

Unabated anorexic and enhanced thermogenic responses to melanotan II in diet-induced obese rats despite reduced melanocortin 3 and 4 receptor expression

G Li¹, Y Zhang^{1,2}, J T Wilsey^{1,2} and P J Scarpace^{1,2}

¹Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida 32610, USA

²Geriatric Research, Education and Clinical Center (182), Department of Veterans Affairs Medical Center, Gainesville, Florida 32608-1197, USA

(Requests for offprints should be addressed to P J Scarpace, Geriatric Research, Education and Clinical Center, Malcolm Randall Veterans Affairs Medical Center, Gainesville, Florida 32608-1197, USA; Email: scarpace@ufl.edu)

Abstract

The effects of the chronic activation of the central melanocortin (MC) system by melanotan II (MTII) were assessed in chow-fed (CH) and high-fat (HF) diet-induced obese (DIO) Sprague–Dawley rats. Six-day central infusion of MTII (1 nmol/day) reduced body weight and visceral adiposity compared with *ad libitum*-fed control and pair-fed groups and markedly suppressed caloric intake in both CH and DIO rats. The anorexic response to MTII was similar in DIO relative to CH rats. MTII induced a sustained increase in oxygen consumption in DIO but a delayed response in CH rats. In both diet groups, MTII reduced serum insulin and cholesterol levels compared with controls. HF feeding increased brown adipose tissue (BAT) uncoupling protein 1 (UCP1) by over twofold, and UCP1 levels were further elevated in MTII-treated CH and DIO rats. MTII lowered acetyl-CoA carboxylase

expression and prevented the reduction in muscle-type carnitine palmitoyltransferase I mRNA by pair-feeding in the muscle of DIO rats. Compared with CH controls, hypothalamic MC3 and MC4 receptor expression levels were reduced in DIO controls. This study has demonstrated that, despite reduced hypothalamic MC3/MC4 receptor expression, anorexic and thermogenic responses to MTII are unabated with an initial augmentation of energy expenditure in DIO versus CH rats. The HF-induced up-regulation of UCP1 in BAT may contribute to the immediate increase in MTII-stimulated thermogenesis in DIO rats. MTII also increased fat catabolism in the muscle of DIO rats and improved glucose and cholesterol metabolism in both groups.

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Introduction

Melanocortins (MCs) are bioactive peptides derived from a common pre-hormone, pro-opiomelanocortin (POMC). Among them, α -melanocyte stimulating hormone (α -MSH) is a major regulator of feeding and body weight via hypothalamic MC3 and 4 receptors (MC3R and MC4R) (Cone 1999). Central infusion of α -MSH or its synthetic agonists causes anorexia and weight loss (Poggioli *et al.* 1986, Fan *et al.* 1997, Grill *et al.* 1998), whereas infusion of MCR blockers or over-production of the endogenous MCR antagonist, agouti-related protein (AgRP), produces hyperphagia and obesity (Fan *et al.* 1997, Graham *et al.* 1997, Ollmann *et al.* 1997, Hagan *et al.* 1999). Knockout studies of the central MC3R and MC4R have identified these receptors as important players in energy homeostasis (Huszar *et al.* 1997, Chen *et al.* 2000). Targeted disruption of the MC4R gene leads to overfeeding and obesity (Huszar *et al.* 1997), whereas MC3R knockouts over-accumulate fat with minimal changes in

caloric intake (Chen *et al.* 2000). Deficiency in POMC also results in increased food intake and morbid obesity in both rodents and humans (Krude *et al.* 1998, Yaswen *et al.* 1999). Apparently, the central MC system has critical functions in the homeostatic regulation of body weight.

One of the hallmarks of obesity, whether it is genetic, diet induced or age related, is leptin resistance. Human obesity as well as many rodent models of obesity is accompanied by elevated serum leptin and leptin resistance, which becomes more pronounced with progressive degrees of obesity. Diet-induced obese (DIO) rodent models, characterized by hyperleptinemia and hyperinsulinemia, somewhat resemble the onset of human obesity and hence provide a valuable tool for investigating leptin resistance in humans. The nature of leptin resistance associated with DIO animals is not well understood. The blunted responsiveness to both endogenous and exogenous leptin has been, in part, attributed to defects in the blood–brain barrier transport system (peripheral resistance) (Burguera *et al.* 2000) and in leptin signal transduction in

the hypothalamic leptin-responsive neurons (central resistance) (El Haschimi *et al.* 2000, Scarpace *et al.* 2001). A growing body of evidence suggests that the MC system is located downstream of the hypothalamic leptin-signaling pathway. Leptin activates POMC- and suppresses AgRP-containing neurons of the ventrolateral and ventromedial arcuate nucleus respectively, resulting in an increase in the expression of POMC and a reduction in AgRP (Schwartz *et al.* 1996, 1997, Cheung *et al.* 1997, Baskin *et al.* 1999, Elias *et al.* 2000). It is possible that the central resistance is partially due to a failure of the leptin signal to activate POMC or suppress AgRP neurons so that the proper regulation of POMC and AgRP expression by leptin is lost. As a result, leptin-initiated MC activation is impaired. In support of this notion, α -MSH and a potent MC agonist, melanotan II (MTII), as well as central POMC gene therapy were effective in obese Zucker rats with defective leptin receptor signaling (Hwa *et al.* 2001, Cettour-Rose & Rohner-Jeanrenaud 2002, Li *et al.* 2003) and in DIO mice with leptin resistance (Pierroz *et al.* 2002). Chronic impairment in MC activation may generate hypersensitivity (or hyper-responsiveness) of the MC pathway to pharmacological MC stimulation, potentially through homeostatic up-regulation of MC3R and MC4Rs. This postulated hypersensitivity has been indicated in two studies, one demonstrating enhanced responses to MTII in obese Zucker rats (Cettour-Rose & Rohner-Jeanrenaud 2002) and another reporting an acute enhanced anorexic response to α -MSH in leptin-resistant DIO rats (Hansen *et al.* 2001). However, a recent study indicated that high-fat (HF) feeding actually decreased the anorexic effects of MTII, which could contribute to diet-induced obesity (Clegg *et al.* 2003). Since most of these previous reports assessed the acute responses to MCs, one of our aims was to determine the chronic effects of central infusion of MTII in a DIO rat model with a normal genetic background. We also examined whether diet-induced obesity alters the expression levels of hypothalamic MC3R and MC4R that may potentially mediate the differential response to MTII in DIO compared with lean animals. Furthermore, because of the scarcity of information on how α -MSH influences energy expenditure and body metabolism (in contrast to plentiful information about the effect of α -MSH and its analogs on food intake), we assessed the thermogenic response as well as fat metabolism in brown adipose tissue (BAT) and skeletal muscle following central MTII administration in both lean and DIO rats.

