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RESEARCH COMMUNICATION

Acute cold-induced suppression of *ob* (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system

Paul TRAYHURN,* Jacqueline S. DUNCAN and D. Vernon RAYNER

Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

The effect of acute exposure to cold on the expression of the *ob* (obese) gene, which encodes a protein that plays a critical role in the regulation of energy balance and body weight, has been examined in epididymal white adipose tissue of mice. Overnight (18 h) exposure of mice to a temperature of 4 °C led to the disappearance of *ob* mRNA in epididymal white fat, and subsequent studies showed that a cold-induced loss of *ob* mRNA could occur in as little as 2–4 h of exposure to 4 °C. When mice exposed to cold for 18 h were returned to the warm (24 °C), there was a rapid stimulation of the expression of the *ob* gene, the mRNA returning within 2.5 h. Administration of noradrenaline led to a reduction in the level of *ob* mRNA in mice maintained in the warm, while isoprenaline resulted in the disappearance of the mRNA; these changes in *ob* mRNA were paralleled by similar changes in lipoprotein lipase mRNA. In contrast to white fat, the level of lipoprotein lipase mRNA in brown adipose tissue was increased by noradrenaline and isoprenaline. It is concluded that there is a cold-induced suppression of *ob* gene expression in white adipose tissue of mice and that this is mediated primarily by the sympathetic system. The profound effect of cold on *ob* gene expression indicates that the *ob* system relates to energy expenditure, as well as to satiety.

INTRODUCTION

The factors responsible for the regulation of body weight and total body fat stores in mammals have been the subject of extensive investigation over a number of years. Positional cloning has recently led to the identification and sequencing of the mutant gene responsible for the development of obesity in the *ob/ob* mouse [1], this animal being one of the most widely used models in studies on the regulation of body weight [2]. The *ob* (obese) gene appears to be expressed exclusively in white adipose tissue [1,3,4], where it codes for a protein with an M_r of 18000 [1]. This protein, now termed leptin [5], is thought to be secreted from the adipocyte as a product with an M_r of 16000 which signals the size of the white adipose tissue depots. Leptin may act as a satiety factor [1,5–7], but there is also evidence that it affects overall energy expenditure [5,6].

Increased expression of the *ob* gene has been observed in several types of obese rodent [1,3,4,8,9] and following the administration of glucocorticoids to rats [10]. Expression of the gene is subject to nutritional regulation, the level of the mRNA falling on fasting and increasing rapidly on subsequent re-feeding [4]. In the present study we have examined the effects of acute exposure to cold on *ob* gene expression. Cold-exposure leads to an immediate and substantial increase in energy expenditure, particularly in small mammals, and there is a net flux of fatty acids from white adipose tissue. In brown fat, the other form of adipose tissue, exposure to cold activates the generation of heat through the mitochondrial proton conductance pathway [11,12].

The results presented here demonstrate that *ob* gene expression is suppressed by acute cold exposure of mice and that this may be mediated by the sympathetic system.

MATERIALS AND METHODS

Animals and tissues

The animals used in this study were 8-week-old male lean mice of the 'Aston' variety, from a colony maintained at the Rowett Research Institute. The mice were killed by cervical dislocation, and epididymal white adipose tissue and interscapular brown adipose removed and frozen in liquid N_2. The tissues were stored at −80 °C until analysis (within 2 weeks). A minimum of five mice were used in each experimental group.

To investigate the effects of acute cold exposure, the mice were housed individually in cages with wire-mesh bottoms and placed in a room at 4 °C with the same 12 h light/12 h dark cycle as the animal house (lights on at 07.00 h). Control animals were maintained at 24 °C. Throughout the study the animals had continuous access to tap water and a rodent diet (Biosure; Special Diet Services) containing 18 % (w/w) protein and 2.4 % (w/w) fat.

