Regulation of leptin receptor and NPY gene expression in hypothalamus of leptin-treated obese (*ob/ob*) and cold-exposed lean mice

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Abstract Leptin receptor gene expression has been measured in arcuate and ventromedial hypothalamic nuclei. Receptor mRNA in both hypothalamic areas was higher in obese mice than in lean littermates. Twice daily leptin administration for 7 days profoundly affected food intake, reduced leptin receptor mRNA in the arcuate nucleus, and had a similar effect on neuropeptide Y gene expression. A single leptin injection was ineffective. Exposure of lean mice to cold for 24 h caused an induction of leptin receptor and NPY mRNA which was normalized when animals were returned to the warm. Regulation of receptor gene expression may be an important component in the reading of the leptin signal.

Key words: Leptin receptor mRNA; Arcuate nucleus; Ventromedial nucleus; Obesity; Leptin injection; Cold exposure

1. Introduction

Mutation of the *obese* gene and consequent leptin deficiency causes profound obesity in *ob/ob* mice [1]. Genetic leptin deficiency gives rise to a number of obesity-related phenotypes which can be corrected by peripheral injection of recombinant murine leptin, resulting in weight loss, reduced food intake, increased metabolic rate, normalization of plasma hormone and metabolite concentrations [2–4], and stimulation of the reproductive endocrine system [5]. Initial evidence that leptin of adipose tissue origin might be active in the central nervous system came from study of the effects of administration into the ventricular system [4], which was followed by expression cloning of a leptin receptor gene from the choroid plexus [6]. Splice variants of the receptor exhibit a common extracellular sequence, but the Ob-Rb variant is distinguished by a long intracellular domain that is thought to be active in signal transduction [6–8]. The hypothalamus, a key integrative centre in energy homeostasis, was shown to express leptin receptor Ob-Rb mRNA, first by RT-PCR [6] and subsequently by in situ hybridization [9]. Leptin receptor gene expression was localized to a number of critical hypothalamic areas, including the arcuate (ARC), ventromedial (VMN) and paraventricular (PVN) nuclei [9]. Neuropeptide Y (NPY), which has potent effects on food intake and energy balance within the hypothalamus [10], is also abundantly expressed in the ARC. *ob/ob* mice exhibit elevated NPY gene expression in the ARC, and in common with the other obesity-related phenotypes described above, leptin administration leads to a normalization of the elevated mRNA level [11,12]. This effect is likely to be through a direct mechanism, as we have shown recently that leptin receptor mRNA is co-expressed in NPY neurons of the arcuate nucleus [13].

Cellular responses to leptin stimulation may be influenced by regulation of leptin receptor gene expression and protein levels. We hypothesized that the lack of functional circulating leptin in the *ob/ob* mouse could lead to differences in receptor gene expression in the hypothalamus between obese *ob/ob* mice and their lean littermates, and that this expression might be modulated by systemically administered leptin. We have also investigated the effect of cold exposure on leptin receptor gene expression. Leptin receptor gene expression was examined by in situ hybridization with probes for the common extracellular domain of the receptor (Ob-R) and its Ob-Rb-specific intracellular domain [9].

2. Materials and methods

2.1. Animals

Lean (+/?) and obese (*ob/ob*) mice of the 'Aston' variety were drawn from a colony maintained at the Rowett Research Institute on a 12/12 h light/dark cycle (lights on 07:00 h). Food (Biosure; SDS) and water were available ad libitum. All animals were killed by cervical dislocation. Recombinant murine leptin was dissolved in bicarbonate buffer according to the manufacturer's instructions (Peprotech, London) and administered to obese animals by intraperitoneal (i.p.) injection. Three injection protocols were employed. In long-term studies of the effects of leptin on body weight and food intake, the peptide was administered either twice daily at 09:00 h and 18:00 h for 7 days (1.25 μg/g body weight), or daily at 18:00 h for 5 days (2 μg/g body weight). The acute effects of a single leptin injection (1.25 μg/g body weight) early in the light phase were examined over a 5 h period in the absence of food. Some of the animals which received twice daily leptin injections for 7 days were injected with buffer vehicle for a further 6 days; control mice in all three leptin administration experiments also received vehicle injections. In a separate experiment, energy flux was manipulated in lean mice by cold exposure, commencing early in the light phase. The effect of maintenance at 4°C for 24 h was compared with control animals housed at 24°C and a group which was replaced at 24°C for 12 h following cold exposure for 24 h.

