Hypothalamic Pro-Opiomelanocortin mRNA Is Reduced By Fasting in *ob/ob* and *db/db* Mice, but Is Stimulated by Leptin

Tooru M. Mizuno, Steven P. Kleopoulos, Hugo T. Bergen, James L. Roberts, Catherine A. Priest, and Charles V. Mobbs

Reduction in the activity of the α-melanocyte–stimulating hormone (α-MSH) system causes obesity, and infusions of α-MSH can produce satiety, raising the possibility that α-MSH may mediate physiological satiety signals. Since α-MSH is coded for by the pro-opiomelanocortin (POMC) gene, we examined if POMC gene expression would be inhibited by fasting in normal mice or in models of obesity characterized by leptin insufficiency (*ob/ob*) or leptin insensitivity (*db/db*). In wild-type mice, hypothalamic POMC mRNA was decreased >60% after a 2-day fast and was positively correlated with leptin mRNA. Similarly, compared with controls, POMC mRNA was decreased by at least 60% in both *db/db* and *ob/ob* mice. POMC mRNA was negatively correlated with both neuropeptide Y (NPY) and melanin-concentrating hormone (MCH) mRNA. Finally, treatment of both male and female *ob/ob* mice with leptin stimulated hypothalamic POMC mRNA by about threefold. These results suggest that impairment in production, processing, or responsiveness to α-MSH may be a common feature of obesity and that hypothalamic POMC neurons, stimulated by leptin, may constitute a link between leptin and the melanocortin system. *Diabetes* 47:294–297, 1997

The obesity of yellow A^y mice is thought to be due to ectopic expression of the product of the agouti gene (1), an antagonist of the action of α-melanocyte–stimulating hormone (α-MSH) acting through the melanocortin-4 (MC-4) receptor (2). Furthermore, disruption of the MCR-4 receptor (3) causes obesity. In addition, α-MSH agonists and antagonists can promote satiety and feeding, respectively (4). Therefore α-MSH is a plausible candidate as a physiological mediator of satiety. Obese A^y mice exhibit elevated expression of the gene coding for the hormone leptin (5). Mutations in leptin cause obesity (6), experimental elevation of leptin reduces body weight and induces satiety (7), and expression of leptin is reduced during fasting (5). The elevation of leptin in obese A^y mice suggests that these mice are resistant to the satiety effects of leptin; in turn, since the obesity of A^y mice appears to be due to antagonism of α-MSH, these data suggest the hypothesis that α-MSH mediates effects of leptin. In contrast to the regulation of neuropeptide Y (NPY) mRNA, which is inhibited by leptin (8), these observations suggest that the synthesis and/or release of α-MSH might be stimulated by leptin, and, conversely, inhibited in fasting mice and in *db/db* and *ob/ob* mice, which are resistant to and deficient in leptin, respectively. α-MSH is produced by proteolytic cleavage and modification of pro-opiomelanocortin (POMC). Therefore, the present study assessed the hypothesis that in models characterized by low leptin (fasting and *ob/ob* mice) and leptin resistance (*db/db*), hypothalamic POMC mRNA would be reduced, and, conversely, that hypothalamic POMC mRNA would be elevated after leptin administration. Such a result would be consistent with the hypothesis that POMC gene products, in particular α-MSH, may mediate some effects of leptin and other satiety factors.

From the Fishberg Center for Neurobiology (T.M.M., S.P.K., C.A.P., J.L.R., C.V.M.), the Neurobiology of Aging Laboratories, and the Department of Geriatrics (T.M.M., S.P.K., C.V.M.), Mount Sinai School of Medicine, New York, New York; and the Department of Anatomy (H.T.B.), University of Manitoba, Winnipeg, Manitoba, Canada.

Address correspondence and reprint requests to Dr. Charles V. Mobbs, Neurobiology of Aging Laboratories, Box 1639, Mt. Sinai School of Medicine, 1425 Madison Ave., New York, NY 10029–6574. E-mail: mobbsc@alum.mit.edu.

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α-MSH, α-melanocyte-stimulating hormone; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; POMC, pro-opiomelanocortin.

