Peripherally administered \([\text{Nle}^4, \text{D-Phe}^7]-\alpha\)-melanocyte stimulating hormone increases resting metabolic rate, while peripheral agouti-related protein has no effect, in wild type C57BL/6 and \(\text{ob}/\text{ob}\) mice

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Abstract

The melanocortin system coordinates the maintenance of energy balance via the regulation of both food intake and energy expenditure. Leptin, a key adipogenic hormone involved in the regulation of energy balance is thought to act by stimulating production, in the hypothalamic arcuate nucleus, of \(\alpha\)-melanocyte stimulating hormone (\(\alpha\)MSH), a potent agonist of MC3/4 melanocortin receptors located in the paraventricular nucleus of the hypothalamus. Additionally leptin inhibits release of agouti-related protein (AgRP), an MC4R antagonist. During periods of caloric restriction, weight loss is not sustained because compensatory mechanisms, such as reduced resting metabolic rate (RMR) are brought into play. Understanding how these compensatory systems operate may provide valuable targets for pharmaceutical therapies to support traditional dieting approaches. As circulating leptin is reduced during caloric restriction, it may mediate some of the observed compensatory responses.

In addition to decreases in circulating leptin levels, circulating AgRP is increased during fasting in rodents while \(\alpha\)MSH is decreased. As central administration of AgRP depresses metabolism, we hypothesised that the peripheral rise in AgRP might be involved in signalling the depression of RMR during food restriction. We hypothesised that changes in plasma AgRP and \(\alpha\)MSH may coordinate the regulation of changes in energy expenditure acting through central MC4 melanocortin receptors via the sympathetic nervous system.

We show here that acute peripherally administered AgRP at supra-physiological concentrations in both lean (C57BL/6) and obese leptin-deficient (\(\text{ob}/\text{ob}\)) mice does not depress RMR, possibly because it crosses the blood–brain barrier very slowly compared with other metabolites. However, \textit{in vitro} AgRP can decrease leptin secretion, by approximately 40\%, from adipocytes into culture medium and may via this axis have an effect on energy metabolism during prolonged caloric restriction. In contrast, peripheral \([\text{Nle}^4, \text{D-Phe}^7]-\alpha\)MSH produced a large and sustained increase in resting energy expenditure (0·15 ml O\(_2\)/min; \(P<0·05\)) with a similar response in leptin-deficient \(\text{ob}/\text{ob}\) mice (0·27 ml O\(_2\)/min) indicating that this effect is independent of the status of leptin production in the periphery. In both cases respiratory exchange ratio and the levels of energy expended on spontaneous physical activity were unaffected by the administration of peripheral \([\text{Nle}^4, \text{D-Phe}^7]-\alpha\)MSH. In conclusion, \(\alpha\)MSH analogues that cross the blood–brain barrier may significantly augment dietary restriction strategies by sustaining elevated RMR.

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Introduction

The primary approach to weight management is to place patients on dietary regimes that induce a caloric deficit (Gibbs \textit{et al}. 2004). When adhered to, diets generating caloric deficit induce successful weight loss, but the initial rate of weight loss is seldom maintained (Gibbs \textit{et al}. 2004). A primary reason for this reduced rate of response is because the body generates compensatory changes in levels...
of expenditure, including activity and resting metabolism, that narrow the deficit between energy intake and expenditure (Ballor 1991, Weinsier et al. 2002). Understanding the factors that regulate resting metabolic rate (RMR) and active metabolic rate are therefore important because they may provide useful targets for pharmaceuticals that could overcome the compensation, and thereby provide adjunct therapies to the traditional dietary approaches. This is particularly so if the targets are located in the periphery.

The melanocortin system plays a key role in the regulation of energy balance. Leptin secreted by adipose tissue regulates energy balance principally through its hypothalamic receptors (Ahima & Osei 2004). Although leptin receptors in the hypothalamus are known to interact with pathways involving a variety of neuropeptides, neurons originating in the arcuate nucleus (ARC) releasing α-melanocyte stimulating hormone (αMSH) in the paraventricular nucleus, form an important part of this system. The melanocortin receptors involved are thought to be primarily MC4R and MC3R. An integral part of this system appears to be an additional leptin-sensitive pathway arising in the ARC releasing agouti-related protein (AgRP), an MC3/4R antagonist. Leptin thus stimulates αMSH and inhibits AgRP in a coordinated response to reduce food intake (see Ahima et al. 2000, MacNeil et al. 2002, Zimanyi & Pelleymouther 2003).

