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Central administration of melanocortin agonist increased insulin sensitivity in diet-induced obese rats

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Abstract **In this study, we examined the effects of intracerebroventricular administration of melanotan II (MTII), a melanocortin agonist, on insulin sensitivity in diet-induced obese (DIO) rats. Although MTII treatment significantly decreased food intake and body weight for 10 days, there was no significant difference in body weight between MTII and pair-fed groups. The insulin tolerance test showed that insulin sensitivity was significantly improved in the MTII group compared to the pair-fed group. Furthermore, MTII treatment increased the number of small-sized adipocytes in epididymal white adipose tissues, suggesting that MTII increased insulin sensitivity through action on the white adipose tissues in DIO rats.**

Keywords: Melanocortin; Central nervous system; Obesity; Insulin sensitivity; Adipocytes; Peroxisome proliferator-activated receptor γ

1. Introduction

The consumption of a high caloric diet is one of the main causes of obesity [1]. Rats fed with a high fat diet show increases in body weight and decreases in insulin sensitivity [2], and these diet-induced obese (DIO) rats have been used for studying the mechanisms of insulin resistance due to obesity. It has been demonstrated that the size and weight of adipose tissues are increased in DIO rats [3], and that the hypertrophy of adipocytes leads to changes in the release of adipocytokines such as leptin and adiponectin regulating insulin sensitivity [4,5]. These morphological changes in adipose tissues as well as changes in plasma levels of adipocytokines have been postulated to be among the causes of insulin resistance in DIO rats [6,7].

The melanocortin systems in the hypothalamus, including a subset of neurons in the arcuate nucleus synthesizing pro-opiomelanocortin (POMC), are one of the key systems in energy homeostasis [8]. POMC neurons in the arcuate nucleus are influenced by humoral factors such as leptin synthesized in adipose tissues as well as neural inputs reflecting energy balance [9], and melanocortins cleaved from POMC exert their effects by binding to members of a family of melanocortin receptors in the brain [10]. While the major action of melanocortin in the brain is to reduce food intake, it is also demonstrated that melanocortin agonists or antagonists injected into the cerebral ventricle affected insulin actions in the periphery while food was withdrawn or the intake was maintained constant [11,12]. These data suggest that the action of melanocortin on insulin sensitivity is, at least partially, independent of energy balance, although the detailed mechanisms have not been fully elucidated.

In the present study, we examined the effects of the activation of central melanocortin pathways on insulin sensitivity, morphological changes in adipocytes and serum levels of adiocytokines in DIO rats.

2. Materials and methods

2.1. Animals and treatment

Male Sprague–Dawley rats (age 7 weeks, body weight 200–225 g; Chubu Science Materials, Nagoya, Japan) were housed in individual cages at 23 °C with lights on from 0900 to 2100 h. Rats fed a high-fat diet (60.1% energy as fat and 15.6% as carbohydrate) for 12 weeks were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg BW), and a 28-gauge stainless steel cannula (Brain infusion kit II; Alzet, Palo Alto, CA) was inserted stereotaxically into the right lateralventricle for an intracerebroventricular (icv) injection. An osmotic mini-pump (Model 2002; Alzet, Palo Alto, CA) connected to the cannula was placed subcutaneously. Vehicle (0.9% saline) was constantly administered into the right lateral ventricle for 2 weeks through icv cannula connected osmotic mini-pumps. After these 2-week recovery periods, on day 0, all rats were anesthetized again with diethyl ether for subcutaneous replacement of another osmotic mini-pump containing vehicle or melanotan II (MTII) (kindly provided by Sankyo Corporation, Tokyo, Japan) dissolved in vehicle. Vehicle or MTII (1 μg/day) was administered constantly into the lateral ventricle for 10 days through the replaced osmotic mini-pumps, and food intake and body weight of the animals were measured daily.

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Abbreviations: MTII, melanotan II; DIO, diet-induced obesity; POMC, pro-opiomelanocortin; icv, intracerebroventricular; ITT, insulin tolerance tests; TG, triglyceride; WAT, white adipose tissue; BAT, brown adipose tissue; PPARγ, peroxisome proliferator-activated receptor γ; aP-2, adipocyte-specific fatty acid binding protein; qRT-PCR, real-time quantitative RT-PCR; SNS, sympathetic nervous systems

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All procedures were performed in accordance with the institutional guidelines for animal care of the Nagoya University Graduate School of Medicine.

