

Chapter 5

Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity: a novel therapeutic approach to insulin resistance

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Submitted

Abstract

A growing body of evidence implicates ceramide and/or its glycosphingolipid metabolites in the pathogenesis of insulin resistance. We have developed a highly specific small molecule inhibitor of glucosylceramide synthase, an enzyme which catalyses a necessary step in the conversion of ceramide to glycosphingolipids. In cultured adipocytes the iminosugar derivative N-(5'-adamantane-1'-ylmethoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), enhanced insulin-stimulated glucose uptake and reversed TNF-alpha induced abnormalities in insulin signal transduction. When administered to mice, AMP-DNM significantly reduced glycosphingolipid but not ceramide concentrations in various tissues. Treatment of *ob/ob* mice with AMP-DNM for 7 days normalised their elevated tissue glucosylceramide levels, markedly lowered circulating glucose levels, improved oral glucose tolerance, improved insulin sensitivity in muscle and liver, reduced hepatic fat deposition and increased cell surface expression of GLUT4 on adipocytes. These findings provide evidence that metabolites of ceramide, rather than ceramide itself, may be involved in mediating the link between obesity and insulin resistance and that interference with glycosphingolipid biosynthesis might present a novel approach to the therapy of states of impaired insulin action such as type 2 diabetes.

Introduction

Impaired responsiveness to insulin is reflected by increased glucose synthesis by the liver and reduced glucose uptake by skeletal muscle and adipose tissue (1-3). In insulin-resistant individuals the translocation of the glucose transporter GLUT4 to the plasma membrane of myocytes and adipocytes is impaired. Obesity is strongly associated with insulin resistance but the underlying pathogenic mechanism is still an enigma. The strong correlation between insulin resistance and intramyocellular lipid levels suggests that excessive exposure to lipids or their metabolites, so called lipotoxicity, may play a crucial role (1-7). The rapid induction of insulin resistance in rodents by infusions with palmitate, and in cultured cells by supplying this fatty acid, has directed attention to the sphingolipid ceramide as a potential mediator of insulin resistance (1,4-7). Palmitate is a critical precursor in the synthesis of ceramide and its enhanced supply inevitably increases sphingolipid formation in tissues (8,9). Increased ceramide concentrations (two-fold) were indeed recently reported for skeletal muscle from obese insulin-resistant individuals⁴. The well-established induction of insulin resistance by the cytokine TNF α may also be attributed to its ability to promote sphingolipid biosynthesis, as has been demonstrated at both mRNA and cellular lipid levels (10-12). In addition, investigations with cultured cells have linked excessive ceramide concentrations to disturbed insulin signalling (6,13-15). Manipulation of ceramide levels in cultured cells was consistently found to affect the insulin signalling pathway downstream at the level of AKT, but conflicting reports exist regarding effects on the insulin receptor, IRS-1 and associated PI 3-kinase activity (6,13-15). Surprisingly, in most studies it has been overlooked that metabolites of ceramide, such as glycosphingolipids, might also play an important role in the development of insulin resistance. Glycosphingolipids are found in specific (detergent-resistant) membrane microdomains in close physical proximity to the insulin receptor, as well as other tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor (5). A regulatory role for glycosphingolipids in hormone sensitivity was first proposed by Bremer and coworkers who showed that EGF-mediated signalling is inhibited by GM3, the simplest ganglioside (16). More recently, Tagami *et al.* reported that addition of GM3 to cultured adipocytes also suppresses phosphorylation of the insulin receptor and its down-stream substrate IRS-1, resulting in reduced glucose uptake (17).

Other observations further substantiate the role of the gangliosides GM3 in responsiveness to insulin. Mutant mice lacking GM3 show an enhanced phosphorylation of the skeletal muscle insulin receptor after ligand binding and are protected from high-fat diet induced insulin resistance (18). Conversely, GM3 levels are elevated in the muscle of certain obese, insulin resistant mouse and rat models (17). Inokuchi and coworkers employed the ceramide-analogue 1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP), an inhibitor of glucosylceramide synthase, to reduce glycosphingolipids in cultured adipocytes. They noted that PDMP counteracted the inhibitory effects of TNF- α on IR and IRS-1 phosphorylation (17). Very recently it was reported by the same researchers that high GM3 levels diminished IR accumulation in detergent-resistant membrane microdomains and insulin-dependent IR internalization (19). Again glycosphingolipid depletion by incubation of cells with PDMP prevented these abnormalities. However, the observations made with PDMP are difficult to interpret since this compound not only inhibits conversion of ceramide to glucosylceramide but also its transacylation to 1-O-acylceramide and consequently increases cellular levels of ceramide (20). Based on the present information it may be therefore conceived that not ceramide itself but rather its glycosphingolipid metabolites are instrumental in the development of insulin resistance. To discriminate between these possibilities we examined in obese mice the effect of reduction of glycosphingolipids by N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), a specific inhibitor

of glucosylceramide synthase (21). Here we show that pharmacological lowering of glycosphingolipids, without significant reduction of ceramide, dramatically reverses insulin resistance in *in vitro* and *in vivo* models.

Materials and methods

Mice

Experimental procedures were all approved by the appropriate Ethics Committee for Animal Experiments. C57Bl/6J and *ob/ob* mice (C57Bl/6J background) were obtained from Harlan (Horst, The Netherlands) and housed in a light- and temperature controlled facility. The animals were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) containing about 6% fat and ~0.01% cholesterol (w/w). The iminosugar AMP-DNM was mixed in the food for most experiments. In indicated experiments animals were used to which the compound had been administered by oral gavage two times daily.