Central nervous system (CNS) administration of αMSH or MTII (both MC4R agonists) decreases food intake and body weight (Rossi et al. 1998, Barsh 1999). Conversely, single central injections of the MC4R antagonists, AgRP, SHU9119 or HS014, stimulate food intake in rodents, and chronic CNS administration leads to obesity (Fan et al. 1997, Rossi et al. 1998, Kask et al. 1999, Raposoinho et al. 2000). Melanocortins may also have a role in the regulation of energy expenditure. CNS administration of MT11 or αMSH increases oxygen consumption (Hwa et al. 2001, Jonsson et al. 2001). Contrasting these data, the MC4R antagonist HS024 has no effect on energy expenditure (Jonsson et al. 2001), but the native antagonist AgRP has been suggested to decrease oxygen consumption, consistent with the effects of αMSH and other agonists of MC4R (Asakawa et al. 2002, Small et al. 2003).

AgRP and αMSH are also present in the systemic circulation (Penny & Thody 1978, Li et al. 2000). The function of circulating AgRP and αMSH along with the peripheral roles of melanocortins and their receptors remain to be elucidated. However, studies in rodents have shown that plasma AgRP is increased, while αMSH is decreased during fasting (Li et al. 2000, Shen et al. 2002, Hoggard et al. 2004a). Recent studies in humans have shown a similar increase in plasma AgRP on fasting (Shen et al. 2002, Hoggard et al. 2004b).

As there is an indication that central administration of AgRP depresses metabolism, we hypothesised that the peripheral rise in AgRP during starvation might be involved in signalling the depression of reduced RMR during food restriction, acting coordinately with the peripheral change in αMSH in the regulation of energy balance. For these reasons we investigated the acute effects of both [Nle⁴,d-Phe⁷]-αMSH and AgRP administered peripherally to mice on energy expenditure (resting O₂ consumption and CO₂ production). We show that acute administration of [Nle⁴,d-Phe⁷]-αMSH increases RMR independently of changes in food intake, when administered i.p. to lean C57BL/6 mice. This effect was reproduced in leptin-deficient ob/ob mice, suggesting [Nle⁴,d-Phe⁷]-αMSH acts independently of leptin. i.p. administration of AgRP did not differ in its effects from i.p. saline.

Materials and methods

Animals

Obese Aston mice (11 weeks) were drawn from colonies maintained at the Rowett Research Institute. C57BL/6 mice were purchased from Harlan UK Ltd (Bicester, Oxon, UK). Animals were maintained on a 12 h light:12 h darkness photoperiod with lights on at 0800 h. Room temperature was controlled at 20 ± 1°C and humidity was regulated to be less than 70%. Food (Biosure CRM-1 pelleted chow; Special Diets Services, Wilham, Essex, UK) and water were available freely unless stated. Following the measurements of metabolic rate all the animals were killed by CO₂ overdose, in the second half of the light phase and a blood sample was then extracted by cardiac puncture. Adipose tissue (gonadal) was dissected from both lean and obese mice then frozen immediately in liquid nitrogen.
and stored at –80 °C until extraction of RNA. All procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and received ethical approval from the University of Aberdeen Ethical Review Committee.

**Energy expenditure**

Measurements of resting metabolism were made using an open-flow indirect calorimetry apparatus as previously described (Hayes et al. 1992). Because our systems include an O2 and a CO2 analyser uniquely dedicated to each metabolic chamber, these systems permit continuous logging of O2 consumption and CO2 production at a user-defined interval – in this case every 15 s. The detailed time course of response allowed us to identify periods when the animals had elevated metabolism due to physical activity and thereby quantify not only resting metabolism but also the expenditure of energy on spontaneous physical activity. The ability to do this is extremely important because CNS administration of AgRP has been suggested to affect the levels of spontaneous physical activity (Tschop 2004). Closed system respirometers and open flow respirometry systems that involve periodic attention to several chambers running in parallel, combined with large chambers and long mixing times are prone to error in short-term measurements when animals differ in their levels of physical activity. Previous reports of effects of MTII and AgRP may therefore have overestimated the effects on ‘resting’ metabolic rates because such effects were confounded with changes in spontaneous physical activity.