2.2. Food intake and body weight measurements

The food intake and body weight of DIO rats were measured daily between 1900 and 2100 h. DIO rats were divided into three groups: (1) ad libitum group, with a mini-pump containing vehicle and allowed to eat 0 ad libitum; (2) MTII group, with a mini-pump containing MTII and free access to food; and (3) pair-fed group, with a mini-pump containing vehicle and pair-fed the same amount of food as those in the MTII group. All groups were fed a high-fat diet throughout the experiments.

2.3. Insulin tolerance test (ITT)

On day 7, ITT was performed between 0900 h and 1200 h on animals fasted for 10 h before the test. To minimize the duration of fast in pair-fed group, the matched amount of food was served to the pair-fed group at 1900 h as well as at 3 h before food was withdrawn on day 6. Human regular insulin (1 U/kg body weight: humalin R; Eli Lilly Japan, Kobe, Japan) was administered intraperitoneally, and blood samples were taken from the tail vein of the conscious rats before and 30, 60, 90 and 120 min after insulin administration. Blood glucose levels were immediately measured with a blood glucose monitor (TERUMO Corporation, Tokyo, Japan).

2.4. Fat pad and tissue analysis

On day 10 all rats were decapitated between 0900 h and 1200 h. Rats in MTII and ad libitum groups had free access to food until sacrifice while those in the pair-fed group were served the matched amount of food 3 h before sacrifice. After sacrificing by decapitation, the epididymal, perirenal, retroperitoneal and inguinal subcutaneous white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) as well as liver were dissected out. The epididymal WAT and liver were immediately frozen in liquid nitrogen and stored at −80 °C for future measurement. One part of the epididymal WAT was immersed in 4% buffered formaldehyde until future analysis.

2.5. Serum adiponectin and leptin measurement

Serum leptin and adiponectin levels were measured with enzyme-linked immunosorbent assay commercial kits (Wako, Osaka, Japan, and Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan, respectively).

2.6. Triglyceride (TG) content in liver

One hundred milligram frozen sections of liver were homogenized, and tissue lipids were extracted with an extract solution (chloroform:methanol = 2:1), and then solubilized in isopropanol after samples were dried. TG content levels measured with an enzymatic commercial kit (Wako).

2.7. Determination of DNA content and mRNA levels by real-time PCR

The genomic DNA was extracted and purified from a piece of 25 mg frozen epididymal WAT using NucleoSpin® Tissue kit (NIPPON Genetics Co., Ltd., Tokyo, Japan). The DNA content of WAT was measured by spectrophotometer method using NanoDrop® ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). The mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), adipocyte-specific fatty acid binding protein (aP-2) and 36B4 were assessed and quantified by real-time quantitative RT-PCR (qRT-PCR). Total RNA was extracted from 100 mg frozen epididymal WAT with the Trizol reagents (Invitrogen Corporation, Carlsbad, CA, USA), and cDNAs were synthesized from 1 μg total RNA using SuperScript II reverse transcriptase (Invitrogen Japan K.K.). The qRT-PCR was carried out in a 20 μl reaction mixture that contained 2 μl of the above cDNA products and SYBR Green I PCR Matrix (Eurogentec, Seraing, Belgium). The levels of PPARγ and aP-2 mRNA were normalized to that of 36B4. The sequence primers were: PPARγ forward 5′-CATGACCAGGGAGTTCCTCAA, reverse 5′-AGCAAACTCAAACTTAGGCTC- CAT; aP-2 forward 5′-GAGTTGGCTTCGCCACCA, reverse 5′-AAGTCCCCTTCTA-CGCTGATGA; 36B4 forward 5′-CCTTCCCACTG-GCTGAAAAG, reverse 5′-CGCAGCCGCAAATGC.

2.8. Histological analysis and morphometry

Epididymal WATs fixed in 4% formaldehyde were embedded in paraffin, cut at a thickness of 10 μm on a microtome, placed on slides, and stained with hematoxylin and eosin. For the quantification of the number and size of adipocytes, the sectional areas of epididymal adipocytes (160000 μm^2per each rat) were traced manually and analyzed with an image analysis system (NEUROLUCIDA® software; MicroBright-Field Japan, Inc., Japan). The numbers of adipocytes of each size from eight rats in each group were shown as histograms.