Plasma and tissue sampling

Animals were sacrificed under isoflurane anaesthesia. A large blood sample was collected by cardiac puncture. Tissues were quickly removed and frozen for further analysis.

Cells

Human adipocytes and appropriate cell culture medium were obtained from Zen-Bio Inc. (Chapel Hill, USA). 3T3-L1 pre-adipocytes were obtained from the ATCC (Manassas, USA). They were propagated and differentiated as previously described (22). Primary adipocytes were prepared from epididymal fat pads as described earlier (23).

Iminosugar

AMP-DNM (N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin) was synthesized as described previously (24). Plasma levels of AMP-DNM were determined by mass spectrometry following high pressure liquid chromatography (Xendo, Groningen, The Netherlands).

Analysis of lipids and measurement of enzyme activities

Lipids were extracted according to Folch *et al* (25). Ceramide and glucosylceramide collected from the chloroform phase were determined by HPLC analysis of orthophthaldehyde-conjugated lipids according to a procedure described previously with some modifications (26). The chloroform layer was thoroughly dried and deacylation of lipids was performed in 0.5 ml 0.1 M NaOH in methanol in a microwave oven (CEM microwave Solids/Moisture System SAM-155). After deacylation 0.5 ml methanol and 2 ml chloroform were added and phase separation was performed. The chloroform layer was dried under N₂ and the deacylated lipids were taken up in 250 µl methanol. Deacylated glycolipids were derivatised on line for 30 min with Ophthalaldehyde. Analysis was performed using an HPLC system (Waters Associates, Milford, MA) and a Hypersil BDS C18 3µ, 150 x 4.6 mm reverse phase column (Alltech). Chromatographic profiles were analysed using Waters Millenium software. All samples were run in duplicate and in every run a reference sample was included.

Ganglioside composition was determined by HPTLC analysis of the acidic glycolipid fraction obtained after Folch extraction using chloroform/methanol/water (65:25:4) as solvent. Gangliosides were visualized by spraying resorcinol reagent followed by heating at 105 °C for 10 min and quantified by densitometry. Total sphingolipids biosynthesis by cultured cells was quantified by exposing cells to [³H]serine or [³H]palmitate as described previously (9).

Palmitate was administered as 1:1 complex with bovine serum albumin. Activity of glucosylceramide synthase and glucocerebrosidase in living cells was determined using as substrates fluorescently labelled NBD-ceramide and NBD-glucosylceramide, respectively (27). Briefly, NBD-ceramide or NBD-glucosylceramide was complexed to fatty acid-free bovine serum albumin in a molar ratio of 1:1. Cells were incubated with 150 μ M lipid and harvested at different time points. Lipids were extracted, separated by thin layer chromatography and NBD-ceramide and NBD-glucosylceramide were quantified (27).

IC₅₀ values of AMP-DNM for various enzyme activities were determined by exposing cells or enzyme preparations to an appropriate range of iminosugar concentrations. IC₅₀ values for glucosylceramide synthase and glucocerebrosidase activities were measured using living cells with NBD-ceramide and NBD-glucosylceramide as respective substrates. Lactase, maltase and sucrase were determined with homogenates of freshly isolated rat intestine using assay conditions described earlier (28). Debranching enzyme (α -1,6-glucosidase) was measured with an erythrocyte preparation as enzyme source as described previously (29).

2-[³H]deoxyglucose uptake

Cultured adipocytes were deprived of serum for 1 h prior to exposure to 0.1 mM 2-[³H]deoxyglucose (0.4 mCi/ml). 2-[³H]deoxyglucose uptake measurements were made in triplicate under conditions when hexose uptake was linear (29). Non-specific absorption of 2-[³H]deoxyglucose, as determined by cell-associated counts in the presence of 5 μ M cytochalasin B, was always less than 10% of the total uptake.

Analysis of insulin signalling in 3T3L1 adipocytes

Fully differentiated 3T3-L1 adipocytes were serum deprived, by incubation in DMEM + 1% BSA, and simultaneously pre-treated with or without AMP-DNM (50 μ M) and/or TNF α (0.6 nM) for 24hrs. Following insulin stimulation (5 min with 100 nM) cells were washed with ice-cold PBS, and lysed in modified RIPA buffer (50 mM Tris-HCL pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1mM EDTA, 50 mM β -glycerol-2-phosphate, 5 μ M AEBSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 mM sodium pervanadate, 1 mM sodium fluoride, 50 nM okadaic acid). Cell lysates were clarified by centrifugation (13,000 rpm for 10 min) and the supernatant collected. Protein concentrations were determined using the BioRad DC protein assay kit. Equal amounts of whole cell lysates were separated by SDS-PAGE and immunoblots performed in parallel using anti-phosphotyrosine antibody (4G10), anti-IRS-1- Upstate Biotech. US), anti-IR (K. Siddle), anti-pSer473 AKT, Anti-AKT (Cell Signalling Technology Inc., US), appropriate HRP-linked secondary Ab (DAKO, US) and an enzymelinked chemiluminescent kit (Amersham, UK).

