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Recombinant human ciliary neurotrophic factor reduces weight partly by regulating nuclear respiratory factor 1 and mitochondrial transcription factor A

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Abstract

Ciliary neurotrophic factor (CNTF) can lead to weight loss by up-regulating energy metabolism and the expression of UCP-1 in mitochondria. To investigate the up-stream regulators of the expression of UCP-1, recombinant human CNTF (rhCNTF) (0.1, 0.3, 0.9 mg/kg/day s.c.) administered to KK-Ay mice for 30 days resulting in reductions in body weight and perirenal fat mass. In brown adipose tissues, the gene expressions of nuclear respiratory factor (NRF)-1, mitochondrial transcription factor A (TFam) and uncoupling protein (UCP)-1 were found up-regulated by rhCNTF. To the best of our knowledge, these effects represent new insights on the mechanisms of action of weight loss by rhCNTF. In addition, we also found that rhCNTF increased the activity of mitochondrial complex IV. The stimulation of NRF-1, TFam, UCP-1 and the enhanced activity of mitochondrial complex IV may be associated with remedying obesity. The result indicates that rhCNTF can enhance the expressions of NRF-1 and TFam, both of which can up-regulate the expression of UCP-1.

Keywords: Recombinant human ciliary neurotrophic factor; Obesity; Uncoupling protein-1; Nuclear respiratory factor-1; Mitochondrial transcription factor A

1. Introduction

The worldwide incidence of obesity has greatly increased. Obesity/abdominal obesity, type 2 diabetes, hypertension and dyslipidemia are closely linked together and clustering of these medical disorders in an individual has been labeled the "metabolic syndrome" (Unger, 2002). Body weight reduction is an important strategy for its treatment.

Ciliary neurotrophic factor (CNTF), a member of gp130 family, is an injury- and metabolic activity-induced up-regulation factor (Watt et al., 2006) and has now been identified as an anti-injury factor and anti-obesity agents (Kelleher et al., 2006; Zvonic et al., 2005). CNTF was found to possess anti-obesity activity now, but the mechanism of this action is unclear. When CNTF was administered to individuals suffering from amyotrophic lateral sclerosis (Guler et al., 2000) and to mice (Lambert et al., 2001), it caused anorexia and weight loss. Furthermore, CNTF-induced-weight loss appears to be mediated through leptin-like and leptin-independent mechanisms. Other studies have also shown that CNTF decreases body weight and food intake in various mouse models of obesity such as leptin deficient *ob*/*ob* mice, leptin-resistant *db*/*db* mice, diet-induced obese AKR/J mice and UCP-1 diphtheria toxin A mice (Sleeman et al., 2003; Bluher et al., 2004). The way in which CNTF affects the gene expression of nuclear or mitochondrial uncoupling protein-1 (UCP-1) has not been reported.

The transcriptional control of mitochondrial biogenesis requires the expression of a large number of genes encoded by the nuclear and mitochondrial genetic systems (Andersson and Scarpulla,

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2001; Franco et al., 2006). Nuclear genes play a predominant role in controlling mitochondrial transcription, translation, and DNA replication (Natalie et al., 2005). The coding genes of the regulating factors for transcription, duplication and the processing of mitochondrial DNA (mtDNA) exist in nuclear DNA (nDNA) (Bergeron et al., 2001). It has been suggested that the transcription factor, nuclear respiratory factor-1 (NRF-1) plays a role in the cellular adaptation to energy metabolism by transducing a metabolic perturbation into an increased capacity to generate energy by transcription factor binding the DNA binding region.

Cytochrome *c* is present in mitochondria and is an essential component of the electron transfer chain involved in the transfer of electrons between complex III and complex IV. It is a marker for content of mitochondria. The activity of complex IV is a marker of mitochondrial respiration. We were also interested in whether the membrane potential of mitochondria changed, because the increased UCP-1 in mitochondrion may lead to low membrane potential.

