Peripherally administered [Nle⁴,D-Phe⁷]- α -melanocyte stimulating hormone increases resting metabolic rate, while peripheral agouti-related protein has no effect, in wild type C57BL/6 and *ob/ob* mice

N Hoggard¹, D V Rayner¹, S L Johnston¹ and J R Speakman^{1,2}

¹Division of Energy Balance and Obesity, Rowett Research Institute, Aberdeen Centre for Energy Regulation and Obesity (ACERO), Aberdeen AB21 9SB, UK

²ACERO, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

(Requests for offprints should be addressed to N Hoggard; Email: nh@rowett.ac.uk)

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 α -melanocyte stimulating hormone (α 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 α 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 α 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 (*ob/ob*) mice does not depress RMR, possibly because it crosses the blood–brain barrier very slowly compared with other metabolites. However, *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 [Nle⁴,D-Phe⁷]- α MSH produced a large and sustained increase in resting energy expenditure (0.15 ml O₂/min; $P < 0.05$) with a similar response in leptin-deficient *ob/ob* mice (0.27 ml O₂/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 [Nle⁴,D-Phe⁷]- α MSH. In conclusion, α MSH analogues that cross the blood–brain barrier may significantly augment dietary restriction strategies by sustaining elevated RMR.

Journal of Molecular Endocrinology (2004) **33**, 693–703

Introduction

The primary approach to weight management is to place patients on dietary regimes that induce a caloric deficit (Gibbs *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 *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 (α MSH) 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 α MSH and inhibits AgRP in a coordinated response to reduce food intake (see Ahima *et al.* 2000, MacNeil *et al.* 2002, Zimanyi & Pelleymounter 2003).

Central nervous system (CNS) administration of α MSH 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, Raposinho *et al.* 2000). Melanocortins may also have a role in the regulation of energy expenditure. CNS administration of MT11 or α MSH 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 α MSH and other agonists of MC4R (Asakawa *et al.* 2002, Small *et al.* 2003).

AgRP and α MSH are also present in the systemic circulation (Penny & Thody 1978, Li *et al.*

2000). The function of circulating AgRP and α MSH 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 α MSH 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 α MSH in the regulation of energy balance. For these reasons we investigated the acute effects of both [Nle⁴,D-Phe⁷]- α MSH and AgRP administered peripherally to mice on energy expenditure (resting O₂ consumption and CO₂ production). We show that acute administration of [Nle⁴,D-Phe⁷]- α MSH 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]- α MSH 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 O_2 and a CO_2 analyser uniquely dedicated to each metabolic chamber, these systems permit continuous logging of O_2 consumption and CO_2 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]- α MSH (a potent analogue of α MSH) (M-8764; Sigma) or 13 μg AgRP (the natural antagonist of α MSH 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]- α MSH (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 CO_2 and reductions in O_2 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 VO_2 and VCO_2 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 CO_2 production divided by the minimum O_2 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

(Trayhurn *et al.* 1995). 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'-GGTCTGAGGCAGGGAGCAGCTCTTGGAGAAGGC-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 1.5×10^5 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*-tests. Results were considered statistically significant if the *P* value was less than 0.05.

Results

Effect of AgRP and [Nle⁴,D-Phe⁷]- α MSH 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

Figure 1 Typical time course of oxygen consumption observed in a C57BL/6 mouse during an experimental run to illustrate typical features of the change in metabolism over time. The animal is placed in the chamber at point A. The oxygen consumption increases at point B, reflecting activity of the animal in the chamber. The animal eventually falls asleep (point C), although during the decline in metabolism there are still occasional excursions reflecting renewed activity (e.g. point D). The pre-injection RMR is indicated by the level E. At F the animal is removed from the chamber, rapidly injected i.p. with the treatment compound (in this case saline) and returned immediately into the chamber. As this is performed within a period of about 10 s, during which the chamber is not ventilated, the analyser does not register a decline in the oxygen consumption. There is a second bout of activity (G) following return to the chamber but the animal settles down more quickly in this case. Occasional elevations in metabolism due to activity are still observed in the post-injection phase (H) and the animal is finally removed at point I. The post-injection level of resting metabolism is indicated by the level J. Each measurement is 15 s apart.

during the final 30 min (approximately) were generally observed to be asleep (Fig. 1 point C). Positive excursions of O_2 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 O_2 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 O_2 consumption underlying these changes due to activity were very different between the three treatments. The mean

O_2 consumption averaged across all individuals in each treatment group is illustrated in Fig. 2A (saline), B (AgRP) and C ($[Nle^4, D-Phe^7]-\alpha$ MSH). 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 O_2 consumption had declined slightly relative to that measured before injection. In the $[Nle^4, D-Phe^7]-\alpha$ MSH-treated mice there was a similar increase in O_2 consumption immediately after injection, following which the O_2 consumption returned to a level almost as low as the pre-injection O_2 consumption for a period of about 30 min (Fig. 2C, point A). However, following this decline there was a sustained increase in O_2 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 O_2 consumption measured over a continuous period of 150 s over the entire post-injection period, there was a significant effect of saline, AgRP and $[Nle^4, D-Phe^7]-\alpha$ MSH on resting O_2 consumption (ANOVA: $F_{2,11}=11.28$, $P<0.002$; Fig. 3A). In saline- and AgRP-treated lean mice the mean O_2 consumption across individuals declined between pre- and post-injection periods by -0.14 and -0.11 ml O_2 /min respectively, while in $[Nle^4, D-Phe^7]-\alpha$ MSH-treated mice O_2 consumption was significantly increased (0.15 ml O_2 /min). Because this analysis isolates the minimal metabolic rate during the entire post-injection period, the period isolated for $[Nle^4, D-Phe^7]-\alpha$ MSH-treated mice inevitably focused on the drop in O_2 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 $[Nle^4, D-Phe^7]-\alpha$ MSH were much more profound (Fig. 3B). On average