In studies on the effects of β-adrenoceptor agonists on *ob* gene expression, mice were injected subcutaneously with either noradrenaline (bitartrate) or isoprenaline (bitartrate). The adrenoceptor agonists (Sigma) were dissolved in 0.9 % sterile saline, the saline alone being administered to control animals. Isoprenaline was injected at a dose of 750 μg/kg of body weight at time 0, with a second dose at 2 h; the mice were killed 4 h after the first injection. Noradrenaline was administered at doses of either 500 or 1000 μg/kg of body weight. In the case of the lower doses, injections were made at 0, 1.5 and 3 h, the animals being killed at 4.5 h. With the higher doses, noradrenaline was injected at 0 and 2 h, and tissues removed 4 h after the first injection.

Extraction of RNA and Northern blotting

Total RNA was extracted using a guanidium isothiocyanate/phenol method [13], and fractionated by agarose gel electrophoresis [14]. The RNA was then transferred to a charged nylon membrane (Boehringer Mannheim) by capillary blotting for 4 h, or by vacuum blotting, and fixed with UV light. The mRNA for *ob* was detected by a chemiluminescence-based procedure [14] utilizing a 33-mer antisense oligonucleotide probe end-labelled

* To whom correspondence should be addressed.

(5′) with a single digoxigenin ligand (Boehringer Mannheim). The oligonucleotide (5′-GGTCTGAGGCAGGGAGCAGCTC-TTGGAGAAGGC), which has been validated previously [4], was based on a region of the mRNA downstream from the site of the primary mutation in *ob/ob* mice and synthesized commercially (R & D Systems Europe).

Pre-hybridization was performed at 42 °C for 1 h in 50% formamide, 5 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate), 2% blocking reagent (Boehringer Mannheim), 50 mM sodium phosphate (pH 7.0), 0.1% *N*-dodecanoylsarcosine and 7% SDS. Hybridization was at 42 °C overnight in pre-hybridization buffer, together with the oligonucleotide (25 ng/ml). Post-hybridization washes were performed as previously [14]. The membranes were incubated with an anti-(digoxigenin) serum–alkaline phosphatase conjugate, and processed essentially as in the protocols provided by Boehringer Mannheim. CDP-*Star* (Tropix) was used as the chemiluminescence substrate [15–17]. Signals were visualized by exposure of membranes to film and quantified by densitometry using NIH Image.

In some studies, blots were stripped and re-probed for lipoprotein lipase mRNA or 18 S rRNA; the latter was used to check the loading and transfer of RNA during blotting. Stripping was performed immediately after exposure of the membrane to film, care being taken to ensure that the membrane did not dry out. The membranes were stripped by washing at 68 °C (2 × 15 min washes) in a buffer containing 50% dimethylformamide, 1% SDS and 50 mM Tris/HCl (pH 9.5). This was followed by a 1 min wash in 100 ml of sterile water and a 1 min wash in 50 ml of 2 × SSC, both washes being at room temperature. The membrane was then transferred to the pre-hybridization solution.

Lipoprotein lipase mRNA and 18 S rRNA were detected by the same chemiluminescence-based procedure as for *ob* mRNA, except that the 31-mer digoxigenin-labelled antisense oligonucleotide (5′-CGCCTGCTGCCTTCCTTGGATGTGGTAG-CCG) used to probe for the 18 S rRNA was employed at a concentration of 10 pg/ml. The sequence of the 30-mer antisense oligonucleotide designed as a probe for lipoprotein lipase mRNA was: 5′-GCCAGCAGCATGGGCTCCAAGGCTGTACCC [14]. A maximum of three probings were performed on each membrane. In control experiments, brown adipose tissue was probed for the mRNA encoding uncoupling protein using a 32-mer antisense oligonucleotide (5′-CGGACTTTGGCGGTGTC-CAGCGGGAAGGTGAT) [18].

The statistical significance of differences between groups was assessed by Student's unpaired *t*-test.

RESULTS

Effect of acute cold exposure on *ob* mRNA

In the first experiments, mice were exposed overnight (18 h) to a temperature of 4 °C and epididymal white adipose tissue removed and probed for *ob* mRNA using an antisense oligonucleotide in conjunction with chemiluminescence detection. Figure 1(a) shows that cold-exposure led to the complete disappearance of *ob* mRNA; even prolonged exposure of the membrane to film did not result in the detection of any signal in the cold-exposed animals. Subsequent stripping and re-probing of the membrane with an antisense oligonucleotide for 18 S rRNA showed that satisfactory transfer of RNA had occurred (Figure 1a), indicating that the absence of a signal in the cold-exposed mice was not due to any problems with gel loading or the efficiency of blotting.