2.9. Statistical analyses

All results are expressed as mean values ± S.E.M. Data were analyzed by one-way (treatment) or two-way (treatment, time) ANOVA. For time course changes in body weight and food intake, repeated measures ANOVA were employed. These analyses were followed by a Fisher protected least significant difference test for post hoc comparisons. Differences were considered statistically significant at $P < 0.05$. The group size was eight in all experiments.

3. Results

3.1. Effect of MTII administration on food intake and body weight

Repeated ANOVA revealed that there was a significant treatment effect on daily food intake [$F(1,14) = 17.4$, $P < 0.01$]. The post hoc analyses revealed that daily food intake in the MTII group was significantly lower compared to the ad libitum group throughout the experiment (Fig. 1A). There was also a significant treatment effect on body weight [$F(2,21) = 20.1$, $P < 0.01$]. However, post hoc analyses revealed that, although body weight in MTII and pair-fed

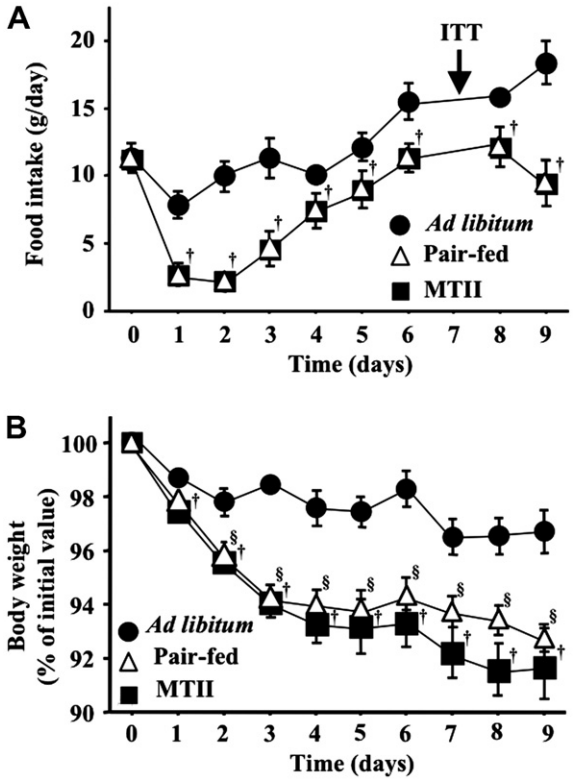

Fig. 1. Changes in daily food intake (A) and body weight (B) of DIO rats. DIO rats were divided into three groups: ad libitum (closed circles), pair-fed (triangles) and MTII (closed squares) groups. †, $P < 0.05$ MTII vs ad libitum; §, $P < 0.05$ pair-fed vs ad libitum.

groups was significantly decreased compared to ad libitum group, there were no significant differences in changes in body weight between MTII and pair-fed groups (Fig. 1B).

3.2. Effect of MTII administration on ITT

There were no significant differences in blood glucose levels before insulin injection between the groups (ad libitum 105.8 ± 15.8 mg/dl, pair-fed 95.9 ± 15.5 mg/dl, MTII 96.2 ± 5.9 mg/dl). Two-way ANOVA revealed a significant treatment effect [$F(2,105) = 87.0$, $P < 0.01$], time effect [$F(4,105) = 328.4$, $P < 0.01$] and treatment-time interaction [$F(8,105) = 5.59$, $P < 0.01$]. The post hoc analyses revealed that changes in blood glucose levels after insulin injection were significantly lower in MTII group than in the ad libitum and pair-fed groups at all time points examined, and that the values in pair-fed groups were significantly lower than in ad libitum groups at 60, 90 and 120 min (Fig. 2).

3.3. Effect of MTII administration on fat pad and TG content in liver

There were no significant differences among ad libitum, pair-fed and MTII groups in the weight of visceral WAT (sum of epididymal, perirenal and retroperitoneal fat, Fig. 3A), subcutaneous WAT (Fig. 3B) or BAT (Fig. 3C). On the other hand, there was a significant treatment effect on TG contents in liver [$F(2,21) = 5.00$, $P < 0.01$]. The post hoc analyses revealed that the TG contents in liver were significantly decreased in MTII group compared to other groups (Fig. 3D).