The aim of the present study was investigating the anti-obesity mechanism of action of recombinant human CNTF (rhCNTF). We investigated the expression of NRF-1, mitochondrial transcription factor A (TFam) and UCP-1 and also determined the content of cytochrome *c*, the activity of complex IV and membrane potential of mitochondria in brown adipose tissue obtained from obese KK-Ay mice. These mice are obese because the central leptin signaling cascade is disrupted causing alimentary-induced obesity and insulin resistance. It is enough for the use in this study because we were focusing upon the effects of CNTF on the regulation of UCP-1.

2. Methods

2.1. Reagents

RhCNTF (molecular mass 21,244 Da) was developed by China C-bons pharmaceutical corporate (Hubei China). It comprised 186 amino acids in the following sequence:MAFTEHSPLTPHRRDLCSRSIWLARKIRSDLTALTESYVKHQGLNKNINLDSADGMPVASTDQWSELTEAERLQENLQAYRTFHVLLARLLEDQQVHFTPTEGDFHQAIHTLLLQVAAFAYQIEELMLLEYKIPRNEADGMPINVGDGGLFEKKLWGLKVLQELSQWTVRSIHDLRFISSHQTG.

TRIzol® and Superscript™ III reverse transcriptase were obtained from Invitrogen Corporation (Life Tech Co., Ltd. TakaRa Taq ™DNA polymerase (TakaRa Biotechnology Co. Ltd (Dalian China), ribonuclease inhibitor and Random Primer were purchased from TakaRa Biotechnology Co., Ltd (Dalian China). Triiodothyronine (3,3′,5′-triiodo-L-thyronine (T3); 98% purity) was purchased from Sigma Chemical Co., Ltd (USA). All other chemicals were of analytical grade and purchased/obtained from Chinese Beijing Xinjingke corporate.

2.2. Animals

The experiments were carried out in compliance with the guidelines for animal care and use of China and the experimental protocols were approved by the animal ethics committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. Fifty-five genetically obese KK-Ay mice (30±3 g, aged 6 weeks, male or female randomly) were single-housed for 30 days in a light (12 h on and 12 h off), temperature (23±2 °C) and humidity (40–60%) controlled specific-pathogen free environment, with a high caloric (4.61 kcal/g) mice chow and water available *ad libitum*. The mice were obtained from and the diet was prepared by the Institute of Laboratory Animal, Chinese Academy of Medical Sciences and Peking Union Medical College.

The mice were divided equally into five groups: a control group (Veh) in which normal saline was injected subcutaneously for 30 days; a positive control group (Con) in which 0.4 mg/kg T3 was injected subcutaneously for 30 days (Mracek et al., 2005); a low dose treatment group (CL) in which 0.1 mg/kg rhCNTF was injected subcutaneously for 30 days; a medium dose treatment group (CM) in which 0.3 mg/kg rhCNTF was injected subcutaneously for 30 days; and a high dose treatment group (CH) in which 0.9 mg/kg rhCNTF was injected subcutaneously for 30 days. Food intakes and body weights were measured every 48 h. Mice were acclimated to single-housing and daily human handling to reduce stress for at least one week prior to experimental procedures (Zhang et al., 2006).

To investigate the short term effects of rhCNTF, five mice in each group were removed from the experiment after three days of treatment, i.e. on the fourth day. These animals were killed by decapitation and the brown adipose tissues between the scapulae were surgically removed and used for preparing mitochondria and the various assays (see later for details). At the end of the 30 days treatment, the remaining mice in each group were killed by decapitation and the body weights and the perirenal fat mass were measured/determined.