To this end, we examined the effects of a 6-day central administration of leptin or MTII on energy balance, BAT thermogenesis, and indicators of skeletal muscle fat metabolism in chow-fed (CH) lean and DIO Sprague-Dawley rats. Food intake, body weight, adiposity, serum hormone and metabolite levels, oxygen consumption, BAT uncoupling protein 1 (UCP1) protein, expression of acetyl-CoA carboxylase (ACC) and muscle-type carnitine palmitoyl-

transferase I (M-CPT I) in soleus muscle and MC3R and MC4R in the hypothalamus were measured.

Materials and Methods

Animals and diets

Fourteen-week-old female Sprague-Dawley rats were obtained from Taconic (Germantown, NY, USA). Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the NIH Guide to the Care and Use of Experimental Animals. Rats were housed individually with a 12 h light:12 h darkness cycle (0700–1900 h). Standard rat chow (15% fat; 3.3 kcal/g diet 2018; Harlan Teklad, Madison, WI, USA) and water were provided *ad libitum*. At the start of the experiment, 30 rats were maintained on chow (CH), whereas 70 rats were switched to an HF diet (60% fat; 5.2 kcal/g D12492; Research Diets, New Brunswick, NJ, USA) for 10 weeks. The top 40% of weight gainers on the HF diet were designated as DIO whereas the rest were removed from the study. DIO and CH animals remained on their respective diets through the conclusion of the study. At the end of the 10-week feeding period, levels of serum leptin (collected from the tail) were assessed in representative subgroups of DIO and CH animals.

Experiment 1: 6-day intracerebroventricular (i.c.v.) administration of leptin

CH or DIO rats were infused with either artificial cerebrospinal fluid (ACSF; 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.4 mM CaCl₂, 1.5 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 0.1 mg/ml bovine serum albumin) (six to seven per group) or recombinant mouse leptin (10 μ g/day, five to seven per group) into the left lateral ventricle by mini-osmotic pumps (Alzet 2001; Durect, Cupertino, CA, USA) for 6 days. Under 6 mg/kg xylazine (Phoenix Pharmaceutical, St Joseph, MO, USA) and 60 mg/kg ketamine (Monarch Pharmaceuticals, Bristol, TN, USA) anesthesia, a cannula (Brain Infusion Kit II; Durect) was stereotaxically placed into the left lateral ventricle using the following co-ordinates: 0.8 mm posterior to bregma, 1.4 mm lateral to the midsagittal suture, and to a depth of 4 mm. The brain infusion cannula was anchored to the skull using acrylic dental cement and a small stainless steel screw. A catheter was connected from the cannula to the mini-osmotic pump flow moderator. The pump was inserted into a subcutaneous pocket on the dorsal surface. Rats were kept warm until fully recovered and were provided with a highly palatable Jell-O mixture consisting of Jell-O (Kraft Foods, Rye Brook, NY, USA), Ensure Plus (Abbott Laboratories, Columbus, OH, USA), STAT (PRN pharmaceutical, Pensacola, FL, USA) and soy protein booster (Naturade; Irvine, CA, USA) at day 1 to

alleviate the anorexic effect elicited by surgical intervention. Chow or HF diet resumed from day 2, and rats were allowed access to food *ad libitum*. Food consumption and body weight were recorded for 6 days. Oxygen consumption was monitored at days 2 and 6. All rats were killed at the end of the dark cycle of day 6 and tissues harvested for analysis.

Experiment 2: 6-day *i.c.v.* administration of MTII

CH and DIO rats were infused with either ACSF (control and pair-fed groups, six to eight per group) or MTII (1 nmol/day, six to nine per group, Phoenix Pharmaceuticals, Belmont, CA, USA) into the left lateral ventricle by mini-osmotic pump (Alzet 2001) for 6 days. Rats were provided with the highly palatable Jell-O mixture at day 1, and chow or HF diet was resumed from day 2. Control and MTII-treated rats were allowed access to food *ad libitum*, whereas pair-fed rats were pair-fed to the amount of food consumed by the MTII-treated rats. Daily food consumption and body weight were recorded for 6 days. Oxygen consumption was monitored at days 2 and 6. All rats were killed at the end of the dark cycle of day 6 and tissues harvested for analysis.

Oxygen consumption

Oxygen consumption was assessed in up to four rats simultaneously with an Oxyscan analyzer (OXS-4; Omnitech Electronics, Columbus, OH, USA) as described previously (Scarpace *et al.* 1992). Flow rates were 2 liters/min with a 30-s sampling time at 5-min intervals. The rats were placed into the chamber for 90 min with the oxygen consumption values for the last 30 min (when their oxygen consumption had reached stable levels) used in the calculations. Food was not available during the measurement. Results are expressed as mass-adjusted consumption (ml/min per kg^{0.75}).

