METABOLISM CLINICAL AND EXPERIMENTAL 61 (2012) 99–107

Available at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Treatment of genetically obese mice with the iminosugar N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin reduces body weight by decreasing food intake and increasing fat oxidation

Mirjam Langeveld[a,*,1], *Sjoerd A.A. van den Berg*[b,1], *Nora Bijl*[a], *Silvia Bijland*[b], *Cindy P. van Roomen*[a], *Judith H. Houben-Weerts*[a], *Roelof Ottenhoff*[a], *Sander M. Houten*[c], *Ko Willems van Dijk*[b], *Johannes A. Romijn*[d], *Albert K. Groen*[a], *Johannes M. Aerts*[a], *Peter J. Voshol*[d,e]

[a] *Department of Medical Biochemistry, Academic Medical Center, 1105 AZ Amsterdam, the Netherlands*
[b] *Department of Human Genetics, Leiden University Medical Center, 2333 ZA Leiden, the Netherlands*
[c] *Laboratory Genetic Metabolic Diseases, Academic Medical Center, 1105 AZ Amsterdam, the Netherlands*
[d] *Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, 2333 ZA, Leiden, the Netherlands*
[e] *Institute of Metabolic Science, University of Cambridge, CB2 0QQ Cambridge, United Kingdom*

ARTICLE INFO

Article history:
Received 22 October 2010
Accepted 9 May 2011

ABSTRACT

Obesity and its associated conditions such as type 2 diabetes mellitus are major causes of morbidity and mortality. The iminosugar *N*-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) improves insulin sensitivity in rodent models of insulin resistance and type 2 diabetes mellitus. In the current study, we characterized the impact of AMP-DNM on substrate oxidation patterns, food intake, and body weight gain in obese mice. Eight *ob/ob* mice treated with 100 mg/(kg d) AMP-DNM mixed in the food and 8 control *ob/ob* mice were placed in metabolic cages during the first, third, and fifth week of the experiment for measurement of substrate oxidation rates, energy expenditure, activity, and food intake. Mice were killed after 6 weeks of treatment. Initiation of treatment with AMP-DNM resulted in a rapid increase in fat oxidation by 129% ($P = .05$), a decrease in carbohydrate oxidation by 35% ($P = .01$), and a reduction in food intake by approximately 26% ($P < .01$) compared with control mice. Treatment with AMP-DNM decreased hepatic triglyceride content by 66% ($P < .01$) and, in line with the elevated fat oxidation rates, increased hepatic carnitine palmitoyl transferase 1a expression. Treatment with AMP-DNM increased plasma levels of the appetite-regulating

Author contributions: Mirjam Langeveld: design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. Sjoerd A.A. van den Berg: design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. Nora Bijl: design and conduct of the study, data collection, and data interpretation. Silvia Bijland: data collection. Cindy P. van Roomen: conduct of the study and data collection. Judith H. Houben-Weerts: data collection. Roelof Ottenhoff: conduct of the study and data collection. Sander M. Houten: data collection and data analysis. Ko Willems van Dijk: data analysis and data interpretation. Johannes A. Romijn: data interpretation and manuscript writing. Albert K. Groen: design of the study, data interpretation, and manuscript writing. Johannes M. Aerts: design of the study, data interpretation, and manuscript writing. Peter J. Voshol: design and conduct of the study, data analysis, data interpretation, and manuscript writing.

* *Corresponding author*. Department of Internal Medicine (F5-166), Division of Endocrinology and Metabolism, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. Tel.: 0031 20 5668890; fax: 0031 20 6917682.
E-mail address: m.langeveld@amc.uva.nl (M. Langeveld).

[1] Equally contributed to this article.

0026-0495/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2011.05.013

peptide YY compared with control mice. Treatment with AMP-DNM rapidly reduces food intake and increases fat oxidation, resulting in improvement of the obese phenotype. These features of AMP-DNM, together with its insulin-sensitizing capacity, make it an attractive candidate drug for the treatment of obesity and its associated metabolic derangements.
© 2012 Elsevier Inc. All rights reserved.

1. Introduction

As the incidence of obesity and its associated morbidities, such as type 2 diabetes mellitus, increases, the search for new treatment modalities to combat these conditions continues. Decreasing caloric intake and increasing physical activity are effective in improving obesity, hypertension, insulin resistance, and dyslipidemia [1-4]. However, the positive effect of these dietary and lifestyle interventions is often short-lasting because permanent changes in lifestyle and diet are hard to achieve [5,6]. Moreover, weight reduction obtained by low-caloric diets induces adaptations in energy metabolism, resulting in a lower resting energy expenditure (EE) [7,8]. If caloric intake is subsequently increased again, fat mass may rapidly increase [9,10]. The emphasis in the treatment of those patients that fail to acquire or maintain weight loss should therefore be on pharmacological interventions aimed at persistent weight loss and the reduction of obesity-associated pathologies.

Obesity and type 2 diabetes mellitus are associated with diminished responsiveness to insulin. Insulin resistance leads to impaired glucose and fat homeostasis; results in damage of organs such as the liver and pancreas due to exposure to inappropriately high insulin, glucose, and fatty acid concentrations [11,12]; and is an independent risk factor for cardiovascular mortality [13]. Moreover, obesity may affect substrate oxidation patterns. In lean individuals, glucose is preferentially oxidized in the fed state and fat in the fasted state. This ability to switch between carbohydrate and fat oxidation, in relation to nutritional status, is referred to as *metabolic flexibility*; and this flexibility is impaired in the obese, insulin-resistant state [14]. Reduced fat oxidation contributes to ectopic fat accumulation, for instance, in liver and muscle, further reducing insulin sensitivity [15]. Improvement of insulin sensitivity is therefore essential for reduction of the negative consequences of obesity.

Ectopic lipid accumulation results in reduced responsiveness to insulin by the formation of specific lipid metabolites that interfere with insulin signalling [16]. Sphingolipids are one class of lipids involved in the induction of insulin resistance. The simplest sphingolipid, ceramide, is formed from palmitoyl-CoA and serine by the enzyme serine-palmitoyltransferase; and the availability of palmitoyl-CoA is rate limiting for ceramide synthesis [17]. From ceramide, glycosphingolipids and subsequently the more complex gangliosides can be formed. Increased availability of the free fatty acid (FFA) palmitate and low-grade inflammation in obesity enhance the synthesis of sphingolipids and gangliosides [18,19]. Ceramide interferes with insulin signaling at the level of PKB-Akt, reducing the metabolic actions of insulin [18,19]. The ganglioside GM3, when present in abundance, is thought to hamper phosphorylation of the insulin receptor, resulting in reduced responsiveness to insulin [20,21].

