The Effects of the Melanocortin Agonist (MT-II) on Subcutaneous and Visceral Adipose Tissue in Rodents

April D. Strader, Haifei Shi, Ryuichi Ogawa, Randy J. Seeley, Ofer Reizes

University of Cincinnati School of Medicine, Cincinnati, Ohio (A.D.S, H.S., R.O., R.J.S).
Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio (O.R.)
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B) Corresponding author:

April D. Strader Ph.D
Southern Illinois University – School of Medicine, Department of Physiology
1135 Lincoln Drive; Carbondale, Illinois 62901
Email: astrader@siumed.edu, Phone – 618-453-1533, Fax – 618-453-1527

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D) Abbreviations

Melanotan II - MT-II
High Fat Diet – HF diet
Low Fat Diet – LF diet
Diet Induced Obese – DIO
Epididymal white adipose tissue – EWAT
Inguinal white adipose tissue – IWAT

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Abstract

The melanocortin system is a critical pathway in the regulation of energy balance. In this study we analyzed the peripheral effects of the synthetic melanocortin agonist melanotan-II (MT-II) in rodents fed a low-fat diet or high-fat diet. MT-II-treated high-fat diet induced obese (DIO) mice lost weight and body fat whereas MT-II-treated low-fat fed mice maintained their original body weight. Specifically, MT-II treatment led to a general reduction in both visceral and subcutaneous adipose tissue in high-fat fed mice compared to Vehicle (ad lib) controls. Vehicle-treated pair-fed DIO mice lost an equivalent amount of body weight compared to MT-II-treated mice, but retained more adipose tissue. Pair-fed mice showed a reduction in visceral adipose tissue and no effect on subcutaneous adipose tissue compared to MT-II-treated mice. Surprisingly, subcutaneous lean mass was significantly reduced in the pair fed mice. The data were replicated in DIO rats and indicated that MT-II treatment led to a generalized reduction in adipose tissue. These results indicate that peripheral MT-II-treatment leads to weight loss that affects both the visceral and subcutaneous fat compartments. This finding illustrates the complexity of analyzing weight-reducing compounds. While the present data suggest that the anorectic effect of MT-II is primarily a consequence of reduced food intake, the body composition data suggest other mechanisms are involved.
Introduction

The melanocortin system is a critical neuronal signaling pathway for the actions of peripheral adiposity signals such as leptin and insulin (Seeley et al., 2004). Energy balance regulated by the central melanocortin system relies on a balance in signaling between the endogenous melanocortin-3 and melanocortin-4 receptor (MC3R and MC4R) antagonist agouti-related peptide (AgRP) and the agonist alpha-melanocyte stimulating hormone (α-MSH) derived from the pro-opiomelanocortin peptide (POMC). Central administration of AgRP results in long-lasting food intake in rats (Hagan et al., 2000), while both central and peripheral administration of α-MSH and the synthetic melanocortin receptor agonist Melanotan-II (MT-II) reduces food intake (Hwa et al., 2001; Obici et al., 2001; Cettour-Rose and Rohner-Jeanrenaud, 2002; Hamilton and Doods, 2002; Pierroz et al., 2002; Choi et al., 2003a; Raposinho et al., 2003; Bluher et al., 2004; Seeley et al., 2005). The vital role of the MC3R and MC4R in maintaining melanocortin tone and energy balance is evident in melanocortin-receptor knockout mice. Both the MC3R and MC4R knockout mice exhibit increased adiposity due to the lack of receptor signaling by α-MSH (Chen et al., 2000a; Chen et al., 2000b).

While it is widely accepted that peripheral administration of MT-II results in a reduction in overall body fat (Pierroz et al., 2002; Choi et al., 2003a; Seeley et al., 2005), few studies have examined reductions in specific fat compartments. For example, peripheral MT-II-treatment in rats selectively reduced visceral adipose tissue such as the retroperitoneal and epididymal fat pads (Choi et al., 2003a) but no changes in subcutaneous fat. The use of a pair-fed control group suggests that the weight-reducing effects of MT-II are primarily a consequence of reduced food intake, since pair-fed and MT-II-treated groups show similar body weights at the end of treatment (Pierroz et al., 2002). In contrast to MT-II, central administration of AgRP or the
synthetic antagonist SHU9119 results in a general increase in adipose tissue such as the epididymal, retroperitoneal and inguinal fat pads (Raposinho et al., 2000; Obici et al., 2001; Small et al., 2001; Korner et al., 2003). Even rats given central AgRP and pair-fed the same number of calories as vehicle-treated controls exhibit a specific increase in the weight of the inguinal fat pad (Korner et al., 2003).

Increased adiposity increases levels of leptin and insulin. Leptin is secreted primarily from subcutaneous fat and plasma levels correlate best with total subcutaneous adipose tissue (Montague et al., 1997; Van Harmelen et al., 1998). Unlike leptin, insulin levels are closely correlated with visceral adiposity (Pouliot et al., 1992; Ross et al., 1996). Central administration of AgRP and SHU9119 results in a disproportionate increase in leptin in ad lib fed rodents compared to AgRP and SHU9119-pair-fed rodents which may be explained by as increase in subcutaneous fat (Adage et al., 2001; Korner et al., 2003). These data indicate that melanocortin signaling can regulate peripheral adipose stores and that some of their effects may be independent of ingestion. Evidence for this shows that melanocortin receptor ligands alter oxygen consumption, body temperature, and lipolysis (Murphy et al., 2000; Hamilton and Doods, 2002; Choi et al., 2003a; Bradley et al., 2005; Song et al., 2005).

