The role of glycosphingolipids in insulin signaling and lipid metabolism

Bijl, N.

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Chapter 5

Treatment of genetically obese mice with the iminosugar AMP-DNM increases fat oxidation and decreases food intake

M. Langeveld[1], S. Van den Berg[2,] N. Bijl[1], S. Bijland[2], C.P. van Roomen[1], J.H. Houben-Weerts[1], R. Ottenhoff[1], S.M. Houten[3], K. Willems van Dijk[2], A. Kalsbeek[4], C.X. Yi[4], A.K. Groen[1], J.M. Aerts[1] and P.J. Voshol[2]

[1]Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands,

[2]Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands

[3]Department of Clinical Chemistry, Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands,

[4]Netherlands Institute for Neuroscience, The Netherlands

Abstract

Objectives and background

Obesity and its associated conditions such as insulin resistance and type II diabetes are a major cause of morbidity and mortality. Crucial in the treatment of obesity-associated pathology is enhancement of insulin sensitivity, which leads to improved nutrient homeostasis. The iminosugar AMP-DNM has been shown to improve insulin sensitivity in rodent models for insulin resistance and type II diabetes by lowering glycosphingolipid levels. In the current study we characterized the impact of AMP-DNM on metabolic parameters in ob/ob mice, by measuring substrate oxidation patterns, food intake and body weight gain.

Results

Eight mice treated with 100 mg/kg/day AMP-DNM mixed in the food and eight control 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. Treatment initiation resulted in a rapid increase in fat oxidation, decrease in carbohydrate oxidation and a reduction in food intake as well as energy expenditure. Mice were sacrificed at day 37 of the experiment. In liver of AMP-DNM treated animals triglyceride content was markedly decreased and, in line with the elevated fat oxidation rate, CPT-1a expression was increased. In a second more acute experiment, four hours after administration of 100 mg/kg AMP-DNM to ob/ob mice no changes were detected in plasma metabolic parameters but immunohistochemical analysis of the brain showed activation of the arcuate nucleus and lateral hypothalamus, both regions known to be involved in the regulation of food intake.

Conclusions

Treatment with AMP-DNM rapidly increases fat oxidation, decrease carbohydrate oxidation, and reduces food intake. These features of AMP-DNM, together with its insulin-sensitizing capacity, makes it an attractive candidate drug for the treatment of obesity and its associated metabolic derangements.

Introduction

As the incidence of obesity and its associated morbidities, such as type II diabetes, increases, the search for new treatment modalities to combat these conditions continues.

Decreasing caloric intake and increasing the amount of physical activity have been shown effective in improving obesity, hypertension, insulin resistance and dyslipidemia[1-4]. However, the positive effect of dietary and life style interventions is often short lasted, due to the fact that permanent changes in life style 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[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 weight loss or the reduction of obesity-associated pathologies.

An important link between obesity and its associated pathology is diminished responsiveness to insulin. Insulin resistance leads to impaired glucose and fat homeostasis and 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]. Moreover, obesity may have an effect on 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 substrates, depending on their availability, is referred to as metabolic flexibility and this flexibility is impaired in the obese, insulin resistant state[13]. Reduced fat oxidation adds to fat accumulation in for example the liver, further reducing insulin sensitivity[14]. Improving insulin sensitivity is therefore essential for reduction of the negative consequences of obesity.

Lipid accumulation results in reduced responsiveness to insulin by the formation of specific lipid metabolites that interfere with insulin signaling[15]. Sphingolipids are one class of lipids involved in the induction of insulin resistance. The most simple 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[16]. From ceramide, glycosphingolipids and subsequently the more complex gangliosides can be formed. The increased availability of the free fatty acid palmitate and the low grade inflammation in obesity results in an enhanced synthesis of sphingolipids and gangliosides[17,18]. Ceramide interferes with insulin signaling at the level of PKB-Akt, reducing the metabolic actions of insulin[17]. The ganglioside GM3, when present in abundance, is thought to hamper phosphorylation of the insulin

receptor, resulting in reduced responsiveness to insulin[19,20].
Inhibition of sphingolipid synthesis, either at the level of ceramide or glucosylceramide, restores insulin sensitivity in mouse models for insulin resistance and type II diabetes[21-23]. The iminosugar N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) reduces glucosylceramide and subsequent ganglioside synthesis and is known to improve both peripheral and hepatic insulin sensitivity[21]. Succeeding studies showed that this drug also improves adipocyte function, reduces inflammation and ameliorates hepatic steatosis in insulin resistant genetically obese ob/ob mice[24,25]. In these studies, a reduction in body weight gain upon treatment with a relatively high dose of a 100 mg/kg/day AMP-DNM was noted[24,25.] The cause of this reduced body weight gain and the course of the metabolic effects of AMP-DNM administration in time has 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, energy homeostasis and activity. In this study we show that treatment with AMP-DNM rapidly increases fat oxidation, reduces carbohydrate oxidation and decreases food intake. The latter effect results in a sustained lower body weight in the AMP-DNM treated animals.