We measured metabolic rate in leptin-deficient obese ob/ob (n=15, 5 per group) and lean wild type C57BL/6 mice (n=15, 5 per group) prior to (1·5 h) and after (4 h) i.p. administration of either saline, 150 µg [Nle4,d-Phe7]-αMSH (a potent analogue of αMSH) (M-8764; Sigma) or 13 µg AgRP (the natural antagonist of αMSH at melanocortin receptors) ((82–131)-NH2, 003–57; Phoenix Peptides, Belmont, CA, USA), both solubilised in saline. Mice were not fed while in the respirometry chamber. Metabolism measurements started between 0900 and 1000 h. We rejected data for one saline-injected lean C57BL/6 animal, which did not settle in the chamber sufficiently to measure a good pre-dose RMR.

Prior to measurements all the mice were weighed and had their body temperatures measured using a rectal probe. Mice (both lean and obese) were placed in the respirometry chamber at 20 °C (the temperature at which the mice were routinely housed) for 90 min to obtain a pre-injection baseline measurement. They were then removed from the chamber and injected i.p. with either [Nle4,d-Phe7]-αMSH (150 µg in saline, n=5), AgRP (13 µg in saline, n=6) or saline (200 µl, n=5) alone and within 30 s replaced in the respirometry chamber. Solutions were injected blind. Metabolism was then monitored for a further 4 h. After 4 h animals were removed from the chamber and their body weights and temperatures re-measured. Animals were then killed and dissected as described above.

Positive excursions of CO2 and reductions in O2 concentration in the traces connected with physical activity were very obvious. To eliminate the effects of activity on metabolism in both the pre- and post-injection phases we measured VO2 and VCO2 as the lowest consecutive 150 s of metabolism. Each animal therefore acted as its own weight-matched control. We defined the treatment effect as the difference between the pre- and post-injection measures of RMR. We used two inclusion zones for the post-injection measures of RMR: first the entire period between placing animals back into the chamber and their removal 4 h later, and second a restricted period of 6000 s commencing 4500 s after the animals were replaced in the chambers. Respiratory exchange ratio (RQ) was calculated as the minimum CO2 production divided by the minimum O2 consumption in pre- and post-injection phases. We estimated the levels of energy expended on physical activity by taking the average metabolic rate throughout the entire pre- and post-injection periods and subtracted from this the RMR. In the post-injection period we performed this analysis both including and excluding the period of elevated activity that mice exhibited for around 15–20 min after being returned to the metabolic chamber. All analyses of metabolism were performed blind of the treatment.

**Northern blotting**

RNA was extracted from gonadal adipose tissue by a guanidinium isothiocyanate–phenol method and fractionated by agarose gel electrophoresis...
It was then transferred by vacuum blotting onto a charged nylon membrane (Boehringer Mannheim), and fixed with u.v. light. 

ob mRNA was detected by a chemiluminescence procedure (Trayhurn et al. 1995), utilising a 33-mer antisense oligonucleotide probe (5'-GGTCTGAGGACGCTTTGGAGAACGC-3') end-labelled (5') with digoxigenin (Boehringer Mannheim). The oligonucleotide was synthesised commercially (R&D Systems Europe, Abingdon, Oxon, UK). Hybridisation was performed overnight at 42 °C in pre-hybridisation buffer containing the oligonucleotide probe (25 ng/ml). Post-hybridisation washes were performed as previously (Trayhurn et al. 1995), and the membranes then incubated with an anti-digoxigenin Fab/alkaline phosphatase conjugate (Boehringer Mannheim). CDP-Star (Roche) was used as the chemiluminescence substrate. Signals were detected on film and quantified by densitometry. After exposure to film (5–60 min) the membranes were stripped and re-probed for 18S rRNA using a 31-mer digoxigenin-labelled antisense oligonucleotide (10 pg/ml) (Trayhurn et al. 1995).