Tissue harvesting and preparation

Rats were killed by cervical dislocation under 85 mg/kg pentobarbital anesthesia. Blood samples were collected by heart puncture and serum was harvested by a 15-min centrifugation in serum separator tubes. The circulatory system was perfused with 30 ml cold saline and BAT, perirenal (PWAT) and retroperitoneal white adipose tissues (RTWAT), and a portion of the red soleus muscle were excised. The hypothalamus was removed by making an incision medial to piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus to a depth of 2–3 mm. Tissues were stored at –80 °C until analysis. For Western analysis, BAT were homogenized in 0.3 ml 10 mM Tris-HCl, pH 6.8, 2% SDS, and 0.08 µg/ml okadaic acid. Protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamide, and 2 µM leupeptin

were also present. Homogenates were immediately boiled for 2 min, cooled on ice, and stored frozen at –80 °C. Protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). BAT samples were filtered through a 0.45 µm syringe filter (Whatman, Clifton, NJ, USA) to remove lipid particles prior to protein measurements.

Serum leptin, insulin, glucose, and cholesterol

Serum leptin levels were measured using a rodent leptin ELISA kit (Crystal Chem, Chicago, IL, USA). Serum insulin levels were measured with rat insulin RIA kits (Linco Research, St Charles, MO, USA). Total cholesterol was determined by enzymatic colorimetric kits from WAKO Chemicals (Neuss, Germany). Serum glucose was assessed via a colorimetric reaction with Trinder, the Sigma diagnostics glucose reagent (St Louis, MO, USA).

BAT UCP1 protein

UCP1 levels were determined by immunoreactivity in BAT homogenates. BAT samples (20 µg) prepared as described above were separated on an SDS-PAGE gel and electrotransferred to nitrocellulose membrane. Immunoreactivity was assessed on separate membranes with an antibody specific to rat UCP1 (Linco Research). Immunoreactivity was visualized by enhanced chemiluminescent detection (Amersham Pharmacia Biotech) and quantified by video densitometry (Bio-Rad).

Relative quantitative RT-PCR

ACC and M-CPT I expression in the soleus muscle and MC3R and MC4R mRNA levels in the hypothalamus were identified by relative quantitative RT-PCR using QuantumRNA 18S internal standards kit (Ambion, Austin, TX, USA). Total cellular RNA was extracted as described (Li *et al.* 2002) and treated with RNase-free DNase (Ambion). First-strand cDNA synthesis was generated from 2 µg RNA in a 20 µl volume using random primers (Life Technologies, Rockville, MD, USA) containing 200 units M-MLV reverse transcriptase (Life Technologies). Relative quantitative PCR was performed by multiplexing corresponding primers (ACC sense 5'-GCTTGCAAACCTCGACCTCTC-3', antisense 5'-CTTGATGATGGCGTTCTTGA-3'; M-CPT I sense 5'-GCAAACCTGGACCGAGAAGAG-3', antisense 5'-CCTTGAAGAAGCGACCTTG-3'; MC3R sense 5'-AGCAACCGGAGTGGCAGT-3', antisense 5'-GGCCACGATCAAGGAGAG-3'; MC4R sense 5'-AGTCTCTGGGGAAGGGGCA-3', antisense 5'-CAACTGATGATGATCCCGAC-3'), 18S primers, and competitors and coamplifying. Linearity for ACC and M-CPT I amplicons was determined to be between 26 and 36 cycles, 18 and 32 for MC3R and 24 to 44 for MC4R. The

Table 1 Body weight (BW) and serum leptin levels of CH and HF DIO rats after 10 weeks of diet. Values are means \pm s.e.

	CH	DIO
BW		
Baseline (g)	291.1 \pm 4.1	291.9 \pm 4.9
Week 10 (g)	333.6 \pm 5.5	372.4 \pm 7.0*
BW gain (g)	42.5 \pm 4.5	80.6 \pm 3.4*
Serum leptin (ng/ml)	5.32 \pm 0.47	11.34 \pm 1.50*

Thirty and 70 rats were started on the chow (3.3 kcal/g) or HF (5.2 kcal/g) diet respectively. The top 40% of weight gainers consuming the HF diet at the end of 10 weeks of feeding were designated as DIO ($n=27$). Serum leptin levels were measured in 7 CH and 10 DIO rats at end of 10 weeks of feeding.

* P at least <0.01 vs CH (unpaired t -test).

optimum ratio of 18S primer to competitor was 1:9 for ACC and MC4R, 1:6 for MC3R, and 2:15 for M-CPT I. PCR was performed at 94 °C denaturation for 60 s, 59 °C annealing temperature for 45 s, and 72 °C elongation temperature for 60 s for 34 cycles for ACC, 32 for M-CPT I, 28 for MC3R, and 40 for MC4R. The PCR product was electrophoresed on acrylamide gel and stained with SYBR green (Molecular Probes, Eugene, OR, USA). Gels were scanned using a STORM fluorescent scanner and data analyzed using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). The relative values of ACC, M-CPT I, MC3R, and MC4R mRNA were derived from dividing the signal obtained for the corresponding amplicon by that for the 18S amplicon.

Statistical analysis

Results are presented as means \pm s.e. Unpaired two-tailed Student's t -test was employed for analysis of body weight and serum leptin after 10 weeks of feeding and hypothalamic MC3R/MCR4 expression levels, as well as for the comparison of the percent baseline levels of caloric intake between MTH-treated CH and DIO rats at day 2. For all other data, one-way or two-way ANOVA was performed. When the main effect was significant, a post hoc test (either Tukey or Bonferroni multiple comparison with the error rate corrected for the number of contrasts) was applied to determine individual differences between means. A value of $P < 0.05$ was considered significant.