Methods

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 start of the experiments, the animals were housed in a temperature controlled room on a 12:12-h light-dark cycle for two 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. In the first experiment 16 mice were subjected to individual indirect calorimetry measurements (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, Columbus Ohio, US) for four consecutive days during week 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. In the first

week, after 38 hours of basal measurements, 8 mice were switched to food containing AMP-DNM at a dose of approximately 100 mg/kg body weight/day. Analyzed parameters included real time food intake and activity. Oxygen consumption (VO_2) and carbon dioxide production rates (VCO_2) were recorded at 7 minute intervals. Respiratory exchange rates (RER) as a measure for metabolic substrate choice as well as quantitative analysis of fat and carbohydrate oxidation were calculated using the following formulas (with VO_2 and VCO_2 in ml/hr):

$RER = VCO_2/VO_2$

$Fat\ oxidation\ (kcal/hr)=(((1.695*VO_2) - (1.701*VCO_2))*9)/1000$

$Carbohydrate\ oxidation\ (kcal/hr)=(((4.585*VCO_2) - (3.226*VO_2))*4)/1000$

Total energy expenditure was calculated as the sum of carbohydrate and fat acid 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 hand held 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 HbA1C concentrations (A1Cnow, Metrika Inc, Sunnyvale, CA) were determined. Subsequently mice were fasted for four hours and anesthetized by ip 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 0.3125 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.

In the second experiment 12 ob/ob mice were fasted for 4 hours. Next, 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. The mice brains were removed and immersion fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. For cryoprotection the brain tissue was equilibrated for 48hrs with 30% sucrose in 0.1M Tris-buffered saline (TBS) before sectioning. Thereafter, the brain tissue was cut into 30 µm sections and divided into two equal vials for immunocytochemical staining.

Plasma and tissue biochemical analysis

Plasma insulin concentrations were determined by ELISA (Crystal Chem Inc, USA). Colorimetric enzymatic kits were used for the measurements of plasma and liver

triglyceride concentrations (Human, Wiesbaden, Germany) and plasma free fatty acid (FFA) concentrations (Wako Chemicals GmbH, Neuss, Germany). To correlate liver lipid values, the protein content of the liver was measured using the BCA 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 derivatised with O-phtaldehyde reagent and separated using the high performance liquid chromatography (HPLC) method as described earlier26.
Part of the EDTA blood from the second experiment was directly mixed with 1M perchloric acid and stored on ice for at least 10 minutes. After centrifugation the supernatant was neutralized using 2M KOH, 0.5M 2-(N-morpholino)ethanesulfonic acid. Metabolite concentrations were measured in the neutralized supernatant after removal of KClO4. 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. Beta-hydroxybutyrate was measured using beta-hydroxybutyrate dehydrogenase.

Gene expression in liver and muscle

Total RNA was extracted from approximately 50 mg frozen tissues using Trizol reagent (Invitrogen, Breda, the Netherlands). For cDNA 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, Breda, The Netherlands). The real time PCR measurement of individual cDNAs 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, USA). 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.

Immunohistochemistry

The two vials of brain sections were incubated overnight at 4°C with either goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for the single Fos staining, or goat anti-Fos and rabbit anti-orexin or rabbit anti-MCH primary antibodies (1:2000; Phoenix Pharmaceuticals, Belmont, CA) for orexin/Fos or MCH/Fos double stainings. Sections were then rinsed in 0.1M TBS, incubated 1 hour in bioti-

nylated horse anti-goat IgG, and then 1 hour in avidin–biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA). The reaction product was visualized by incubation in 1% diaminobenzidine (DAB) with 0.01% hydrogen peroxide for 5-7 minutes. Nickel ammonium sulphate (0.05%) was added to the DAB solution to darken the reaction product (DAB/Ni). For double stainings, after Fos immunostaining, sections were rinsed in 0.1M TBS, incubated 1 hour in biotinylated goat anti-rabbit IgG, and then 1 hour in ABC. This reaction product was visualized by DAB staining only. All sections were mounted on gelatine-coated glass slides, dried, run through ethanol and xylene and covered for observation by light microscopy.

