Peripheral Administration of the Melanocortin-4 Receptor Antagonist NBI-12i Ameliorates Uremia-Associated Cachexia in Mice

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ABSTRACT

We have recently shown that genetic or pharmacological blockade of the melanocortin-4 receptor (MC4-R) attenuates uremia-associated cachexia. However, the potential clinical utility of this approach has been limited by the need to deliver a peptide MC4-R antagonist into the ventricles of the brain. NBI-12i is a recently developed small molecule MC4-R antagonist, with high affinity and selectivity that penetrates the central nervous system after peripheral administration. We tested whether NBI-12i would also be effective in attenuating uremia-associated cachexia in a mouse model. Intraperitoneal administration of NBI-12i stimulated food intake and weight gain in uremic mice. Furthermore, NBI-12i–treated uremic mice gained lean body mass, fat mass, and had a lower basal metabolic rate compared to vehicle-treated and diet-supplemented uremic mice, which lost both lean body mass and fat mass and had an increase in basal metabolic rate. We found that NBI-12i normalizes the expression of uncoupling protein, which is normally upregulated in uremic mice, and we speculate that this may contribute to the drug’s protective effect. These data underscore the importance of melanocortin signaling in the pathogenesis of uremia-associated cachexia and demonstrate the potential of peripheral administration of MC4-R antagonists as a novel therapeutic approach.


Cachexia is common in patients with chronic illnesses such as chronic kidney disease (CKD) and is correlated with quality of life as well as mortality and morbidity in these patients. Anorexia, hypoalbuminemia, muscle wasting, and loss of both lean body mass and fat mass are the predominant clinical features. To date, there is no effective therapy for uremic cachexia. Nutritional strategies such as caloric supplementation and appetite stimulants have largely been unsuccessful. There is, therefore, an urgent need for the development of new therapeutic agents for this potentially fatal complication of CKD.

One important mechanism for cachexia in chronic disease states is that an elevation in circulating proinflammatory cytokines acts on the central nervous system to regulate the release and function of a number of key neurotransmitters, thereby altering both appetite and metabolic rate. Several neuronal pathways, including the leptin and central melanocortin systems, have been identified as targets of cytokine action in the hypothalamus, which is an essential regulator of food intake and energy homeostasis. Pro-opiomelanocor-
tin (POMC) is a propeptide precursor that is produced in neurons found in the hypothalamic arcuate nucleus. POMC neurons are thought to provide an important tonic inhibition of food intake and energy storage, primarily via production and release of α-melanocyte–stimulating hormone, which is derived from the POMC precursor. α-Melanocyte–stimulating hormone binds to melanocortin receptors 3 and 4 (MC3-R and MC4-R) and inhibits food intake, primarily via the MC4-R.6 Recently, we demonstrated that uremia-associated cachexia in mice could be ameliorated by genetic or pharmacologic blockade of central melanocortin signaling via the MC4-R.7 Our findings may form the basis of a novel therapeutic strategy for uremic cachexia.

Although promising, our previous results with AgRP have limited clinical utility, because this peptide needs to be given intracerebroventricularly. NBI-12i, a small-molecule MC4-R antagonist with high affinity, selectivity, and central nervous system penetration after peripheral administration, was recently developed.7 Subsequent studies have demonstrated that NBI-12i improves appetite and prevents weight loss and loss of lean body tissues in tumor-bearing mice.8

The aim of this study was to determine whether peripheral administration of NBI-12i attenuates uremia-associated cachexia. Our results suggest that intraperitoneal delivery of NBI-12i improves food intake, ameliorates weight loss, and decreases basal metabolic rate in uremic mice. In view of the recent findings that increased metabolic rate in cancer cachexia was associated with elevation of uncoupling protein (UCP) gene expression, we further investigated the relationship between beneficial effects of NBI-12i treatment and UCP expression.

RESULTS

Cachexia in Nephrectomized Mice

Male c57BL/6j mice were used for this study. Subtotally nephrectomized (N) mice, fed 17% protein diets, were uremic but not acidic. N mice had higher levels of blood urea nitrogen (BUN) and creatinine (71.6 ± 3.0 and 0.6 ± 0.1 mg/dl, respectively; n = 9) than sham-operated (S) mice (29.7 ± 2.4 and 0.3 ± 0.1 mg/dl, respectively; n = 9; P < 0.0001; Table 1). Blood bicarbonate levels were not different in N mice (26.7 ± 0.3 mmol/L) and S mice (26.5 ± 0.2 mmol/L; Table 1).