2.3. Detection of nuclear and mitochondrial NRF-1, TFam, UCP-1 gene transcripts by RT-PCR

Total RNAs were extracted from brown adipose tissues removed from mice treated for three days with TRIzol® and centrifuged at 12,000 g for 10 min at 4 °C. The RNA pellets were stored at −40 °C until use. For conducting semi-quantitative RT-PCR to assess the expression of 1 μg total RNA and first-strand cDNA synthesis, the pellets were re-suspended in sterile ribonuclease-free water and incubated at 95 °C for 4 min. RT-PCR was performed according to the directions included with the SuperScript™ system in a 25 μl reaction volume using the Techne Genius PCR equipment (Made by Techne Ltd. DUXFORD Cambridge England), PCR amplifications employed 29 cycles with steps at 95 °C for 40 s, 52 °C for 45 s, and 72 °C for 45 s followed by elongation at 72 °C for 7 min. The presence of specific PCR products was confirmed by visualization on a 1.5% agarose gel stained with ethidium bromide, and analyzed by the Kodak digital imaging system (Kodak DC120, Digital Science 1 D System, USA). The results are expressed as ratios relative to β-actin (density of PCR product/density of β-actin). The forward and reverse sequences used for RT-PCR are listed in Table 1. They are all identified in GeneBank.

Table 1
Primer pairs used in RT-PCR

Gene	Sequence	Product
β-actin	Forward 5′-GTCGTACCACAGGCATTGTGATGG-3′ Reverse 5′-GCAATGCCTGGGTACATGGTGG-3′	493 bp
UCP-1	Forward 5′-TGTTTGGGCATTCTGGCTGAGG-3′ Reverse 5′-TTCTGGGGGCGTTTTCTGTGCT-3′	353 bp
NRF-1	Forward 5′-CAA ACT GAA CAC ATG GCT AC-3′ Reverse 5′-TTG AAG ACA GGG TTG GGT TT-3′	412 bp
TFam	Forward 5′-CTGTATTCCGAAGTGTTTTTCAAG-3′ Reverse 5′-GAATCATCCTTTGCCTCCTG-3′	410 bp

2.4. Preparation of mitochondria from brown adipose tissue

The preparation of mitochondria was done in accordance to the method used by Du (Du et al., 1999). Following its excision, brown adipose tissue was weighed, cut into small pieces with scissors and washed thoroughly with ice cold MSETB buffer (210 mM mannitol, 70 mM sucrose, 0.5 mM EDTA, 10 mM Tris–HCl and 0.2% bovine serum albumin, pH 7.4). The tissues were then suspended in ice-cold MSETB buffer (1 g/10 ml), homogenized in a glass homogenizer using a hand pestle and the homogenate filtered through four layers of gauze. The filtrate was centrifuged at 3000 g for 1.5 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 17,500 g for 4.5 min at 4 °C. The resulting supernatant was discarded and the pellet was re-suspended into the same medium (5 ml/g tissue). The suspension was centrifuged at 15,500 g for 4.5 min at 4 °C. Upon completion of the centrifugation and the supernatant discarded, the resulting pellet was re-suspended in SET buffer (280 mM sucrose, 0.5 mM EDTA and 10 mM Tris–HCl, pH 7.4) and centrifuged at 17,500 g for 4.5 min at 4 °C. The mitochondrial suspension was obtained by suspending the pellet in SET buffer to give a final concentration of approximately 10 mg protein/ml. Mitochondrial protein was determined using Coomassie brilliant blue with bovine serum albumin as the standard.

2.5. Determination of the activity of mitochondrial complex IV

The activity of complex IV was determined spectrophotometrically by measuring the extent of oxidation of cytochrome *c* assayed in a 200 μl reaction mixture containing 8.8 mM potassium phosphate buffer (pH 7.0) and 0.1% reduced cytochrome *c* by vitamin *c* (OD550/OD565>12) for 2 min. Mitochondria concentration was 250 μg/ml, complex IV activity was calculated by measuring the decrease in absorbance due to oxidation of cytochrome *c* at 550 nm (Davis et al., 1985).

2.6. Determination of mitochondrial membrane potential

Fluorescence was determined in a 200 μl reaction volume of microwell plate (137 mM KCl, 3.6 mM NaCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 4 mM Hepes, 1 mg/ml D-glucose, 1% MEM, pH 7.4, 1 mM Rhodamin 123). After being incubated for 30 min in 37 °C, the fluorescence was detected in 485 nm (ex) and 520 nm (em). The quenched fluorescence of mitochondria was the detected fluorescence minus the base fluorescence which was determined before adding mitochondria.