Cell culture

Fibroblastic preadipocytes were isolated from adipose tissue as previously described (Mitchell et al. 1997). The inguinal fat pads from 14-day-old male Hooded Lister rats (eight per group) were removed under sterile conditions. The resultant cell preparation (mainly fibroblastic preadipocytes) was adjusted to a density of 1.5 x 10^5 cells/ml in Medium 199 with 10% fetal calf serum (Gibco BRL). Cells (1.5 ml well) were plated into six-well plates. After 4 days in culture at 37 °C in an atmosphere of 5% CO₂, differentiation was induced by the addition of medium supplemented with 0.5 mM isobutylmethylxanthine (Sigma), 0.25 mM dexamethasone (Sigma) and 10 mg/ml insulin (CP Pharmaceuticals, Wrexham, UK). After 48 h, the induction medium was removed and replaced by Medium 199 containing 10% fetal calf serum supplemented with insulin (10 mg/ml) alone. This medium was changed every 2 days. Eight days after differentiation cells were incubated in medium without serum for 24 h. This was followed by a further 24 h incubation with or without AgRP in serum-free medium. Following incubation, samples were retained and centrifuged at 150 g for 10 min; the supernatant was stored at –80 °C for the assay of leptin. Cells were lysed directly on the plate for determination of protein by the Bio-Rad Protein Assay (Catalogue No. 500–0006; Bio-Rad).

Leptin ELISA

Plasma leptin was determined by an in-house chemiluminescent ELISA, as previously described (Hardie et al. 1996, Crabtree et al. 2000). Chemiluminescence was achieved by the addition of 100 µl/well CDP-Star substrate in enhancer solution (Sapphire-II enhancer in DEA buffer; 1:10) and quantified in a MLX luminometer (Dynex, Worthing, UK) in terms of relative light units. Recombinant murine leptin standards (NIBSC, Potters Bar, UK) were run in triplicate and appropriate blanks of culture medium were included. Results were expressed as a percentage of the control after correction for minor differences in protein levels in the wells. Each incubation was carried out in six individual wells.

Statistics

We explored the effects of treatment on the change in metabolism using one-way ANOVA and probed the pairwise differences using Tukey’s test. Other between-group comparisons were made using unpaired Student's t-test. Results were considered statistically significant if the P value was less than 0.05.

Results

Effect of AgRP and [Nle⁴,D-Phe⁷]-αMSH administered acutely to lean C57BL/6 mice on O₂ consumption and CO₂ production

Mice were randomly allocated to the treatment groups. There were no significant differences in body mass or pre-injection RMR between the treatment groups. A typical time course of O₂ consumption for a single individual mouse injected with saline is shown in Fig. 1. This plot illustrates several features that were apparent in the traces of all animals. During the initial phase after being introduced to the chamber all the mice exhibited exploratory behaviour and their levels of O₂ consumption remained elevated for around 50–60 min (Fig. 1 point B). They then settled and
during the final 30 min (approximately) were generally observed to be asleep (Fig. 1 point C). Positive excursions of O₂ consumption for brief periods coinciding with periods of activity were evident on the recordings (Fig. 1 point D). Following injection of the compounds the mice exhibited a second increase in O₂ consumption coinciding with elevated activity on return to the chambers. Following this period, which lasted 20–30 min, the mice again settled down and positive excursions of the traces due to activity were again evident (Fig. 1 point H). The pattern of change in the baseline resting O₂ consumption underlying these changes due to activity were very different between the three treatments. The mean O₂ consumption averaged across all individuals in each treatment group is illustrated in Fig. 2A (saline), B (AgRP) and C ([Nle⁴,d-Phe⁷]-αMSH). In these plots, periods when all mice were active or had elevated resting metabolism show as positive excursions while individual bouts of activity that were not coincident across individuals are damped by the average. In the pre-injection phase all three plots appear identical as would be expected since the groups were matched for body mass and at this point their treatments did not differ. In the saline- (Fig. 2A) and AgRP-injected (Fig. 2B) mice the metabolic rate increased following return to the chamber after injection but then settled to a level where the minimal O₂ consumption had declined slightly relative to that measured before injection. In the [Nle⁴,d-Phe⁷]-αMSH-treated mice there was a similar increase in O₂ consumption immediately after injection, following which the O₂ consumption returned to a level almost as low as the pre-injection O₂ consumption for a period of about 30 min (Fig. 2C, point A). However, following this decline there was a sustained increase in O₂ consumption (Fig. 2C, range B) that lasted at least 3–4 h. Direct observations of the mice confirmed that the mice were asleep and inactive during most of the post-injection phase, and this increase in metabolic rate was not therefore due to elevated physical activity.