Results

Body weight and serum leptin levels in CH and DIO rats after 10 weeks of diet

At the beginning of the HF feeding period, the average body weight of CH and DIO rats were very similar (Table 1). By the end of the 10 weeks of HF feeding, DIO animals had gained almost twice as much weight and were nearly 40 g heavier than CH animals (Table 1). Serum

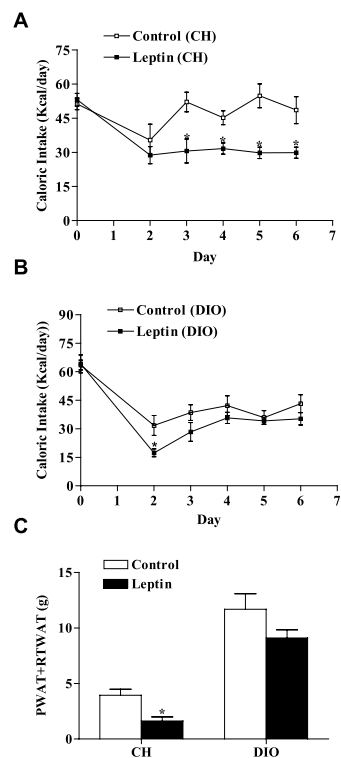


Figure 1 (A and B) Daily caloric intake and (C) visceral adiposity following 6 days of i.c.v. administration of vehicle or leptin (10 μ g/day) in CH and DIO rats. The vehicle was ACSF. Values are means \pm s.e. of five to seven animals per group. Visceral adiposity levels are represented by the sum of PWAT+RTWAT. * P at least <0.05 vs corresponding controls.

leptin levels were elevated in DIO rats by more than twofold compared with CH rats.

Experiment 1: effects of 6-day i.c.v. leptin infusion on food intake and visceral adipose tissues in CH and DIO rats

To test whether the DIO rats were leptin resistant, we examined the responsiveness to central leptin infusion (10 μ g/day) in CH and DIO rats for 6 days. Compared with vehicle solution (ACSF), leptin induced a sustained suppression in food consumption in CH rats, starting at day 3 and persisting throughout the 6-day experimental period (Fig. 1A). In contrast, only a transient anorexic response to leptin was observed (day 2) in DIO rats (Fig. 1B).

The fat-reducing effect of leptin was also investigated in two major visceral fat depots, PWAT and RTWAT. The sum of PWAT and RTWAT of DIO control rats was threefold higher than that of CH controls (Fig. 1C). This result and the significant elevation of serum leptin levels in the obese rats indicated a dramatic increase in visceral adiposity and presumably total adiposity levels in DIO animals following HF feeding. The 6-day leptin infusion reduced the sum of PWAT and RTWAT as expected by

59% in CH rats compared with controls (Fig. 1C). On the contrary, visceral adiposity levels decreased only slightly in DIO rats given leptin (Fig. 1C).

Experiment 2: 6-day *i.c.v.* MTII infusion in CH and DIO rats

Food consumption and body weight To determine if the DIO rats with leptin resistance are responsive to central MC activation, the MC agonist MTII (1 nmol/day) or ACSF was infused into the lateral ventricle for 6 days in CH or DIO rats. MTII induced a significant reduction in caloric intake in CH rats compared with the *ad libitum*-fed control animals (Fig. 2A). Specifically, the MTII-treated CH rats ate only 32% of calories consumed by control rats at day 2, and 66% at day 6. Despite the fact that DIO animals were more susceptible to the surgical intervention compared with CH ones in term of food intake (Fig. 2A and B), a phenomenon also observed in experiment 1 (Fig. 1A and B), MTII still effectively suppressed caloric intake in DIO rats to a magnitude similar to that in CH rats (Fig. 2B): MTII administration reduced caloric intake by 84% in DIO rats at day 2 and 31% at day 6 when compared with DIO controls.

In addition to marked anorexia, MTII also reduced body weight significantly in both CH and DIO rats (Fig. 2C). MTII-treated CH rats weighed 7.9% less at day 6 compared with *ad libitum*-fed CH controls (Fig. 2C). In DIO rats, the average body mass decreased by 6.5% at day 6 compared with *ad libitum*-fed DIO controls (Fig. 2C). Pair-feeding in this study revealed that the MTII-induced weight loss was not merely caused by the suppression in food intake. Even though both pair-fed CH and DIO groups lost more body weight than their respective *ad libitum*-fed counterparts at the end of the experiment, the MTII-treated rats on both CH and HF diets exhibited a further reduction in their body weight compared with their respective pair-fed groups (Fig. 2C).

Visceral adiposity, serum hormones, and metabolites As shown in Table 2, relative to the respective *ad libitum*-fed controls, central MTII infusion reduced visceral adiposity (represented by the sum of PWAT and RTWAT) significantly in CH (48%) and DIO (44%) rats with similar efficacy. In contrast, pair-feeding failed to produce a significant reduction in visceral fat mass in either CH or DIO rats (Table 2). Whilst circulating leptin concentrations were similar between *ad libitum*-fed control and pair-fed groups on either CH or HF diets, serum leptin decreased by 59% and 69% in MTII-treated CH and DIO rats respectively (Table 2).

MTII administration also lowered serum insulin levels in CH and DIO rats and decreased serum glucose in DIO rats while pair-feeding itself only reduced serum glucose and insulin in DIO animals. Total serum cholesterol levels were decreased by 43% and 18% in CH and DIO rats

respectively, whereas pair-feeding induced a similar effect in CH but only a tendency towards a decrease in DIO rats with respect to serum cholesterol levels (Table 2).