Reduction of sphingolipid synthesis restores insulin sensitivity in mouse models for insulin resistance and type 2 diabetes mellitus [22-24]. The iminosugar N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) reduces glucosylceramide and subsequent ganglioside synthesis and improves both peripheral and hepatic insulin sensitivity [23]. Subsequent studies showed that this drug also improves adipocyte function, reduces inflammation, and ameliorates hepatic steatosis in insulin-resistant genetically obese *ob/ob* mice [25,26]. In these studies, a reduction in body weight gain upon treatment with a relatively high dose of 100 mg/kg/d AMP-DNM was noted [25,26]. The cause of this reduced body weight gain and the course of the metabolic effects of AMP-DNM administration in time have not been reported so far. To monitor the kinetics of the effect of AMP-DNM on metabolism in *ob/ob* mice, we have now performed an investigation using a setup of computerized metabolic cages, which allows in-depth analysis of the effects of the drug on substrate oxidation, food intake (FI), energy homeostasis, and activity. In this study, we show that treatment with AMP-DNM rapidly increases fat oxidation, reduces carbohydrate oxidation, and decreases FI. These changes result in a sustained lower body weight in the AMP-DNM–treated animals.

2. Methods

2.1. *Animals, diets, and indirect calorimetry*

Experiments were all approved by the ethics committee for animal experiments of the Academic Medical Center or Leiden University Medical Center. Leptin-deficient *ob/ob* mice (C57Bl/6J background), 6 weeks old, were purchased from Charles River Laboratories (Maastricht, the Netherlands). Before the start of the experiments, the animals were housed in a temperature-controlled room on a 12:12-hour light-dark cycle for 2 weeks. They were fed ad libitum with rodent AM-II chow (Arie Blok Diervoeders, Woerden, the Netherlands) containing 24.8% crude protein, 6.6% crude fat (0.018% wt/wt cholesterol), 3.6% crude fiber, and 4.5% minerals.

Indirect calorimetry measurements were performed as described previously [27-29]. In short, in the first experiment, 16 mice were subjected to individual indirect calorimetry measurements (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, Columbus, OH) for 4 consecutive days during weeks 1, 3, and 5 of the experiment. A period of 24 hours was included at the start of the experiment to allow acclimatization of the animals to the cages and the single housing. Food and water were available ad libitum during the whole experiment, and intake was analyzed every 10 seconds. In the first week, after 38 hours of basal measurements, 8 mice were switched (without disruption of calorimetric measurements) to food containing AMP-DNM at a

dose of approximately 100 mg/kg body weight per day. Voluntary physical activity was measured in real time as infrared beam breaks in x and z direction. Experimental analysis started at 9:00 AM and continued for 72 hours. Oxygen consumption (V_{O_2}) and carbon dioxide production rate (V_{CO_2}) measurements were performed at intervals of approximately 5 minutes throughout the whole period. Respiratory exchange rate (RER) as a measure for metabolic substrate choice was calculated as the ratio between V_{CO_2} and V_{O_2}. Carbohydrate and fat oxidation rates were calculated according to Perronnet and Massicote [30] using V_{O_2}/V_{CO_2} values. Total EE was calculated from the sum of carbohydrate and fat oxidation.

Body weight was measured at the beginning of the first, third, and fifth week and at the end of the experiment. Temperature was measured rectally, and blood glucose concentration was determined with a handheld meter (Accu-Check; Roche Diagnostics, Mannheim, Germany) at the end of the first, third, and fifth week upon exiting the metabolic cages. On day 37 (week 6), mice were weighed; and blood glucose and hemoglobin A_{1c} concentrations (A1Cnow; Metrika, Sunnyvale, CA) were determined. Subsequently, mice were fasted for 4 hours and anesthetized by intraperitoneal injection with a combination of 6.25 mg/kg acetylpromazine (Sanofi Santé Nutrition Animale, Libourne Cedex, France), 6.25 mg/kg midazolam (Roche, Mijdrecht, the Netherlands), and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, the Netherlands). Tissues were quickly removed and immediately placed in liquid N_2 and stored at –80°C until further analysis. EDTA plasma was stored at –20°C. Duration of the experiment was chosen based on previous studies showing effects in *ob/ob* mice on body weight and insulin sensitivity after 4 and 5 weeks of treatment with 100 mg/kg body weight per day AMP-DNM [25,26].

In the second experiment, 12 *ob/ob* mice were fasted for 4 hours. Subsequently, a single dose of 100 mg/kg body weight AMP-DNM was administered to 6 mice by gavage. Six control mice received vehicle (water) by gavage. Four hours later, mice were anesthetized; and blood and tissues were handled as in the first experiment.

In a third experiment, 6 *ob/ob* mice were treated for 5 weeks with a daily dose of 100 mg/kg body weight of AMP-DNM by gavage; and 6 control mice received vehicle (water) daily by gavage. Mice were group housed according to treatment, and FI was measured per cage of 6 mice approximately every 3 days (days 3, 7, 10, 14, 21, 24, 28, and 35 of treatment).

2.2. Plasma and tissue biochemical analysis

Plasma insulin concentrations were determined by enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove, IL). Colorimetric enzymatic kits were used for the measurements of plasma and liver triglyceride concentrations (Human, Wiesbaden, Germany) and plasma FFA concentrations (Wako Chemicals, Neuss, Germany). Total plasma peptide YY (PYY) concentrations (PYY 1-36 and PYY 3-36) were measured using a commercially available kit (Phenix Pharmaceuticals, Burlingame, CA). To correlate liver lipid values, the protein content of the liver was measured using the bicinchoninic acid method (Pierce, Perbio Science, Etten-Leur, the Netherlands).

For the analysis of ceramide and glucosylceramide, lipids from 50 μL of 4-times-diluted liver homogenate were extracted according to Folch, followed by deacylation in 500 μL 0.1 mol/L NaOH in methanol using a microwave oven (CEM Microwave Solids/Moisture System SAM-155). The deacylated lipids were derivatized with *O*-phtaldehyde reagent and separated using the high-performance liquid chromatography method as described earlier [31].