2.2. In situ hybridization

Leptin receptor gene expression in the ARC and VMN and NPY mRNA in the ARC of the mouse hypothalamus was examined in 20 μm coronal sections (corresponding to Fig. 1c in [9]; equivalent to Bregma −3.3 to −2.8 in the rat brain according to [14]). In situ hybridization to sections mounted on poly(L-lysine)-coated slides employed techniques described in detail elsewhere [9,15]. Adjacent brain sections were hybridized with riboprobes for the common extracellular domain of the leptin receptor (Ob-R), the long form of the receptor, Ob-Rb, and preproNPY mRNA. Leptin receptor cDNAs were cloned from the mouse as described previously [9]. NPY probes were generated from a 0.5 kb fragment of a rat NPY cDNA, kindly supplied by Dr. Stephen Sabol [15]. Autoradiographic images were quantified using the Bio Image system (Millipore). Data were standardized with ^{14}C autoradiographic scales (Amersham, UK). Gene expression was measured as the integrated intensity of the autoradiographic signal (background-corrected OD integrated over all pixels in the hybrid-

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ization area). Two sections were analyzed from each brain and data from each hypothalamic region were averaged to yield a single value for expression of each mRNA species in each animal. Adjacent sections incubated with sense riboprobes yielded weak, non-specific, film images [9].

2.3. Statistical analysis

Data were analyzed by *t*-tests or one-way analysis of variance followed by multiple comparison procedures, as appropriate. Differences were considered statistically significant if $p < 0.05$. Data are presented as means ± S.E.

3. Results

Brain sections which contained both the ARC and VMN were used in the quantification of leptin receptor gene expression. Probes for the common extracellular domain of the leptin receptor (Ob-R) and the intracellular domain, Ob-Rb, revealed a significant elevation in receptor gene expression in obese (*ob/ob*) mice compared with their lean, age-matched, littermates; receptor mRNA levels were increased in both the ARC and VMN (Fig. 1). Obese mice also exhibited elevated NPY gene expression in the ARC. Elevated expression of the leptin receptor gene in the *ob/ob* mouse may be due to the absence of functional leptin in this obese model. This possibility was investigated by administration of recombinant murine leptin or buffer vehicle to obese mice.

When leptin was administered by twice daily injection for 7 days (LEP-treated), voluntary food intake fell rapidly from a daily intake of approx. 8 g to a new, relatively stable, level of 1.5–2.5 g (Fig. 2). When leptin was withdrawn, but injection of vehicle continued for a further 6 days (LEP/VEH-treated), intake returned to levels exhibited by obese control mice (VEH-treated controls; Fig. 2). These changes in intake contributed to a 19% reduction in body weight during the leptin treatment period (mean body weights, 76.4 g vs 61.9 g); approx. 40% of this weight loss was restored during the subsequent 6 days of vehicle injections. Using the Ob-R probe, there was a significant effect of treatment on leptin receptor gene expression in the ARC, but not in the VMN (Fig. 3). Mice treated with leptin for 7 days (LEP-treated) had lower

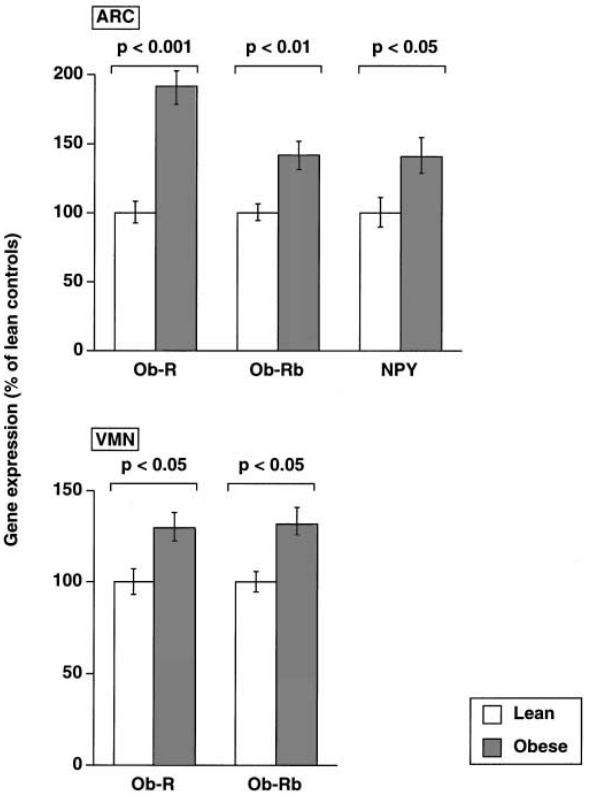

Fig. 1. Leptin receptor and NPY gene expression in the ARC and VMN of lean and obese (*ob/ob*) mice. Leptin receptor mRNA was measured with probes to the common leptin receptor sequence, Ob-R, and the Ob-Rb splice variant. Data from obese mice are expressed as a percent of lean controls. Groups contained 5 animals.