3.4. Effect of MTII administration on adipocytes in epididymal WAT

Histological analysis revealed that MTII treatment increased the population of smaller-sized adipocytes in epididymal WAT of DIO rats (Fig. 4). There was a significant treatment effect on the DNA content in epididymal WAT [$F(2,21) = 8.08$, $P < 0.01$], and the post hoc analyses revealed that the DNA content was significantly increased in the MTII group compared to the ad libitum and pair-fed groups, whereas there were no significant differences in the DNA content between the pair-fed and ad libitum groups (Fig. 5A).

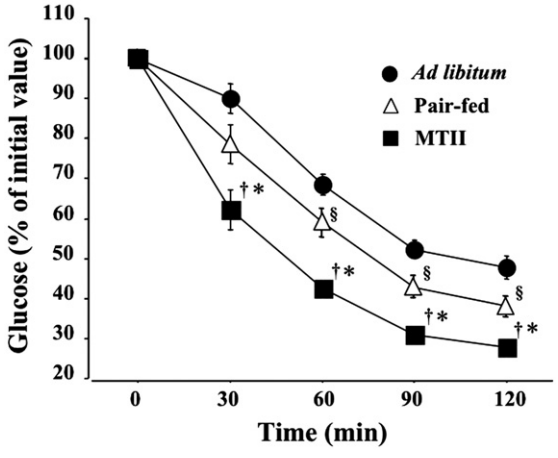

Fig. 2. Intraperitoneal insulin tolerance test in DIO rats on day 7. DIO rats were divided into three groups: ad libitum (closed circles), pair-fed (triangles) and MTII (closed squares) groups. After being fasted for 10 h, rats were injected with human regular insulin (1.0 U/kg body weight) intraperitoneally, and blood glucose levels were measured. Levels relative to initial values were shown. †, $P < 0.05$ MTII vs ad libitum; *, $P < 0.05$ MTII vs pair-fed; §, $P < 0.05$ pair-fed vs ad libitum.

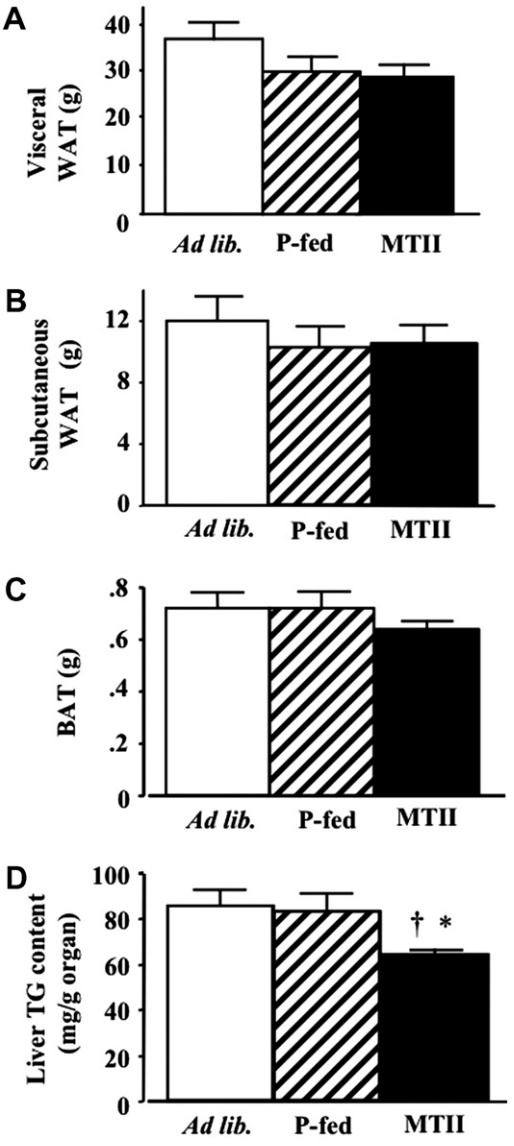

Fig. 3. Effects of icv infusion of MTII on adipose weight (A–C) and TG content in liver (D) in DIO rats. Visceral WAT weight is sum of epididymal, perirenal and retroperitoneal fat weight, while subcutaneous WAT is from inguinal subcutaneous fat. †, $P < 0.05$ MTII vs ad libitum; *, $P < 0.05$ MTII vs pair-fed.