Mice were then exposed to the cold for shorter periods of time. Even after only 2 h in the cold, no signal for *ob* mRNA was detected (Figure 1b). Stripping and re-probing for 18 S rRNA again showed that satisfactory blotting had taken place (Figure 1b).

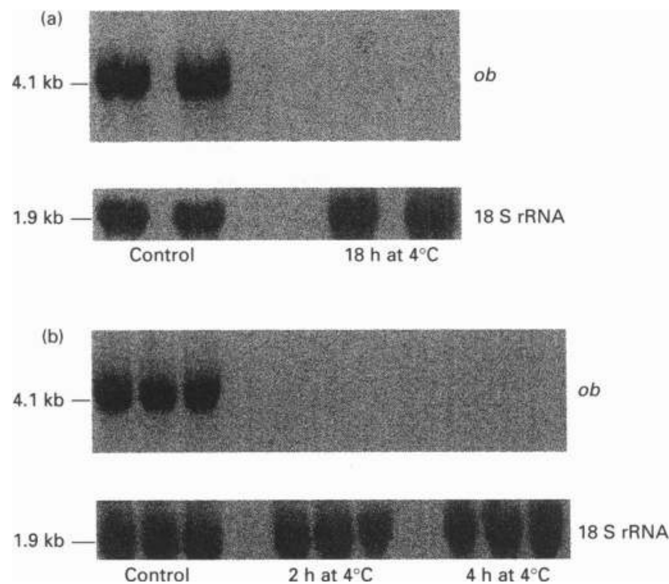

Figure 1 Northern blot of effect of exposure to cold on *ob* mRNA in mouse white adipose tissue

Mice were exposed to a temperature of 4 °C, or maintained at 24 °C, and epididymal white fat removed. Total RNA was extracted and Northern blotting performed. The blots were probed for *ob* mRNA with a 33-mer antisense oligonucleotide, and hybridization detected with the chemiluminescence substrate, CDP-*Star*. The blots were stripped and reprobed with a 31-mer antisense oligonucleotide for 18 S rRNA. In (**a**), mice were exposed overnight (18 h) to 4 °C. In (**b**), mice were exposed to 4 °C for 2 or 4 h. Representative blots are shown, with 2–20 min exposure to film.

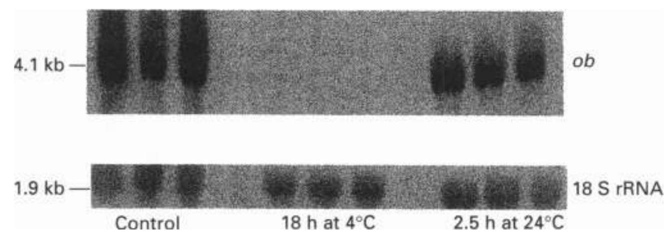

Figure 2 Northern blot of reversibility in the warm of cold-induced loss of *ob* mRNA in mouse white adipose tissue

Mice were exposed overnight (18 h) to a temperature of 4 °C, and half were then transferred to 24 °C for 2.5 h, control animals remaining in the warm throughout. Epididymal white fat was removed, total RNA extracted and Northern blotting performed. The blots were probed for *ob* mRNA with a 33-mer antisense oligonucleotide, and hybridization detected with CDP-*Star*. The blots were stripped and reprobed with a 31-mer antisense oligonucleotide for 18 S rRNA. Representative blots are shown, with 1–20 min exposure to film.

The reversibility of the cold-induced loss of *ob* mRNA was next examined. Mice were exposed to a temperature of 4 °C overnight, and half the animals were returned to the warm (24 °C) and the epididymal white fat removed 2.5 h later. Figure 2 shows that *ob* mRNA had again disappeared following cold-exposure, but a strong signal was evident after transfer to the warm for 2.5 h. Indeed, at 2.5 h in the warm the level of *ob* mRNA was similar to that in the control animals maintained in the warm throughout.