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 food intake. Diurnal data were generated during the light period between 07:00 and 19:00 and nocturnal data were generated during the dark period between 19:00 and 07:00.

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 $p<0.05$.

Results

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 bodyweight/day mixed in food for 5 weeks. Parallel, control ob/ob mice (n=8) received identical food without AMP-DNM. In accordance with earlier studies, AMP-DNM was well tolerated and caused no overt side effects. Prior to treatment initiation, respiratory exchange ratio (RER), carbohydrate and fatty acid oxidation rates, energy expenditure (figure1) and food intake (not shown) were comparable in both groups.

Exposure to AMP-DNM rapidly and significantly decreased the RER (nocturnal: AMP-DNM 0.88±0.07 vs control 0.95±0.05, $p=0.03$; diurnal: AMP-DNM 0.84±0.08 vs control 0.92±0.06, $p=0.04$), indicating an increase in fat to carbohydrate oxidation ratio (figure 1A, Table 1). During the first two days of exposure to AMP-DNM, total fat oxidation was higher in AMP-DNM treated animals, both during the nocturnal

period (AMP-DNM 0.16±0.09 vs control 0.07±0.06 kcal/hr, p=0.05) and the diurnal period (AMP-DNM 0.19±0.10 vs control 0.10±0.08 kcal/hr, p=0.08)(figure 1B, Table 1). The high fat oxidation rate in the treated animals was maintained during the third (nocturnal: AMP-DNM 0.18±0.0 kcal/hr; diurnal: AMP-DNM 0.19±0.06) and fifth week (nocturnal: AMP-DNM 0.15±0.06 kcal/hr; diurnal: AMP-DNM 0.16±0.05) of treatment (table 1).

Carbohydrate oxidation was markedly lower in AMP-DNM treated animals during the first (nocturnal: AMP-DNM 0.28±0.12 vs control 0.43±0.08 kcal/hr, p=0.01; diurnal: AMP-DNM 0.20±0.34 vs control 0.34±0.09 kcal/hr, p=0.01) and third week (nocturnal: AMP-DNM 0.26±0.07 vs control 0.42±0.08 kcal/hr, p=0.01; diurnal: AMP-DNM 0.20±0.08 vs control 0.39±0.08 kcal/hr, p<0.01) of the experiment (figure 1C and Table 1).

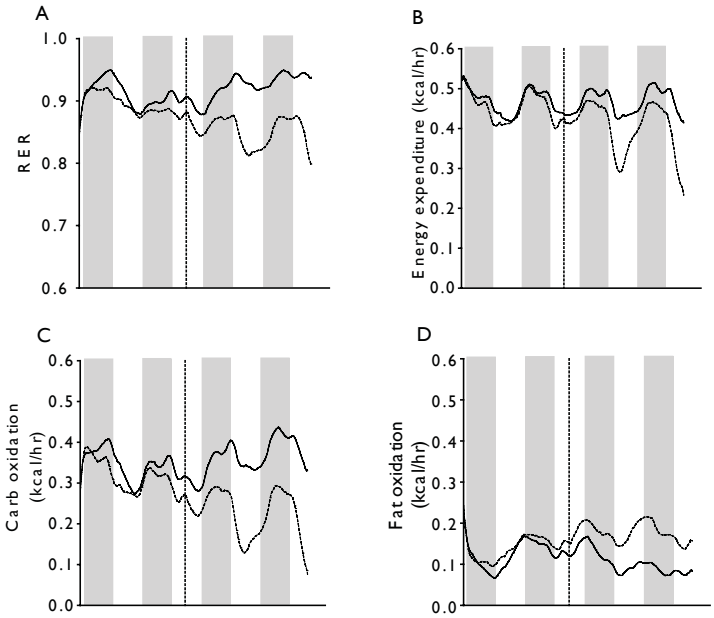

Figure 1 Exposure to AMP-DNM leads to rapid changes in metabolic substrate selection and total energy expenditure. Vertical dotted lines in all first four graphs indicate start of treatment. Grey areas represent the dark (12 hrs) and white areas the light periods (12 hrs). AMP-DNM exposed animals (dotted line) have an increased fat to carbohydrate oxidation ratio (A). Absolute fat oxidation (B) is higher and absolute carbohydrate oxidation (C) and energy expenditure (C) lower in the AMP-DNM fed animals in both the dark and light periods. RER=Respiratory Exchange Ratio, Carb oxidation=Carbohydrate oxidation.