Energy expenditure Whole body oxygen consumption was assessed at day 2 and day 6 following MTII or ACSF infusion by indirect calorimetry in both CH and DIO animals. Oxygen consumption was elevated at day 6 (Fig. 3B) but not at day 2 (Fig. 3A) in MTII-infused CH rats compared with either *ad libitum*-fed control or pair-fed rats. In contrast, MTII induced a rapid and significant increase in oxygen consumption at day 2 in DIO rats (Fig. 3A). The decrease in food intake brought about by MTII should normally suppress energy expenditure; however, elevation of oxygen consumption occurred despite anorexia and persisted through day 6 of MTII infusion (Fig. 3B). By observation, all rats were sedentary during oxygen consumption measurements, and there were no observed differences in activity levels between groups.

Because body weight was considerably greater in the DIO compared with the CH rats, and MTII significantly reduced body weight in both CH and DIO rats, we also calculated oxygen consumption per rat rather than adjusted to lean body weight. By this calculation, there was also a significant increase in oxygen consumption with MTII compared with control or pair-fed rats (data not shown).

Induction of UCP1 in BAT is an important marker for enhanced thermogenesis or energy expenditure in rodents (Scarpace *et al.* 1997). In the present study, we examined the UCP1 protein levels following MTII treatment. Basal BAT UCP1 was greater than twofold higher in DIO controls compared with CH controls (Fig. 4), indicating that HF feeding induced UCP1 synthesis in BAT. In response to MTII administration, UCP1 levels in both CH and DIO rats increased significantly, whereas UCP1 levels in pair-fed rats remained unchanged as compared with respective *ad libitum*-fed controls (Fig. 4).

Expression of enzymes regulating fat metabolism in skeletal muscle M-CPT I and ACC are two important enzymes involved in fat metabolism in skeletal muscle. Increases in M-CPT I and decreases in ACC are consistent with enhanced fat catabolism (Jeukendrup 2002). We conducted relative quantitative RT-PCR to determine the expression levels of these two enzymes in red soleus muscle with respect to MTII treatment. The mRNA levels of either enzyme were similar among *ad libitum*-fed, MTII-treated and pair-fed CH rats. However, in DIO rats, MTII infusion prevented the dramatic reduction in M-CPT I expression by pair-feeding (Fig. 5A), and slightly but significantly reduced the mRNA levels of ACC relative to both *ad libitum*-fed controls and pair-fed rats (Fig. 5B). These data suggested that MTII induces fat catabolism in skeletal muscle.

Expression of MC3R and MC4Rs in the hypothalamus The initial enhanced anorexic and thermogenic responses to MTII in the HF-fed rats prompted us to examine the expression of MC3R and MC4R in the hypothalamus. MC3R and MC4R expression levels were determined by relative quantitative RT-PCR in CH and DIO *ad libitum*-fed control rats. Surprisingly, 10 weeks of HF feeding significantly decreased MC3R and MC4R by 18% and 26% respectively (Fig. 6).

Discussion

The present study assessed the effects of MTII, a potent MC3R/MC4R agonist, on several aspects of energy regulation in a DIO rodent model. After 10 weeks of HF feeding, 40% of the female Sprague–Dawley rats became obese and displayed leptin resistance to centrally infused leptin. However, subsequent central MTII infusion circumvented leptin resistance in these DIO rats, leading to suppressed food intake, reduced body weight, and visceral adiposity.

Although in agreement with most of the earlier reports in that genetically obese and DIO animals respond robustly to α -MSH or MTII treatment (Hansen *et al.* 2001, Cettour-Rose & Rohner-Jeanraud 2002, Pierroz *et al.* 2002), our study has provided an extension of the previous knowledge. First, despite a reduction in hypothalamic MC3R and MC4R expression levels in DIO rats, the animals responded to MTII administration with similar efficacy to that of CH rats. Moreover, an increase in the initial energy expenditure was evident as early as day 2 in DIO, but not apparent in CH rats until day 6. There is speculation that central MC receptor up-regulation might contribute to an enhanced anorexic response in DIO animals (Hansen *et al.* 2001). For instance, enhanced responses to MTII in obese Zucker rats are linked to increased hypothalamic MC4R densities (Harrold *et al.* 1999). Yet the same study also noted that MC4R densities in specific hypothalamic regions involved in energy regulation were actually diminished in DIO rats with a normal genetic background. Our data together with this report seem to argue against the homeostatic MC3R/MC4R up-regulation theory.

The present report also suggested that an increase in energy expenditure contributes to the loss of body weight and visceral adiposity following MTII treatment in both CH and DIO rats. Central MTII infusion markedly reduced body weight and visceral adiposity in obese DIO rats compared with their respective *ad libitum*-fed or pair-fed animals. Because pair-feeding accounts for changes due to reduced food intake, our observation is suggestive of a food intake-independent component in the fat-trimming effect of MTII. The elevation of oxygen consumption at day 2 in DIO and day 6 in CH and DIO