Measurement of whole-body insulin sensitivity

Whole-body insulin sensitivity of *ob/ob* mice was determined using hyperinsulinemic euglycaemic clamps as described previously (31,32). Male *ob/ob* mice were treated for 7 days with a diet with or without MZ21 (25 mg/kg BW). At the start of the diet, mice were equipped with a permanent catheter in the right atrium via the jugular vein. Nine hours before the start of the clamp experiment, food was withdrawn but mice still had free access to water. Mice were kept in metabolic cages during the experiment, allowing frequent collection of bloodspots from the tail under conscious and unrestrained conditions. The mice were infused for six hours with two solutions. The first infusate contained 220 mU/ml insulin (Actrapid, Novo Nordisk., Bagsvaerd, Denmark), 40 μ g/ml somatostatin (UCB, Breda, The Netherlands), 10 mg/ml BSA (Sigma, St. Louis, MO). To prevent high infusion rates leading

to higher morbidity, this solution contained, when given to treated mice, 100 mg/ml glucose, from which 3% was [U-¹³C]-glucose (99% ¹³C APE) (Cambridge Isotope Laboratories Inc., Andover, MA). The solution was infused at a rate of 0.3 ml/h. The second infusate was a 30% (treated mice) or 15% (untreated mice) glucose solution, from which 3% was [U-¹³C]-glucose. Its infusion rate was adjusted according to measured blood glucose levels to maintain euglycemic conditions. Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium) in a small tail blood sample that was drawn every 15 minutes. Just before the start of the experiment, a small blood sample was obtained by tail bleeding. Blood samples were centrifuged and the obtained plasma was stored at -20°C until analysed. Every hour, a bloodspot was obtained by tail bleeding. After the clamp, animals were sacrificed by cardiac puncture under anaesthesia. Plasma was obtained by centrifugation. The liver was removed, weighed and frozen in liquid N₂ until further analysis.

Calculation of endogenous glucose production and metabolic clearance rate under clamped conditions

Analytical procedures for extraction of glucose from bloodspots from filterpaper, derivatization glucose to its aldonitril pentaacetate derivative and GC-MS were essential according to Van Dijk *et al.* (31,32). The measured fractional isotopomer distribution by GCMS (m₀-m₆) was corrected for the fractional distribution due to natural abundance of ¹³C, by multiple linear regression to obtain the excess mole fraction of mass isotopomers M₀-M₆ due to isotope dilution of infused labelled glucose. Two solutions with [U-¹³C]-glucose were infused with different rates. Therefore, the total rate of appearance of glucose into plasma (Ra(glc;whole body)) was calculated as follows:

$$Ra(glc;whole\ body) = (M_6(glc)_{glucose} \times infusion(glc;M_6)_{glucose} + M_6(glc)_{insulin} \times infusion(glc;M_6)_{insulin}) / M_6(glc)_{blood}$$

in which M₆(glc)_{glucose} and M₆(glc)_{insulin} are the excess mole fractions of infused [U-¹³C]-glucose in the glucose and insulin solution, respectively, and infusion(glc;M₆)_{glucose} and infusion(glc;M₆)_{insulin} are the infusion rates of [U-¹³C]-glucose of the glucose and insulin solution, respectively. The rate of endogenous plasma glucose (Ra(glc;endo)) was calculated as follows:

$$Ra(glc;endo) = Ra(glc;whole\ body) - infusion(glc;M_6)_{glucose} - infusion(glc;M_6)_{insulin}.$$

The metabolic clearance rate of glucose (MCR) was calculated according to:

$$MCR = Ra(glc;whole\ body) / [glc],$$

where [glc] is the blood glucose concentration (mM).

Glucose tolerance test

The tolerance test was performed in fasted mice (13–15 h) with oral gavage of glucose (2 g of glucose per kg of body weight). Blood glucose values were measured immediately before and 10, 20, 30, 60, 90 and 120 min after glucose injection. AUCs (areas under the curve) were determined for individual mice.

Cell surface expression of GM3 and GLUT4

Flow cytometry using monoclonal anti-GM3 antibody (Seikagu, Japan) or monoclonal anti-GLUT4 antibody (Abcam, USA) and FITC-or Alexa 488-conjugated rabbit anti-mouse secondary antibody were employed according to the procedure described earlier (17).

Statistical testing

Values presented in figures represent mean +/- SEM. Statistical analysis of two groups was assessed by Student's t-test (one-tailed) or ANOVA for repeated measurement (clamp experiment). Level of significance was set at $p < 0.05$.

Results

Modulation of glycosphingolipids with AMP-DNM

N- (5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM) is a very potent inhibitor of glucosylceramide synthase activity in all cell types tested. As measured using fluorescently labelled C6-NBD-ceramide as substrate, exposure of cells to AMP-DNM results in swift and marked inhibition of synthesis of glucosylceramide. As determined in this manner, IC50 values in cultured macrophages, myoblasts, melanoma cells, HepG2 cells and skin fibroblasts are about 150, 220, 200, 150 and 220 nM, respectively. Cellular levels of glucosylceramide and its metabolites lactosylceramide and gangliosides are gradually reduced by AMP-DNM, reaching equilibrium within 36 hours. Ceramide levels however remain constant upon exposure to AMP-DNM. Figure 5.1 shows an illustrative response in lipid levels of cultured macrophages and melanoma cells after exposure to 200 nM AMP-DNM.