N mice were fed ad libitum (53.8 ± 0.5 g), whereas S mice were pair-fed (53.8 ± 0.5 g; Figure 1A) for the study period of 14 d. N mice were cachectic. N mice gained less weight (gain of 0.4 ± 0.1 g) compared with pair-fed S mice (gain of 1.9 ± 0.1 g; P < 0.0001; Figure 1B). N mice continued to lose lean body mass and fat mass (loss of 3.7 ± 0.5 and 5.4 ± 0.8%, respectively), whereas S mice gained lean body mass and fat mass (gain of 3.7 ± 0.6 and 3.6 ± 0.6%, respectively; P < 0.0001; Figure 2, A and B).

Nutritional Effect of NBI-12i on Uremia-Associated Cachexia

After successful nephrectomy or sham operation, 3 mg/kg NBI-12i or saline was given to N or S mice intraperitoneally, twice per day, for a period of 14 d. N mice that were treated with NBI (N-NBI) had higher BUN and creatinine levels (81.2 ± 5.2 and 0.6 ± 0.1 mg/dl, respectively; n = 9) compared with those in S mice that were treated with saline (S-V; 28.9 ± 1.4 and 0.3 ± 0.1 mg/dl respectively; n = 9; P < 0.0001; Table 1). Blood bicarbonate levels in N-NBI mice (26.7 ± 0.2 mmol/L) were comparable to those in S-V mice (26.2 ± 0.3 mmol/L; Table 1).

N-NBI mice resisted N-induced uremic cachexia. N-NBI mice were fed ad libitum, whereas S-V mice were pair-fed with the N-NBI mice (Figure 1C). The cumulative food intake of the N-NBI mice was significantly increased compared with N mice (60.2 ± 1.2 versus 53.8 ± 0.5 g; P < 0.001; Figure 1, A and C). The weight gain in N-NBI and S-V mice was not different (gain of 1.8 ± 0.1 versus 2.1 ± 0.1 g; NS; Figure 1D). There was also no difference in lean body mass gain between N-NBI and S-V mice (gain of 2.2 ± 1.4 versus 3.5 ± 1.9%; NS). N-NBI mice gained fat mass (gain of 2.2 ± 0.5%), although less than S-V mice (gain 5.6 ± 0.7%; P < 0.001; Figure 2, C and D).

To investigate the potential metabolic effects of NBI-12i beyond its nutritional effects, the difference in daily caloric intake between individual N-NBI and N mice was calculated

Table 1. Serum chemistry of experimental micea

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N (n = 9)</th>
<th>S (n = 9)</th>
<th>N-NBI (n = 9)</th>
<th>S-V (n = 9)</th>
<th>N-Supp (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>21.1 ± 0.3</td>
<td>20.9 ± 0.5</td>
<td>19.5 ± 0.4</td>
<td>21.6 ± 0.4</td>
<td>20.2 ± 0.3</td>
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<tr>
<td>Serum chemistry</td>
<td></td>
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</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>71.6 ± 3.0b</td>
<td>29.7 ± 2.4</td>
<td>81.2 ± 5.2b</td>
<td>28.9 ± 1.4</td>
<td>69.1 ± 3.2b</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.6 ± 0.1b</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1b</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.1b</td>
</tr>
<tr>
<td>Bicarbonate (mmol/L)</td>
<td>26.7 ± 0.3</td>
<td>26.5 ± 0.2</td>
<td>26.7 ± 0.2</td>
<td>26.2 ± 0.3</td>
<td>26.6 ± 0.3</td>
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aData are means ± SEM. Five groups of wild-type mice were included: N versus S and N-NBI and S-V versus N-Supp. All mice were killed at the end of a 14-d period.