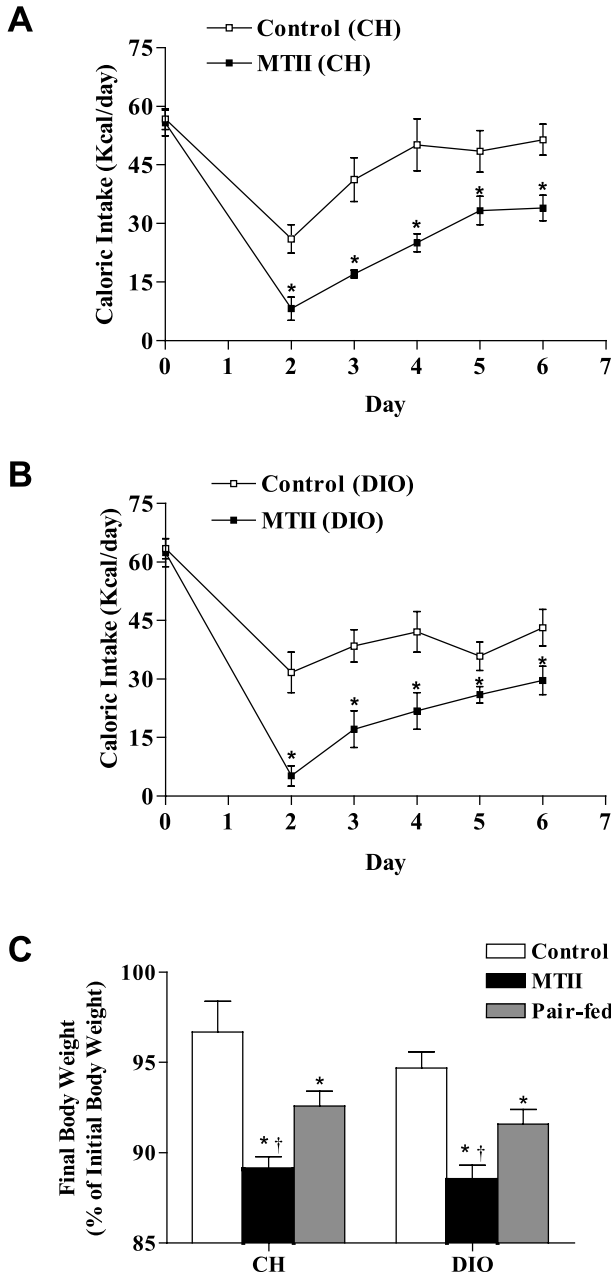


Figure 2 (A and B) Caloric intake and (C) body weight of CH and DIO rats following 6 days of i.c.v. administration of vehicle or MTII (1 nmol/day). The vehicle was ACSF. Control animals were either fed *ad libitum* (control) or pair-fed to the amount of calories consumed by the MTII-treated groups (pair-fed). The final body weight is expressed as the percentage of initial body weight prior to the start of the experiment (day 0). Values are means \pm s.e. of six to nine animals per group. During the entire experimental period, caloric intake of pair-fed and MTII-treated rats was significantly different from the controls by repeated measures ANOVA. **P* at least <0.05 vs controls and †*P* at least <0.05 vs pair-fed by post hoc Tukey's test.

Table 2 Effects of 6-day i.c.v. MTII administration on visceral adiposity, serum leptin, glucose, insulin, and total cholesterol levels in CH and DIO rats. Values were measured at the end of the 6-day experimental period and are means \pm S.E. of six to nine animals per group.

	CH			DIO		
	Control	MTII	Pair-fed	Control	MTII	Pair-fed
PWAT+RTWAT (g)	4.61 \pm 0.71	2.41 \pm 0.48*	4.47 \pm 1.25	10.91 \pm 1.45	6.12 \pm 0.92* [†]	9.91 \pm 1.37
Leptin (ng/ml)	2.63 \pm 0.47	1.09 \pm 0.24*	2.49 \pm 1.12	9.74 \pm 2.19	3.02 \pm 0.61* [†]	7.13 \pm 1.51
Glucose (mg/dl)	84.1 \pm 2.6	79.9 \pm 1.8	73.8 \pm 8.2	140.5 \pm 9.7	124.2 \pm 3.9 [†]	92.6 \pm 5.4*
Insulin (ng/ml)	3.69 \pm 0.56	1.17 \pm 0.63*	2.73 \pm 0.54	4.37 \pm 0.82	2.05 \pm 0.34*	2.48 \pm 0.45*
Cholesterol (mg/dl)	91.7 \pm 8.2	51.9 \pm 6.3*	62.2 \pm 5.1*	120.7 \pm 6.0	99.1 \pm 5.0*	109.3 \pm 3.9

MTII (1nmol/day) or vehicle (ACSF) was infused into the left lateral ventricle for 6 days. Control animals were either fed *ad libitum* (control) or pair-fed to the amount of food consumed by the MTII-treated groups (pair-fed). Visceral adiposity is represented by the sum of PWAT and RTWAT.

**P* at least <0.05 vs control.

[†]*P*<0.05 vs pair-fed by one-way ANOVA with post hoc Tukey's test.

rats during central MTII infusion further argues that an increase in energy expenditure is involved. Non-shivering thermogenesis in BAT represents an essential element in adaptive energy expenditure in rodents, and the UCP1 protein level is one indicator of the thermogenic status of BAT (Nedergaard *et al.* 2001). A previous report indicated that animals treated with MTII had elevated levels of BAT UCP1 expression (Cettour-Rose & Rohner-Jeanraud 2002). Similarly, in the present study, MTII greatly enhanced UCP1 protein levels in BAT. This substantial increase in UCP1 may well be the mediator for the elevated thermogenesis following MTII treatment. The long-term HF feeding (10 weeks) also increased basal BAT UCP1 protein levels by more than twofold. This up-regulation of basal UCP1 in BAT may serve as one explanation for the immediate increase in the MTII-induced energy expenditure (at day 2) in DIO rats.