Part of the EDTA blood from the second experiment was directly mixed with 1 mol/L perchloric acid and stored on ice for at least 10 minutes. After centrifugation, the supernatant was neutralized using 2 mol/L KOH and 0.5 mol/L 2-(*N*-morpholino) ethanesulfonic acid. Metabolite concentrations were measured in the neutralized supernatant after removal of $KClO_4$. Blood glucose was measured using hexokinase and glucose-6-phosphate dehydrogenase. Pyruvate was measured using lactate dehydrogenase. For lactate, we used lactate dehydrogenase and glutamate pyruvate transaminase. β-Hydroxybutyrate was measured using β-hydroxybutyrate dehydrogenase.

2.3. Gene expression in liver and muscle

Total RNA was extracted from approximately 50 mg frozen tissues using Trizol reagent (Invitrogen, Breda, the Netherlands). For complementary DNA synthesis, RNA was treated with RQ1 RNase-free DNase (Promega, Leiden, the Netherlands) and reverse transcribed with SuperScript II Reverse Transcriptase and random hexamers (Invitrogen). The real-time polymerase chain reaction (PCR) measurement of individual complementary DNAs was performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System using the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The PCR primers were designed on the basis of Primer Express 1.7 software (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The expression data were normalized by calculating the ratio with cyclophilin B (Ppib), a housekeeping gene.

2.4. Statistical analysis

Data are represented as mean ± SD. Statistical analysis for the metabolic cage data were performed on 12-hour averages per parameter, based on the light-dark cycle, apart from total accumulated FI. Data were generated for the light period between 7:00 AM and 7:00 PM and for the dark period between 7:00 PM and 7:00 AM. Normality checks were performed; and in case of normal distribution of the data, comparisons were made using unpaired *t* tests. In all other cases, Mann-Whitney tests were used. Significance was set at $P < .05$.

3. Results

3.1. Exposure to AMP-DNM rapidly increases fat oxidation and decreases carbohydrate oxidation

Leptin-deficient *ob/ob* mice (n = 8) were exposed to 100 mg AMP-DNM/kg body weight per day mixed in food for 5 weeks. In parallel, control *ob/ob* mice (n = 8) received identical food

without AMP-DNM. In accordance with earlier studies [23,25,26], AMP-DNM was well tolerated and caused no overt adverse effects, as further strengthened by the equal activity levels in treated and untreated animals throughout the experiment (Fig. 1D and Supplemental Figure 1). Before treatment initiation, RER, EE, activity, and FI (Fig. 1) were comparable in both randomized groups.

Exposure to AMP-DNM rapidly decreased the RER (dark period: AMP-DNM 0.88 ± 0.07 vs control 0.95 ± 0.05, $P = .03$; light period: AMP-DNM 0.84 ± 0.08 vs control 0.92 ± 0.06, $P = .04$), indicating an increase in the relative fat to carbohydrate oxidation ratio (Fig. 1B, Table 1). The RER remained lower during the third week of treatment (dark period: AMP-DNM 0.84 ± 0.05 vs control 0.93 ± 0.05, $P < .01$; light period: AMP-DNM 0.86 ± 0.04 vs control 0.93 ± 0.05, $P < .01$). During the first 2 days of exposure to AMP-DNM, total fat oxidation was higher in AMP-DNM–treated animals during both the dark period (AMP-DNM 0.16 ± 0.09 vs control 0.07 ± 0.06 kcal/h, $P = .05$) and the light period (AMP-DNM 0.19 ± 0.10 vs control 0.10 ± 0.08 kcal/h, $P = .08$) (Table 1). The high fat oxidation rate in the treated animals was maintained during the third (dark period: AMP-DNM 0.18 ± 0.0 kcal/h; light period: AMP-DNM 0.19 ± 0.06) and fifth week (dark period: AMP-DNM 0.15 ± 0.06 kcal/h; light period: AMP-DNM 0.16 ± 0.05) of treatment (Table 1).

In agreement with the lower RER, carbohydrate oxidation was markedly lower in AMP-DNM–treated animals during the first week (dark period: AMP-DNM 0.28 ± 0.12 vs control 0.43 ± 0.08 kcal/h, $P = .01$; light period: AMP-DNM 0.20 ± 0.34 vs control 0.34 ± 0.09 kcal/h, $P = .01$) and third week (dark period: AMP-DNM 0.26 ± 0.07 vs control 0.42 ± 0.08 kcal/h, $P = .01$; light period: AMP-DNM 0.20 ± 0.10 vs control 0.39 ± 0.08 kcal/h, $P < .01$) of the experiment (Table 1). During the fifth week of the experiment, substrate oxidation rates were no longer different between the treated and control animals. Dark-period and light-period RER did not differ significantly between treated and control animals (dark period: AMP-DNM 0.86 ± 0.05 vs