levels of Ob-R mRNA in the ARC than VEH-treated controls, however, differences between these groups and mice where leptin treatment was followed by 6 days of vehicle treatment (LEP/VEH-treated) were not statistically significance. Adjacent sections were hybridized with the Ob-Rb-spe-

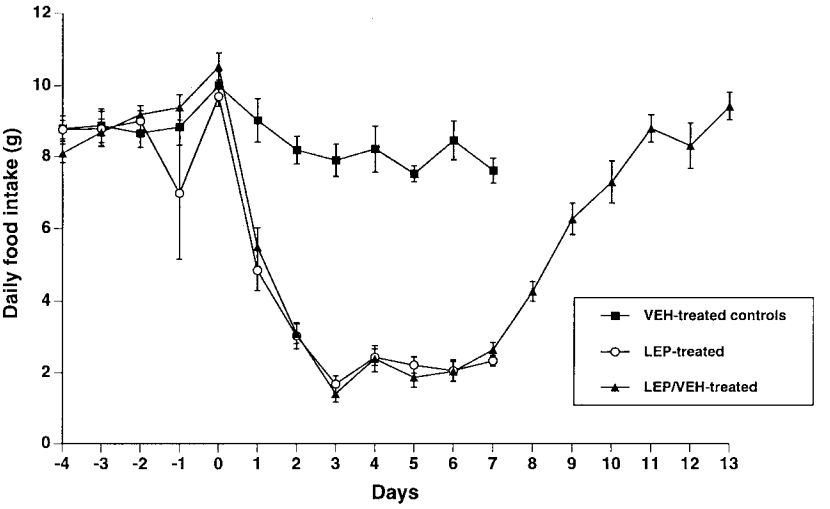

Fig. 2. Effect of twice daily i.p. injections of leptin on food intake of obese *ob/ob* mice. Injections started on day 0. Mice injected with leptin (1.25 μg/g body weight) twice daily for 7 days (LEP-treated) were compared with controls injected with buffer vehicle (VEH-treated), and with animals where leptin treatment was followed by vehicle injections for a further 6 days (LEP/VEH-treated). LEP-treated and VEH-treated groups contained 5 animals. The LEP/VEH-treated group contained 4 animals.

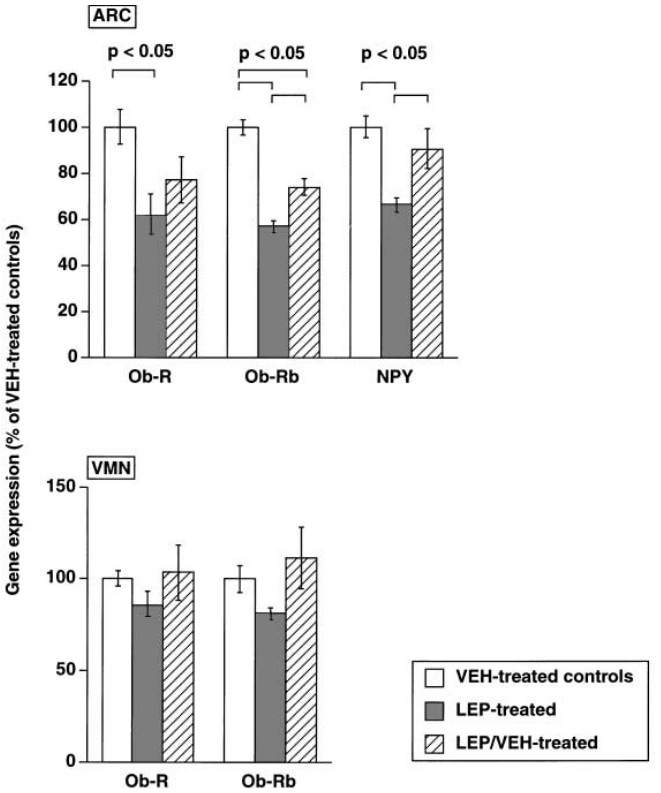

Fig. 3. Effect of twice daily i.p. injections of leptin on leptin receptor and NPY gene expression in the ARC and VMN of obese *ob/ob* mice. Mice injected with leptin (1.25 μg/g body weight) twice daily for 7 days (LEP-treated) were compared with controls injected with buffer vehicle (VEH-treated), and with animals where leptin treatment was followed by vehicle injections for a further 6 days (LEP/VEH-treated). Data from LEP-treated and LEP/VEH-treated mice are expressed as a percent of VEH-treated controls. Other details as in legend to Fig. 2.