RESEARCH DESIGN AND METHODS

Animals. C57Bl/6J, CBA, *db/db* (along with control C57Bl/KsJ), and *ob/ob* (along with control C57Bl/6J) mice were obtained at 2 months of age from The Jackson Laboratory. Mice were individually housed with free access to feed and water under 12:12 h light-dark cycle (lights on at 0700). All studies had been approved by the appropriate Institutional Animal Review Board. To assess effects of nutritional status, mice were fasted for 48 h or fed ad lib (n = 4–12 per group); in some cases, food was removed from cages of ad lib fed mice for 7 h during the day to ensure acute nutritional status was equivalent in all mice. To assess effects of leptin replacement, male *ob/ob* mice and wild-type controls were injected intraperitoneally with 3 μg/g body wt daily for 5 days; in this study, POMC mRNA was assessed by Northern blots. In a second study, female *ob/ob* mice were either fed ad lib or fasted for 48 h; during the period of fasting, the ad lib fed mice and the fasting mice were injected every 12 h with saline or leptin (0.5 μg/g body wt, i.p.) with the final, fifth injection 30 min before being killed; in this study POMC mRNA was assessed by in situ hybridization. Mice were killed just before lights out (except for the mice in the leptin replacement study, who were killed 2 h after lights on) under anesthesia by CO_2 narcosis. Brain blocks were removed, frozen on dry ice and stored at –70°C until sectioning on a cryostat or preparation of RNA.

Template production and probe labeling. POMC template was prepared by amplifying a source plasmid DNA using polymerase chain reaction (PCR) with N-terminal primer: 5′-CCTGTGAAGGTGTACCCCAATGTC-3′ and C-terminal primer: 5′-CACGTTCTTGATGATGGCGTTC-3′. NPY template was prepared in the same way with N-terminal primer: 5′-CTAGGTAACAAACGAATGGGG-3′ and C-terminal primer: 5′-CCACATGGAAGGGTCTTCAAG-3′. MCH template was prepared from mouse hypothalamic RNA by reverse transcription-polymerase chain reaction (RT-PCR) with N-terminal primer: 5′-CCAAGTCCATCAGGAACGTA-3′ and C-terminal primer: 5′-TTGTGGACCAGCAGGTATCA-3′. Leptin template was prepared as described previously (5). The amplified fragments were gel-purified, diluted to 50 ng/μl and stored at –20°C. Single-stranded internally labeled DNA

probes were produced by amplified primer extension labeling by using only C-terminal primer (5).

Northern blot analysis. RNA from mediobasal hypothalamus (microdissected as described previously [9]) was extracted in TRIzol (GIBCO BRL, Gaithersburg, MD) and 3 μg of total RNA (determined by UV absorbance corroborated by ethidium bromide-stained integrity gels) was subjected to Northern blot analysis to detect POMC mRNA and NPY mRNA in the hypothalamus, and leptin mRNA in adipose tissue from gonadal fat pad. Northern blot analysis was performed as described previously (5). To monitor RNA loading, membranes were reprobed and hybridized with ^{32}P-labeled probe encoding 18S ribosomal RNA to ensure equal loading in all lanes.

In situ hybridization histochemistry. Frozen coronal sections (10 μm thick) through the mouse hypothalamus were cut, fixed in 3% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.0) containing 0.03% DEPC, dehydrated, and stored at –20°C until use. Sorting on the basis of histology was carried out to ensure sections were matched for anterior-posterior level. Sections were prehybridized in 2 X SSC, 5 mmol/l EDTA, 2.5 × Denhard's solution, 5 mmol/l DTT, 100 mg/ml herring sperm DNA, 100 mg/ml yeast tRNA, 5 mg/ml single-stranded calf thymus DNA and 50% deionized formamide for 2 h at 42°C. Hybridization was carried out in the same buffer containing 10% dextran sulfate and labeled probe (^{32}P: 2 × 10^6 dpm per 20 μl per section) at 42°C overnight. Sections (two sections per matched A-P level) were washed twice in 1 × SSC for 15 min and in 0.1 × SSC overnight at room temperature, followed by a final wash in 0.1 × SSC for 1 h at 55°C. Slides were dehydrated, air dried, and apposed to autoradiography film.

Data analysis. The total integrated densities of hybridization signals were determined by computerized densitometric scanning (MCID System, St. Catherine's, Ontario, Canada) as described previously (10). Statistical analysis was performed by analysis of variance (ANOVA) followed by Tukey-Kramer pair-wise comparisons, or by linear regression, with criterion for significance as $P < 0.05$.