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) $[Nle^4,D-Phe^7]-\alpha$ MSH. In saline- and AgRP-injected mice there was no difference between the pre-injection and post-injection levels of resting metabolism; however, in the $[Nle^4,D-Phe^7]-\alpha$ MSH-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).

during this restricted period the mice injected with saline had reduced their O_2 consumption relative to pre-injection levels by -0.077 ml/min, in AgRP-treated mice the decline was -0.087 ml/min, but in $[Nle^4,D-Phe^7]-\alpha$ MSH-treated mice the change was an increase of 0.211 ml/min. The treatment effect was highly significant ($F_{2,13}=15.81$, $P<0.001$) and the increase in the $[Nle^4,D-Phe^7]-\alpha$ MSH-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 $[Nle^4,D-Phe^7]-\alpha$ MSH significantly increased resting O_2 consumption when compared with the effect of saline or AgRP (Tukey, $P<0.05$). The effect of AgRP on O_2 consumption was not significantly different from that of the saline control.

Effect of AgRP and $[Nle^4,D-Phe^7]-\alpha$ MSH administered acutely to *ob/ob* mice on O_2 consumption and CO_2 production

To determine if the effect of peripheral $[Nle^4,D-Phe^7]-\alpha$ MSH on O_2 consumption and CO_2 production was independent of leptin status, either saline, AgRP or $[Nle^4,D-Phe^7]-\alpha$ MSH 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 and $[Nle^4,D-Phe^7]-\alpha$ MSH on the change in resting O_2 consumption between before and after injection (ANOVA: $F_{2,12}=5.67$, $P=0.018$; Fig. 5). In saline- and AgRP-injected *ob/ob* mice, O_2 consumption declined between pre- and post-injection periods by -0.106 and -0.238 ml O_2 /min respectively, but in $[Nle^4,D-Phe^7]-\alpha$ MSH-injected *ob/ob* mice, O_2 consumption increased (0.274 ml O_2 /min) (Fig. 5). As with lean C57BL/6 mice, RQ was unaffected (Fig. 4) and the levels of energy expended on spontaneous physical

A

B

Figure 3 Change in the average minimum oxygen consumption $\pm$ 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 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 [Nle⁴,D-Phe⁷]- α 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.

activity were also unaffected by the treatment. [Nle⁴,D-Phe⁷]- α MSH significantly increased O_2 consumption when compared with the effect of AgRP in obese *ob/ob* mice (Tukey, $P<0.05$), but not

when compared the effect of saline in these mice. The effect of AgRP in decreasing O_2 consumption was not significantly different from that of the saline control.

Figure 4 Mean $RQ \pm S.E.M.$ recorded prior to and following i.p. injection with saline, AgRP and $[Nle^4, D-Phe^7]-\alpha$ 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.

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 $[Nle^4, D-Phe^7]-\alpha$ MSH on leptin mRNA expression in gonadal white adipose tissue, extracted from the mice at the end of the experiments (Fig. 6).

Figure 5 Change in the average minimum oxygen consumption $\pm S.E.M.$, 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.

Figure 6 Effect of saline, AgRP or [Nle⁴,D-Phe⁷]- α MSH on leptin gene expression as determined by Northern blotting, when administered i.p. into obese leptin-deficient *ob/ob* mice after 4 h. $n=5$ per group expressed as a percentage of the control $\pm$ S.E.M.

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.

Figure 7 Addition of AgRP at 200 and 500 nM in serum-free medium to primary cultures of differentiated adipocytes, which were incubated in the absence of serum for 24 h prior to treatment, for 24 h significantly ($P<0.05$) inhibits leptin protein secreted into the cell culture medium compared with untreated control cells, as determined by a leptin specific ELISA. $n=6$ for each point expressed as a percentage of the control $\pm$ S.E.M. * $P<0.05$.

Discussion

It has recently been shown that circulating AgRP is increased while α MSH 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 α MSH, 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 (Tschop 2004). Here we show in agreement with the MTII studies that acute administration of [Nle⁴,D-Phe⁷]- α MSH, another potent analogue of α MSH, 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 [Nle⁴,D-Phe⁷]- α MSH acts independently of leptin. This supports an effect of α MSH independent of any action of leptin, but cannot rule out an additional effect of α MSH via the leptin axis.

In contrast to the effects of α MSH, 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 & Pelkeymounter 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 [Nle⁴,D-Phe⁷]- α MSH may therefore be the result of α MSH crossing the blood–brain barrier stimulating the sympathetic nervous system via MC3R and MC4R in the hypothalamus. However, a peripheral role for α 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, α 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 α MSH also inhibits leptin expression in differentiated adipocytes (Hoggard *et al.* 2004a). It has been suggested that this may form part of a more complicated feedback mechanism regulating energy balance (Hoggard *et al.* 2004a).

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 from 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 O₂ 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 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 [Nle⁴,D-Phe⁷]- α 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 α MSH analogues that cross the blood–brain barrier may significantly augment dietary restriction strategies by sustaining elevated RMR.

Acknowledgements

This work was supported by a grant from the Scottish Executive Environment and Rural Affairs Department (SEERAD). We are grateful to Drs Paula Redman, Gillian Dalgleish and Ela Krol for assistance dissecting the animals and Shabina Bashir for technical support.

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Received 5 August 2004

Accepted 17 August 2004

Made available online as an Accepted Preprint
27 August 2004