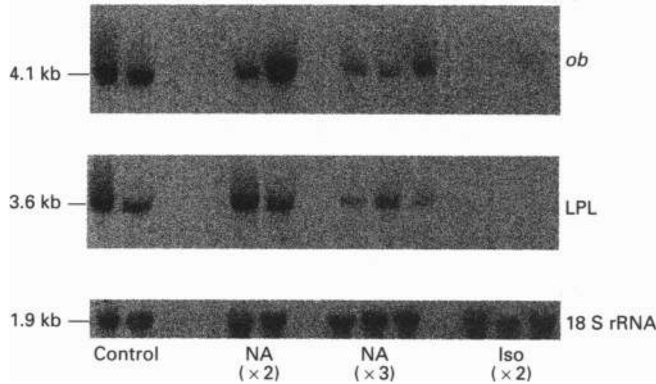

Figure 3 Northern blot of effect of administration of noradrenaline or isoprenaline on *ob* mRNA in mouse white adipose tissue

Mice were injected with two doses of noradrenaline (NA, 1000 μg/kg of body weight) at 0 and 2 h and tissues removed at 4 h, or with three doses (500 μg/kg body weight) at 0, 1.5 and 3 h, and tissues removed at 4.5 h. Isoprenaline (Iso, 750 μg/kg of body weight) was administered at 0 and 2 h and tissues removed at 4 h. Control mice received saline injections. Total RNA was extracted and Northern blotting performed. The blots were probed for *ob* mRNA with a 33-mer antisense oligonucleotide, and hybridization detected with CDP-*Star*. The blots were then stripped and reprobed sequentially with a 30-mer antisense oligonucleotide for lipoprotein lipase mRNA (LPL) and a 31-mer antisense oligonucleotide for 18 S rRNA. Representative blots are shown, with 5–40 min exposure to film.

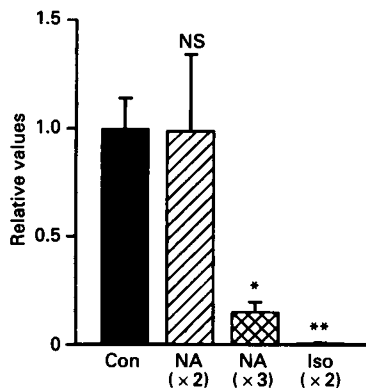

Figure 4 Quantification of effects of administration of noradrenaline or isoprenaline on *ob* mRNA levels in mouse white adipose tissue

Blots, obtained as described in the legend to Figure 3, were quantified by densitometry. Con, controls; NA, noradrenaline; Iso, isoprenaline. The results are given as mean values ± S.E.M. (bars) for 4–5 mice in each group. NS, not significant ($P > 0.05$), *$P < 0.01$, **$P < 0.001$, compared with the control mice treated with saline. Values in parentheses indicate the number of doses administered.

Effects of β-adrenoceptor agonists on *ob* mRNA

Further experiments were performed to determine whether the cold-induced disappearance of *ob* mRNA is mediated by the sympathetic system. Mice maintained in the warm were given injections of either noradrenaline or the β-adrenoceptor agonist, isoprenaline. The administration of three doses, though not two, of noradrenaline led to a marked fall in the level of *ob* mRNA (Figure 3). The most striking results were obtained with isoprenaline, two injections of which resulted in the disappearance of the mRNA. Stripping and re-probing the blots with the antisense oligonucleotide for 18 S rRNA indicated that the loading and transfer was similar for each of the groups (Figure 3).

The films were quantified by densitometry, and the level of *ob* mRNA in each of the treated groups related to that of the control animals (Figure 4). In the mice treated with two doses of noradrenaline the level of the mRNA was 99 % of the control values, while for those treated with three injections the level was decreased to 16 % of the controls. The mRNA level in the latter group was significantly different from that of the controls ($P < 0.01$). The values obtained from densitometry for the isoprenaline-treated mice were < 1 % of those of the control animals.

The level of lipoprotein lipase mRNA was also measured in this experiment, the blot being sequentially probed for *ob* mRNA, lipoprotein lipase mRNA and 18 S rRNA. The effects of the β-adrenoceptor agonists on lipoprotein lipase mRNA levels in epididymal white adipose tissue paralleled the responses in *ob* mRNA (Figure 3). Lipoprotein lipase mRNA was undetectable in white adipose tissue of mice treated with isoprenaline.