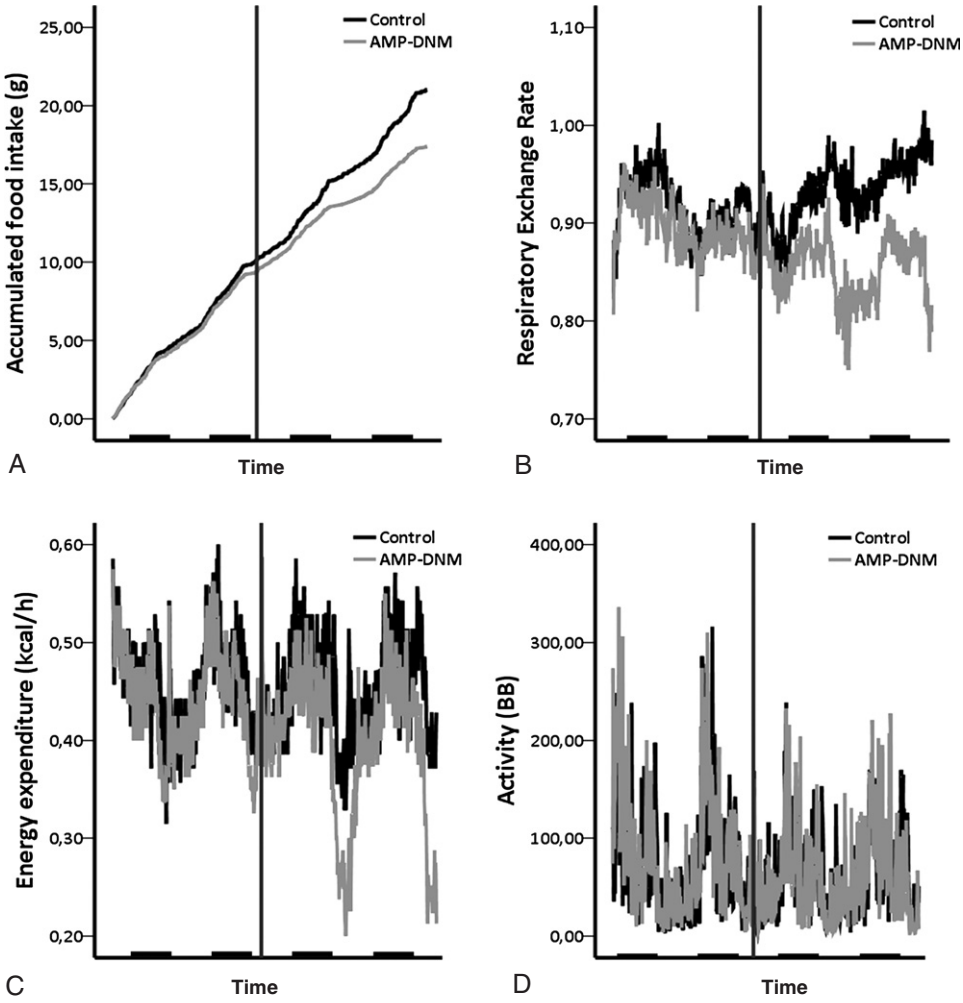

Fig. 1 – Initiation of treatment with AMP-DNM results in rapid reduction in FI (A) and in changes in metabolic substrate selection (B) and total EE (C), without changes in activity (D). Lines represent the mean values in 8 treated mice (gray lines) and 8 control mice (black lines). Vertical lines indicate start of AMP-DNM treatment. Black lines under the x-axis represent the dark (12 hours) and white areas the light periods (12 hours).

Table 1 – Oxidative metabolism and FI data of control and AMP-DNM–treated animals

	Week 1		Week 3		Week 5	
	AMP-DNM	Control	AMP-DNM	Control	AMP-DNM	Control
RER dark	0.88 ± 0.07	0.95 ± 0.05*	0.84 ± 0.05	0.93 ± 0.05†	0.86 ± 0.05	0.87 ± 0.04
RER light	0.84 ± 0.08	0.92 ± 0.06*	0.86 ± 0.04	0.93 ± 0.05†	0.90 ± 0.04	0.90 ± 0.04
FAox dark	0.16 ± 0.09	0.07 ± 0.06*	0.18 ± 0.0	0.10 ± 0.07*	0.15 ± 0.06	0.17 ± 0.07
FAox light	0.19 ± 0.10	0.10 ± 0.08	0.19 ± 0.06	0.10 ± 0.07*	0.16 ± 0.05	0.20 ± 0.07
CHox dark	0.28 ± 0.12	0.43 ± 0.08*	0.26 ± 0.07	0.42 ± 0.08†	0.37 ± 0.09	0.43 ± 0.10
CHox light	0.20 ± 0.10	0.34 ± 0.09*	0.20 ± 0.08	0.39 ± 0.08†	0.25 ± 0.08	0.34 ± 0.09
EE dark	0.44 ± 0.06	0.50 ± 0.05*	0.43 ± 0.06	0.52 ± 0.04†	0.52 ± 0.08	0.60 ± 0.05*
EE light	0.39 ± 0.05	0.45 ± 0.04*	0.40 ± 0.07	0.45 ± 0.03†	0.41 ± 0.08	0.54 ± 0.05†
FI	8.02 ± 0.61	10.9 ± 1.47†	8.07 ± 1.18	11.83 ± 1.08†	8.39 ± 1.13	9.88 ± 1.72*

Values are presented as mean ± SD for 8 mice in each group. Values for FI are the total grams of food consumed during the final 48 hours of each period in the metabolic cages. Respiratory exchange rate, glucose and fat oxidation rates, and EE are the mean values of all measurements during either the dark or light period in the metabolic cages. Fat oxidation rate, carbohydrate oxidation rate, and EE are expressed in kilocalories per hour; FI is expressed in grams per 48 hours. FAox indicates fat oxidation rate; CHox, carbohydrate oxidation rate.

* $P < .05$.

† $P < .01$.

control 0.87 ± 0.04, $P = 1.00$; light period: AMP-DNM 0.90 ± 0.04 vs control 0.90 ± 0.04, $P = .73$); and absolute fat oxidation and glucose oxidation rates were similar in both groups (Table 1; Supplemental Figure 2).

3.2. *Exposure to AMP-DNM decreases FI and total EE*

Concomitantly with the changes in substrate oxidation patterns, FI decreased after initiation of treatment (Table 1). Total caloric intake was lower in treated compared with control animals in the first (grams consumed during 48 hours: AMP-DNM 8.0 ± 0.6 vs control 10.9 ± 1.5, $P < .01$), third (grams consumed during 48 hours: AMP-DNM 8.1 ± 1.8 vs control 11.8 ± 1.1, $P < .01$), and fifth week of treatment (grams consumed during 48 hours: AMP-DNM 8.4 ± 1.1 vs control 9.9 ± 1.7, $P = .04$) (Table 1) (Supplemental Figure 2). The reduction in FI was confirmed in a separate experiment, where FI was monitored every 3 days for a period of 5 consecutive weeks (Fig. 3B).

Total EE was lower in treated animals during the first (dark period: AMP-DNM 0.44 ± 0.06 vs control 0.50 ± 0.05 kcal/h, $P = .03$; light period: AMP-DNM 0.39 ± 0.05 vs control 0.45 ± 0.04 kcal/h, $P = .02$) (Fig. 1C), third (dark period: AMP-DNM 0.43 ± 0.06 vs control 0.52 ± 0.04 kcal/h, $P < .01$; light period AMP-DNM 0.40 ± 0.07 vs control 0.45 ± 0.03 kcal/h, $P < .01$), and fifth week of treatment (dark period: AMP-DNM 0.52 ± 0.08 vs control 0.60 ± 0.05 kcal/h, $P = .02$; light period: AMP-DNM 0.41 ± 0.08 vs control 0.54 ± 0.05 kcal/h, $P < .01$) (Table 1; Supplemental Figure 2). Total animal activity did not differ between the treatment groups at any point during the entire experiment, confirming that treatment with AMP-DNM did not result in malaise (Supplemental Figure 1).