2.7. Measurement of cytochrome c content in mitochondria

Mitochondrial cytochrome *c* content was measured spectrophotometrically according to the method of Du (Du et al., 1999). Mitochondria were sonicated by ultrasound before being added into phosphate buffered solution PBS to 0.1 mg/ml, and 2 μl saturated sodium hydrosulfite were added to remove oxygen. Optical density was screened in SpectraMax M_5 (Molecular BIO. USA.) after vibration. Standard curve was built with cytochrome *c*.

2.8. Statistical analysis

Values are mean±S.D. Statistical differences were calculated with an analysis of variance and $P \leq 0.05$ was considered significant.

3. Results

3.1. Effect of rhCNTF on the body weights of obese KK-Ay mice

The body weights of fasting KK-Ay mice were decreased following administration of rhCNTF or T3 for 30 days. The body weights of Con, CL, CM and CH mice decreased by 9.9%, 5.6%, 8.8% and 9.5% respectively compared with vehicle-treated mice (Fig. 1).

3.2. Effects of rhCNTF on the mass of perirenal fat

Administration of rhCNTF reduced perirenal fat mass in the KK-Ay mice when compared to that of the vehicle-treated group (Fig. 2). The perirenal fat mass of vehicle-treated group was 1.01± 0.18 g and that of CL, CM and CH groups were 0.78±0.11 g ($P<0.05$), 0.77±0.10 g ($P<0.05$) and 0.70±0.11 g ($P<0.01$), respectively.

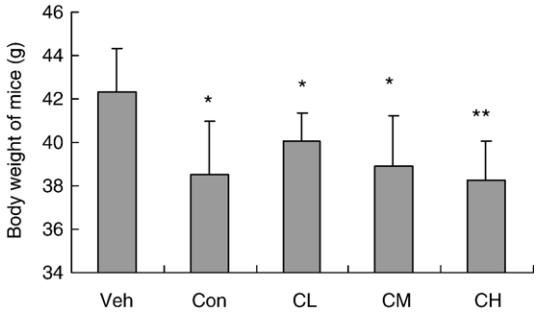

Fig. 1. The effect of 30 days treatment with rhCNTF on the body weights of KK-Ay mice. In all treatment groups (CL — 0.1 mg/kg/day s.c.; CM — 0.3 mg/kg/day s.c.; CH — 0.9 mg/kg/day s.c.), body weight fell significantly. The body weights of the mice treated with 0.4 mg/kg T3 for 30 days (Con, T3, 0.4 mg/kg/day s.c.) also dropped significantly. Each column represents the mean±S.D. of body weights of 6 mice. *$P<0.05$, **$P<0.01$ represent the significance of the difference from the control group (Veh).

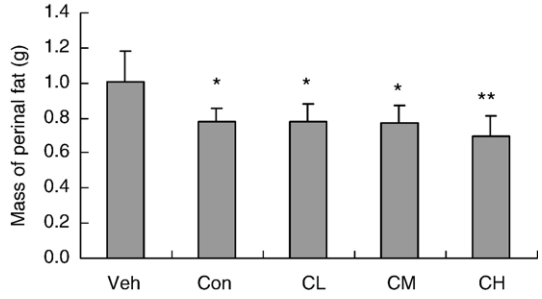

Fig. 2. The effect of 30 days treatment with rhCNTF on the mass of perirenal fat of KK-Ay mice. In all treatment groups (CL — 0.1 mg/kg/day s.c.; CM — 0.3 mg/kg/day s.c.; CH — 0.9 mg/kg/day s.c.), the mass of perirenal fat fell significantly. The mass of perirenal fat of the mice treated with 0.4 mg/kg T3 for 30 days (Con) also dropped significantly. Each column represents the mean±S.D. of body weights of 6 mice. *$P<0.05$, **$P<0.01$ represent the significance of the difference from the control (saline group (Veh)).