Table 1 Substrate oxidation rates, energy expenditure and food intake during 5 weeks of AMP-DNM treatment.

week 1	control	AMP-DNM	p-value
RER nocturnal	0.95±0.05	0.88±0.07	0.03
RER diurnal	0.92±0.06	0.84±0.08	0.04
Fat oxidation nocturnal (kcal/h)	0.07±0.06	0.16±0.09	0.05
Fat oxidation diurnal (kcal/h)	0.10±0.08	0.19±0.09	0.08
Glucose oxidation nocturnal (kcal/h)	0.43±0.08	0.28±0.12	0.01
Glucose oxidation diurnal (kcal/h)	0.34±0.09	0.20±0.10	0.01
Energy expenditure nocturnal (kcal/h)	0.50±0.05	0.44±0.06	0.03
Energy expenditure diurnal (kcal/h)	0.39±0.05	0.45±0.04	0.02
Food intake (g in 48 h)	10.87±1.47	8.02±0.61	<0.01
week 3			
RER nocturnal	0.93±0.05	0.84±0.05	<0.01
RER diurnal	0.93±0.05	0.86±0.04	<0.01
Fat oxidation nocturnal (kcal/h)	0.10±0.07	0.18±0.00	0.01
Fat oxidation diurnal (kcal/h)	0.10±0.07	0.19±0.06	0.02
Glucose oxidation nocturnal (kcal/h)	0.42±0.08	0.26±0.07	<0.01
Glucose oxidation diurnal (kcal/h)	0.39±0.08	0.20±0.08	<0.01
Energy expenditure nocturnal (kcal/h)	0.52±0.04	0.43±0.06	<0.01
Energy expenditure diurnal (kcal/h)	0.45±0.03	0.40±0.07	<0.01
Food intake (g in 48 h)	11.83±1.08	8.07±1.18	<0.01
week 5			
RER nocturnal	0.87±0.04	0.86±0.05	1.00
RER diurnal	0.90±0.04	0.90±0.04	0.73
Fat oxidation nocturnal (kcal/h)	0.17±0.07	0.15±0.06	0.59
Fat oxidation diurnal (kcal/h)	0.20±0.07	0.16±0.05	0.24
Glucose oxidation nocturnal (kcal/h)	0.43±0.10	0.37±0.09	0.24
Glucose oxidation diurnal (kcal/h)	0.34±0.09	0.25±0.08	0.08
Energy expenditure nocturnal (kcal/h)	0.60±0.05	0.52±0.08	0.02
Energy expenditure diurnal (kcal/h)	0.54±0.05	0.41±0.08	<0.01
Food intake (g in 48 h)	9.88±1.72	8.39±1.13	0.04

Values are presented as mean ± SD for eight mice in each group. RER=Respiratory Exchange Ratio. Values for food intake are the total grams of food consumed during the final 48 hours of each period in the metabolic cages. RER, glucose and fat oxidation rates and energy expenditure are the mean values of all measurements during either the nocturnal or the diurnal periods in the metabolic cages in which the mice were exposed to AMP-DNM.

Table 2 Body weight, body weight gain and temperature during 5 weeks of AMP-DNM treatment.

Week 1	AMP-DNM	control	P-value
Weight (g)	41.0±2.4	39.9±2.1	0.33
Temperature (°C)	33.8±3.8	36.8±1.6	0.13
Week 3			
Weight (g)	43.5±2.8	45.5±2.3	0.14
Body weight gain (g)*	2.1±2.3	5.6±0.6	<0.01
Percentage body weight gain (%)*	5.0±5.3	14.1±1.6	<0.01
Temperature (°C)	36.0±0.5	37.1±0.7	<0.01
Week 5			
Weight (g)	44.3±3.3	49.5±3.0	<0.01
Body weight gain (g)*	3.0±3.1	9.7±2.3	<0.01
Percentage body weight gain (%)*	7.1±6.8	24.2±4.0	<0.01
Temperature (°C)	32.5±3.7	36.5±0.8	<0.01

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

Table 3 Metabolic blood/plasma parameters during 5 weeks of AMP-DNM treatment.

Blood parameters week 1,3,5	AMP-DNM	control	P-value
Glucose week 1 (mmol/L)	13.9±5.2	19.5±8.6	0.14
Glucose week 3 (mmol/L)	6.8±1.2	10.8±3.7	0.01
Glucose week 5 (mmol/L)	6.5±2.0	12.6±5.1	0
Blood/plasma parameters week 6			
Glucose (mmol/L)	6.9±1.1	10.9±2.3	0
Insulin (mg/ml)	7.8±2.6	9.7±3.4	0.22
HbA1C (%)	4.3±0.3	6.1±0.7	<0.00
Triglycerides (mmol/L)	0.85±0.22	0.62±0.11	0.02
FFA (mmol/L)	1.2±0.20	0.66±0.10	<0.00

Values are presented as mean ± SD for eight mice in each group. Glucose values at week 1,3,5 and 6 were measured in non-fasted animals. All other parameters at week 6 were measured after 4 hour fasting.