Using the criterion of minimal resting O₂ consumption measured over a continuous period of 150 s over the entire post-injection period, there was a significant effect of saline, AgRP and [Nle⁴,d-Phe⁷]-αMSH on resting O₂ consumption (ANOVA: F₂,11=11·28, P<0·002; Fig. 3A). In saline- and AgRP-treated lean mice the mean O₂ consumption across individuals declined between pre- and post-injection periods by −0·14 and −0·11 ml O₂/min respectively, while in [Nle⁴,d-Phe⁷]-αMSH-treated mice O₂ consumption was significantly increased (0·15 ml O₂/min). Because this analysis isolates the minimal metabolic rate during the entire post-injection period, the period isolated for [Nle⁴,d-Phe⁷]-αMSH-treated mice inevitably focused on the drop in O₂ consumption to a minimum, marked A on Fig. 2C, that occurred immediately following the post-injection increase. If the analysis was restricted to the period spanning 6000 s, commencing 4500 s after injection the effects of [Nle⁴,d-Phe⁷]-αMSH were much more profound (Fig. 3B).
during this restricted period the mice injected with saline had reduced their O₂ consumption relative to pre-injection levels by –0·077 ml/min, in AgRP-treated mice the decline was –0·087 ml/min, but in [Nle⁴,D-Phe⁷]-αMSH-treated mice the change was an increase of 0·211 ml/min. The treatment effect was highly significant (F₂,13=15·81, P<0·001) and the increase in the [Nle⁴,D-Phe⁷]-αMSH-treated mice was significantly greater than both AgRP- and saline-treated mice (Tukey pairwise comparisons P<0·05 in both cases).

RQ in the post-injection phase was not significantly (P>0·05) affected by treatment (Fig. 4). Levels of energy expended on spontaneous physical activity were also not significantly different either prior to injection (P>0·05) or during the post-injection phase (P>0·05) whether the immediate post-injection activity period was included or not.

In summary [Nle⁴,D-Phe⁷]-αMSH significantly increased resting O₂ consumption when compared with the effect of saline or AgRP (Tukey, P<0·05). The effect of AgRP on O₂ consumption was not significantly different from that of the saline control.

**Effect of AgRP and [Nle⁴,D-Phe⁷]-αMSH administered acutely to ob/ob mice on O₂ consumption and CO₂ production**

To determine if the effect of peripheral [Nle⁴,D-Phe⁷]-αMSH on O₂ consumption and CO₂ production was independent of leptin status, either saline, AgRP or [Nle⁴,D-Phe⁷]-αMSH was administered i.p. in obese (ob/ob) mice using the same protocol we used for lean mice.

The pattern of response in obese mice was similar to that in lean wild type C57BL/6 mice. There was a significant overall effect of saline, AgRP or [Nle⁴,D-Phe⁷]-αMSH was administered i.p. in obese (ob/ob) mice using the same protocol we used for lean mice.

The pattern of response in obese mice was similar to that in lean wild type C57BL/6 mice. There was a significant overall effect of saline, AgRP or [Nle⁴,D-Phe⁷]-αMSH on the change in resting O₂ consumption between before and after injection (ANOVA: F₂,12=5·67, P=0·018; Fig. 5). In saline- and AgRP-injected ob/ob mice, O₂ consumption declined between pre- and post-injection periods by –0·106 and –0·238 ml O₂/min respectively, but in [Nle⁴,D-Phe⁷]-αMSH-injected ob/ob mice, O₂ consumption increased (0·274 ml O₂/min) (Fig. 5). As with lean C57BL/6 mice, RQ was unaffected (Fig. 4) and the levels of energy expended on spontaneous physical