Humans with excessive fat deposition in the body have a high risk of various obesity-related disorders such as type 2 diabetes, heart diseases, and stroke (Gasteyger & Tremblay 2002). MTII produced an impressive reduction in visceral adiposity in the present study, which was not matched by pair-feeding. Even though chronic caloric restriction has been shown to decrease visceral adiposity in rodents and humans (Barzilai *et al.* 1998, Tchernof *et al.* 2002), we did not observe a significant decrease in visceral fat mass in either CH or DIO animals pair-fed to MTII treatment. Such a discrepancy could result from the transient anorexic response to MTII. Unlike constant food restriction, pair-fed rats in our experiment were restricted to a small amount of food during the initial days of the experiment but then provided with much more food towards the later days of the treatment. This pair-feeding pattern resembles caloric restriction followed by partial refeeding. Humans and animals under this kind of feeding pattern often undergo a greater weight gain and fat repletion (Soriquer *et al.* 2003). In our case, a significant loss in visceral adiposity was likely prevented by this variable pair-feeding. In contrast to pair-feeding, MTII treatment clearly reduced visceral adiposity in both CH

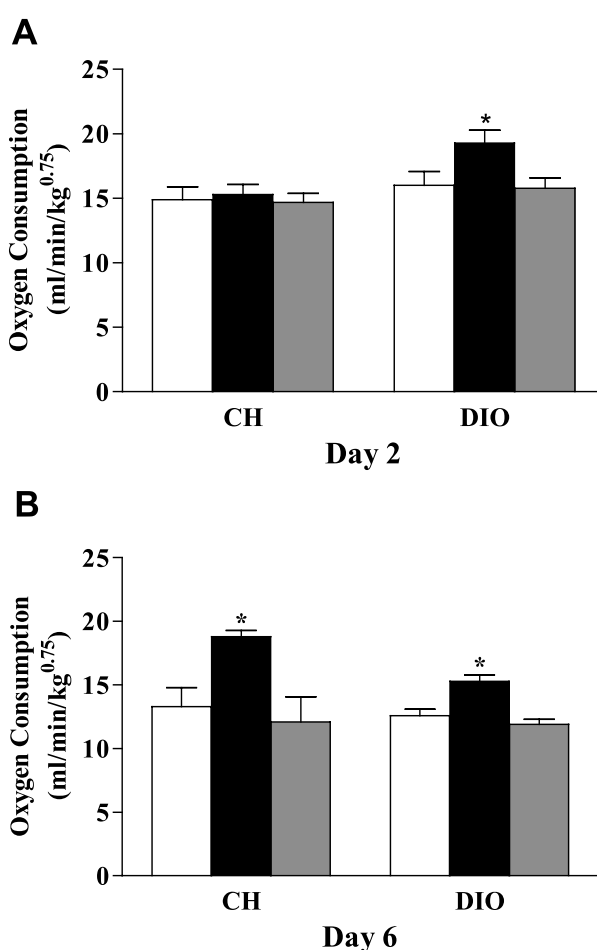


Figure 3 Energy expenditure of CH and DIO rats following 6 days of i.c.v. administration of vehicle or MTII (1 nmol/day; solid bars). Control animals were either fed *ad libitum* (control; open bars) or pair-fed to the amount of calories consumed by the MTII-treated groups (pair-fed; shaded bars). Oxygen consumption was measured at (A) day 2 and (B) day 6. Values are means \pm S.E. of six to nine animals per group. **P* at least <0.05 vs corresponding control and pair-fed groups by one-way ANOVA with post hoc Tukey's test.

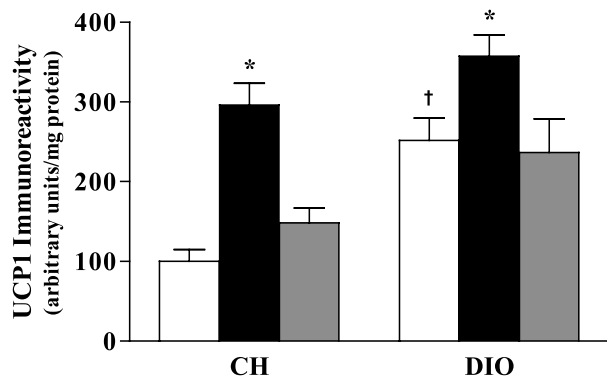


Figure 4 BAT UCP1 levels in CH and DIO rats following 6 days of i.c.v. administration of vehicle or MTII (1 nmol/day; solid bars). The vehicle used was ACSF. Control animals were either fed *ad libitum* (control; open bars) or pair-fed to the amount of calories consumed by the MTII-treated groups (pair-fed; shaded bars). Values are means \pm S.E. of six to nine animals per group. UCP1 levels are expressed in arbitrary units with levels in CH controls set to 100 and the S.E. adjusted proportionally. * P at least <0.05 vs controls and pair-fed; † P <0.01 vs CH controls by two-way ANOVA with post hoc Bonferroni test.

and DIO groups in spite of the temporal change in food intake. Therefore, the food-independent effect of MTII, presumably the increased energy expenditure as reflected by both elevated oxygen consumption and BAT UCP1, plays a crucial part in fat catabolism.

MTII has been shown to increase the expression of liver CPT I in lean and obese Zucker rats as compared with their respective pair-fed controls (Cettour-Rose & Rohner-Jeanrenaud 2002). CPT I is a key enzyme in fat catabolism that controls the transfer of long-chain fatty acyl-CoA molecules into mitochondria where they are oxidized. Another important enzyme is ACC, the rate-limiting enzyme for malonyl-CoA formation. Malonyl-CoA is an allosteric inhibitor of CPT I and, thus, a reduction in ACC is consistent with promotion of fat catabolism (Jeukendrup 2002). In our study, MTII not only prevented the decrease in muscle CPT I expression associated with pair-feeding, but also reduced ACC mRNA in skeletal muscle in DIO rats. The simultaneous changes in the expression of ACC and M-CPT I indicate an overall increase in fatty acid oxidation in skeletal muscles, implying that the increased fat catabolism in muscle could be an additional factor in mediating the fat-reducing action of MTII. Considering the relatively small amount of BAT versus the large volume of skeletal muscles in humans, the MTII-evoked muscle fat metabolism seems to offer a much more promising target for any potential clinical application.