Over-consumption of a high-fat diet results in generalized increased adiposity (Woods et al., 2003) that can be partially reversed when rodents are given MT-II (Pierroz et al., 2002; Seeley et al., 2005). Melanocortin-induced (MT-II) anorexia is most pronounced when animals are obese or have been made obese following consumption of a high-fat diet compared to a low-fat diet (Hwa et al., 2001; Cettour-Rose and Rohner-Jeanrenaud, 2002; Hamilton and Doods, 2002; Pierroz et al., 2002; Bluher et al., 2004). Traditionally, it is believed that the accumulation of visceral adipose tissue poses a greater risk for co-morbid conditions such as type-2 diabetes.
and heart disease while the presence of subcutaneous adipose tissue is viewed as less harmful (Despres, 1993; Lebovitz, 2003). Therefore, an important effect of MT-II treatment or behavioral methods such as caloric restriction is the determination of what fat compartment is actually being reduced. Because weight loss induced by MT-II in rodents exposed to a high-fat diet is incomplete and never results in a level of adiposity possessed by rodents having never been on a high-fat diet, we hypothesized that the adipose tissue loss during MT-II treatment is a result of differential loss from the two main adipose compartments, visceral and subcutaneous. To test this hypothesis we implanted mice and rats with 14 day osmotic mini-pumps and examined depot-specific weight loss using nuclear magnetic resonance.

**Methods**

**Animals**

Male mice (C57Bl/J6) and male rats (Long Evans) were obtained from Harlan (Indianapolis, IN) for two separate studies. Animals were group housed when they arrived at the Laboratory Animal Facility at the University of Cincinnati until the start of each study. All animals were maintained on a 12:12 light:dark cycle and at constant temperature. All of the studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Research Diet and Treatments**

For both of the studies described below mice and rats were given *ad libitum* access to various research diets. After a prolonged period of access to the diets, rats and mice were separated into various treatment groups and individually housed for the studies. The treatment
drugs (PBS and MT-II) were generously provided by Procter & Gamble Pharmaceuticals. Melanotan-II (MT-II) is a synthetic non-selective melanocortin receptor agonist. Based on previous studies we selected a dose of MT-II that would reliably reduce food intake and body weight in animals made overweight with unlimited access to high-fat diet. We have previously determined that the adipose-reducing effects of MT-II were the same for 0.3 and 3 mg/kg/day in diet-induced obese rats (Seeley et al., 2005). Other studies examining MT-II and food intake have used similar and identical doses (Cettour-Rose and Rohner-Jeanrenaud, 2002; Hamilton and Doods, 2002; Choi et al., 2003a; Seeley et al., 2005). Specifically, the study by Hamilton and Doods administered 1 mg/kg/day of MT-II to rats given unlimited access to a cafeteria diet (Hamilton and Doods, 2002).

In the first experiment, 5-week old mice (n=51) were given either a high-fat (HF) diet (n=31; 45% lard; Research Diets) or a low-fat (LF) diet (n=20; 11% lard). The mice that were given the HF diet were divided into three groups: 1) Vehicle – PBS (phosphate buffered saline) – ad lib; (n=10), 2) MT-II (1 mg/kg/day; n=12), 3) Vehicle - PBS-pair-fed to MT-II group (n=9). The mice that were given the LF diet were separated into two groups: 1) Vehicle-PBS-ad lib; (n=8) and 2) MT-II (1mg/kg/day; n=12). Mice were placed on this diet regimen for a period of ten weeks.

In the second experiment adult rats (225-250g) were given ad libitum access to HF diet only. Rats were separated into three groups: 1) Vehicle-PBS-ad lib; (n=10), 2) MT-II (1mg/kg/day; n=10), or 3) Vehicle-PBS-pair-fed to the MT-II group (n=10).
Both mice and rats were surgically implanted with osmotic mini-pumps (Alzet) filled with PBS or MT-II (1mg/kg/day) for 14 days. The osmotic mini-pumps were placed subcutaneously between the scapulae. During the surgical procedure rats and mice were anesthetized with Isoflurane gas. The animals were shaved and cleaned for surgery and a small incision was made between the scapulae. Using a blunt instrument a tunnel was made subcutaneously for insertion of the mini-pump. The pumps were inserted and a wound clip was used to close the site of incision. Animals were removed from the isoflurane gas and placed in their home cage.

Experimental Designs

Experiment 1: Effects of MT-II on Adipose Tissue in Mice

During experiment 1 mice were placed on the HF or LF diet for 10 weeks. After ad libitum access to the HF or LF diet the mice were surgically implanted with osmotic mini-pumps filled with either Vehicle (PBS) or MT-II (1mg/kg/day). These pumps allow for constant continuous infusions for at least a 14 day period. As described above, the HF diet groups consisted of 1) ad libitum fed Vehicle (PBS)(n=10), 2) ad libitum fed MT-II (n=12)and a 3) pair-fed Vehicle (PBS) group (n=9). The pair-fed group was given the average amount of kcal consumed each day by the MT-II treated group. The LF diet groups consisted of 1) ad libitum Vehicle (PBS)(n=8) and 2) ad libitum MT-II (n=12). During the experiment, food and body weights were measured daily during the 14-day treatment period. At the end of the 14 day treatment period mice were placed into an Echo MRI NMR to measure total body fat and lean mass. Mice were then sacrificed with FetalPlus (Vortech Pharmaceuticals, Dearborn, MI; 100
mg/kg, intraperitoneally) and dissected for measurements of subcutaneous and visceral adipose and lean tissue content (as described below).