3.3. Effect of rhCNTF on the expressions of NRF-1, TFam and UCP-1

Administration of rhCNTF to KK-Ay mice for 3 days significantly increased the expressions of NRF-1, TFam and UCP-1 in brown adipose tissue (Fig. 3). Compared to the vehicle-treated group, the expression of (a) NRF-1 in Con, CL, CM and CH groups increased to 2.01, 1.33, 2.12 and 3.31 fold, respectively, (b) TFam in Con, CL, CM, and CH groups increased to 1.85, 1.57, 2.23 and 2.84 fold, respectively and (c) UCP-1 in Con, CL, CM, and CH groups increased to 1.89, 1.98, 2.36 and 3.12 fold, respectively.

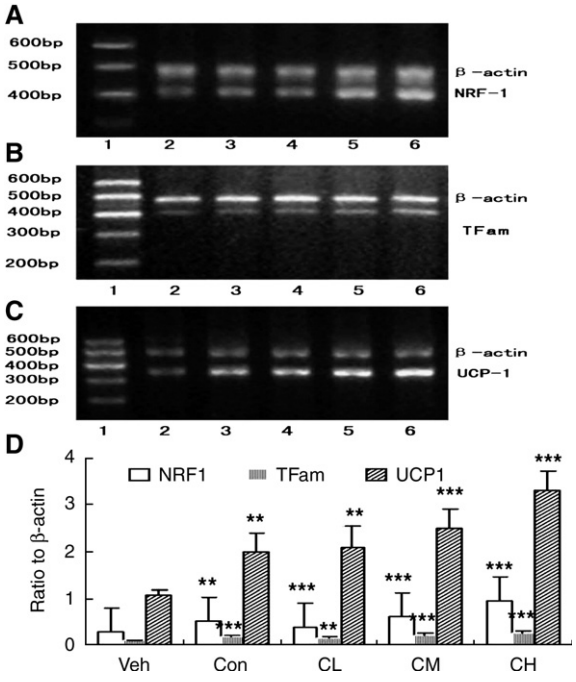

Fig. 3. The effect of 3 days treatment with rhCNTF on the expression of NRF-1, TFam and UCP-1 in KK-Ay mice (A, B and C). In all treatment groups (CL — 0.1 mg/kg/day s.c.; CM — 0.3 mg/kg/day s.c.; CH — 0.9 mg/kg/day s.c.), body weight fell significantly. A positive control group (Con) was given T3 (0.4 mg/kg/day s.c.) lane 1, molecular mass marker; lane 2, vehicle-treated mice (Veh, saline, s.c.); lane 3, positive control T3 0.4 mg/kg/day s.c treated mice (Con,); lane 4, low dose rhCNTF treated mice (CL); lane 5, medium dose rhCNTF treated mice (CM); lane 6, high dose rhCNTF treated mice (CH). (D) Ratios of PCR products relative to β-actin. Each column represents the mean±S.D. from 5 mice. *$P<0.05$, **$P<0.01$ represent the significance of the difference from the control (saline (vehicle)-treated) group (Veh, saline).

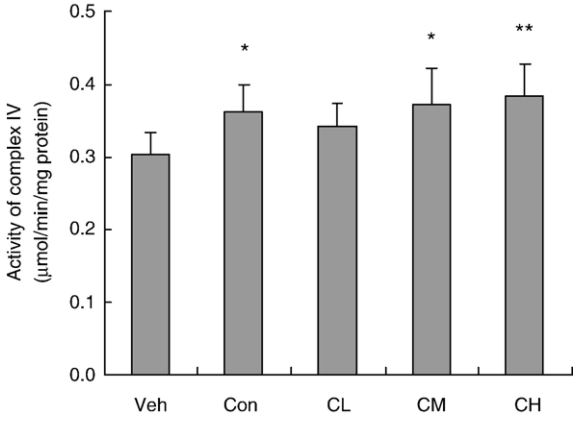

Fig. 4. The effect of 3 days treatment with rhCNTF on the activity of mitochondrial complex IV in brown adipose tissues of KK-Ay mice. In CM and CH groups (CL — 0.1 mg/kg/day s.c.; CM — 0.3 mg/kg/day s.c.; CH — 0.9 mg/kg/day s.c.), the activity of complex IV was greater than that of the saline vehicle-treated control group (Veh). Each column represents the mean±S.D. ($n=5$). *$P<0.05$, **$P<0.01$ represent the significance of the difference from the control (saline (vehicle)-treated) group (Veh, saline). A positive control group of mice (Con) was treated with T3 (0.4 mg/kg/day s.c.).