3.5. Effect of MTII administration on gene expressions in epididymal WAT

There were significant treatment effects on the expression levels of PPARγ mRNA [$F(2,21) = 35.7$, $P < 0.01$] and aP-2 [$F(2,21) = 20.2$, $P < 0.01$]. The post hoc analyses revealed that the expression levels of PPARγ mRNA as well as aP-2 were significantly higher in the MTII group compared to the ad libitum and pair-fed groups, whereas there were no significant differences in the levels between the ad libitum and pair-fed groups (Fig. 5B and C).

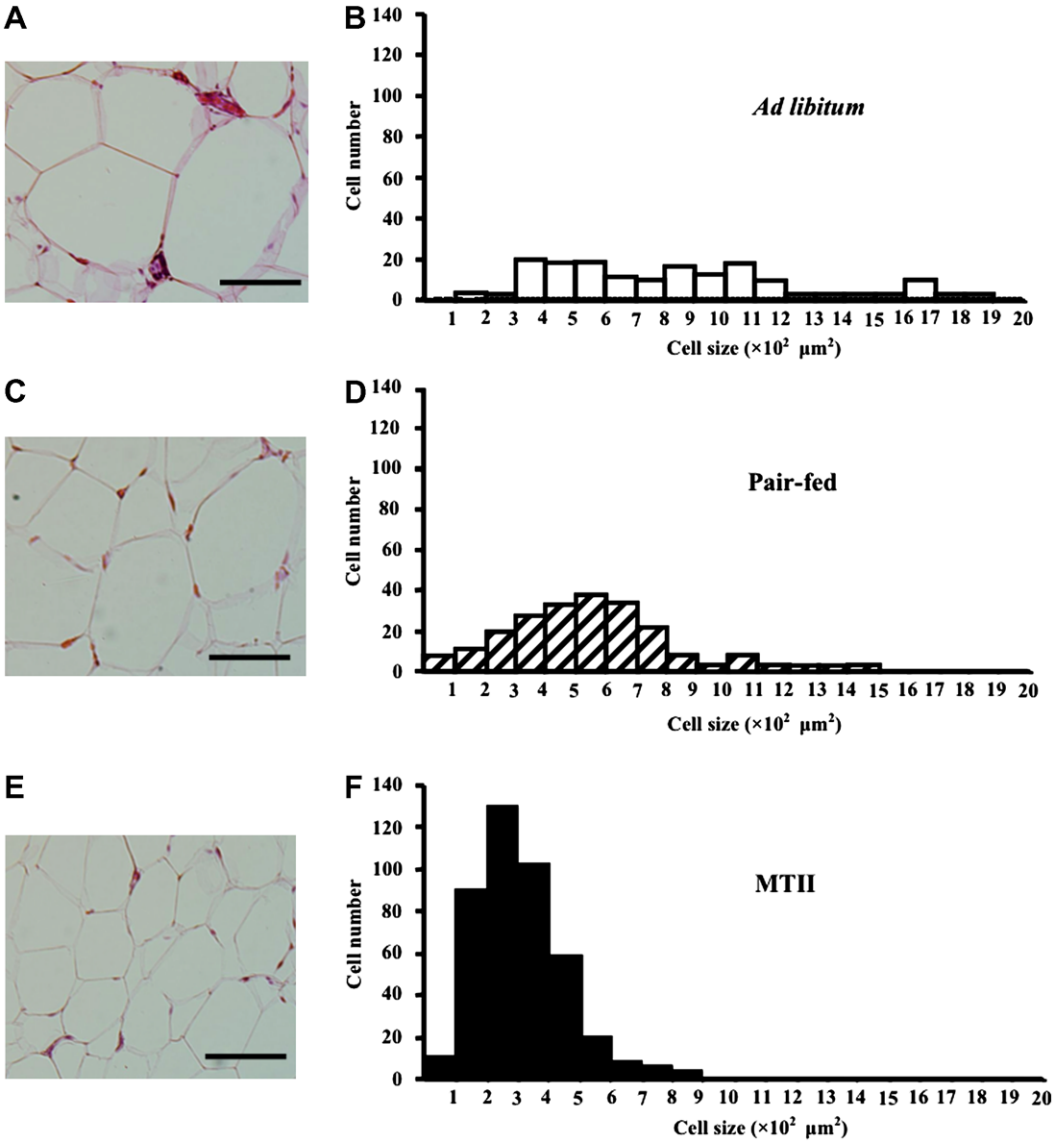

Fig. 4. Histology of epididymal WAT and quantification of cell sizes and numbers in ad libitum (A,B), pair-fed (C,D) and MTII (E,F) groups. Black bars in panels indicate 100 μm. Sectional areas (160000 μm^2 per rat) were analyzed with an image analysis system, and numbers of adipocytes of each size from eight rats in each group were shown as histograms (B,D,F).