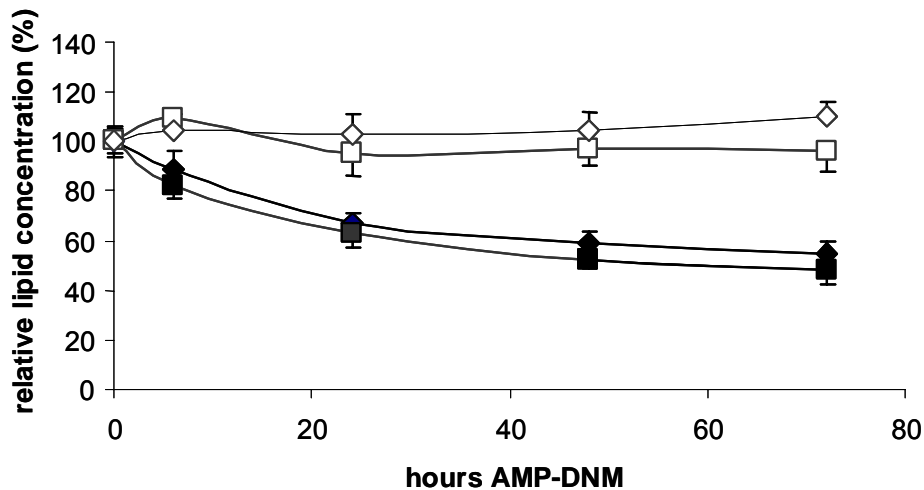


Figure 5.1. Effect of AMP-DNM on ceramide and glucosylceramide concentrations in cultured cells. Macrophages derived from peripheral blood monocytes and melanoma cells were cultured in the absence or presence of 250 nM AMP-DNM. At different time points, cells were harvested and ceramide and glucosylceramide content were determined. Lipid levels are expressed as percentage of those of cells not exposed to AMP-DNM. Macrophages: glucosylceramide (closed squares), ceramide (open squares); melanoma cells: glucosylceramide (closed diamonds), ceramide (open diamonds). Error bars indicate standard deviation.

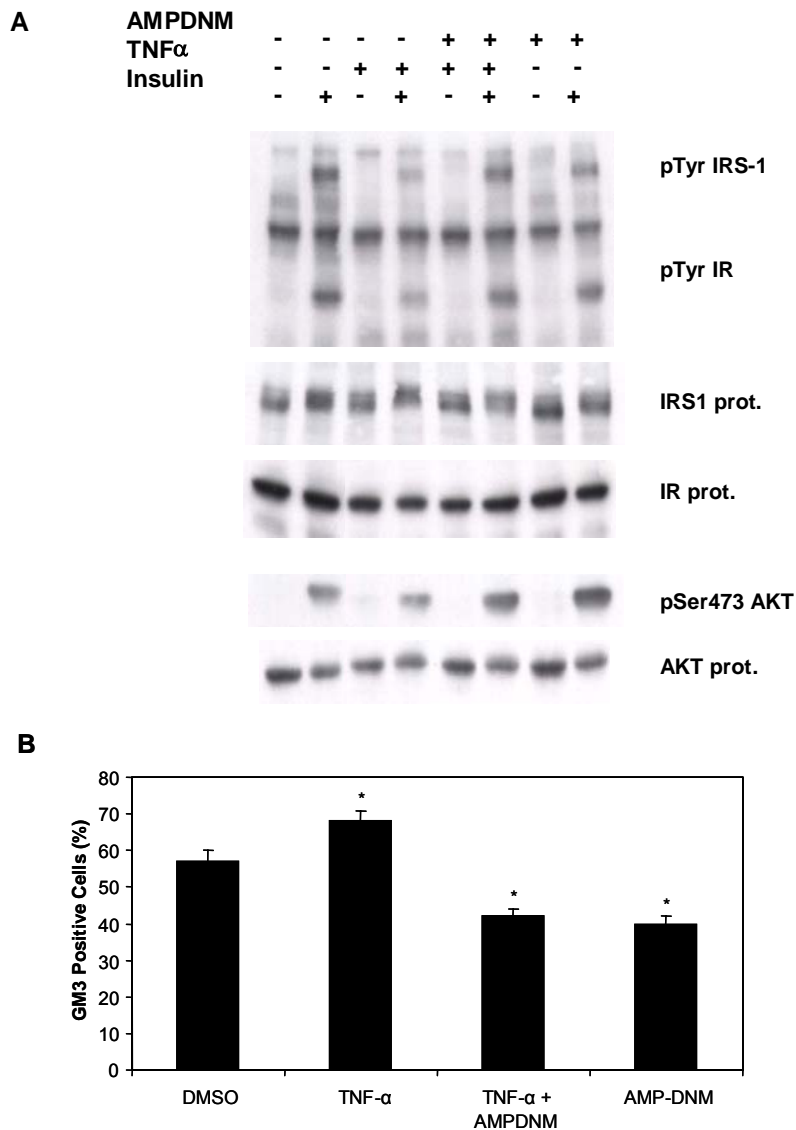


Figure 5.2. AMP-DNM reverses TNF-alpha induced insulin resistance (A) and surface expression of GM3 (B) in 3T3-L1 adipocytes. Serum starved 3T3-L1 adipocytes were treated with either vehicle control, AMP-DNM (50 μ M) and/or TNF-alpha (0.6 nM) for 24hrs prior to stimulation with or without insulin (100nM for 5 min.) (A) Immunoblots of whole cell lysates were performed in parallel as described in materials and methods. Representative blots are shown from one of 3 independent experiments. (B) Cell surface expression of GM3 was determined on basal adipocytes (not stimulated with insulin) by FACS analysis. Values represent mean (\pm SE) % of viable cells that stained positive for GM3. * indicates statistical significance ($p < 0.05$) and was observed in four independent experiments.

Table 5.1. 2- 3 H]deoxyglucose uptake by cultured human adipocytes in the presence and absence of insulin.