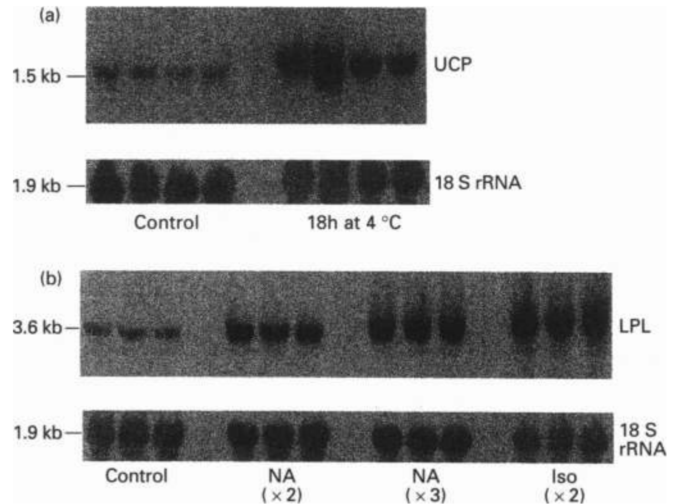

Figure 5 Northern blot of effects of cold-exposure or administration of noradrenaline or isoprenaline on gene expression in mouse brown adipose tissue

In (**a**), mice were exposed to 4 °C for 18 h or maintained at 24 °C, as described in the legend to Figure 1, and interscapular brown fat removed. Total RNA was extracted and Northern blotting performed. The blots were probed for uncoupling protein mRNA (UCP) with a 32-mer antisense oligonucleotide. In (**b**), mice were treated with noradrenaline (NA) or isoprenaline (Iso) as described in the legend to Figure 3. Total RNA was extracted and Northern blotting performed. The blots were probed for lipoprotein lipase mRNA (LPL) with a 30-mer antisense oligonucleotide. Hybridization was detected with CDP-*Star*. In both (**a**) and (**b**) blots were stripped and reprobed with a 31-mer antisense oligonucleotide for 18 S rRNA. Representative blots are shown, with 2–30 min exposure to film. Values in parentheses indicate the number of doses administered.

Brown adipose tissue

Exposure to cold has a major stimulatory effect on the thermogenic activity of brown adipose tissue (see [11,12]), and the expression of key genes such as those encoding the tissue-specific mitochondrial uncoupling protein [19–21] and lipoprotein lipase [22] is increased. To ensure that the inhibition of *ob* gene expression by cold-exposure and β-adrenoceptor agonists was not due to adverse effects of these treatments on the mice, gene expression was also examined in brown adipose tissue. Overnight exposure to cold resulted in an increase in the level of the mRNA

for uncoupling protein in brown fat (Figure 5a), in the same animals in which *ob* mRNA disappeared from white adipose tissue.

In contrast to white fat, the administration of both noradrenaline and isoprenaline induced an increase in lipoprotein lipase mRNA level in brown adipose tissue (Figure 5b). This is consistent with previous work demonstrating a sympathetic stimulation of lipoprotein lipase gene expression in brown fat [22], and indicates that the effects of these agents on *ob* mRNA at the doses used are not a reflection of a generalized inhibition of gene expression.

DISCUSSION

The mRNA for *ob* was detected in the present study by a rapid chemiluminescence-based procedure using a digoxigenin-labelled antisense oligonucleotide. This general strategy has been used for the detection of several mRNAs [14], and the measurement of *ob* mRNA with the 33-mer antisense oligonucleotide probe employed here has been validated previously [4]. The *ob* gene is expressed in white adipose tissue [1,3,4,9], and differences between different fat depots have been observed [4,9]. Whether the gene is also expressed in brown adipose tissue is not yet clear. Both the absence of expression, and low expression, have been observed in brown adipose tissue [3,4,9], with one study reporting that the level of *ob* mRNA in the tissue was 50-fold lower than in white fat [9]. The occasional low level of *ob* expression in brown adipose tissue may in practice reflect contamination, or infiltration, of the tissue by white adipocytes [4].