During the fifth week of the experiment, differences in substrate oxidation rates between the treated and control animals were no longer detectable. Nocturnal and diurnal RER did not differ significantly between treated and control animals (nocturnal: AMP-DNM 0.86±0.05 vs control 0.87±0.04, p=1.00; diurnal: AMP-DNM 0.90±0.04 vs control 0.90±0.04, p=0.73) and absolute fat oxidation and glucose oxidation rates were similar in both groups (table 1). This was due to an increase in the fat oxidation rate in untreated animals and an increase in glucose oxidation rate in the AMP-DNM treated animals between week 3 and 5 of the experiment. From two weeks of treatment onwards, non-fasted plasma glucose levels were significantly lower in the AMP-DNM treated animals. The improved glucose homeostasis was reflected in lower glycated haemoglobin (HbA1C) concentrations in the treated animals (table 3).

Exposure to AMP-DNM decreases food intake and total energy expenditure

Concomitant with the changes in substrate oxidation patterns, food intake started to decrease after treatment initiation (table 1). Total caloric intake was significantly lower in treated compared to control animals in the first (calories consumed during 48 hrs: AMP-DNM 8.0±0.6 vs control 10.9±1.5 kcal/hr, p<0.01), third (calories consumed during 48 hrs: AMP-DNM 8.1±1.2 vs control 11.8±1.1 kcal/hr, p<0.01) and the fifth week of treatment (calories consumed during 48 hrs: AMP-DNM 8.4±1.1 vs control 9.9±1.7 kcal/hr, p=0.04) (table 1). Total energy expenditure was lower in treated animals during the first (nocturnal: AMP-DNM 0.44±0.06 vs control 0.50±0.05 kcal/hr, p=0.03; diurnal: AMP-DNM 0.45±0.04 vs control 0.39±0.05 kcal/hr, p=0.02), third (nocturnal: AMP-DNM 0.43±0.06 vs control 0.52±0.05 kcal/hr, p<0.01; diurnal AMP-DNM 0.40±0.07 vs control 0.45±0.03 kcal/hr, p<0.01) and fifth week of treatment (nocturnal: AMP-DNM 0.52±0.08 vs control 0.60±0.05 kcal/hr, p=0.02; diurnal: AMP-DNM 0.41±0.08 vs control 0.54±0.05 kcal/hr, p<0.01)(table 1). Activity did not differ between the treatment groups at any point during the whole experiment (data not shown). Lower body weight gain in the treated animals was noted during the first two weeks (gain as percentage of initial body weight: AMP-DNM 5.0±5.3% vs control 14.1±1.6%, p=0.00) and the second two weeks of treatment (table 2). This had not yet resulted in a significantly lower body weight at the beginning of the third week of treatment, probably due to 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

vs control 51.3 gram, p=0.01) body weight was significantly lower in the treated animals. 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 tended to be lower in the AMP-DNM treated animals (table 2).

Treatment with AMP-DNM results in upregulation in beta-oxidation genes in liver and reduction hepatic fat content

Given the finding of an increased fat oxidation rate in AMP-DNM treated ob/ob mice, we decided to study the expression of genes involved in fatty acid oxidation (CPT-1a, LCAD, PDK-4) in liver and muscle and the effects of treatment on liver lipid content. Mice were sacrificed after five weeks of treatment with AMP-DNM. We found increased hepatic expression of carnitine palmitoyl transferase-1a (CPT-1a, AMP-DNM 0.36±0.13 vs control 0.12±0.06, p<0.01) (figure 2D), which is in line with the increased fatty acids oxidation rates. Expression of LCAD and PDK4 in liver was not significantly changed (figure 2E and F).