Experiment 2: Effects of MT-II on Adipose Tissue in Rats

In experiment 2 adult male rats were placed on ad libitum access to HF diet for a period of 10 weeks. After ad libitum access to the HF and LF diet the rats were surgically implanted with osmotic mini-pumps filled with either Vehicle (PBS) or MT-II (1mg/kg/day). As described above, the rat study consisted of 1) ad libitum fed Vehicle (PBS)(n=10), 2) ad libitum fed MT-II (n=10) and a 3) pair-fed Vehicle (PBS)(n=10). The pair-fed group was given the average amount of kcal consumed by the MT-II treated group at the same time each day. During the experiment, food and body weights were measured daily during the 14-day treatment period. After the two-week treatment period rats were placed in the Echo MRI NMR for body fat and lean measurements. Rats were then sacrificed and dissected for analysis of subcutaneous and visceral adipose and lean tissue content. Trunk blood was obtained by decapitation after rats were deeply anesthetized with FetalPlus (Vortech Pharmaceuticals, Dearborn, MI; 100 mg/kg, intraperitoneally) euthanasia solution. The entire epididymal fat pads (EWAT) were removed and weighed from each rat at the time of sacrifice.

Body Fat Analysis

To measure total body fat and lean mass living rats and mice were placed into a plexiglass tubular holder that was then inserted into the Echo MRI NMR (Echo Medical Systems, Houston, TX) machine. Because of the dramatic size difference between mice and rats we used a mouse and rat-specific MRI machine to measure body composition. Animals were not stressed
during this period as the measurement period lasts less than 60 seconds. Immediately after total body composition analysis, rats and mice were sacrificed and the animals were then separated into two parts to determine subcutaneous and visceral fat and lean composition. Briefly, the skin of the mouse and rat is carefully dissected away from the remaining carcass. Fat tissue that remains within the “pelt” contains the inguinal fat pads (IWAT) and any other subcutaneous fat. Fat that was on the surface of any skeletal muscle, specifically on the dorsum of the rat, was removed and placed with the pelt. The remaining carcass then contained all the internal fat pads and intra-myocellular adipose tissue. The pelt and carcass were then wrapped tightly in a piece of saran wrap and placed again into the Echo MRI machine to measure the amount of fat and lean tissue in the subcutaneous (pelt) and visceral (carcass) compartments. For completeness, the grams of fat measured by weighing the epididymal fat pads (EWAT) were added back to the final fat measurements from the carcass (rat study). The epididymal pads were not removed from mice and were analyzed with the carcass. For analysis, the amount of fat within each piece (pelt or carcass) was divided by the weight of the piece to determine percent fat within the pelt or carcass.

Assays

Trunk blood was collected during sacrifice of the rats and analyzed for glucose, insulin, and leptin. Glucose was measured in triplicate using the glucose oxidase method while insulin and leptin were measured in duplicate using RIA kits (Linco Research, St. Charles, MO). The coefficients of variation within and between the assays for leptin are 3% and 4%, for insulin are 5% and 7%. The sensitivity for leptin assay is 0.5 ng/mL and for insulin assay is 3 pmol/L.
Because sufficient trunk blood could not be obtained from the mouse study, these measurements were performed only following the rat study.

**Real-time PCR (RT-PCR) for MC4R**

RNA from rat epididymal (EWAT) and rat inguinal (IWAT) adipose tissue was isolated using TRI-Reagent (MRC Inc., Cincinnati, OH). Samples from rats given ad lib access to HF diet were used the analysis. Following DNAase treatment (Ambion, Austin, TX), cDNA was synthesized using an iScript kit (BioRad, Hercules, CA). PCR primer sets were optimized such that the correlation coefficients were 0.99-1.0 and the PCR efficiency was 90-100%. The rat MC4R PCR forward primer is 5'-ACG CGC TCC AGT ACC ATA AC (nucleotides 866-886), reverse primer is 5'-AAA GAA CGC CCG ATA CTG TG (nucleotides 958-978), product size 92 bp. Primers were selected using Primer3 web-based primer design. The sequence for which the primers were selected was from GenBank Accession #U67863. PCR was performed using a BioRad iCycler (BioRad, Hercules, CA) with 2-step amplification (95°C for 10 seconds, annealing temperature of 58.7°C for 30 seconds) for 40 cycles. Agarose gel electrophoresis (1.5%) in the presence of ethidium bromide confirmed the presence of a single band of the expected size. A negative control using water revealed no band (data not shown).