cific riboprobe. Significant differences were observed between all three treatment groups in expression of the long splice variant of the leptin receptor gene in the ARC (Fig. 3); trends in the VMN again failed to attain statistical significance. The reduction in Ob-Rb mRNA in the ARC of LEP-treated mice and its relative normalization when leptin treatment was terminated (LEP/VEH-treated) was accompanied by similar changes in NPY gene expression in this nucleus; LEP-treated mice had lower levels of NPY mRNA than the other two treatment groups (Fig. 3).

Once daily injection of obese *ob/ob* mice with a larger dose of recombinant leptin for 5 days also decreased food intake, body weight, and leptin receptor gene expression in the ARC (results not shown). Changes in food intake were less pronounced than with twice daily injection and body weight decreased by 6% in leptin-treated mice. 5 h after administration, there was no effect of a single injection of recombinant murine leptin on leptin receptor or NPY gene expression in obese *ob/ob* mice (results not shown).

Energy balance and energy flux were manipulated by housing lean mice at 4°C for 24 h (cold-exposed). A second group of animals was returned to 24°C for 12 h following cold exposure (rewarmed). Exposure to cold had a significant effect on Ob-R ($p < 0.05$) and NPY ($p < 0.01$) gene expression in the ARC (Fig. 4); cold-exposed mice had higher levels of Ob-R and NPY mRNA than controls or animals returned to the warm. The induction of leptin receptor gene expression by cold exposure was apparent throughout the ARC, although the data presented here relate to a discrete region of this nucleus; increased receptor mRNA in the ARC of the obese mouse (Fig. 1) and reductions induced by leptin administration (Fig. 3) were also uniform changes. Differences in leptin receptor gene expression in the VMN due to cold exposure, and in the ARC when probed for the Ob-Rb splice variant, failed to attain statistical significance (Fig. 4).

4. Discussion

The failure of the obese *ob/ob* mouse to synthesize functional leptin releases the leptin receptor from its normal stimulus, and this presumably accounts for elevated receptor mRNA levels in the hypothalamus of the obese animal; both the ARC and the VMN exhibit this effect (Fig. 1). Neuropeptide Y gene expression is also elevated in the ARC of obese *ob/ob* mice, and previous studies have demonstrated that systemic leptin administration to the *ob/ob* mouse reduces this elevated expression of NPY [11,12]. We have extended this observation by demonstrating a reduction in leptin receptor gene expression in the ARC when leptin is administered either twice or once daily over a period of several days (Fig.