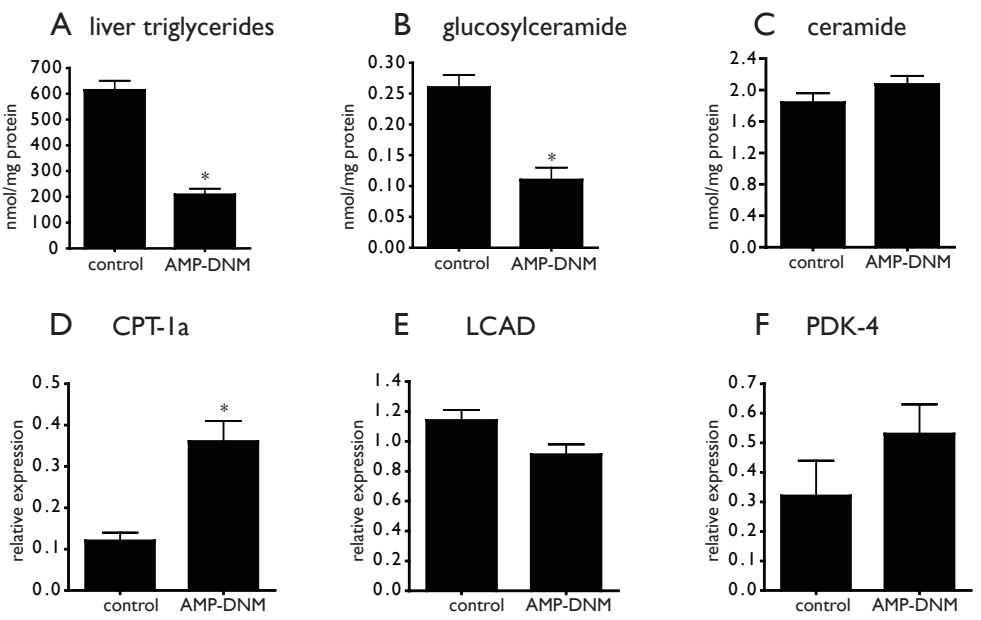

Figure 2 Long term treatment with AMP-DNM results in reduction in liver triglyeride (A) and glucosylceramide (B) content, while the ceramide (C) concentration does not change significantly. CPT-1a expression, regulating fatty acid entry into mitochondrion for oxidation, is significantly increased (D), whereas LCAD (E) and PDK4 (F) expression are not significantly changed. Data presented as mean ± SEM. *p=0.001.

Expression of the examined fatty acid oxidation genes in muscle was not changed after five weeks of AMP-DNM treatment (CPT-1a; AMP-DNM 0.15±0.03 vs control 0.23±0.10 p=0.09, LCAD; AMP-DNM 2.4±0.80 vs control 3.1±1.6 p=0.37, PDK4; AMP-DNM 4.5±2.3 vs control 3.3±0.9 p=0.19).
As described previously[21,24] AMP-DNM lowered glucosylceramide in liver (AMP-DNM 0.11±0.02 vs control 0.27±0.06 nmol/mg protein, p=0.00)(Fig 2B), without altering ceramide levels (figure 2C). Liver triglyceride content was drastically lowered (AMP-DNM 208±64 vs control 614±91 nmol/mg protein, p=0.00) (figure 2A), most likely as a result of both the earlier observed decreased lipogenesis[25] and the here observed increased fat oxidation.

Four hour exposure to AMP-DNM does not change metabolic parameters but activates brain regions involved in appetite regulation

To further characterize the rapid effects of AMP-DNM on fat and glucose oxidation, we studied metabolic parameters and expression of fatty acid oxidation genes in liver and muscle in ob/ob animals four hours after administration of 100 mg/kg AMP-DNM. Groups of six ob/ob mice that had either received AMP-DNM or vehicle by gavage were compared. Four hour exposure to AMP-DNM did not change plasma levels of glucose, insulin, FFAs, triglycerides nor of the intermediate metabolites pyruvate, lactate and the ketone β-hydroxybutyrate (table 4). Expression of fatty acid oxidation genes in liver and muscle was unchanged after this short treatment span (table 4). Due to the short treatment duration, liver glucosylceramide concentrations were not yet reduced in the treated animals (AMP-DNM 0.17±0.02 vs control 0.16±0.05, p=0.70).

Table 4 Plasma metabolic parameters and liver and muscle fatty acid oxidation gene expression after 4 hours of AMP-DNM exposure

Plasma parameters	AMP-DNM	control	P-value
Glucose (mmol/L)	12.0±2.3	15.0±3.4	0.1
Insulin (ng/ml)	15.5±8.6	9.2±1.9	0.11
Triglycerides (mmol/L)	0.83±0.12	0.79±0.12	0.55
FFA (mmol/L)	0.60±0.20	0.49±0.22	0.41
Pyruvate (mmol/L)	109±26	130±40	0.3
Lactate (mmol/L)	1.73±0.75	1.38±0.50	0.36
b-hydroxybutyrate (mmol/L)	66±33	54±51	0.64
Gene expression	AMP-DNM	control	P-value
Liver			
CPT I	0.23±0.14	0.23±0.14	0.98
LCAD	0.77±0.43	0.97±0.20	0.33
PDK-4	0.11±0.03	0.12±0.07	0.65
Muscle			
CPT I	0.14±0.06	0.15±0.04	0.72
LCAD	3.1±1.0	3.3±1.0	0.78
PDK-4	7.1±3.0	5.0±2.4	0.22

Values are presented as mean ± SD for six mice in each group.