The present study demonstrates that expression of the *ob* gene is rapidly inhibited by acute exposure to cold. The fact that *ob* mRNA was undetectable within 2–4 h of cold-exposure suggests that the half-life of the mRNA is short (a matter of minutes), unless its stability as well as expression are substantially reduced in the cold. The response to cold-exposure is more profound than that observed with fasting, which also leads to a fall in the level of *ob* mRNA [4]. A recent study has shown that *ob* mRNA levels in epididymal white fat are decreased 4-fold in mice fasted for 24 h, relative to fed mice [4]. The cold-induced inhibition of *ob* gene expression was rapidly reversed following transfer to the warm. Since thermoregulatory thermogenesis and total energy expenditure fall quickly in the warm, this further indicates a close link between energy expenditure and *ob* gene expression.

In brown adipose tissue, noradrenaline secreted from sympathetic innervation plays a central role in both the acute and chronic activation of thermogenesis in response to exposure to cold (see [23]). White adipose tissue is also sympathetically innervated [24], albeit less extensively than brown fat, and the sympathetic system is thought to be the main mechanism for promoting fat mobilization in situations where there is a sudden demand for energy, such as in cold exposure (see [25]). Treatment of mice with noradrenaline (three doses) induced a substantial decrease in *ob* mRNA level, while administration of isoprenaline, a specific β-adrenoceptor agonist, resulted in the disappearance of the mRNA. This suggests that the cold-induced suppression of *ob* gene expression is mediated by catecholamines through β-adrenoceptors. Given that white adipose tissue is sympathetically innervated, noradrenaline secreted locally is likely to be the key factor physiologically in the suppression, rather than circulating adrenaline.

The fact that administration of three doses of noradrenaline led to a decrease in *ob* mRNA level, but that no effect was evident with two doses, requires comment. The most likely explanation is that high circulating levels of noradrenaline were maintained more effectively with three doses rather than two, particularly since the time between injections and the gap between the final injection and removal of adipose tissues was shorter with the larger number of doses. This is consistent with the greater suppressive effect of isoprenaline, which is a long-acting β-adrenoceptor agonist, and the rapid re-appearance of *ob* mRNA in cold-exposed mice on transfer to the warm.

Analysis of the lipoprotein lipase mRNA changes in white adipose tissue shows a close association with the relative level of *ob* mRNA. The specificity of both probes has been validated previously [4,14], and an additional check showed that *ob* mRNA was detected in white adipose tissue but not in the heart. Lipoprotein lipase mRNA, on the other hand, was detected in both tissues with a higher level in the heart than in white fat (results not shown). The apparent association between *ob* mRNA and lipoprotein lipase mRNA may reflect the fact that lipoprotein lipase activity and gene expression will be decreased when adipocytes are exporting considerable amounts of fatty acid, and expression of the *ob* gene should fall with decreasing cell size; conversely, the expression of lipoprotein lipase and *ob* would be expected to rise with increasing adipocyte cell size [9].

A central issue is the physiological significance of a cold-induced suppression of *ob* gene expression by the sympathetic system. Exposure to cold induces a substantial increase in metabolic rate and overall energy expenditure, without which lethal hypothermia would ensue. Initially in the cold, the animal will exhibit a net loss of body fat and be in negative energy balance. It follows, therefore, that if leptin acts as a satiety factor, or regulates some component of energy expenditure in relation to energy balance, then its expression should be suppressed by acute cold. Given the evidence that the protein may stimulate energy expenditure [5,6], as well as inhibit food intake [5–7], there would appear to be a feedback loop between some component of expenditure and the adipocyte, involving the sympathetic system.

Prolonged exposure of rodents to cold results in a gradual increase in food intake and the restoration of body fat stores (although energy expenditure remains elevated), at which point the sympathetic suppression of *ob* gene expression would be expected to fall. Further studies are needed to determine whether this is indeed the case. Additional work is also required to characterize the nature of the β-adrenoceptor subtype(s) responsible for the sympathetically-mediated inhibition of *ob* gene expression. White adipocytes from laboratory rodents and mouse pre-adipose cell lines contain β1-, β2- and β3-adrenoceptor subtypes [26], each of which could be involved in the inhibitory response.

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