3.6. Effect of MTII administration on serum adipocytokine levels

There were significant treatment effects on the serum levels of adiponectin [$F(2,21) = 35.4$, $P < 0.01$] and leptin [$F(2,21) = 7.48$, $P < 0.01$]. The post hoc analyses revealed that the serum adiponectin levels in the MTII group were significantly higher compared to either ad libitum or pair-fed groups, while there were no significant differences in the levels between ad libitum and pair-fed groups (Fig. 6A). On the other hand, the serum leptin levels in MTII group were significantly higher compared to the pair-fed group, while the levels in the pair-fed group were significantly lower compared to the ad libitum group (Fig. 6B).

4. Discussion

It has been shown that the activation or blockade of central MC4R pathways affected insulin sensitivity in the periphery via mechanisms other than changing the energy balance [11,12]. Our data demonstrated, for the first time to our knowledge, that the activation of central MC4R pathways could also ameliorate insulin resistance induced by a high-fat diet and that the action was independent of energy balance. As the size of adipocytes in WAT, which is closely related to insulin sensitivity in adipose tissues [7], was decreased by MTII treatment, it is suggested that the melanocortin systems increased insulin sensitivity at the level of WAT in DIO rats, at least to some degree. Our data also demonstrated that TG content in liver was significantly decreased by MTII treatment. Together with a previous study showing that chronic administration of α-MSH increased tyrosine phosphorylation of insulin receptor substrate-1, 2 in liver in rats [12], it is suggested that activation of central melanocortin systems leads to increases in insulin sensitivity at the level of liver as well. Furthermore, it is reported that an acute injection of MTII increased GLUT4 mRNA expression in skeletal muscle in mice [11].

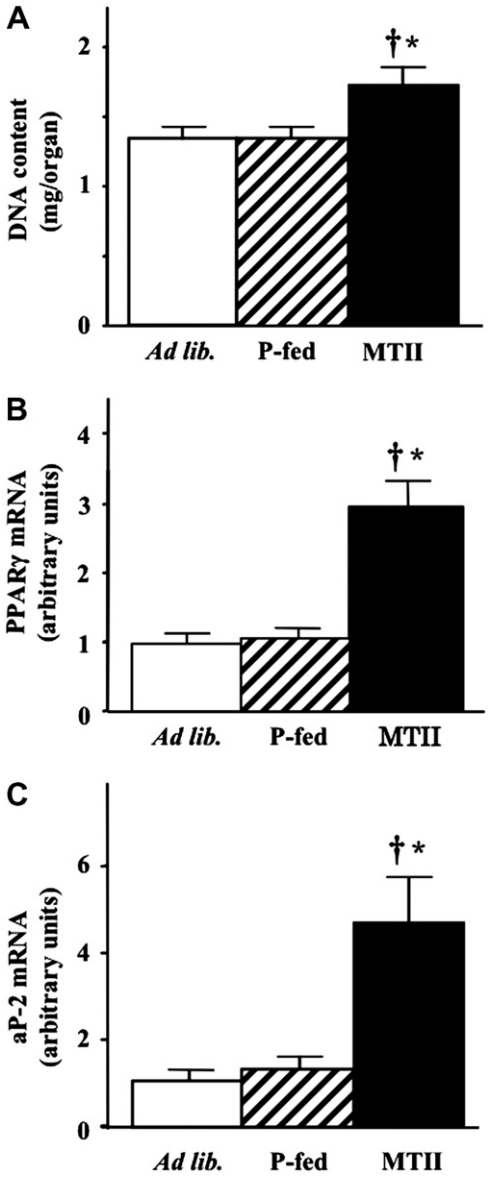

Fig. 5. DNA content (A) and gene expression of PPARγ (B) and aP-2 (C) in epididymal WAT of ad libitum (Ad lib.), pair-fed (P-fed) and MTII groups. †, $P < 0.05$ MTII vs ad libitum; *, $P < 0.05$ MTII vs pair-fed.