Given the immediate effect of AMP-DNM on food intake in the first experiment, we analyzed activation of brain regions involved in appetite regulation in the four hour treated animals. We looked at staining for Fos, an immediate early gene and marker of neuronal activation. In the AMP-DNM treated animals, compared to vehicle treated mice (figure 3B, D and F), the immunohistochemical analysis of the brains showed increased staining for Fos in the lateral hypothalamic area (figure 3A) and arcuate nucleus (figure 3C) in the hypothalamus, as well as the xiphoid region in the thalamus (figure 3E). Both the lateral hypothalamus and the arcuate nucleus are well known for their involvement in appetite regulation. Two well known orexigenic neuropeptides that are produced in the lateral hypothalamus are orexin and melanin-concentrating hormone (MCH). Although the observed Fos immuno-reactivity was located in the same region where these two populations of orexigenic neurons reside, double staining for Fos with orexin (figure 3F) and MCH (figure 3G) did not show any co-localization. This is compatible with the reduced food intake in response to AMP-DNM treatment since activation of these orexigenic neurons is expected to increase in food intake.

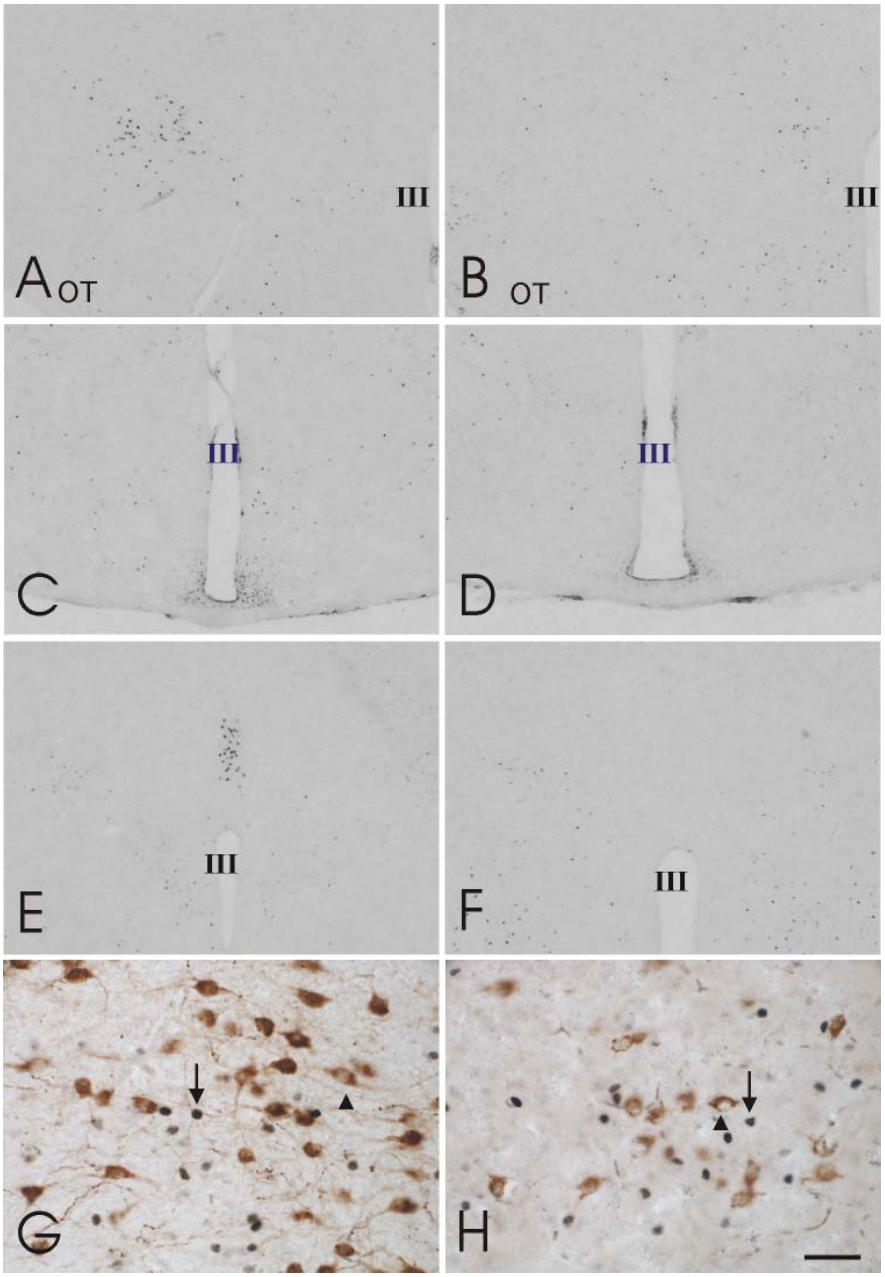

Figure 3 Hypothalamic Fos immunoreactivity in response to AMP-DNM treatment. Increased Fos expression, 4-h after gavage with AMP-DNM (100 mg/kg bodyweight), is observed specifically in the lateral hypothalamic area (A), the arcuate nucleus (C) as well as the xiphoid nucleus in thalamus (E). The panels on their respective right side (B, D and F) show the absence of Fos immunoreactivity in these areas in vehicle treated mice. Despite their close location, Fos positive nuclei (arrow) in the lateral hypothalamus are not colocalized with either orexin (G) or MCH containing neurons (H) (arrowheads in G and H). III, third ventricle, OT: optic tract. Scale bar: A-F: 200μm, G, H: 50 μm.

Discussion

Previous studies on the effect of AMP-DNM treatment in ob/ob mice showed marked improvement of insulin resistance, hepatic steatosis and inflammation[21,24,25]. 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. The change in substrate oxidation pattern does not seem to result from a reduced glucose availability, since plasma glucose levels were not significantly different after four and 36 hours of AMP-DNM treatment.

Food intake is also immediately affected by treatment. How the reduction in food intake and the changes in substrate oxidation patterns are related remains to be clarified. Reduction in body weight, as a result of a low caloric diet, has been shown to induce an increase in fat oxidation in the fasted state in some studies, though others report no effect of weight loss on fatty acid oxidation[13]. In the current study, changes in body weight do not seem to be responsible for the altered substrate oxidation pattern, as the increase in fat oxidation precedes the reduction in body weight gain.