	Uptake in cpm/ μ g protein		
	10 $^{-7}$ M insulin	10 $^{-8}$ M insulin	No insulin
0 μ M AMP-DNM	27.6 \pm 3.0	23.4 \pm 2.9	24.3 \pm 2.8
10 μ M AMP-DNM	50.7 \pm 4.5	46.4 \pm 6.8	29.7 \pm 3.1

Reduction of glycosphingolipids in normal mice by AMP-DNM

Normal, six week old C57Bl/6J mice (n=4) were treated with or without AMP-DNM at a dose of 25 mg/kg body weight/day for 14 days. The plasma concentration of AMP-DNM was 108 ± 6 nM and remained stable during the period of treatment. Exposure to AMP-DNM was well tolerated by the animals as reflected by unchanged body weight gain. After two weeks treatment, liver ceramide and glucosylceramide concentrations were analysed by HPLC following microwave-mediated deacylation. The hepatic ceramide content of C57Bl/6J mice was not changed by the AMP-DNM diet, being 0.24 ± 0.02 and 0.26 ± 0.05 $\mu\text{mol/liver}$ of treated and untreated animals. In sharp contrast, liver glucosylceramide (85 ± 15 nmol/liver) was reduced by 41 ± 5 % by the AMP-DNM diet. Analysis by HPTLC showed that the ganglioside GM2 was concomitantly reduced by ~30%. Similar effects on sphingolipids were observed in muscle tissue of treated animals (not shown). The pronounced reduction by AMP-DNM of glycosphingolipids levels in mouse tissues without concomitant change in ceramide concentrations is comparable to the observed effect of the iminosugar in cultured cells.

Effect of AMP-DNM on insulin sensitivity of cultured adipocytes

In order to study the effect of AMP-DNM on insulin sensitivity in normal cells, we examined basal and insulin-stimulated glucose uptake in cultured human adipocytes. Basal uptake of 2- ^3H deoxyglucose glucose was not significantly affected by AMP-DNM treatment. However uptake of 2- ^3H deoxyglucose in the presence of 10^{-7} M insulin was increased by 84 ± 12 % after 16 h exposure to $10 \mu\text{M}$ AMP-DNM (table 5.1). Glucosylceramide levels in the treated cells were reduced to 63 ± 8 % of basal but ceramide levels were unaffected (not shown). In order to study the effects of AMP-DNM on insulin signalling in an insulin-resistant cellular model we examined 3T3-L1 adipocytes exposed to TNF-alpha. As expected TNF-alpha impaired insulin receptor autophosphorylation, IRS-1 tyrosine phosphorylation and the serine phosphorylation of AKT at residue 473. All of these abnormalities were significantly ameliorated by the simultaneous exposure to AMP-DNM (figure 5.2a). TNF-alpha increased cell surface levels of GM3 gangliosides and this was blocked by AMP-DNM (figure 5.2b).

Ceramide and glycosphingolipids in ob/ob mice before and after treatment with AMP-DNM

In *ob/ob* mice, the hepatic concentration of glucosylceramide was higher (115 ± 16 nmol/liver) compared to wild types animals (85 ± 15 nmol/liver; $p=0.045$). Increased concentrations of gangliosides were also noted upon HPTLC separation of lipids and charring densitometry. The ganglioside GM2 content was 36 ± 12 % increased, a finding consistent with previous studies in other tissues of *ob/ob* mice (17). The ceramide content of liver of *ob/ob* mice was also higher than that in wild type animals (3.15 ± 0.72 and 0.24 ± 0.02 $\mu\text{mol/liver}$, respectively). In muscle tissue of *ob/ob* mice glucosylceramide was again elevated compared to wild type mice (2.5 ± 0.5 vs. 1.5 ± 0.4 nmol/mg protein, $p=0.031$), however ceramide concentrations were similar (1.2 ± 0.4 and 1.4 ± 0.6 nmol/mg protein respectively, n.s.).

Two weeks treatment with AMP-DNM did not result in any significant changes in ceramide levels in muscle or liver from *ob/ob* mice (figure 5.3). In sharp contrast, glucosylceramide content of muscle and liver from *ob/ob* mice decreased by 45 ± 23 % and 45 ± 11 % with AMP-DNM, reaching values similar to those in wild-type animals (figure 5.3).