RESULTS

As assessed by Northern blots in C57Bl/6J mice, a 48-h fast significantly reduced hypothalamic POMC mRNA by ~60% (Fig. 1*A*, *D*) and at the same time induced hypothalamic NPY mRNA by about fourfold (Fig. 1*B*, *E*), with no change in the 18S ribosomal RNA band (Fig. 1*C*, *F*). In these mice, leptin mRNA was reduced ~80% by the 48-h fast (FED: 100 ± 9%; FASTED: 16 ± 2%, expressed as percentage of fed controls). POMC mRNA was positively correlated with leptin mRNA ($r = 0.89$, $P < 0.01$), and negatively correlated with NPY mRNA ($r = -0.88$, $P < 0.01$). These data were corroborated in a separate study and using an RNAse protection assay for POMC mRNA, which also demonstrated a 60% decrease by fasting (data not shown). The effects of fasting were also corroborated by in situ hybridization in a separate study in which fasting reduced POMC mRNA by over 80% throughout the arcuate nucleus (Fig. 1*G*). In the same mice, NPY was increased over 300% (Fig. 1*H*), and leptin mRNA was reduced by fasting over 90% (FED: 100 ± 11%; FASTED: 7.3 ± 2%).

Leptin resistance in nonfasted *db/db* mice was associated with significant reduction in POMC mRNA of ~80%, compared with nonfasted wild-type C57/KsJ mice (Fig. 2*A–C*). In wild-type KsJ mice, fasting (Fig. 2*C*) was associated with significant decreases in POMC mRNA. In *db/db* mice, fasting was associated with a further significant reduction in POMC mRNA (Fig. 2*C*). NPY mRNA was elevated in *db/db* mice compared with wild-type controls under all conditions, and, interestingly, fasting caused a further elevation in *db/db* mice (Fig. 2*D*). Similarly, MCH mRNA was significantly elevated in every circumstance in which POMC mRNA was reduced (Fig. 2*E*). POMC mRNA was significantly negatively correlated with both NPY mRNA ($r = -0.79$, $P < 0.001$) and MCH mRNA ($r = -0.59$, $P < 0.05$), while NPY mRNA and MCH mRNA were significantly positively correlated with each other ($P = 0.46$, $P < 0.05$).

As assessed by Northern blot analysis, hypothalamic POMC mRNA was reduced by ~90% in leptin-deficient nonfasted *ob/ob* male mice, compared with wild-type controls (Fig. 3*A*). Injection of leptin over 5 days significantly elevated hypothalamic POMC mRNA in *ob/ob* mice (Fig. 3*A*), although at these doses over this period of time, POMC mRNA was not restored completely to wild-type levels. As expected, NPY mRNA was elevated in *ob/ob* mice, but like POMC mRNA, at this dose and period of injection, NPY mRNA was only partially corrected (Fig. 3*B*). These results were corroborated in fed and fasted female *ob/ob* mice, in which leptin injection elevated hypothalamic POMC mRNA to about the same degree as observed in male mice as assessed by Northern blots (Fig. 3*C*).

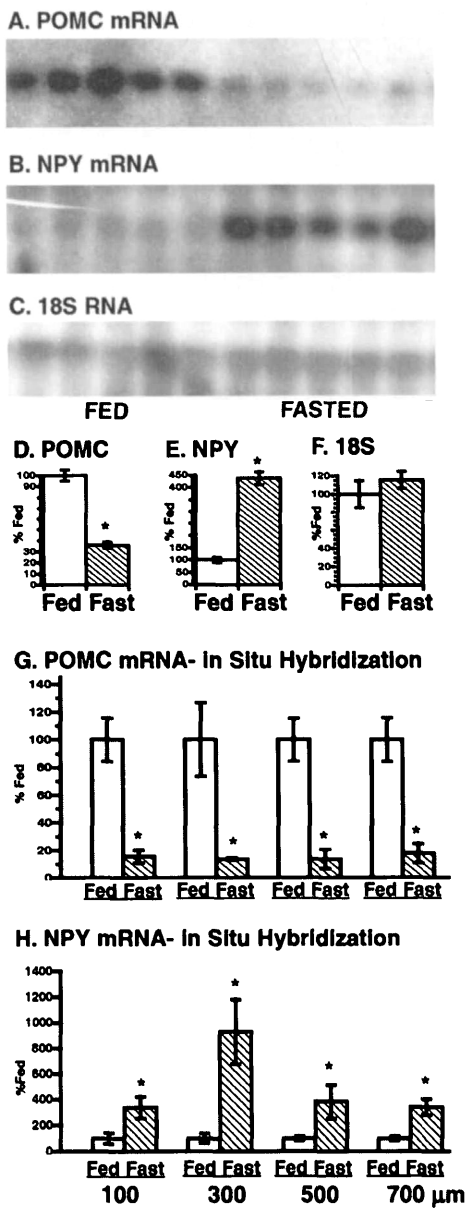

FIG. 1. Effect of a 48-h fast on hypothalamic POMC (*A*), NPY (*B*), and 18S RNA (*C*). Quantification of Northern blots shown in *A–C*: POMC (*D*), NPY (*E*), or 18S RNA (*F*) band, mean ± SE, expressed as percentage of fed controls. Quantification of POMC (*G*) or NPY (*H*) mRNA signal after in situ hybridization; in fed or fasted mice; $n = 4$–12 per group. Abscissa in *G* and *H* indicates number of microns from beginning af arcuate nucleus, thus representing almost the entire extent on the nucleus in mice. *Significant ($P < 0.05$) effect of fast versus comparable fed.

