2 h period, higher concentrations of the ob-gene product inhibited food intake during the

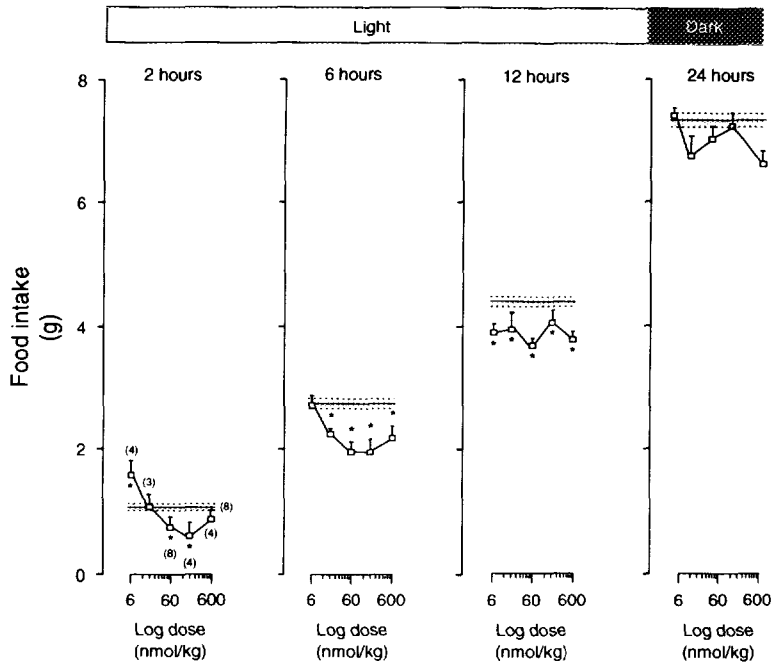

Figure 2. Effect of differing concentrations of the ob-gene product on food intake in 24 h fasted mice. The ob-gene product was injected at time 0 and food intake measured at the intervals shown after food was returned to the mice. Food intake is cumulative at each time point. Results are expressed as mean + S.E. Horizontal line and accompanying broken lines are the mean ± S.E. of the food intake in the control group of animals. Numbers of animals used at each dose are shown in parentheses. Food intake from control values was significantly altered by the ob-gene product at 2, 6 & 12 hours after returning the food to the animals. There were no statistically significant differences between any of the groups after 24 hours.

light phase. These observations provide compelling evidence that this protein can act as a satiety factor in fasted mice. Although injected intravenously, the effect of the ob-gene product on food intake took approximately 6 hours to become maximally effective. The maximum decrease in food intake at this time was approximately 28%. The inhibition of food intake 4 and 8 hours after returning food to the animals was slightly less for each dose of the ob-gene product than at 6 hours (results not shown for clarity). Thereafter, food intake gradually returned to control values over the following 24 hour period. Although mimicking the normal situation where the ob-gene product appears in the circulation after release from adipocytes, intravenous injection cannot define a site of action. The most likely direct target is the brain but a peripheral site of action with a secondary effect upon the brain cannot be ruled out.

It has been suggested that the quantity of ob-gene product in the circulation is proportional to the body fat mass (3,6,8). In this regard, the peptide is believed to function as an afferent signal from the adipocyte to the brain helping to maintain a

constant body fat mass by regulating feeding. However, recently published studies have shown ob-gene expression in adipocytes to be rapidly influenced by various stimuli (13). For example, after 3 hours of cold exposure or after a 24 hour fast ob-gene product expression in the adipocytes rapidly falls to very low levels (13). These observations may suggest that ob-gene product functions as a short-term rather than a long-term regulator of food intake.

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