bP < 0.0001.
and an appropriate amount of aqueous nutritional diet with the differential calories was given as a supplement by oral gavage to another group of N mice (Figure 1C). Calorie-supplemented N (N-Supp) mice were uremic but not acidotic. N-Supp mice had higher levels of BUN and creatinine (69.1 ± 3.2 and 0.7 ± 0.1 mg/dl, respectively; n = 9) than did S-V mice (P < 0.0001; Table 1). Blood bicarbonate levels were not different in N-Supp mice (26.6 ± 0.3 mmol/L) compared with those in S-V mice (Table 1). N-Supp mice were cachectic, despite receiving the same amount of total calories as N-NBI or pair-fed S-V mice. N-Supp mice gained less weight (gain of 0.7 ± 0.1 g) compared with both N-NBI and pair-fed S-V mice (P < 0.002; Figure 1D). N-Supp mice continued to lose lean body mass and fat mass (loss of 0.2 ± 0.2 and 0.7 ± 0.4%, respectively), whereas both N-NBI and S-V mice gained lean body mass and fat mass (P < 0.0001 and P < 0.0001, respectively; Figure 2, C and D).

Metabolic Effect of NBI-12i on Uremia-Associated Cachexia

Previous studies have demonstrated that blockade of the MC4-R is associated with a decrease in basal oxygen consumption, whereas melanocortin agonists increase basal oxygen consumption.9,10 We tested whether NBI-12i has additional metabolic advantages beyond the nutritional effects of stimulating appetite by measuring the basal metabolic rate of individual mice.

Basal metabolic rate was increased in N mice (4182 ± 48 ml/kg per h) compared with S mice (3930 ± 24 ml/kg per h; P < 0.007; Figure 3A). Conversely, efficiency of food consumption (calculated as the cumulative weight gain [in grams] divided by total food consumption [in grams]) was decreased in N mice (0.007 ± 0.001) compared with that in pair-fed S mice (0.036 ± 0.001; P < 0.0001; Figure 3B). NBI-12i significantly decreased energy expenditure. Basal metabolic rate was still increased in N-Supp mice (4214 ± 33 ml/kg per h) compared with that of N-NBI and pair-fed S-V mice (3839 ± 29 and 3867 ± 21 ml/kg per h; P < 0.0001; Figure 3C). Both N-NBI and pair-fed S-V mice had higher efficiencies of food consumption (0.029 ± 0.001 and 0.036 ± 0.002, respectively) than did N-Supp mice (0.012 ± 0.001; P < 0.001) (Figure 3D).

Proinflammatory Cytokines in Uremia-Associated Cachexia

Proinflammatory cytokines may play important roles in the development and progression of cachexia in the uremic milieu. We measured the serum levels of IL-6 in this study. Serum IL-6 levels were significantly increased in N mice (12.2 ± 1.6 pg/ml)
than in S mice (2.3 ± 0.3 pg/ml; *P < 0.0001; Figure 4A). N-NBI and N-Supp mice had higher levels of serum IL-6 (17.7 ± 3.4 and 16.8 ± 4.2 mg/dl, respectively) than did S-V mice (3.6 ± 0.1 mg/dl; *P < 0.001; Figure 4B).

Effects of NBI-12i on UCP Expression in Uremia-Associated Cachexia

UCP-1 mRNA expression was significantly increased in brown adipose tissue (BAT) of N mice (7.94 ± 0.78) compared with pair-fed control S mice (1.00 ± 0.12; *P < 0.001; Figure 5A). NBI-12i decreased BAT UCP-1 mRNA expression in uremic mice. BAT UCP-1 mRNA content was higher in N-Supp mice (8.00 ± 0.46) compared with that of NBI mice and pair-fed S-V mice (2.30 ± 0.13 and 1.02 ± 0.11; *P < 0.001; Figure 5C). UCP-1 protein content was determined by Western blotting in BAT to allow a comparison between the respective UCP-1 mRNA and protein levels. In four animals from each group of mice, we determined the UCP-1 protein levels in BAT. UCP-1 protein level in BAT was higher in N mice than in S mice (65% higher; Figure 5B). The relative amount of UCP-1 protein in BAT was comparable in N-NBI versus S-V mice, whereas BAT UCP-1 protein content was higher in N-Supp mice (60% higher; Figure 5D).