**Statistical Analysis**

All results are presented as mean ± SEM. For both studies a two-way ANOVA was utilized to assess changes in body weight and food intake over the two-week treatment period. One-way ANOVA was used to compare total, subcutaneous, visceral, and epididymal (EWAT) adipose and lean percentages and weights between the groups. A t-test was used to compare
absolute fat grams between ad lib-PBS vs. pair-fed-PBS groups as well as ad lib-PBS vs. MT-II-treated groups. Body fat percentages were compared using an unpaired-t-test for the mice on the LF diet (PBS-ad lib vs. MT-II). Plasma concentrations of leptin, insulin and glucose were also analyzed using a one-way ANOVA. Epididymal fat pad weights were compared using a one-way ANOVA. In all cases where significance was found a Tukey’s Multiple Comparison test was performed to make post hoc group comparisons. A p-value of less than 0.05 was considered significant for all tests.

Results

MT-II reduces food intake and body weight in HF-fed rodents but not LF-fed rodents

MT-II at a dose of 1 mg/kg/day was chronically administered by subcutaneous implanted osmotic mini-pumps and food intake and body weight was monitored for 14 days. HF-fed mice treated with MT-II lost significant amounts of body weight compared to the PBS (ad lib) controls (Figure 1C) (p<0.05) while MT-II-treated mice maintained on a low fat diet did not (Figure 1A). Similarly, high-fat fed PBS (pair-fed) mice also lost significant body weight (Figure1C) compared to PBS (ad lib) controls. Daily food intake for the mice that were on the high-fat diet and treated with MT-II was significantly lower compared to the mice treated with PBS (Figure 1D). The two-way ANOVA revealed a significant main effect of MT-II and also a significant interaction with time (p<0.05). Food intake was reduced initially for the first four days of treatment with MT-II and then returned to the level of the PBS (ad lib) controls (p<0.05; Figure 1D) for the remainder of the infusion period in mice. Because the final body weights of the mice given mini-pumps filled with MT-II (33.40 ± 0.24) were nearly identical to the PBS (pair-fed)
mice (33.28 ± 0.18) the reduction in body weight appears to be largely due to a reduction in food intake.

In contrast to the mice on the high-fat diet, mice that were maintained on a low-fat diet for the 10 weeks and given subcutaneous MT-II did not lose body weight at the end of the 14 day infusion period (Figure 1A). The two-way ANOVA revealed that the body weights were significantly different between the PBS (ad lib) and MT-II-treated mice on day 2 and 3 of treatment (p<0.05), however body weights were identical throughout the remainder of the two-week treatment period (Figure 1A). Daily food intake of the low-fat diet was not significantly different during any day of the 14 day treatment period (Figure 1B).

Because of the pre-existing literature and our current finding that mice fed a LF diet were less sensitive to the anorectic effects of MT-II, the second experiment with rats involved pre-exposure to a high-fat diet only. Similar to the findings in the first experiment with mice, rats that were raised on a high-fat diet for 10 weeks were extremely sensitive to the anorectic effects of MT-II (Figure 2B). The ability of MT-II to reduce food intake was more pronounced in the second experiment using rats. Daily food intake was significantly reduced in MT-II-treated rats compared to PBS (ad lib) controls for the first 11 days of the study (p<0.05; Figure 2B). Although a two-way ANOVA did not yield any significant difference in body weight over the 14 day infusion period, a student’s t-test on final body weight revealed that the rats infused with MT-II weighed less than rats infused with PBS (ad lib)(p<0.05). Similarly, the PBS (pair-fed) rats also weighed less than the PBS (ad lib) controls (p<0.05).

**MT-II reduced both Visceral and Subcutaneous Adiposity in Rodents***
An important specific aim of the present study was to identify the specific sites of action for the MT-II-induced reduction in body fat. To accomplish this we measured total body fat in both mice and rats immediately prior to sacrifice on the final day of the mini-pump treatment. First, no effect on body fat was detected in the low-fat mouse study (Table 1). However, for both mice and rats on the high-fat diet, MT-II reduced total body fat percent compared to the PBS (ad lib) group (Figure 3A and Figure 4A; p<0.05). Neither the mouse nor rat PBS (pair-fed) groups showed significant changes in total body fat (Figure 3A and Figure 4A) in comparison to the PBS (ad lib) group. The magnitude of the fat loss for the MT-II-treated groups compared to the PBS (ad lib) group was identical in both studies. Mice and rats given MT-II lost 28% of their total body fat when compared to the PBS (ad lib) group (calculated from Table 2). Pair-feeding resulted in adipose loss but the magnitude was not as pronounced as in the MT-II-treated groups, with PBS (pair-fed) mice losing 18% and rats losing 11% of their total body fat compared to PBS (ad lib) controls (calculated from Table 2). As stated above, total body fat at the end of the 14 day infusion period was not significantly different between the PBS (ad lib) and PBS (pair-fed) in either the mouse or rat study (Figure 3A and Figure 4A).