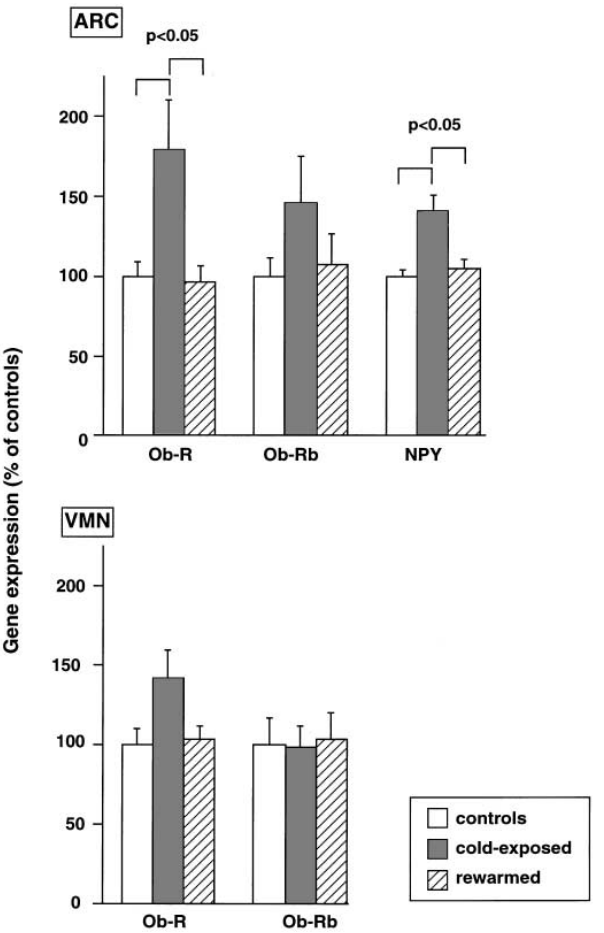

Fig. 4. Effect of cold-exposure (cold-exposed) or rewarming following cold exposure (rewarmed) on leptin receptor and NPY gene expression in the ARC and VMN of lean mice. Data from cold-exposed and rewarmed mice are expressed as a percent of controls maintained at 24°C throughout. Groups contained 5 animals.

3). Although similar trends were observed, the failure of the VMN to exhibit significant changes in receptor gene expression following leptin injection may be due to lower concentrations of leptin reaching this nucleus. Following intravenous injection, [^{125}I]leptin is taken up by the ARC, median eminence and choroid plexus [16], but outside these areas, uptake is diffuse. When leptin treatment was terminated, the inhibitory regulation of leptin receptor and NPY gene expression was removed (Fig. 3). A single systemic injection of leptin early in the light phase failed to reduce leptin receptor or NPY gene expression, and the duration of leptin administration (or dose) required to induce changes in gene expression remains to be determined; it is possible that transport across the blood-brain barrier is rate-limiting.

Although leptin regulates receptor mRNA level in the *ob/ob* mouse, the response of gene expression to fluctuating plasma leptin concentrations in the normal lean animal may be more significant. We employed cold exposure in the lean mouse as an energetic manipulation which rapidly alters leptin signals arising from adipose tissue. Exposure of lean mice to cold leads to complete disappearance of *ob* mRNA in adipose tissue within hours [17], a change which is more profound than that observed with fasting and which is likely to reduce leptin synthesis. This is demonstrated by lean rats, where plasma leptin is reduced by cold-exposure [18]. Effects of cold on *ob* mRNA and plasma leptin are reversible during short periods at 24°C [17,18]. The increase in Ob-R mRNA in the ARC of cold-exposed lean mice, and the reversible nature of this induction, supports the contention that leptin receptor gene expression is regulated by plasma leptin concentration in the lean mouse. However, an indirect effect of cold exposure on receptor gene expression, such as via the stress axis, cannot be ruled out. We also observed elevated NPY gene expression in the ARC of the cold-exposed mouse (Fig. 4), which was normalized on return to the warm. These data, reflecting negative energy balance, are consistent with increases in NPY peptide concentration in a number of hypothalamic regions in rats exposed to cold for 18 h [19].

A major component of the biological activity of leptin appears to involve interactions within the hypothalamus. Our data from the *ob/ob* mouse (Figs. 1 and 3) show regulation of hypothalamic receptor mRNA in response to leptin. Although the presumptive leptin binding protein in the choroid plexus does not possess the intracellular signaling domain, binding studies in *ob/ob* and *db/db* mice suggest that leptin deficiency or severe hyperleptinemia might regulate receptor numbers [20,21]. Thus, at least in the obese mouse, there is evidence that receptor activation results in regulation both at the protein level and at the level of gene expression. These changes are likely to be transient in nature, being reversible once leptin is withdrawn (Fig. 3). The rapid and reversible induction of hypothalamic leptin receptor gene expression in leptin-competent animals in which the leptin signal was manipulated by exposure to cold (Fig. 4) indicates that receptor regulation is likely to be an important mechanism for regulating the leptin signal and thus subsequent cellular activation in the normal animal. It is recognized, however, that the speed of receptor turnover will be an important component in determining whether relatively rapid changes in leptin receptor mRNA are translated into changes in receptor density. Differences in the degree of induction of receptor mRNA detected with either the Ob-R or the Ob-Rb probes in obese mice (Fig. 1) and cold-exposed lean mice (Fig. 4) may reflect a mixed population of receptor mRNA splice variants in the ARC. The fact that NPY gene expression changes in parallel to leptin receptor mRNA under different circumstances provides further evidence for an interaction between these signaling pathways in the hypothalamus [13]. In conclusion, regulation of the receptor gene expression may prove to be an important component in the regulation of the leptin signal.

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