There was no difference in UCP-3 mRNA levels between N mice (0.90 ± 0.18) and S controls (1.00 ± 0.12; Figure 6A). Similarly, there was no difference in UCP-3 mRNA levels among N-NBI, S-V, and N-Supp mice (0.93 ± 0.09 versus 0.94 ± 0.11 versus 0.94 ± 0.09; Figure 6C). However, BAT UCP-3 protein content was 27% higher in N mice than in S mice (Figure 6B). BAT UCP-3 protein content is 34% higher in N-Supp mice than in N-NBI and pair-fed S-V mice (Figure 6D).

Figure 3. Basal metabolic rate (A and C) and efficiency of food consumption (B and D) were measured in experimental mice at the end of the study. Basal metabolic rate (ml/kg per h) is calculated as the mean of three lowest readings obtained during the recording period. Efficiency of food consumption is calculated as the cumulative weight gain (in grams) divided by total food consumption (in grams). Data are means ± SEM.

Figure 4. Serum IL-6 was measured in experimental mice at the end of the study. LINCOplox mouse cytokine/chemokine kit was used. Data are means ± SEM.

Figure 5. UCP-1 mRNA and protein content in BAT. Expression of UCP-1 mRNA in five groups of mice was compared (A and C). The comparative 2−ΔΔCt method was used to determine the relative quantification of UCP-1 versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA. Data are means ± SEM. Final results are expressed as arbitrary units. UCP-1 mRNA (A and C) and protein content (B and D) was evaluated. Equal amount of BAT mitochondrial protein extract from four individual mice within the same experimental group was admixed. For the Western blot, UCP-1 protein was tagged with polyclonal antibodies recognizing UCP-1. Lane 1, N; lane 2, S; lane 3, N-NBI; lane 4, S-V; lane 5, N-Supp. Autoradiograph was subjected to scanning densitometry. Final results are expressed as arbitrary units.
**DISCUSSION**

The hallmark clinical features of cachexia are loss of appetite and an inability to conserve energy, with significant loss of fat and lean body mass. Using an established uremia model, we demonstrated that subtotal nephrectomy in mice led to reduction in food intake and weight gain, as well as loss of lean body mass and fat mass compared with respective pair-fed sham controls. Appetite is a key indicator of nutritional status and clinical outcome in patients with ESRD.

NBI-12i, a potent MC4-R antagonist, stimulated food intake in normal mice and attenuated cachexia in tumor-bearing mice.7,8 Our data demonstrate that peripheral administration of NBI-12i stimulated food intake and improved weight gain, as well as loss of lean body mass and fat mass compared with respective pair-fed sham controls. Appetite is a key indicator of nutritional status and clinical outcome in patients with ESRD.

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Elevated metabolic rate is another important pathophysiologic feature of cachexia.12–14 Previously, we and others demonstrated that intracranial injection of AgRP significantly attenuated inappropriately elevated metabolic rate in cachectic mice.5,15 The effects of nephrectomy on increasing basal metabolic rate were similarly attenuated in N-NBI mice in this study. The most striking observation was that there was no difference in basal metabolic rate between N-NBI and pair-fed S-V mice, whereas basal metabolic rate was still significantly elevated in N-Supp mice. That N-Supp mice gained less weight and continued to lose lean body mass and fat mass compared with N-NBI mice, despite the same intake of total calories, suggested that NBI-12i has additional metabolic advantages beyond the nutritional effects of stimulating appetite in uremic mice. Our results are consistent with a recent report in which peripheral administration of NBI-12i decreased basal metabolic rate in normal and tumor-bearing mice.8 The protection of NBI-12i against cachexia is likely to be due to the combination of appetite stimulation and energy expenditure reduction in uremic mice.

Inflammation is closely associated with increased energy expenditure in patients with CKD.16 Elevated levels of proinflammatory cytokines may lead to increased protein catabolism, enhanced lipolysis, suppression of appetite, and increased energy expenditure in patients with CKD. We showed that serum IL-6 levels were significantly higher in uremic mice, including the mice that were treated with NBI-12i. Thus, the reversal of cachexia by NBI-12i is not mediated by the change in IL-6 levels but rather is due to the effects of signaling through the MC4-R.