To examine the adipose reducing effects of MT-II even further the sacrificed mice and rats were carefully divided into a carcass and a pelt. The carcass contains the visceral adipose tissue (retroperitoneal, peri-renal, mesenteric, epididymal, and intra-myocellular adipose tissue), while the pelt contains the subcutaneous adipose tissue (primarily inguinal and epidermal fat). Each of these compartments was analyzed using the Echo MRI small animal body fat analyzer. No changes were detected in any fat or lean compartment in the mouse low-fat diet study when analyzed using a Student’s t-test (Table 1). In contrast, rodents that were made obese with the high-fat diet showed significant adipose compartmental changes following subcutaneous MT-II
treatment compared to PBS (ad lib) controls and these findings are outlined below. The fat mass for each compartment are presented in both graphic (percentage of final body weight) (Figure 3 and 4) and tabular (grams) (Table 2) form to highlight the magnitude and absolute amount of adipose and lean tissue loss following the MT-II treatment. The results of a one-way ANOVA revealed that MT-II reduced visceral adipose tissue in both mice and rats when compared to the PBS (ad lib) controls (Figure 3B; p<0.01 and 4B; p<0.05). Pair-feeding also significantly reduced visceral fat as well, although not as much as MT-II, and was only detected in the mouse study (Figure 3B; p<0.05). Interestingly, the amount of fat that was within the pelt (subcutaneous adipose) was consistently reduced in both mice and rats treated with MTI-II (Figure 3C and Figure 4C; p<0.05). This effect is independent of the prominent effect of MT-II on food intake since the pair-fed mice and rats showed no reductions in subcutaneous adipose tissue (Figure 3C and Figure 4C).

Because fat mass was not significantly reduced in the pair-fed cohorts an analysis of the lean mass was performed. In the mouse study, the total and compartmental lean body mass was significantly greater in the MT-II-treated group, a finding consistent with a lower level of body adiposity (p<0.01 and p<0.05; Figure 3D-F). In contrast, the PBS (pair-fed) mouse group showed a significant decrease in subcutaneous lean mass (p<0.05; Table 2) compared to the PBS (ad lib) controls. However, in the rat study, a trend (p=0.07; Table 2) but no significant difference in percentage or absolute amount of lean mass was detected in any depot (Table 2 and Figure 4D-F).

Lastly, in the rat experiment the epididymal fat pads were weighed and a significant reduction in weight was detected only in the group treated with MT-II (Figure 5; p<0.01 vs AL...
and p<0.05 vs PF). The epididymal fat pad weight in the PBS (pair-fed) or PBS (ad lib) groups were not significantly different (Figure 5).

**MT-II reduced plasma leptin, insulin and glucose more than pair-fed controls**

Because various circulating adiposity signals are a reflection of the amount of adipose tissue mass, plasma leptin, insulin, and glucose were measured in the rat study. These plasma levels were measured only in the rat study as opposed to the mouse study due to the ability to obtain a larger volume of plasma from the rats. Plasma leptin was greatly reduced (more than halved) following MT-II treatment (p<0.001) and following pair-feeding (p<0.01) compared to the PBS (ad lib) controls (Table 3). The difference in leptin between the MT-II-treated and PBS (pair-fed) rats approached significance (p=0.07; Table 3). Plasma insulin was reduced to a similar extent in both the MT-II-treated and PBS (pair-fed) rats (p<0.05; Table 3) compared to the PBS (ad lib) controls. In contrast to leptin and insulin, non-fasting plasma glucose was reduced only in the MT-II-treated rats (p<0.05; Table 3).

**Melanocortin-4 receptor mRNA within Subcutaneous and Epididymal Adipose Tissue**

The differential effect of MT-II and pair-feeding on adipose tissue is perplexing and may be indicative of peripheral effect of MT-II pharmacology. Previous data indicated that melanocortin receptors are expressed on various adipose pads, thus an analysis of MC4R transcript expression was performed. Because we observed a significant effect of MT-II on the amount of subcutaneous and epididymal adipose tissue, we questioned whether MC4R were present in the inguinal and epididymal fat pads in the rat study. We used RT-PCR and primers specific to MC4R. PCR product revealed a single peak and the ethidium bromide-stained gel for
MC4R PCR products indicated that both the cDNAs of the subcutaneous (inguinal) and the epididymal fat pads had the presence of the band of the expected size (92 bp) for MC4R gene (Figure 6).

Discussion

Weight loss through chronic administration of the melanocortin receptor agonist MT-II is readily achieved in rodents (Obici et al., 2001; Pierroz et al., 2002; Choi et al., 2003a; Choi et al., 2003b; Seeley et al., 2005). The current findings support existing data and extend the literature in many ways. First, the data corroborate previous studies in that MT-II elicits weight loss most effectively in DIO rodents (Hwa et al., 2001; Cettour-Rose and Rohner-Jeanrenaud, 2002; Pierroz et al., 2002; Seeley et al., 2005). In contrast, rodents raised on low-fat diets are less sensitive to peripheral MT-II (Cettour-Rose and Rohner-Jeanrenaud, 2002; Pierroz et al., 2002). One of the primary mechanisms for MT-II-induced weight loss is reduced food intake. A critical point in understanding the mechanism for weight loss maintenance during chronic MT-II delivery is that once a new adiposity level is reached and plasma leptin has fallen, food intake increases to reflect the reduced inhibitory signaling of leptin on CNS circuits that regulate food intake. Specifically, AgRP neurons are targets for leptin and when leptin levels are low, the inhibitory effect of leptin on AgRP is reduced which allows AgRP to be more effective in increasing intake. In the present study, high-fat-fed rodents given MT-II reduced food intake for a period of time shorter than the length of delivery by the minipump. At the time-point during which food intake increases, weight loss had already reached its peak, therefore, the increase in food intake that followed while the rodents maintained a lowered body weight is a behavioral response that reflects a new lowered level of leptin signaling, rather than a waning of the
anorectic effect of MT-II. This effect of MT-II and other peptides such as amylin on food intake has been reported by others (Hamilton and Doods, 2002; Raposinho et al., 2003; Seeley et al., 2005; Roth et al., 2006). To summarize, MT-II reduces food intake until lowered leptin levels are attained, and once a new body fat level is reached, food intake returns to normal (Seeley et al., 2005). Similarly, if adipose stores are low, as in the rodents on the low-fat diets, MT-II is relatively ineffective in reducing food intake and lowering body fat.