**Figure 2** Time courses of oxygen consumption averaged across five C57BL/6 mice in each group injected with either (A) saline, (B) AgRP or (C) [Nle⁴,D-Phe⁷]-αMSH. In saline- and AgRP-injected mice there was no difference between the pre-injection and post-injection levels of resting metabolism; however, in the [Nle⁴,D-Phe⁷]-αMSH-treated mice (panel C) the post-injection elevation of metabolism was followed by a brief nadir (point A) followed by a sustained elevation of metabolic rate (point B).
activity were also unaffected by the treatment. [Nle\textsuperscript{4},d-Phe\textsuperscript{7}]\textalpha{MSH} significantly increased \textit{O}\textsubscript{2} consumption when compared with the effect of AgRP in obese \textit{ob/ob} mice (Tukey, \(P<0.05\)), but not when compared the effect of saline in these mice. The effect of AgRP in decreasing \textit{O}\textsubscript{2} consumption was not significantly different from that of the saline control.

**Figure 3** Change in the average minimum oxygen consumption\(_{\pm SE.M.}\), defined as the lowest oxygen consumption observed over a continuous 150 s period, between the pre-injection period and the post-injection period of recording for C57BL/6 mice (\(n=5\) per group). In (A) the difference is relative to the minimum oxygen consumption observed over the entire period after injection. For mice injected with [Nle\textsuperscript{4},d-Phe\textsuperscript{7}]\textalpha{MSH} this minimum in the post-injection period was co-incident with the drop in metabolism following the post-injection activity (refer to Fig. 2C, point A). In (B) the difference between pre- and post-injection periods refers to the minimum in the post-injection period that was observed during a portion of the post-injection period starting 4500 s after injection and lasting for 6000 s.
Although \( ob/ob \) mice do not produce functional leptin protein they do express elevated levels of leptin mRNA in adipose tissue (Zhang et al. 1994). There was no significant effect of administration of either AgRP or \([\text{Nle}^4, \text{d-Phe}^7]-\alpha\text{MSH}\) on leptin mRNA expression in gonadal white adipose tissue, extracted from the mice at the end of the experiments (Fig. 6).

![Graph showing mean RQ±S.E.M. recorded prior to and following i.p. injection with saline, AgRP and \([\text{Nle}^4, \text{d-Phe}^7]-\alpha\text{MSH}\) in C57BL/6 mice and \( ob/ob \) mice (\( n=5 \) per group). RQ tended to decline between the pre- and post-injection measurements but this difference was not significant in either group. There were no treatment effects on RQ or change in RQ between pre- and post-injection periods.](image1)

**Figure 4**

![Graph showing change in the average minimum oxygen consumption±S.E.M. defined as the lowest oxygen consumption observed over a continuous 150 s period, between the pre-injection period and the post-injection period of recording averaged across five \( ob/ob \) mice in each treatment group.](image2)

**Figure 5**
Effect of AgRP on leptin secretion into primary adipocyte culture medium

The effect of AgRP on leptin secretion into the medium was determined on differentiated primary culture of rodent adipocytes (Fig. 7). Cells were incubated with AgRP for 24 h in serum-free medium and compared with control (serum-free medium only). AgRP at both 200 and 500 nM decreased leptin secretion, approximately by 40% at the higher dose, into the culture medium compared with the control adipocytes (\(P<0.05\)). Similar results were obtained in two further experiments.

**Discussion**

It has recently been shown that circulating AgRP is increased while αMSH is decreased during fasting in rodents (Li et al. 2000, Shen et al. 2002, Hoggard et al. 2004a). The function of the change in these hormones peripherally in response to acute energy deficits is currently unknown. We hypothesised that these changes may coordinate changes in energy expenditure acting through central melanocortin receptors. MTII, a potent analogue of αMSH, can increase energy expenditure when administered peripherally (Hamilton & Doods 2002, Pierroz et al. 2002, Bluher et al. 2004). However, the respirometry systems employed to monitor such effects cannot easily resolve changes in spontaneous activity from changes in resting metabolism, which is important because AgRP has been implicated recently as an inhibitor of spontaneous activity levels (Tschoop 2004). Here we show in agreement with the MTII studies that acute administration of [Nle\(^4\), d-Phe\(^7\)]-αMSH, another potent analogue of αMSH, does increase resting energy expenditure, as opposed to changes in spontaneous activity levels, independently of changes in food intake when administered peripherally to lean wild type C57BL/6 mice. The effect can be reproduced in leptin-deficient obese ob/ob mice suggesting [Nle\(^4\), d-Phe\(^7\)]-αMSH acts independently of leptin. This supports an effect of αMSH independent of any action of leptin, but cannot rule out an additional effect of αMSH via the leptin axis.