3.4. Effect of rhCNTF on the activity of complex IV in mitochondria in brown adipose tissue

The activity of mitochondria complex IV in vehicle-treated rats was 0.30±0.03 μmol/min/mg protein. Daily subcutaneous administration of T3, 0.3 mg/kg and 0.9 mg/kg rhCNTF for 30 days increased the activity of mitochondria complex IV (0.36±0.04 μmol/min/mg protein, 0.37±0.04 μmol/min/mg protein and 0.38±0.04 μmol/min/mg protein respectively) (Fig. 4).

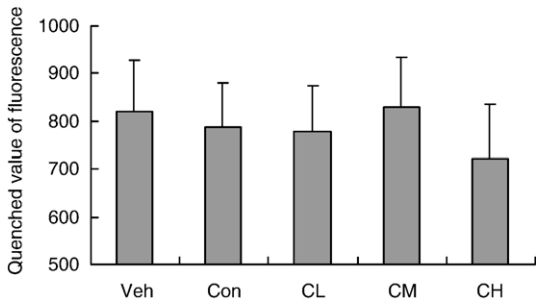

Fig. 5. Effect of rhCNTF on quenched value of fluorescence (marker of mitochondria membrane potential) of mitochondria in mice administered with rhCNTF for 3 days. All mice in Veh and Con group were administered with saline or T3 respectively for 3 days. The quenched fluorescence values in mitochondria of brown adipose tissue in CH group (rhCNTF, 0.1 mg/kg/day s.c.), CM group (0.3 mg/kg/day s.c.) and CH (0.9 mg/kg/day s.c.) group had no statistics difference compared with that of vehicle group (Veh, saline). The data were presented as mean±S.D. ($n=5$).

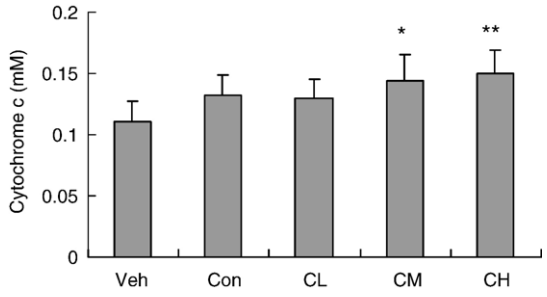

Fig. 6. The effect of 3 days treatment with rhCNTF on the mitochondrial content of cytochrome *c* in brown adipose tissues of KK-Ay mice. In CM and CH groups (CM — 0.3 mg/kg/day s.c.; CH — 0.9 mg/kg/day s.c.), the content was greater than that of the saline vehicle-treated control group (Veh). Each column represents the mean±S.D. ($n=5$). *$P<0.05$, **$P<0.01$ represent the significance of the difference from the control (saline (vehicle)-treated) group (Veh, saline). A positive control group of mice (Con) was treated with T3 (0.4 mg/kg/day s.c.).

3.5. Effect of rhCNTF on the membrane potential of mitochondria in brown adipose tissue

Daily subcutaneous administration of T3, 0.1 mg/kg, 0.3 mg/kg and 0.9 mg/kg rhCNTF for 30 days had no effect on quenched value of fluorescence of mitochondrial. It inferred that the membrane potential was not affected by rhCNTF or T3 (Fig. 5).

3.6. Effect of rhCNTF on the cytochrome c content of mitochondria in brown adipose tissue

The cytochrome *c* content in brown adipose tissue mitochondria was increased in CM, CH group, but not in CL and Con groups. Daily subcutaneous administration of 0.3 mg/kg and 0.9 mg/kg rhCNTF for 30 days significantly increased the content of cytochrome *c* by 30% and 35% respectively (Fig. 6).