Finally, MTII also appeared to improve glucose and cholesterol metabolism and insulin sensitivity. Central MCR activation can reduce insulin release from the pancreas and enhance glucose metabolism (Fan *et al.* 1997, Obici *et al.* 2001). However, the results in obese animal

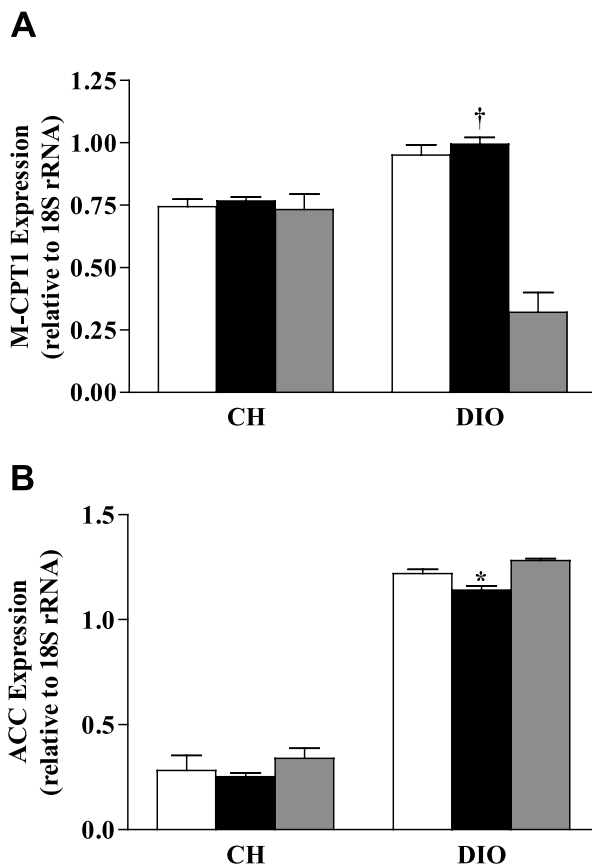


Figure 5 (A) M-CPT I and (B) ACC expression in red soleus muscle of CH and DIO rats following 6 days i.c.v. administration of vehicle or MTII (1 nmol/day; solid bars). The vehicle used was ACSF. Control animals were either fed *ad libitum* (control; open bars) or pair-fed to the amount of calories consumed by the MTII-treated groups (pair-fed; shaded bars). Values are means \pm S.E. of six to nine animals per group. Results are normalized to 18S rRNA. * P <0.05 vs corresponding control and pair-fed groups; † P <0.01 vs pair-fed by one-way ANOVA with post hoc Tukey's test.

models are controversial: one study suggested that peripheral but not central MTII improved insulin resistance in DIO mice (Pierroz *et al.* 2002), whereas another reported that 3-day peripheral MTII administration had no effect on serum insulin in obese Zucker rats (Cettour-Rose & Rohner-Jeanrenaud 2002). In our present study, central MTII infusion resulted in significant reductions in serum insulin levels in CH and DIO rats and serum glucose levels in DIO rats, suggesting improved glucose metabolism and insulin sensitivity by MTII. Besides its impact on insulin and glucose, MTII also reduced total serum cholesterol levels in CH and DIO rats. How MTII lowers cholesterol is currently unknown. It is conceivable that, in addition to the cholesterol-reducing effect of hypophagia, insulin-mediated stimulation of cholesterol synthesis diminishes following a fall in circulating insulin levels (Horton *et al.* 2002).

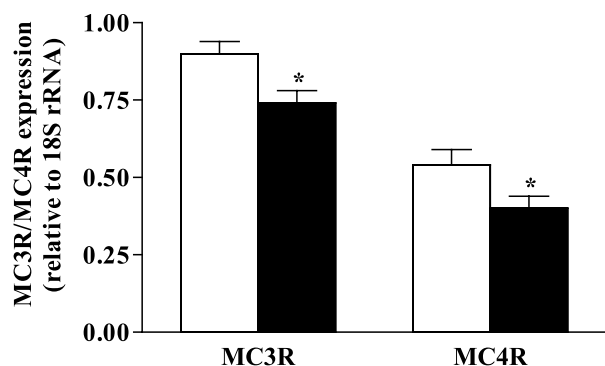


Figure 6 Hypothalamic MC3R and MC4R expression in CH (open bars) and DIO (solid bars) rats following 10 weeks of diet. Values are means \pm s.e. of six animals per group. Results are normalized to 18S rRNA. * $P < 0.05$ vs controls by unpaired *t*-test.

In summary, the present study has demonstrated that central MC activation by the MC3R/MC4R agonist MTII circumvents leptin resistance associated with DIO, resulting in a reduction in body mass and visceral adiposity. Despite reduced hypothalamic MC3R/MC4R expression, the anorexic and thermogenic responses to MTII are unabated with an even more rapid onset for the increase in energy expenditure in DIO versus CH rats. The HF-induced up-regulation of BAT UCP1 in DIO rats may account for this immediate increase in energy expenditure following central MTII infusion. Furthermore, MTII appears to increase fat catabolism in skeletal muscle, and improve glucose and cholesterol metabolism and insulin sensitivity in both CH and DIO rats. The hypophagia and/or increased energy expenditure are the likely mechanisms underlying these improvements.

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