We investigated the molecular mechanism of the protective effect of NBI-12i. UCP, a subgroup of the mitochondrial anion transporter superfamily, are the regulators of two critical mitochondrial functions: ATP synthesis and the production of reactive oxygen species. Uncoupling of mitochondrial electron transport chain activity from the phosphorylation of ADP dissipates the electrochemical energy that is generated during mitochondrial respiration as heat, resulting in thermogenesis.17 UCP-1 and UCP-3 are key regulators of energy expenditure such as nonshivering thermogenesis in rodents as well as in humans.18

BAT UCP-1 mRNA was increased in N mice, suggesting the activation of nonshivering thermogenesis in uremic mice. Nonshivering thermogenesis in BAT contributes significantly to resting energy expenditure in rodents.19,20 Increased thermogenic activity of BAT, as assessed by GDP binding, has been demonstrated in cancer cachexia models.21,22 Our results suggested that inappropriate increased energy expenditure in BAT was associated with uremia-associated cachexia. Elevated BAT UCP-1 expression was attenuated by NBI-12i treatment. BAT UCP-1 mRNA content was significantly higher in N-Supp mice compared with that...
of N-NBI mice and pair-fed S-V mice (Figure 5C). The relative amount of BAT UCP-1 protein was 60% higher in N-Supp mice than in N-NBI or S-V mice (Figure 5D).

UCP-3 has also been related to the efficiency of energy metabolism in BAT. Recent reports suggested that UCP-3 gene expression was increased in cachectic states such as cancer.24,25 Upregulation of UCP-3 gene in murine and human myotube cell culture activates proteolytic pathways, suggesting a possible role for UCP-3 in cachexia.26 Most studies on regulation of UCP-3 expression have investigated changes in UCP-3 mRNA levels. UCP-3 expression is regulated at the posttranscriptional level. Hence, UCP-3 expression at the mRNA level may not necessarily correlate with the expression at the protein levels.10 We showed that BAT UCP-3 protein content was 34% higher in N-Supp mice than in N-NBI and pair-fed S-V mice (Figure 6D). This was associated with a 10% higher basal metabolic rate in N-Supp mice compared with N-NBI and pair-fed S-V mice (Figure 3C). Our data suggest that mitochondrial proton leak mediated by BAT UCP-3 may be associated with increased energy expenditure in uremia-associated cachexia.

In summary, we have demonstrated the potential of peripheral administration of MC4-R antagonists as a novel therapeutic approach. We have provided evidence that melanocortin signaling and UCP may be important in the pathogenesis of uremia-associated cachexia.

CONCISE METHODS

Animals
Male c57BL/6J mice from Jackson Laboratory (Bar Harbor, ME), aged 8 to 10 wk, were used. Mice were raised in a 12-h light/12-h dark cycle. Individual mice were housed, and food intake was estimated by measurement of the weight of powdered food remaining in feeding chambers designed to maximize spill capture. In this study, N mice were fed ad libitum with powdered mouse diet 5015 containing 17% crude protein (LabDiet, St. Louis, MO) that was weighed and replaced daily, and S mice were pair-fed with N mice. N-NBI mice were fed ad libitum, whereas S-V were pair-fed with N-NBI mice. N-NBI mice consumed 4 to 5 g whereas N-mice consumed 3 to 4 g of diet 5015, daily. The physiologic fuel value of mouse diet 5015 is 3.83 kcal/g. The difference in daily caloric intake between individual N-NBI and N mice was then subtracted. An aqueous nutritional diet, Magnacal Renal (Mead Johnson Nutritionals, Evansville, IN), with a physiologic fuel value of 3 kcal/ml, contains 15% protein and was given to individual N-Supp mouse by using a curved, 18-G feeding needle (Harvard Apparatus, Holliston, MA). The volume of supplemented diet (in ml) was calculated as the differential caloric intake (in kcal) divided by 3 kcal/ml. Efficiency of food consumption was calculated as the cumulative weight gain (in grams) divided by total food consumption (in grams). All studies complied with Institutional Animal Care and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Standard Nephrectomy and Sham Operation
Uremia was induced in the mice by standard subtotal nephrectomy operation, in a two-stage procedure as described previously.27,28 For each successful nephrectomy, a subsequent sham-control operation was performed in a control mouse.