An important contribution of the present study is the identification of specific adipose compartments reduced by peripheral MT-II and that the weight loss is partially independent of food intake. Many studies have examined the effects of peripheral and central melanocortin agonists and antagonists on adiposity. For example, in separate studies peripheral and central administration of MT-II or α-MSH decreased visceral adipose tissue (Obici et al., 2001; Choi et al., 2003a; Choi et al., 2003b). Interestingly, Choi et al identified a decrease in subcutaneous fat following central administration of MT-II. In contrast, central administration of SHU9119 or AgRP increased retroperitoneal, epididymal, and inguinal fat (Adage et al., 2001; Obici et al., 2001; Small et al., 2001), an effect partially independent of food intake because antagonist-treated pair-fed rats had more total body fat than vehicle controls. In contrast to central administration, we show for the first time that chronic peripheral MT-II reduced visceral and subcutaneous adipose compartments. The present data are consistent with previous findings in that peripheral MT-II reduced visceral fat in MT-II-treated rodents. Interestingly, the effect on body fat was greatest in the MT-II-treated groups compared to pair-fed controls. In fact, in our rat study and in the study by Choi et al, visceral fat was not reduced in the pair-fed controls as it was in the mouse study (Choi et al., 2003a). Similar effects have been noted by Chen and Heiman in which leptin-induced weight loss was examined in rats and fat loss in pair-fed and ad-
lib-vehicle groups was identical (Chen and Heiman, 2000). Only leptin treatment resulted in a loss of visceral fat in the above mentioned study. One explanation for the discrepancy in visceral weight loss in the pair-fed controls between rats and mice may be a difference in metabolic rate. When pair-fed rodents are given a single allocation of food they typically consume the majority of that food shortly following presentation. Because mice have a higher metabolic rate compared to rats, during the period when the pair-feeding allocation is totally consumed and fuel stores need to be mobilized, mice may be more adept at mobilizing fat stores from the visceral adipose depot than rats. In spite of this, the present study identified a generalized additional decrease in subcutaneous fat in both mice and rats. The current findings also identified a decrease in the epididymal fat only in MT-II-treated rats, which corroborates previous studies (Choi et al., 2003a).

This study highlights the utility of the NMR for analyzing total and specific fat compartments. Most studies assess fat depot changes by dissection and weighing each specific fat pad. By comparison, utilization of the NMR to assess compartmental fat and lean mass is more accurate and complete. Not only were all the specific fat pads contained within each compartment, but non-dissectible fat, such as intramyocellular, epidermal, or organ adipose tissue was included as well. Lastly, the utilization of the NMR technology is powerful in that it revealed potential confounding effects of the pair-feeding control group.

Mice that were pair-fed to the MT-II-treated group displayed reduced lean body mass compared to the ad lib fed vehicle-treated controls despite achieving similar final body weights. Pair-fed mice not only lost visceral body fat, but they also lost lean mass in the subcutaneous compartment. This finding highlights the complexities of parceling out the respective contribution of a therapeutic weight loss treatment, e.g. food intake dependent and independent
effects. These data and recent data from other studies (Larsen et al., 2001; Roth et al., 2006) suggest that pair-feeding may not be an optimal control for anorectic pharmacological agents. For example, in a recent paper by Roth et al., amylin was chronically infused via a mini-pump into rats as in the current study and significant reductions in fat was observed (Roth et al., 2006). In this study a pair-fed control group was included and total adipose and lean body mass were reduced. Although pair-feeding is the current standard for dissociating the anorectic versus metabolic effects of a compound, the physiological relevance of this control group must be evaluated carefully.

The depot-specific reduction in adipose tissue also parallels the plasma levels of two adiposity signals, leptin and insulin (Woods and Seeley, 2001). In both the mouse and rat study visceral fat was decreased following MT-II treatment. It is known that central administration of SHU9119 and α-MSH affects insulin secretion (Adage et al., 2001; Obici et al., 2001). Centrally-administered SHU9119 resulted in a 3-fold increase in plasma insulin compared to pair-fed SHU9119-treated controls; but because the pair-fed SHU9119-treated rats were leaner than the ad lib fed SHU9119-treated rats, the increased adipose tissue in the SHU9119-treated ad lib rats was the cause of the increase in plasma insulin rather than SHU9119-treatment itself. In contrast, we observed a decrease in plasma insulin in MT-II-treated and vehicle-treated pair-fed rats. This finding is in agreement with the peripheral administration of MT-II in the study by Choi et al (Choi et al., 2003a), in which a similar decrease in plasma insulin was noted in both the MT-II and pair-fed rats. In the present study, despite reduced insulin, pair-fed rats did not differ from ad lib-fed controls in respect to visceral fat mass.