The reduction in food intake resulting from AMP-DNM treatment may be regulated at the level of the hypothalamus, since two areas involved in food intake regulation show specific activation after short-term treatment. This precedes changes in metabolic parameters such as blood glucose levels and changes in expression of genes involved in fatty acid oxidation in liver and muscle. Thus, the hypothalamic activation seems not to occur secondary to peripheral metabolic effects of AMP-DNM, suggesting that there is either a direct effect of the compound in the involved brain areas or the effect is mediated by a specific signal from for example the gut to these brain regions. Double staining for the orexigenic neuropeptides orexin and MCH showed no co-localization of the neurons activated by AMP-DNM treatment and these markers. This fits with a reduction in food intake by AMP-DNM, since release of oxerigenic neuropeptides would have had the opposite effect, namely an increase in food intake[27].

One of the peptides involved in appetite regulation that could be responsible for the AMP-DNM effect on both food intake and fat oxidation is peptide YY 3-36 (PYY 3-36). This peptide is released by L cells in the intestine in response to food intake and subsequently reduces appetite and slows gastric emptying[28]. Peripheral administration of PYY 3-36 leads to increases in c-Fos immunoreactivity in the arcuate nucleus

and PYY binding to the Y2-receptor in arcuate nucleus mediates its inhibitory effect on feeding[29]. Peripheral acute and chronic PYY 3-36 administration stimulates fatty acid oxidation[30]. Thus, an increase in PYY 3-36 release from the gut in response to AMP-DNM could explain the observed acute effects of AMP-DNM treatment. In a previous unpublished study in diabetic Zucker (fa/fa) rats, plasma PYY levels were significantly elevated in animals treated for 35 days with AMP-DNM 50 mg/kg/day compared to both control animals and animals treated with 3 mg/kg Rosiglitazone, a PPARγ-agonist. As was found in the mice in the current study, food intake and body weight gain were also considerably lower in the AMP-DNM treated rats. Whether PYY indeed mediates the AMP-DNM effect on fat oxidation and food intake in ob/ob mice remains to be established.

In conclusion, treatment with AMP-DNM rapidly increases fat oxidation and decreases glucose oxidation and food intake. This may be regulated by stimulation of specific brain areas, either directly by AMP-DNM or by an intermediate messenger. Added to the insulin sensitizing capacities of AMP-DNM, the ability of this drug to reduce body weight gain and increase fat oxidation makes it an attractive candidate for the treatment of obesity and its metabolic complications.

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