3.3. *Exposure to AMP-DNM decreases body weight gain*

Lower body weight gain in the treated animals was noted during the first 2 weeks (gain as percentage of initial body weight: AMP-DNM 5.0% ± 5.3% vs control 14.1% ± 1.6%, $P < .01$) and the second 2 weeks of treatment (gain as percentage of initial body weight: AMP-DNM 7.1% ± 6.8% vs control 24.2% ± 4.0%, $P < .01$) (Table 2). This had not yet resulted in a significantly lower body weight at the beginning of the third week of treatment, probably because of a slightly higher body weight in the treated group at the start of the experiment (Table 2). At the beginning of the fifth week (Table 2) and at the end of the experiment (day 37: AMP-DNM 46.2 g vs control 51.3 g, $P = .01$), body weight was significantly lower in the treated animals.

Table 2 – Weight and temperature characteristics of control and AMP-DNM–treated animals

	Week 1		Week 3		Week 5	
	AMP-DNM	Control	AMP-DNM	Control	AMP-DNM	Control
Weight (g)	41.0 ± 2.4	39.9 ± 2.1	43.5 ± 2.8	45.5 ± 2.3	44.3 ± 3.3	49.5 ± 3.0†
Temperature (°C)	33.8 ± 3.8	36.8 ± 1.6	36.0 ± 0.5	37.1 ± 0.7†	32.5 ± 3.7	36.5 ± 0.8†
ΔWeight (g)			2.1 ± 2.3	5.6 ± 0.6†	3.0 ± 3.1	9.7 ± 2.3†
ΔWeight (%)			5.0 ± 5.3	14.1 ± 1.6†	7.1 ± 6.8	24.2 ± 4.0†

Values are presented as mean ± SD for 8 mice in each group. Body weight gain and percentage body weight gain compared with initial body weight at the start of the experiment.

† $P < .01$.

Core body temperature did not differ significantly between both groups after 48 hours of treatment; but at the end of both the third and fifth week of treatment, it was lower in the AMP-DNM–treated animals (Table 2).

3.4. *Treatment with AMP-DNM improves glucose homeostasis*

From 2 weeks of treatment onward, nonfasted plasma glucose levels were significantly lower in the AMP-DNM–treated animals. The improved glucose homeostasis upon AMP-DNM treatment was reflected in lower glucose concentrations throughout the experiment (glucose at weeks 2, 3, and 6 in AMP-DNM vs control: 6.8 ± 1.2 vs 10.8 ± 3.7 mmol/L, 6.5 ± 2.0 vs 12.6 ± 5.1 mmol/L, and 6.9 ± 1.1 vs 10.9 ± 2.3 mmol/L; $P < .05$ for all comparisons). Furthermore, glycated hemoglobin levels were significantly lower after 6 weeks of treatment (hemoglobin A_{1c}: AMP-DNM 4.3% ± 0.3% vs control 6.1% ± 0.7%, $P < .01$), confirming previously reported data [23,25,26].

3.5. *Treatment with AMP-DNM results in upregulation in ß-oxidation genes in liver and reduction hepatic fat content*

Given the observation of increased fat oxidation rates in AMP-DNM–treated *ob/ob* mice, we decided to study the expression of genes involved in fatty acid oxidation (carnitine palmitoyl transferase–1a [CPT-1a], long-chain Acyl-CoA dehydrogenase [LCAD], pyruvate dehydrogenase kinase isozyme 4 [PDK-4]) in liver and muscle and the effects of treatment on liver lipid content. Mice were killed after 5 weeks of treatment with AMP-DNM. We found increased hepatic expression of CPT-1a (AMP-DNM 0.36 ± 0.13 vs control 0.12 ± 0.06, $P < .01$) (Fig. 2D), suggesting an increased hepatic fatty acid oxidation rate in AMP-DNM–treated animals. Expression of LCAD and PDK-4 in liver was not significantly affected (Fig. 2E, F).

Table 3 – Blood/plasma parameters 4 hours after gavage of control and AMP-DNM–treated animals

	AMP-DNM	Control
Glucose (mmol/L)	12.0 ± 2.3	15.0 ± 3.4
Insulin (ng/mL)	15.5 ± 8.6	9.2 ± 1.9
TG (mmol/L)	0.83 ± 0.12	0.79 ± 0.12
FFA (mmol/L)	0.60 ± 0.20	0.49 ± 0.22
Pyruvate (μmol/L)	109 ± 26	130 ± 40
Lactate (mmol/L)	1.73 ± 0.75	1.38 ± 0.50
β-Hydroxybutyrate (μmol/L)	66 ± 33	54 ± 51

Values are presented as mean ± SD. TG indicates triglyceride.

Expression of the examined fatty acid oxidation genes in muscle was not changed after 5 weeks of AMP-DNM treatment (CPT-1a: AMP-DNM 0.15 ± 0.03 vs control 0.23 ± 0.10, $P = .09$; LCAD: AMP-DNM 2.4 ± 0.80 vs control 3.1 ± 1.6, $P = .37$; PDK-4: AMP-DNM 4.5 ± 2.3 vs control 3.3 ± 0.9, $P = .19$).