4. Discussion

CNTF is a member of gp130 family and hematopoietic cytokines superfamily. It can improve the survival of neurons and are also important in the differentiation of neurons and neuroglial cells. It is also known that neurotrophins, including CNTF, can augment energy metabolism. The latter activities are referred to here as the metabotrophic potential of neurotrophins (Chaldakov et al., 2003). The anti-obesity effect of CNTF was first described in patients with lateral sclerosis of spinal cord. Treatment with $CNTF_{Ax15}$, a modified form of CNTF causes weight loss in obese C57BL/6J mice. CNTF appears to be a modulator of mitochondrial UCP-1 (Bluher et al., 2004) and is an anti-obesity target but the mechanism of its action is still unclear (Russell et al., 2004).

CNTF is a powerful reagent for weight loss. Blusher has confirmed the drug action of CNTF in C57BL/6J mice(Bluher et al., 2004). In our experiment, administration of rhCNTF decreased body weight by 5.2%, 8.1% and 10% in CL, CM and CH groups compared with Veh group (control KK-Ay mice). RhCNTF decreased perirenal fat mass by about 39% contrast to Veh group (control KK-Ay mice). It has powerful anti-obesity effects to the body weight and perirenal fat mass of KK-Ay mice. This effect correlates with enhanced expression of UCP-1, NRF-1 and TFam. UCPs are proteins located in the inner membrane of mitochondria of brown adipose tissue, central nerve system, muscle and skin. They have important physiological functions, namely the translocation of protons from the mitochondrial intermembrane space to the mitochondrial matrix, thereby uncoupling the proton gradient and potential. UCP-1 is a protein involved in energy metabolism and body temperature regulation. When the content of UCP-1 is increased, energy consumption is enhanced by facilitated proton leakage across the mitochondrial membrane. Yasushi and his colleagues demonstrated that the ectopic expression of UCP-1 in liver of obese mice results in augmented energy metabolism (Yasushi et al., 2005). Natalie and her colleagues demonstrated that coordinated regulation of nucleus-encoded mitochondrial transcription factors by members of the NRF family is essential to the control of mitochondrial biogenesis (Natalie et al., 2005). Nuclear DNA and mitochondrial genes for NRF-1, TFam and UCP-1 may be involved in the mechanism (Lin et al., 2002; Kelly and Scarpulla, 2004).

In 2003, Baar reported that the expression of cytochrome *c* increased approximately two fold and delta-aminolevulinate synthase increased approximately 50% in muscle of NRF-1 knock-in mice. The investigator also showed that the levels of some mitochondrial proteins, namely cytochrome *c*, respiratory chain subunits, and delta-aminolevulinate, increased 50–60% (Baar et al., 2003). In addition, other investigators reported that co-activation of NRF-1 following its binding with peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) causes up-regulation of TFam thereby representing the pathway that links an external physiological stimulus to the regulation of mitochondrial replication (Bergeron et al., 2001; Cheng et al., 2004).

In our experiment, we demonstrated that administration of rhCNTF enhanced the expressions of NRF-1, TFam and UCP-1 in brown adipose tissue. The enhanced UCP-1 expression may be increased by the NRF-1 and TFam. To our knowledge, this is the first report of such an action of UCP-1. We also found the activity of complex IV and content of cytochrome *c* was enhanced by rhCNTF.

NRF-1 may also regulate the expression of complex IV and cytochrome *c* (Nakagawa et al., 2006). The administration of rhCNTF had no effect on mitochondrial membrane potential.

In summary, this is the first report that the expression of UCP-1 maybe up-regulated by NRF-1 and TFam. At the same time, we also showed that rhCNTF can increase the activity of complex IV without altering the membrane potential of mitochondria in brown adipose tissue.

These results uncover new areas of research that include the upstream regulation of UCP-1 and NRF-1 involving peroxisome proliferator-activated receptors (PPARs), peroxisome proliferator-activated receptor coactivators (PGCs) and retinoid×receptor (R×R). Whether rhCNTF can become a useful agent for the treatment of obesity needs further study.

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