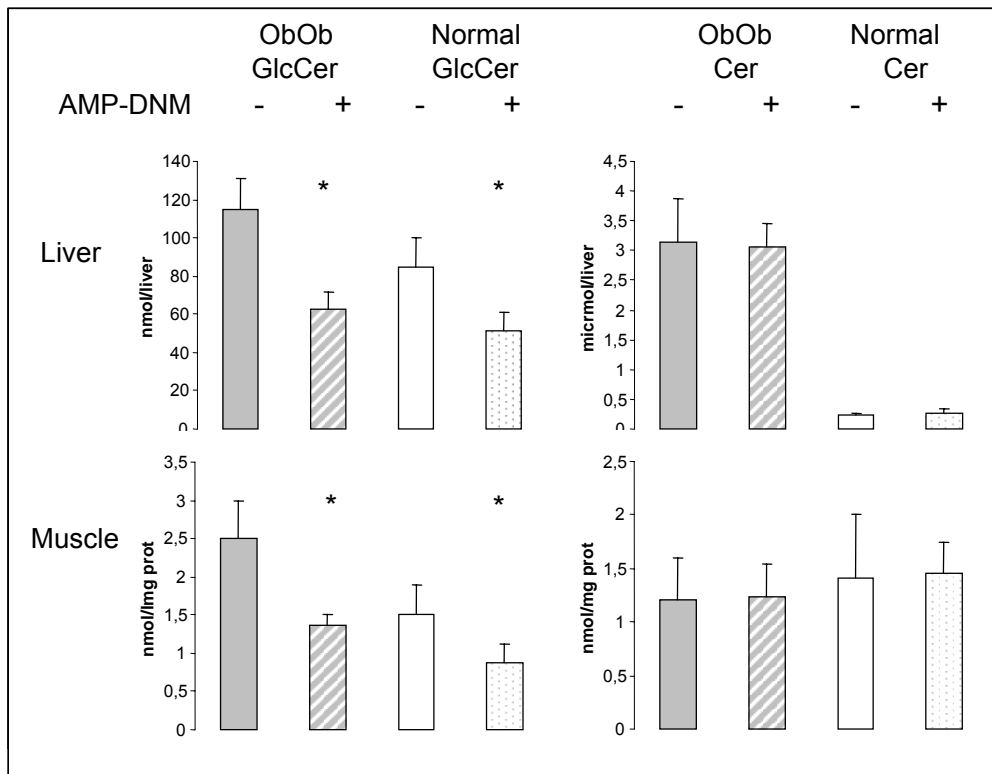


Figure 5.3. Ceramide and glucosylceramide levels in liver and muscle of normal and *ob/ob* mice treated with AMP-DNM. Animals were fed for two weeks with or without 25 mg AMP-DNM/kg. Liver (upper panel) and muscle tissue (lower panel) was collected and analysed on ceramide and glucosylceramide content. *, $P < 0.05$ by Student's t test.

Beneficial effects of AMP-DNM on metabolism in ob/ob mice

Prominent effects of AMP-DNM on glucose homeostasis were noted when six week old C57Bl/6J mice ($n=4$) were treated at a dose of 25 mg/kg body weight/day. AMP-DNM had no significant effect on body weight or food intake (figure 5.4a). Concomitantly, *ob/ob* mice treated with AMP-DNM showed a dramatic reduction in blood glucose levels ($p=0.008$) (figure 5.4b). Hyperinsulinemic euglycemic clamp studies demonstrated that this was associated with a marked improvement in whole body insulin sensitivity ($p=0.003$) (figure 5.4c). This was due in part to a significant ($\sim 26\%$) decrease in hepatic glucose production (Ra) ($p=0.035$) as well as a significant ($\sim 29\%$) increase in glucose disposal (Rd) ($p=0.0028$) (figure 5.4d).

The beneficial effect of AMP-DNM was also revealed by glucose tolerance tests. Animals fed with 25 mg/kg AMP-DNM for 8 days showed a better response to oral glucose administration as compared to untreated mice. The AUCs of blood glucose levels were significantly lower ($p=0.023$) in AMP-DNM treated mice (see insert figure 5.5).

Hepatic fat accumulation was apparent in untreated *ob/ob* mice and this was visibly reduced by AMP-DNM therapy. This was confirmed by direct measurement of triglyceride levels in livers of AMP-DNM fed *ob/ob* mice which significantly decreased from 107 ± 13 to 80 ± 11 nmol/mg liver ($p = 0.035$).

Adipocytes were isolated from epididymal fat pads of *ob/ob* mice treated with or without 25 mg AMP-DNM/kg for 2 weeks. GLUT 4 cell surface expression was examined using FACS analysis. Cell surface levels of GLUT4 protein were on average 3.2 fold increased in adipocytes from AMP-DNM treated mice as compared to those of controls (not shown).