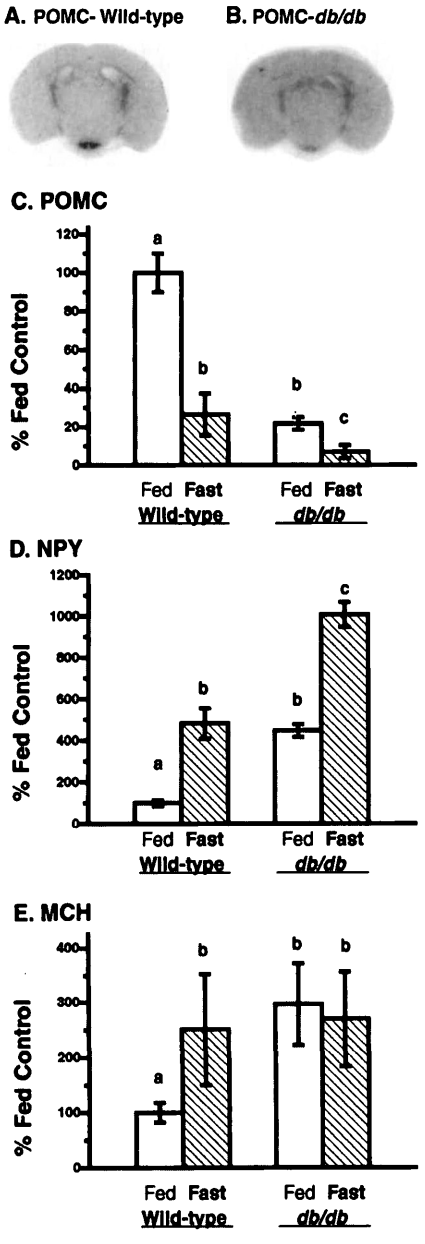

FIG. 2. Effect of *db/db* genotype or fasting on POMC, NPY, and MCH mRNA. *A* and *B*: POMC mRNA, indicating in situ hybridization in groups as indicated. Quantification of POMC (*C*), NPY (*D*), or MCH mRNA (*E*), mean ± SE, n = 6–8/group, expressed as percentage of fed, wild-type controls. Groups with different letters are statistically different ($P < 0.05$).

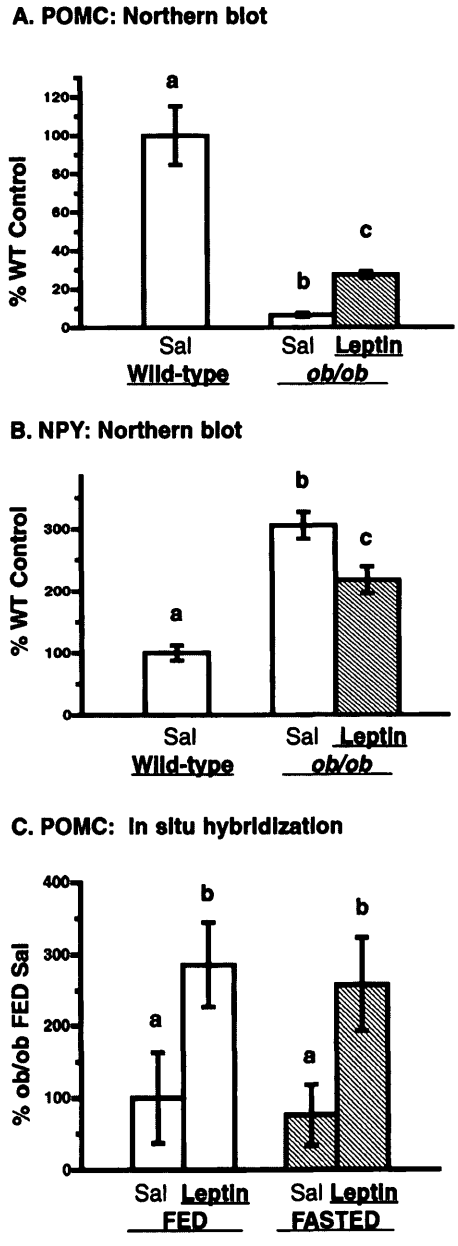

FIG. 3. Effect of *ob/ob* genotype and leptin replacement on POMC (*A*) and NPY (*B*) mRNA levels, as assessed by Northern blot analysis in male mice, and POMC mRNA in fed and fasted *ob/ob* female mice (*C*). Mean ± SE, n = 4–8/group, expressed as percentage of wild-type, saline-injected (Sal) mice (*A* and *B*), or percentage of fed *ob/ob* saline-injected mice (*C*). Groups with different letters are statistically different ($P < 0.05$, ANOVA, followed by Tukey-Kramer).