In contrast to insulin, plasma leptin levels were increased in the SHU9119-treated groups independent of body weight in the study by Adage et al (Adage et al., 2001). SHU9119-
treatment in ad lib and pair-fed rats resulted in significant increases in circulating leptin, 6-fold and 2-fold respectively. Because leptin is traditionally associated with subcutaneous fat it is tempting to speculate that SHU9119 increased subcutaneous adipose tissue and consequently resulted in an increase in plasma leptin. However, only visceral and epididymal adipose tissue changes were measured and both were increased (Adage et al., 2001). In support of this speculation, Obici et al infused SHU9119 centrally and found increased visceral and subcutaneous fat in rats. These findings combined with the present data support the hypothesis that changes in subcutaneous fat predict circulating leptin levels. We show that MT-II-treatment resulted in a selective decrease in subcutaneous adipose mass in both mice and rats and in the rat study plasma leptin was lowest in the MT-II vs. pair-fed and ad lib controls. These findings are consistent with data by Choi et al who demonstrated that central MT-II-treatment results in a decrease in subcutaneous fat (Choi et al., 2003b) and that peripheral MT-II resulted in non-detectable levels of leptin (Choi et al., 2003a).

To determine whether MT-II could be acting directly upon adipocytes it is important to anatomically link melanocortin receptors to specific adipose compartments. In the present study epididymal and inguinal adipose showed expression of the MC4R. These data are consistent with a previous study that also found the MC4R is expressed in inguinal adipose (Hoggard et al., 2004). But because MT-II is a non-selective agonist, it is possible that MT-II may be acting on other melanocortin receptors and some of these are expressed on adipocytes (Boston and Cone, 1996; Hoch et al., 2007). Additionally, α-MSH or MT-II treatment results in adipocyte lipolysis (Bradley et al., 2005). Although melanocortin receptors are on adipocytes, this does not definitively mean that lipolysis is a direct effect of MT-II. Recently, MC4R mRNA was extensively mapped to neural sites responsible for sympathetic outflow to the inguinal fat pad.
(Song et al., 2005). Together these findings suggest that MT-II may act on peripheral or central substrates to reduce levels of subcutaneous fat.

The melanocortin system is arguably a promising target for the development of a pharmacological treatment for obesity, however many studies demonstrate dramatic weight loss through non-conventional delivery methods such as intracranial injections. The present data are significant since they provide evidence for peripherally-delivered MT-II to reduce both compartments of adipose tissue, which when reduced undeniably result in a more promising health outcome.
Acknowledgements

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Hoggard N, Hunter L, Duncan JS and Rayner DV (2004) Regulation of adipose tissue leptin secretion by alpha-melanocyte-stimulating hormone and agouti-related protein: further


Raposinho PD, Castillo E, d'Alleves V, Broqua P, Pralong FP and Aubert ML (2000) Chronic blockade of the melanocortin 4 receptor subtype leads to obesity independently of neuropeptide Y action, with no adverse effects on the gonadotropic and somatotropic axes. *Endocrinology* **141**:4419-4427.


Legends for Figures

Figure 1. Daily body weight and food intake changes of low-fat (LF) (A, B) and high-fat (HF) (C, D) fed mice treated for 14 days with osmotic mini-pumps filled with either MT-II (1 mg/kg/day)(black circles), Vehicle (PBS) (white circles), or Vehicle (pair-fed)(white inverted triangles). Food intake for the Vehicle pair-fed group is not shown since it is identical to the amount of the MT-II-treated group.

Figure 2. Daily body weight (A) changes and daily food intake (B) of high-fat (HF) fed rats treated for 14 days with osmotic mini-pumps filled with either MT-II (1 mg/kg/day)(black circles) or Vehicle (PBS)(white circles), or Vehicle (pair-fed)(white inverted triangles).

Figure 3. Total body, visceral, and subcutaneous fat (A-C) and lean (D-F) percentages from HF fed mice treated with Vehicle (PBS)(AL –white bar), MT-II (1mg/kg/day) (black bar), or Vehicle (pair-fed)(hatched bar) for 14 days.

Figure 4. Total body, visceral, and subcutaneous fat (A-C) and lean (D-F) percentages from HF fed rats treated with Vehicle (PBS)(AL –white bar), MT-II (1mg/kg/day) (black bar), or Vehicle (pair-fed)(hatched bar) for 14 days.

Figure 5. Average epididymal fat pad weight of rats treated with MT-II (1mg/kg/day) compared to Vehicle (PBS) and Vehicle (pair-fed).
Figure 6. RT-PCR ethidium bromide-stained gel showing the presence of MC4R gene within subcutaneous inguinal (IWAT) and epididymal white adipose (EWAT) tissue in the rats. The expected PCR product is 92bp for the MC4R.
<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Total Body Fat (%)</th>
<th>Total Body Lean (%)</th>
<th>Visceral Fat (%)</th>
<th>Visceral Lean (%)</th>
<th>Subcutaneous Fat (%)</th>
<th>Subcutaneous Lean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse/LF</td>
<td>13.38 ± 0.87</td>
<td>82.32 ± 0.82</td>
<td>8.79 ± 0.52</td>
<td>88.24 ± 0.51</td>
<td>22.70 ± 2.02</td>
<td>72.69 ± 1.80</td>
</tr>
<tr>
<td>Vehicle-AL</td>
<td>12.26 ± 0.89</td>
<td>83.94 ± 0.84</td>
<td>8.47 ± 0.59</td>
<td>89.68 ± 0.64</td>
<td>19.68 ± 1.90</td>
<td>75.19 ± 1.77</td>
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<tr>
<td>MT-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