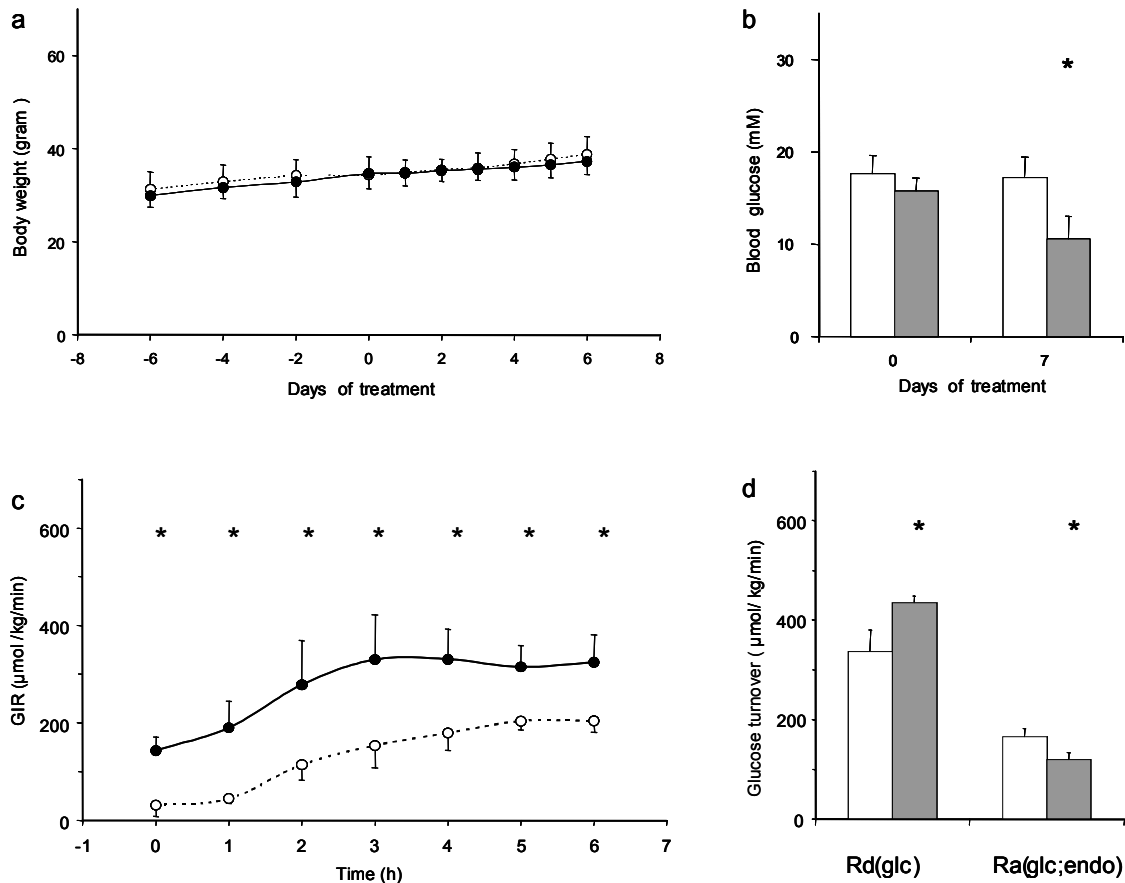


Figure 5.4. Beneficial effects of iminosugar feeding on glucose metabolism in *ob/ob* mice. *Ob/ob* mice were fed either with control chow (open circles and white bars) or with chow providing a dose of 25 mg AMP-DNM per kg bodyweight/day (closed circles and grey bars). Body weight (panel a), blood glucose level (panel b), were determined at indicated time points. During the clamp, glucose infusion rate (GIR) was monitored (panel c), the rate of glucose disposal (Rd(glc)) and the rate of endogenous hepatic glucose production (Ra(glc;endo)) were calculated (panel d). Data presented are means \pm SD. Significant differences between both groups (*) are indicated. Statistical significance of differences were determined using Student's t test, except in calculations over time where ANOVA with repeated measurements was used; $p < 0.05$ was considered as statistically significant.

Discussion

In these studies we have demonstrated that AMP-DNM, a potent inhibitor of glucosylceramide synthase, enhances insulin stimulated glucose uptake in normal adipocytes studies *ex vivo* and reverses the insulin signalling defect produced by exposure of adipocytes to TNF- α . More importantly, AMP-DNM has dramatic beneficial effects on the insulin resistance and hyperglycaemia seen in *ob/ob* mice, via a mechanism that does not require a reduction in food intake or loss of body weight.

A key question that arises relates to the specificity of AMP-DNM as an inhibitor of glucosylceramide synthase. At a concentration $<1 \mu\text{M}$, AMP-DNM causes no significant inhibition of activity of the lysosomal enzymes glucocerebrosidase and acid α -glucosidase or the ER trimming α -glucosidases. The cytosolic debranching enzyme and glycogen synthase are also unaffected at such concentrations (data not shown). AMP-DNM was originally developed as an inhibitor of a non-lysosomal glucosylceramidase (24). The molecular identity of the latter enzyme and its physiological function has still not been established. The enzyme

activity is already inhibited by AMP-DNM in the picomolar range ($IC_{50} \sim 1$ nM). Cultured adipocytes or *ob/ob* mice exposed to low nanomolar concentrations of AMP-DNM that completely inhibit the non-lysosomal glucosylceramidase activity but do not effectively reduce glucosylceramide synthase activity, do not show improved responsiveness to insulin. It therefore can be concluded that inhibition of the non-lysosomal glucosylceramidase by AMP-DNM is not sufficient, and possibly even not needed, to increase insulin sensitivity. Iminosugars can also significantly inhibit intestinal glycosidases, thus reducing the rate of digestion and absorption of oligo- and polysaccharides. This may additionally contribute to the beneficial effects of AMP-DNM on glucose homeostasis in *ob/ob* mice. However, AMP-DNM is only a low affinity inhibitor of sucrase and maltase, with IC_{50} values for these enzymes of $4.5 \mu\text{M}$ and $18 \mu\text{M}$, respectively (personal observations). Therefore it appears highly unlikely that at the dose used AMP-DNM would have significant impact on intestinal glycosidases. Finally, it is unlikely that AMP-DNM had significant non-specific toxic effects as food intake remained unchanged in treated *ob/ob* mice and no abnormalities were noted upon histological examination of liver, kidney and brain of *ob/ob* or wild-type mice fed for up to 4 weeks with 25 mg/kg body weight/day AMP-DNM. Only modest (30-50%) increases of liver glycogen were observed during the first week of feeding of *ob/ob* mice with AMP-DNM diet. Subsequently, glycogen levels remained stable in the normal range of C57Bl/6J animals, suggesting that there was no marked inhibition of debranching enzyme.