As described previously [22,24], AMP-DNM lowered glucosylceramide in liver (AMP-DNM 0.11 ± 0.02 vs control 0.27 ± 0.06 nmol/mg protein, $P < .01$) (Fig. 2B) without altering ceramide levels (Fig. 2C). Treatment with AMP-DNM decreased liver triglyceride content by approximately 66% (AMP-DNM 208 ± 64 vs control 614 ± 91 nmol/mg protein, $P < .01$) (Fig. 2A), most likely as a result of both the earlier

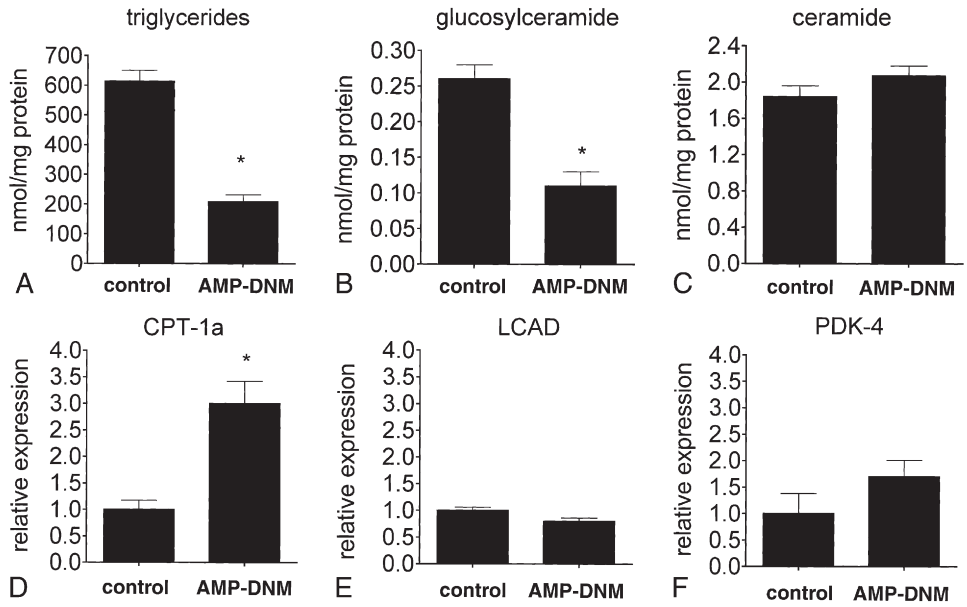

Fig. 2 – Long-term treatment with AMP-DNM results in reduction in hepatic triglyceride (A) and glucosylceramide (B) content, whereas hepatic ceramide (C) concentration does not change significantly. The CPT-1a expression, regulating fatty acid entry into mitochondria for oxidation, is significantly increased in AMP-DNM–treated compared with control mice (D), whereas LCAD (E) and PDK-4 (F) expressions are not significantly changed (all expression relative to the housekeeping gene cyclophilin B [Ppib] and expression in the control mice). Data presented as mean ± SEM. *P< .01.

observed decreased lipogenesis [26] and the here observed increased fat oxidation.

3.6. Four-hour exposure to AMP-DNM does not change metabolic parameters

A switch from carbohydrate to fat oxidation may be explained by limitation of the availability of glucose for oxidation. To study this possibility, groups of 6 *ob/ob* mice received AMP-DNM or vehicle by gavage and were killed after 4 hours. This short-term exposure to AMP-DNM did not change plasma levels of glucose, insulin, FFAs, and triglycerides and of the intermediate metabolites pyruvate, lactate, and the ketone β-hydroxybutyrate (Table 3). A decrease in substrate availability is therefore unlikely to be the cause of acute effects of AMP-DNM on substrate oxidation patterns.

3.7. Treatment with AMP-DNM results in increased levels of the regulatory protein PYY

In search for a mechanism by which AMP-DNM increases fat oxidation and decreases FI (Fig. 3B), we observed that these effects are very similar to the effects of PYY. This short protein is released by L cells in the intestine in response to FI and decreases appetite and increases fatty acid oxidation. We therefore speculated that AMP-DNM treatment would increase plasma levels of PYY; and indeed, after 5 weeks AMP-DNM treatment, plasma PYY levels were elevated in the treated compared with control mice (AMP-DNM 0.65 ± 0.10 vs control 0.43 ± 0.05 ng/mL, $P < .01$) (Fig. 3A).

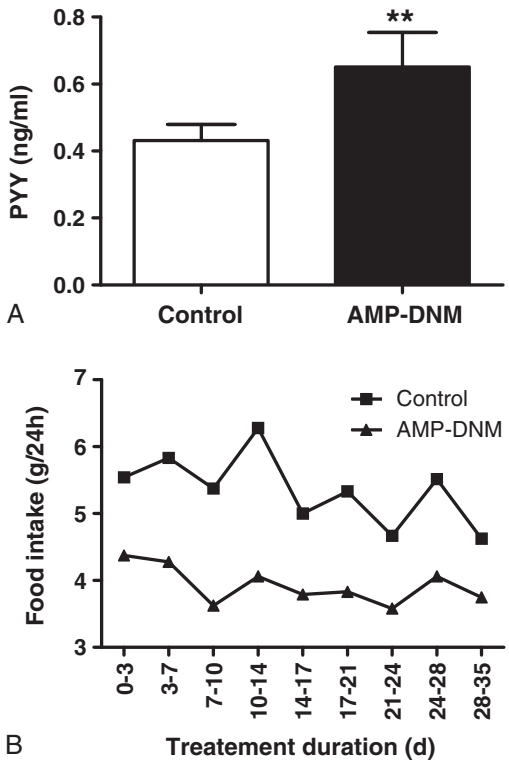

Fig. 3 – Plasma PYY levels after 35 days of treatment were significantly elevated in the AMP-DNM–treated animals compared with controls. Food intake was reduced throughout the experiment. Data presented as mean ± SEM. *P < .01.

4. Discussion

Previous studies on the effect of AMP-DNM treatment in *ob/ob* mice showed marked improvement of insulin resistance, hepatic steatosis, and inflammation [23,25,26]. This prompted us to investigate the effect of this compound on substrate oxidation patterns and energy homeostasis. In the current study, we show that treatment of *ob/ob* mice with AMP-DNM rapidly increases fat oxidation and lowers carbohydrate oxidation. This change in substrate oxidation pattern did not merely result from reduced plasma glucose availability or increased plasma FFA availability because AMP-DNM did not affect plasma glucose, FFA, or insulin levels after 4 or 36 hours of treatment. In addition, AMP-DNM immediately reduced FI. Because this reduction in FI could have been the result of taste aversion resulting from mixing the compound in the food, we performed an experiment in which AMP-DNM was administered to *ob/ob* mice in a similar dose by gavage. In the AMP-DNM–treated mice compared with control mice receiving water by gavage, a decrease in FI was observed starting the first days after treatment initiation (Supplemental Figure 3), indicating an effect of AMP-DNM on FI independent of taste. These observations, in addition to our previous observation regarding the insulin-sensitizing effects of AMP-DNM, suggest that AMP-DNM and related compounds may be relevant therapeutical approaches to improve the pathophysiology of obesity and insulin resistance.