In contrast to the effects of αMSH, we found no effect of AgRP when administered peripherally at supra-physiological concentrations on either resting energy expenditure or the levels of energy expended on spontaneous physical activity in both lean wild type C57BL/6 mice and obese ob/ob mice.

The melanocortins play a role not only in the control of food intake, but are also able to alter energy expenditure and appear to mediate some of the effects of leptin on energy homeostasis. In particular, melanocortins appear to have the ability centrally to alter energy balance by activating sympathetic nervous system-induced thermogenic mechanisms as well as by affecting food intake (Ahima et al. 2000, MacNeil et al. 2002, Zimanyi & Pelleymounter 2003). Central administration of melanocortins has been shown to activate the sympathetic nervous system in renal and lumbar
beds as well as brown adipose tissue. These effects can be inhibited by SHU9119, arguing for the involvement of the MC3/4R receptors in sympathetic nervous system activation (Haynes et al. 1999). The increase in energy expenditure as a result of acute administration of [Nle\textsuperscript{4},\textit{d}-Phe\textsuperscript{7}]-\textalpha\textit{MSH} may therefore be the result of \textalpha\textit{MSH} crossing the blood–brain barrier stimulating the sympathetic nervous system via MC3R and MC4R in the hypothalamus. However, a peripheral role for \textalpha\textit{MSH} cannot be ruled out, and melanocortin receptors have been reported in a number of peripheral tissues (Zimanyi & Pelleymounter 2003). In particular, several melanocortin receptor subtypes are present in adipose tissue (MCR 1, 2, 4 and 5) (Boston & Cone 1996, Chagnon et al. 1997). In addition, \textalpha\textit{MSH} is known to be a potent lipolytic agent in a number of species (Kastin et al. 1975, Forbes et al. 2001) and recently we have shown that \textalpha\textit{MSH} also inhibits leptin expression in differentiated adipocytes (Hoggard et al. 2004\textit{a}). It has been suggested that this may form part of a more complicated feedback mechanism regulating energy balance (Hoggard et al. 2004\textit{a}). Although AgRP did not appear to have a direct effect on energy expenditure when administered peripherally to mice, the increase in AgRP during fasting may also act via the melanocortin receptors on adipose tissue to regulate leptin expression and indirectly regulate energy metabolism. Consistent with this hypothesis, AgRP in primary cultures of rat adipocytes in serum-free medium can reduce the secretion of leptin into the culture medium, which may in turn have an effect on energy metabolism. The physiological function of AgRP on leptin expression remains to be elucidated, particularly as this effect is only seen in serum-free medium and we could not show a significant decrease in leptin gene expression from adipose tissue when AgRP was administered peripherally to ob/ob mice, although a similar trend was evident. However, the time delay of 4 h between administration of the compound and excision of the tissues may be important in this context. A recent publication has suggested that there is a complex interaction between AgRP and leptin expressed form the adrenal, testis and fat, which is tissue-dependent, strain-dependent and regulated by the feeding status of the mouse (Charbonneau et al. 2004). We found no effect of AgRP administered peripherally to lean CL57BL/6 mice on leptin gene expression in adipose tissue (data not shown).

In conclusion, these data suggest that peripherally administered AgRP at supra-physiological concentrations does not depress resting O\textsubscript{2} consumption, possibly because it fails to cross the blood–brain barrier in sufficient quantities. AgRP has previously been shown to cross the blood–brain barrier, but very slowly compared with other metabolites (Kastin et al. 2000). However, in \textit{vitro} AgRP can decrease leptin secretion into adipocyte cell culture medium and may via this axis have an effect on energy metabolism during caloric restriction. In contrast, peripheral [Nle\textsuperscript{4},\textit{d}-Phe\textsuperscript{7}]-\textalpha\textit{MSH} produced a large and sustained increase in resting energy expenditure independently of the status of leptin production in the periphery. This effect suggests that \textalpha\textit{MSH} analogues that cross the blood–brain barrier may significantly augment dietary restriction strategies by sustaining elevated RMR.

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