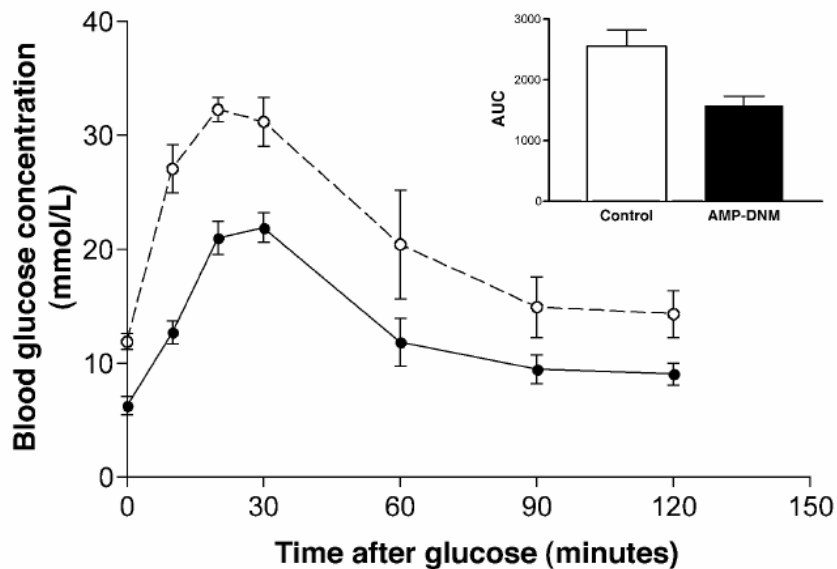


Figure 5.5. Improved glucose tolerance in AMP-DNM treated *ob/ob* mice. Glucose tolerance tests of male *ob/ob* mice, 8 weeks old, treated for 8 days with or without 25 mg AMP-DNM/kg ($n = 6$ each) administered by gavage two times daily. Animals were starved for 16 h prior to test. Values are expressed \pm SD. $P = 0.023$ by ANOVA.

Our studies with this pharmacological inhibitor may help to clarify the relationship between obesity, ceramides and insulin resistance and suggest a molecular mechanism for “lipotoxic” insulin resistance. Firstly, we confirmed previous observations that liver tissue from insulin resistant *ob/ob* mice had elevated levels of ceramide, glucosylceramide and gangliosides and demonstrated that muscle tissue from these animals had increased glucosylceramide but normal ceramide concentrations. We then demonstrated that AMP-DNM had dramatic beneficial effects on insulin sensitivity without any change in ceramide levels but with a marked reduction in tissue levels of glucosylceramide. These observations strongly suggest that it is unlikely to be the elevation in ceramide itself that mediates the insulin resistance seen in *ob/ob* mice, but that downstream metabolites of ceramide are more likely to be critically involved. This explanation is consistent with recent observations by other researchers implicating GM3 gangliosides in the pathogenesis of insulin resistance.

Our studies in cultured adipocytes provide some potential clues to the mechanism whereby AMP-DNM enhances insulin sensitivity. Thus, in normal human adipocytes, AMP-DNM has no effect in basal glucose uptake but significantly enhances insulin stimulated glucose uptake, suggesting an effect in insulin signalling. This is supported by the studies of TNF-alpha treated murine adipocytes in which AMP-DNM reversed the adverse effects of TNF-alpha on several steps in the insulin signal transduction pathway. This effect was demonstrable at a proximal step, *i.e.* insulin receptor autophosphorylation, of interest because of the previously reported localisation of GM3 gangliosides in plasma membrane subdomains containing the insulin receptor and the effect of genetic depletion of GM3 on insulin receptor autophosphorylation.

The pharmacological manipulation of the cellular levels of ceramide and its metabolites seems, at first sight, to be an intrinsically problematic therapeutic avenue since these sphingolipids are implicated in many crucial cellular processes. Ceramide analogues like PDMP that inhibit glycosphingolipid biosynthesis and concomitantly cause an increase in ceramide are known to exert cytotoxic effects. However, our finding that pharmacological manipulation of glycosphingolipid levels with AMP-DNM reverses insulin resistance without causing changes in ceramide concentrations may hold promise for the treatment of insulin resistance. One hydrophobic iminosugar, N-butyldeoxynojirimycin is already registered for the treatment of type 1 Gaucher patients suffering from a deficiency in lysosomal degradation of glucosylceramide (33). Although this compound inhibits glucosylceramide synthase, compared to AMP-DNM, it is neither a potent nor a selective inhibitor. The IC₅₀ value of N-butyldeoxynojirimycin for glucosylceramide formation is close to 100 μ M when measured with a fluorescent ceramide-analogue in living cells (21). The relatively poor bioavailability of the compound in mice requires oral administration of large quantities (>5 g/kg body weight/day) to reach a plasma concentration consistent with the IC₅₀ value. At such high dosages intestinal glycosidases are likely to be inhibited given the measured IC₅₀ values of 0.5 μ M and 9 μ M for sucrase and maltase, respectively. Intestinal complaints are a relatively common side-effects reported by Gaucher patients receiving N-butyldeoxynojirimycin. Interestingly, a more hydrophilic iminosugar, N-hydroxyethyldeoxynojirimycin (Miglitol), is already registered as an oral agent for treating diabetes (34). The ancient use of iminosugar-rich mulberry leaves in the Far East to control hyperglycaemia stimulated the development of this drug (35). The presumed mode of action of Miglitol is inhibition of intestinal glycosidase activities, thereby buffering monosaccharide assimilation (34). We have established that Miglitol does not inhibit glucosylceramide synthase in intact cells or homogenates (personal observations). However, since Miglitol is well absorbed it is possible that some proportion of the compound is metabolized to some structure that is capable of inhibiting glucosylceramide synthase.

In summary, our investigations indicate that the beneficial effects of hydrophobic iminosugars like AMP-DNM on hyperglycaemia are largely mediated by reduction of excessive glycosphingolipids in tissues. These findings support the notion that glycosphingolipids may play a critical role in the mediation of lipotoxic insulin resistance and should encourage the further evaluation of this class of iminosugar-based compounds for the treatment of human insulin resistance and diabetes.

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