DISCUSSION

In the present studies, two forms of genetic obesity (*db/db* and *ob/ob*) were associated with decreased hypothalamic POMC mRNA. Since mutations in the leptin gene have now been associated with obesity in humans (11), and mutations in the leptin receptor also produce obesity in rats as well as in *db/db* mice (12), it will be of interest to assess if hypothalamic POMC mRNA is also reduced in genetic obesity of these species. Mice in which the basic helix-loop-helix Nhlh2 gene has been deleted become obese, and these mice also exhibit reduced hypothalamic POMC mRNA, whereas other hypothalamic gene products such as NPY, tyrosine hydroxylase, galanin, and arginine vasopressin were not influenced in these obese mice (13). Another form of genetic human obesity entails mutation of the PC-1 peptide processing enzyme which leads (among other impairments) to impaired ability to process the POMC precursor peptide into its components (14), presumably leading to impaired production of α-MSH. Similarly, the obesity of the *fatty/fatty* mouse is due to a mutation in another processing enzyme, carboxypeptidase E (15), which leads (among other impairments) to impaired processing of the POMC precursor (16). Finally, the obesity of the yellow A^y mouse entails overproduction (1) of an antagonist of the action of α-MSH (2). Similarly, disruption of the MC-4 receptor through which α-MSH acts also causes obesity (3).

Several forms of acquired obesity are also associated with decreased hypothalamic POMC mRNA. A syndrome of obesity produced by distemper-related virus is associated with reduced hypothalamic POMC mRNA (17). Finally, the process of aging, particularly in rats, is associated with the development of obesity, and hypothalamic POMC mRNA decreases during aging (18). Taken together, these studies indicate that impaired production, processing, or sensitivity to α-MSH is a feature common to many forms of obesity.

The present study suggests that the effect of fasting to reduce hypothalamic POMC mRNA is robust, since results were similar in several strains of wild-type mice, consistent with previous studies in rats (19–20). The following evidence from the present study suggested that the effect of fasting to reduce POMC mRNA is mediated in part by a fall in leptin: *1*) leptin decreased with fasting in normal mice, and POMC mRNA was positively correlated with leptin; *2*) leptin resistance (in *db/db* mice) was associated with reduced hypothalamic POMC mRNA; *3*) leptin insufficiency (in *ob/ob* mice) was associated with reduced POMC mRNA; and *4*) leptin injection significantly stimulated hypothalamic POMC mRNA in *ob/ob* mice. The effect of leptin replacement was independent of effects on food intake and body weight, since the effects were comparable in fed and in fasted *ob/ob* mice, and leptin did not significantly reduce body weight or plasma glucose beyond the effect of fasting (not shown). The hypothesis that hypothalamic POMC gene products mediate some effects of leptin is consistent with other reports, including the following: *1*) in agouti mice, in which the basis of the obesity is an antagonism of α-MSH, leptin is elevated (5), suggesting a leptin resistance in agouti mice which has been recently directly demonstrated (21); *2*) the peri-arcuate nuclear region, the main site of POMC synthesis (22) in the brain, is the area most sensitive to the satiety effects of leptin (23); and *3*) leptin receptors colocalize with POMC-containing neurons (24). Nevertheless, since POMC mRNA was reduced (and NPY was elevated) by fasting in *db/db* mice, it is plausible that other factors, such as insulin and glucose, also stimulate POMC mRNA and inhibit NPY mRNA. The present studies, in which MCH mRNA was elevated in *db/db* and fasted mice, were also consistent with a role for MCH, which stimulates feeding and is elevated in *ob/ob* mice (25), in fasting and obesity. MCH can antagonize the effect of α-MSH on behavior (26). Therefore, a plausible hypothesis is that products of the POMC gene and the MCH gene are inversely expressed and act in opposition (though possibly through different receptors) to regulate body weight. Such a hypothesis suggests that α-MSH agonists may, like leptin (27), reverse or attenuate some effects of fasting.

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