**Table 1.** Total and compartmental fat and lean percentages from LF-fed mice treated with Vehicle (PBS) or MT-II (1mg/kg/day) for 14 days.
Table 2. Total, subcutaneous, and visceral fat and lean mass (grams) for diet-induced obese (DIO) mice and rats treated with Vehicle (PBS), MT-II (1mg/kg/day), or (PBS)-pair-fed for 14 days.

<table>
<thead>
<tr>
<th></th>
<th>SQ fat (g)</th>
<th>Visceral fat (g)</th>
<th>Total fat (g)</th>
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<tbody>
<tr>
<td><strong>Mouse HF Diet</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Vehicle – AL</td>
<td>3.13±0.25</td>
<td>3.38±0.26</td>
<td>6.51±0.45</td>
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<tr>
<td>MTII</td>
<td>2.17±0.20*</td>
<td>2.48±0.26**</td>
<td>4.66±0.44*</td>
</tr>
<tr>
<td>Vehicle - PF</td>
<td>2.69±0.31</td>
<td>2.68±0.31*</td>
<td>5.37±0.60*</td>
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<tr>
<td><strong>Rat HF Diet</strong></td>
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<td></td>
</tr>
<tr>
<td>Vehicle – AL</td>
<td>5.02±0.25</td>
<td>16.19±0.49</td>
<td>21.21±0.72</td>
</tr>
<tr>
<td>MTII</td>
<td>4.64±0.11</td>
<td>15.93±0.23</td>
<td>20.56±0.29</td>
</tr>
<tr>
<td>Vehicle - PF</td>
<td>4.42±0.19*</td>
<td>15.71±0.27</td>
<td>20.13±0.44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>SQ lean (g)</th>
<th>Visceral lean (g)</th>
<th>Total lean (g)</th>
</tr>
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<tbody>
<tr>
<td><strong>Mouse HF Diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle – AL</td>
<td>5.02±0.25</td>
<td>16.19±0.49</td>
<td>21.21±0.72</td>
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<tr>
<td>MTII</td>
<td>4.64±0.11</td>
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<tr>
<td>Vehicle - PF</td>
<td>4.42±0.19*</td>
<td>15.71±0.27</td>
<td>20.13±0.44</td>
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<tr>
<td><strong>Rat HF Diet</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle – AL</td>
<td>93.78±3.34</td>
<td>305.56±6.76</td>
<td>399.35±9.28</td>
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<tr>
<td>MTII</td>
<td>87.22±3.87</td>
<td>307.56±7.91</td>
<td>394.78±11.22</td>
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<tr>
<td>Vehicle - PF</td>
<td>86.94±3.01*</td>
<td>296.58±8.94</td>
<td>383.53±9.89</td>
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</table>

*p<0.05 vs. Ad lib-PBS; **p<0.01 vs. Ad lib- PBS, *p=0.07
Table 3. Plasma leptin, insulin and glucose from DIO rats treated with Vehicle (AL), Vehicle (PF) MT-II (1mg/kg/day) for 14 days.

<table>
<thead>
<tr>
<th>Treatment/Group</th>
<th>Leptin (ng/ml)</th>
<th>Insulin (pmol/l)</th>
<th>Glucose (mg/dl)</th>
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</thead>
<tbody>
<tr>
<td>Rat-HF Diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle-AL</td>
<td>3673.0 ± 329.5</td>
<td>780.9 ± 124.5</td>
<td>183.1 ± 12.1</td>
</tr>
<tr>
<td>MT-II</td>
<td>1546.8 ± 142.2 *</td>
<td>495.4 ± 47.9 *</td>
<td>153.9 ± 5.6</td>
</tr>
<tr>
<td>Vehicle-PF</td>
<td>2417.0 ± 308.3 **</td>
<td>481.0 ± 37.854 *</td>
<td>180.3 ± 4.70</td>
</tr>
</tbody>
</table>

*p<0.05 vs. Vehicle-AL  
**p<0.01 vs. Vehicle-AL  
***p<0.001 vs. Vehicle-AL
Figure 1.

A

B

C

D

Vehicle (AL)

MTII

Vehicle (AL)

MTII

Vehicle (AL)

MTII and Vehicle (PF)

*p<0.05

*p<0.05
Figure 2.

A

- Vehicle (AL)
- MTII
- Vehicle (PF)

Body Weight (g)

Time (days)

B

- Vehicle (AL)
- MTII and Vehicle (PF)

Food Intake (g)

Time (days)

*p < 0.05
Figure 3.
Figure 5.

Epididymal Fat Pads (g)

* $p < 0.05$ vs. MTII

** $p < 0.01$ vs. MTII

* $p < 0.01$ vs. MTII

Treatment Group

AL  MTII  PF
Figure 6:

- Epididymal Fat Pad
- Inguinal Fat Pad

92 bp