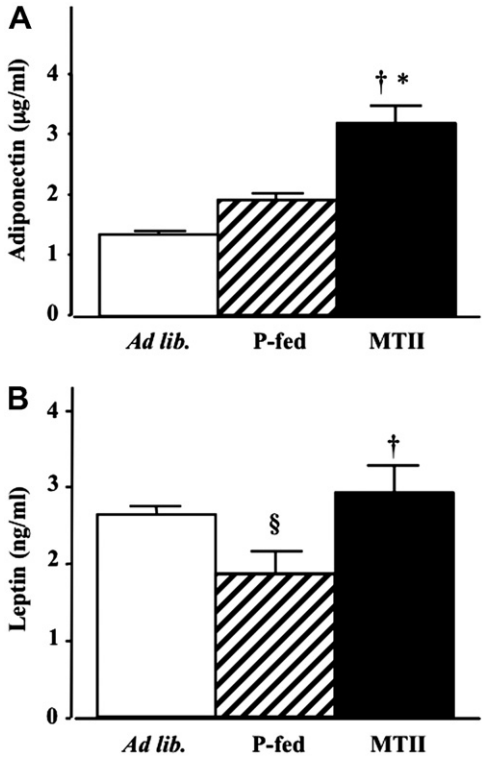

Fig. 6. Serum levels of adiponectin (A) and leptin (B) in ad libitum (Ad lib.), pair-fed (P-fed) and MTII groups. †, $P < 0.05$ MTII vs ad libitum; *, $P < 0.05$ MTII vs pair-fed; §, $P < 0.05$ pair-fed vs ad libitum.

Thus, it is likely that the effects of the activation of the central melanocortin systems on the regulation of insulin sensitivity in the periphery could be diverse.

Our data showed that MTII treatment significantly increased the PPARγ gene expression, which is upregulated when adipocytes are proliferated and differentiated [13]. This was further confirmed by significant increases in aP-2, which is shown to be downstream of PPARγ in WAT [14]. Together with the significant increase in DNA content in the epididymal WAT, our results suggest that the total number of adipocytes in the epididymal WAT was increased by MTII treatment. Although the regulation of the differentiation and proliferation of adipocytes has not been fully elucidated, there is evidence that the stimulation of melanocortin receptors activated the sympathetic nervous system (SNS) [15], which innervates WAT [16]. Thus, it is possible that MTII increased the number of small-sized adipocytes by inducing cell proliferation and differentiation in WAT through SNS, although further studies are necessary to prove this.

Adiponectin synthesized in and secreted from adipose tissues increases insulin sensitivity through its receptors in liver and muscle [17,18]. It is reported that the plasma levels of adiponectin were decreased in DIO rats, and that administration of adiponectin improved their insulin resistance [18]. Decreases in plasma adiponectin levels are also reported in obese patients and thus have been postulated as one of the possible causes of insulin resistance in metabolic syndrome [19]. In the present study, MTII treatment significantly increased the release of adiponectin in DIO rats. These changes could be due to decreases in the size of adipocytes [4], and possibly contributed, at least partially, to the improvement in insulin sensitivity induced by MTII.

Leptin is also one of the adipocytokines whose role in energy balance has been extensively studied [20–22]. Leptin decreases food intake and increases energy expenditure through its receptors in the hypothalamus [20,21], and central melanocortin systems are, to some extent, involved in leptin action [8,21]. In contrast to adiponectin, plasma levels of leptin as well as its synthesis in WAT increase in parallel with adipose stores [5]. Plasma leptin levels were increased in DIO rats as well as in obese patients [2,22,23], results that have been interpreted as leptin resistance. In the present study, while the size of adipocytes in WAT was significantly decreased, serum levels of leptin were significantly increased by MTII treatment, suggesting that MTII stimulated leptin release via mechanisms indepen-

dent of morphological changes in WAT. Thus, our data suggest that melanocortin systems are not only downstream of leptin but also could regulate leptin release. Elucidation of the detailed mechanisms of mutual regulation between central melanocortin systems and adipose tissues is an important task for future study.

In conclusion, we demonstrated that activation of the central melanocortin systems increased insulin sensitivity through its action on the white adipose tissues in DIO rats.

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