During the last week of the experiment, substrate oxidation rates were no longer different between AMP-DNM–treated and control animals. However, fat oxidation rates did not return to the level observed before treatment initiation; rather, the fat oxidation rates in untreated animals increased during the last 2 weeks of the experiment to the level observed in the treated animals. This was most likely due to the increased obesity state in the control group. Indeed, the gross expansion of the fat stores in the control group (+24% body weight compared with +7% in the AMP-DNM–treated group) would result in increased fat oxidation [32]. The higher glucose oxidation rates in the AMP-DNM group possibly reflect the improved insulin sensitivity. Food intake during the fifth week of treatment was comparable to the earlier 1- and 3-week treatment, but glucose oxidation rates were higher. This indicates that the metabolic flexibility of the treated animals (the switch of the oxidative response upon carbohydrate intake) is significantly improved. A greater metabolic flexibility upon a mixed meal intake is associated with a better insulin sensitivity [14].

It remains to be clarified how the reduction in FI and the changes in substrate oxidation patterns are related. A reduction in body weight, as a result of a low-caloric diet, is associated with an increase in fat oxidation in the fasted state in some studies, although others report no effect of weight loss on fatty acid oxidation [14]. In the current study, the altered substrate oxidation pattern clearly preceded the reduction in body weight. This points toward effects of AMP-

DNM on substrate oxidation patterns independent of changes in body weight.

Treatment with AMP-DNM increased fat oxidation, reflected by increased hepatic expression of genes involved in fat oxidation. In accordance with increased fat oxidation rates, AMP-DNM reduced hepatic steatosis in *ob/ob* mice. This inverse relationship between the rate of fat oxidation and the hepatic accumulation of triglycerides is also supported by other studies. For instance, tetrladecylthioacetic acid increases β-oxidation by the induction of increased CPT-1 activity, which protects against diet-induced obesity and insulin resistance, and lowers liver triglyceride content [33]. Conversely, methyl palmoxirate acutely inhibits β-oxidation by inhibition of CPT-1 activity, which results in increased liver triglyceride content even within 6 hours [34]. Therefore, the stimulation of the expression of CPT-1 by AMP-DNM is in line with the reduction in hepatic triglyceride stores.

Core temperature (as assessed by rectal temperature measurement) was not significantly different at the end of the first week of the experiment, but was significantly lower in the AMP-DNM–treated animals at the end of the third and fifth week. *Ob/ob* mice are known to have defective brown adipose tissue thermogenesis due to their leptin deficiency. As has been shown in a study by Himms-Hagen [35], this thermogenic deficiency is aggravated when FI is restricted, resulting in low core body temperature. We therefore hypothesize that the lower core temperature in our treated mice is secondary to the reduction in FI. Because this effect of food restriction on thermogenesis is very pronounced in the *ob/ob* (leptin-deficient) model, we speculate that this effect will be much less prominent in other models or even clinical settings. The lower core temperature could be an adverse effect of treatment with AMP-DNM. However, AMP-DNM treatment is not associated with increased circulating aspartate aminotransferase\alanine aminotransferase levels [26]. Furthermore, we did not see any reduction in physical activity levels in AMP-DNM–treated animals compared with controls, suggesting that AMP-DNM treatment did not result in malaise.

Four-hour treatment with AMP-DNM did not change blood glucose, FFA, and intermediate substrate concentrations; nor did it change expression of genes involved in fatty acid oxidation in liver and muscle (Supplemental Figure 3). Thus, the decrease in FI and the increase in fatty acid oxidation are not secondary to peripheral metabolic effects of AMP-DNM. This suggests that there may be a direct effect of the compound on mediators of satiety signals such as intestinal hormones. In the current study, we found that treatment with AMP-DNM increased the plasma concentration of one of these satiety-regulating intestinal hormones, plasma PYY. This peptide is released by L cells in the intestine in response to FI. Peptide YY reduces appetite and slows gastric emptying, most likely because of stimulation of the Y2 receptor in the hypothalamus [36,37]. Peripheral short- and long-term PYY 3-36 administration stimulates fatty acid oxidation [38] and reduces body weight in rodent models of diet-induced obesity [39]. Thus, the increase in PYY release from the gut in response to AMP-DNM might explain, at least in part, the observed effects of AMP-DNM treatment.

The studies described in this article add to our knowledge of the beneficial effects of iminosugar treatment on obesity and insulin resistance by showing directs effects of iminosugar treatment on FI and substrate oxidation patterns. However, this article does not provide a full mechanistic explanation of the observed effects. Future studies are needed in which PYY and other intestinal hormones are measured at different time points after AMP-DNM treatment, in relation to FI and substrate oxidations patterns, to confirm the role of satiety hormones as mediators of the reduced FI induced by AMP-DNM treatment.

In conclusion, treatment with AMP-DNM rapidly increases fat oxidation and decreases FI. This is associated with an increase in PYY release from the L cells in the gut. Prolonged treatment with AMP-DNM significantly improves glucose homeostasis and metabolic flexibility and reduces body weight gain. Furthermore, prolonged treatment with AMP-DNM reduced hepatic steatosis. Together, this makes AMP-DNM an attractive candidate for the treatment of obesity and its metabolic complications.

Supplementary materials related to this article can be found online at doi:10.1016/j.metabol.2011.05.013.

Funding

This work was supported by grants from the Center of Medical Systems Biology and the Netherlands Consortium for Systems Biology established by the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research.

Conflicts of Interest

No conflicts of interest were reported.

REFERENCES

[1] Pi-Sunyer X, Blackburn G, Brancati FL, et al. Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial. Diabetes Care 2007;30:1374-83.
[2] DeFronzo RA, Sherwin RS, Kraemer N. Effect of physical training on insulin action in obesity. Diabetes 1987;36: 1379-85.
[3] Kraus WE, Houmard JA, Duscha BD, et al. Effects of the amount and intensity of exercise on plasma lipoproteins. N Engl J Med 2002;347:1483-92.
[4] Weinstein AR, Sesso HD, Lee IM, et al. The joint effects of physical activity and body mass index on coronary heart disease risk in women. Arch Intern Med 2008;168: 884-90.
[5] Douketis JD, Feightner JW, Attia J, et al. Periodic health examination, 1999 update: 1. Detection, prevention and treatment of obesity. Canadian Task Force on Preventive Health Care. CMAJ 1999;160:513-25.
[6] Catenacci VA, Wyatt HR. The role of physical activity in producing and maintaining weight loss. Nat Clin Pract Endocrinol Metab 2007;3:518-29.
[7] Wadden TA, Vogt RA, Andersen RE, et al. Exercise in the treatment of obesity: effects of four interventions on body composition, resting energy expenditure, appetite, and mood. J Consult Clin Psychol 1997;65:269-77.