NBI-12i Preparation and Administration
The detailed procedure for NBI-12i preparation and administration was as described previously.7,8 Each mouse was handled daily for 3 consecutive days before the initiation of the experiment, simulating the restraint used during the administration of the compound. NBI-12i was dissolved in normal saline and administered to the intraperitoneal cavity of the experimental animals. During 14 d of study, 3 mg/kg NBI-12i was injected, twice daily at 8:00 a.m. and 4:00 p.m., using a disposable 0.5-ml syringe (Becton Dickinson, Franklin Lakes, NJ). Food intake and body weight were measured daily, and the dosages were normalized to individual animal body weight.

Body Composition
Body composition was determined at the start and the end of the experiments by dual-energy x-ray (DEXA) using a PIXIimus mouse densitometer (MEC Lunar Corp, Minster, OH). The instrument was calibrated at the start of each recording session with a murine calibration standard. All mice were fasted for 12 h before DEXA analysis to minimize the effect of undigested food on the DEXA analysis.

Indirect Calorimetry
Consumption of oxygen and production of carbon dioxide were simultaneously determined by indirect Oxymax calorimetry (Columbus Instruments, Columbus, OH) while mice were housed in separate chambers at 24 ± 1°C. All mice were first acclimatized to the chambers for 2 d. In addition, NBI-12i was administrated to N-NBI mice while saline was given to S-V and N-Supp mice at 8:00 a.m. before the initiation of the measurement. Measurements were recorded for 3 h during the light cycle (9:00 a.m. to 12:00 p.m.). Samples were recorded every 3 min with the room air reference taken every 30 min and the airflow to chambers 500 ml/min. Basal metabolic rate (ml/kg per h) was determined by averaging the three lowest measurements obtained during the day of observation.

Blood Chemistry Analysis
After 14 d of observation, mice were killed and blood samples were collected for subsequent analysis. BUN and blood bicarbonate levels were assayed by standard laboratory methods. Serum creatinine levels were analyzed using Quantichrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA) with the minimal detection concentration of 0.10 mg/dl. Serum IL-6 was measured using LINCOPlex mouse cytokine/chemokine kit (Linco Research, Billerica, MA). The minimal detection concentration for serum IL-6 was 0.7 pg/ml.

UCP mRNA Expression
BAT total RNA was extracted and reverse-transcribed with standard procedures. Appropriate primers and probes for mouse UCP-1, UCP-3, and endogenous control glyceraldehyde-3-phosphate dehydrogenase were obtained. Identities for mouse Taqman Gene Expres-
sion Assays-On-Demand (ABI Applied Biosystems, Foster City, CA) for UCP-1, UCP-3, and glyceraldehyde-3-phosphate dehydrogenase were Mm00494069_m1, Mm00494074_m1, and 4352339E. The PCR amplification was performed in a final volume of 25 μl, containing 4 μl of cDNA, 1.25 μl of Taqman Gene Expression Assay for target gene, and 1× Taqman Universal Master Mix. The parameters for ABI Prism 7000 Sequence Detection System (Applied Biosystems) were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Comparative \(2^{-\Delta\Delta C_T}\) method was used to determine the relative quantification of target gene. The results are expressed in arbitrary units, with 1 U being the mean mRNA level determined in the pair-fed, sham-operated control group.

UCP Protein Expression

BAT mitochondrial protein was extracted according to Jimenez et al.\(^9\)

Protein concentration was determined using Bio-Rad Dc Assay. Equal amount of total protein (approximately 3 μg) was analyzed on a 10% SDS-PAGE gel in reduced condition and electrotransferred to a nitrocellulose paper. The UCP proteins on blot were detected using polyclonal UCP-1 and UCP-3 antibodies, respectively (RDI-Fitzgerald, Concord, MA). The blot was developed with ECL enhanced chemiluminescence system (Pierce, Rockford, IL). Relative expression of UCP-1 and UCP-3 was quantified by using Quantityone program and GS-700 scanning densitometry (Bio-Rad, Hercules, CA).

Statistical Analyses

Data are expressed as means ± SEM. Results were analyzed by \(t\) test when two groups were included or one-way ANOVA with \(post hoc\) analysis when three groups were included. Data sets were analyzed for statistical significance using SPSS 11.0 software package (SPSS, Chicago, IL).

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DISCLOSURES

None.

REFERENCES