[8] Foster GD, Wadden TA, Swain RM, et al. Changes in resting energy expenditure after weight loss in obese African American and white women. Am J Clin Nutr 1999;69:13-7.
[9] van DD, Saris WH, ten HF. Weight maintenance and resting metabolic rate 18-40 months after a diet/exercise treatment. Int J Obes 1990;14:347-59.
[10] Summermatter S, Mainieri D, Russell AP, et al. Thrifty metabolism that favors fat storage after caloric restriction: a role for skeletal muscle phosphatidylinositol-3-kinase activity and AMP-activated protein kinase. FASEB J 2008;22:774-85.
[11] Bays HE. "Sick fat," metabolic disease, and atherosclerosis. AmJ Med 2009;122:S26-37.
[12] Muniyappa R, Iantorno M, Quon MJ. An integrated view of insulin resistance and endothelial dysfunction. Endocrinol Metab Clin North Am 2008;37:685-711.
[13] Godsland IF, Lecamwasam K, Johnston DG. A systematic evaluation of the insulin resistance syndrome as an independent risk factor for cardiovascular disease mortality and derivation of a clinical index. Metabolism 2011, doi:10.1016/j.metabol.2011.02.012.
[14] Corpeleijn E, Saris WH, Blaak EE. Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. Obes Rev 2009;10:178-93.
[15] Westerterp KR. Dietary fat oxidation as a function of body fat. CurrOpinLipidol 2009;20:45-9.
[16] Szendroedi J, Roden M. Ectopic lipids and organ function. Curr Opin Lipidol 2009;20:50-6.
[17] Merrill Jr AH, Wang E, Mullins RE. Kinetics of long-chain (sphingoid) base biosynthesis in intact LM cells: effects of varying the extracellular concentrations of serine and fatty acid precursors of this pathway. Biochemistry 1988;27:340-5.
[18] Holland WL, Summers SA. Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. Endocr Rev 2008;29: 381-402.
[19] Langeveld M, Aerts JM. Glycosphingolipids and insulin resistance. Prog Lipid Res 2009;48:196-205.
[20] Tagami S, Inokuchi JJ, Kabayama K, et al. Ganglioside GM3 participates in the pathological conditions of insulin resistance. J Biol Chem 2002;277:3085-92.
[21] Kabayama K, Sato T, Saito K, et al. Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. Proc Natl Acad Sci USA 2007;104: 13678-83.
[22] Zhao H, Przybylska M, Wu IH, et al. Inhibiting glycosphingolipid synthesis improves glycemic control and insulin sensitivity in animal models of type 2 diabetes. Diabetes 2007;56: 1210-8.
[23] Aerts JM, Ottenhoff R, Powlson AS, et al. Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. Diabetes 2007;56:1341-9.
[24] Holland WL, Brozinick JT, Wang LP, et al. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. Cell Metab 2007;5:167-79.
[25] van EM, Aten J, Bijl N, et al. Reducing glycosphingolipid content in adipose tissue of obese mice restores insulin sensitivity, adipogenesis and reduces inflammation. PLoS One 2009;4:e4723.
[26] Bijl N, Sokolovic M, Vrins C, et al. Modulation of glycosphingolipid metabolism significantly improves hepatic insulin sensitivity and reverses hepatic steatosis in mice. Hepatology 2009;50:1431-41.
[27] van den Berg SA, Nabben M, Bijland S, et al. High levels of whole-body energy expenditure are associated with a lower coupling of skeletal muscle mitochondria in C57Bl/6 mice. Metabolism 2010;59:1612-8.
[28] de Vogel-van den Bosch J, van den Berg SA, Bijland S, et al. High-fat diets rich in medium- versus long-chain fatty acids induce distinct patterns of tissue specific insulin resistance. J Nutr Biochem 2011;22:366-71.
[29] van den Berg SA, Guigas B, Bijland S, et al. High levels of dietary stearate promote adiposity and deteriorate hepatic insulin sensitivity. Nutr Metab (Lond) 2010;27:7-24.
[30] Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. Can J Sport Sci 1991;16:23-9.
[31] Groener JE, Poorthuis BJ, Kuiper S, et al. HPLC for simultaneous quantification of total ceramide, glucosylceramide, and ceramide trihexoside concentrations in plasma. Clin Chem 2007;53:742-7.
[32] Flatt JP. The difference in the storage capacities for carbohydrate and for fat, and its implications in the regulation of body weight. Ann NY Acad Sci 1987;499:104-23.
[33] Madsen L, Guerre-Millo M, Flindt EN, et al. Tetradecylthioacetic acid prevents high fat diet induced adiposity and insulin resistance. J Lipid Res 2002;43:742-50.
[34] Duivenvoorden I, Teusink B, Rensen PC, et al. Acute inhibition of hepatic beta-oxidation in APOE*3Leiden mice does not affect hepatic VLDL secretion or insulin sensitivity. J Lipid Res 2005;46:988-93.
[35] Himms-Hagen J. Food restriction increases torpor and improves brown adipose tissue thermogenesis in *ob/ob* mice. Am J Physiol 1985;248:E531-9.
[36] Karra E, Chandarana K, Batterham RL. The role of peptide YY in appetite regulation and obesity. J Physiol 2009;587:19-25.
[37] Batterham RL, Cowley MA, Small CJ, et al. Gut hormone PYY (3-36) physiologically inhibits food intake. Nature 2002;418: 650-4.
[38] van den Hoek AM, Heijboer AC, Voshol PJ, et al. Chronic PYY3-36 treatment promotes fat oxidation and ameliorates insulin resistance in C57BL6 mice. Am J Physiol Endocrinol Metab 2007;292:E238-45.
[39] Vrang N, Madsen AN, Tang-Christensen M, et al. PYY(3-36) reduces food intake and body weight and improves insulin sensitivity in rodent models of diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 2006;291:R367-75.