Glycosphingolipids and atherosclerosis
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CHAPTER X

Discussion
General Discussion

AMP-DNM and atherosclerosis

In the last years, information has become available linking excessive glycosphingolipids (GSLs) with various symptoms of the metabolic syndrome, in particular insulin resistance. Firstly, mice deficient in GM3 synthase, an enzyme involved in the biosynthesis of the ganglioside GM3, are protected against diet-induced insulin resistance (Yamashita, 2003). In addition, several studies showed that the pharmacological lowering of excessive glycosphingolipids in rodent models for type-2 diabetes has a beneficial effect on insulin sensitivity. Most of these studies have been conducted with the iminosugar AMP-DNM (N-(5’-adamantane-1’-ylmethoxy)-pentyl-1-deoxynojirimycin), a well-tolerated inhibitor of the enzyme glucosylceramide synthase (GCS) catalyzing the initial step in glycosphingolipid biosynthesis. AMP-DNM treatment was shown to not only improve glucose homeostasis (Aerts, 2007), but also to reduce chronic inflammation (Shen, 2004; Van Eijk, 2009), prevent hepatosteatosis (Bijl, 2009), improve satiety (Langeveld, 2012) and prevent pancreatic beta-cell loss (Aerts, 2007). All these metabolic derangements are components of the metabolic syndrome. The primary aim of this thesis was to evaluate whether treatment of mice with AMP-DNM can beneficially affect atherosclerosis, a pathology commonly developing in time in individuals with metabolic syndrome. In the different studies presented in this thesis we used as models for atherosclerosis APOE*3 Leiden and LDLR(-/-) mice. Mice were fed diets high in cholesterol and fatty acids for several weeks in order to induce hyperlipidemia and atherosclerosis. To study the effect of AMP-DNM, animals were fed diets containing the iminosugar at concentrations causing a partial inhibition of glycosphingolipid synthesis in tissues. In chapter II, we describe that AMP-DNM treatment prevents lesion formation. When mice were fed diets containing sufficient AMP-DNM almost no plaques were formed. The anti-atherogenic effect of AMP-DNM was seen both in APOE*3 Leiden and LDLR(-/-). The protective effect was accompanied by absence of hypercholesterolemia despite a high fat/cholesterol diet. AMP-DNM treatment successfully kept plasma cholesterol levels low by stimulating bile formation and secretion, and increasing cholesterol output into the feces. The lipoprotein profile of the mice receiving AMP-DNM resembled the one of animals on normal chow diet, being low in atherogenic APOB-containing lipoproteins. Moreover, AMP-DNM treatment also decreased tumor necrosis factor-α (TNF-α) and monocyte-chemoattactrant protein-1 (MCP-1/CCL2) expression. This anti-inflammatory action of AMP-DNM most likely also contributes to its strong anti-atherogenic effect.
LDLR(-/-) and APOE*3 Leiden mice show a marked increase in plasma cholesterol as well as GSL in lipoproteins when fed a high cholesterol/fat diet. AMP-DNM treatment prevents the high plasma levels of both types of lipids by a reduction of lipoproteins, in particular VLDL/LDL. The exact mechanism(s) by which AMP-DNM accomplishes such reductions in lipoproteins is not yet elucidated. To get some insight we compared the effect of treating LDLR(-/-) mice with either AMP-DNM or its L-ido analogue (L-ido-AMP-DNM). Both compounds inhibit on a par GCS, and they are both potent inhibitors of GBA2, the non-lysosomal glucocerebrosidase (Boot, 2007). However, L-ido-AMP-DNM, in contrast to AMP-DNM, does not inhibit GBA1, the lysosomal glucocerebrosidase that is deficient in Gaucher disease patients. Interestingly, AMP-DNM was found to reduce the concentration of plasma lipoproteins in LDLR(-/-) mice on a high cholesterol/fat diet more than L-ido-AMP-DNM. AMP-DNM consequently prevents atherosclerosis stronger than L-ido-AMP-DNM. The main difference between the two iminosugars is the unique ability of AMP-DNM to stimulate bile production and secretion of biliary lipids. Increasing bile salt and biliary cholesterol excretion increases reverse cholesterol transport which inhibits atherosclerotic lesion formation (Bhat, 2003; Hageman, 2010). Of note, the anti-atherogenic effect of voluntary wheel running in mice was also found to be associated with increased bile flow and biliary bile salt and cholesterol secretion (chapter V). Interestingly, Gaucher patients show signs of increased biliary cholesterol excretion leading to a high incidence of cholesterol-rich gall stones (Taddei, 2010). These patients also show an altered lipoprotein metabolism (Le, 1988) leading to low LDL and HDL cholesterol (Pocovi, 1998). Gaucher patients show no increased risk for atherosclerosis and cardiovascular disease despite their low HDL levels (De Fost, 2009). In these aspects Gaucher patients resemble the situation in AMP-DNM treated mice. Our experimental findings with the two iminosugars suggest, but do not proof, that the inhibition of GBA1 caused by AMP-DNM, but not its idose analogue, is essential for the observed stimulation of bile flow and thus adds to the anti-atherogenic effect of AMP-DNM. An indication that the lowering of plasma cholesterol by iminosugar treatment is key in prevention and/or regression of the atherosclerotic lesion was obtained in the conducted intervention study (chapter IV). Here, animals were first fed a high cholesterol diet for 12 weeks and after lesion formation AMP-DNM treatment was started for 6 weeks. We observed that once the animals are already hypercholesterolemic and present advanced lesions, AMP-DNM treatment was somewhat less efficient in generating low plasma cholesterol and it did not result in complete regression and or inhibition of further progression of atherosclerotic lesions. In hindsight, prior to the intervention with iminosugars mice had already developed very advanced lesions and the chosen duration of iminosugar treatment was relatively short.
We can presently not exclude that iminosugars treatment may lead to some reversion of less severe lesions. The levels of plasma cholesterol (in various lipoproteins) are the most used parameter to predict the risk for atherosclerosis, both in rodents and in humans. In the search for different circulating markers of plaques, we investigated the plasma concentration of chitotriosidase, a macrophage-derived chitinase that is markedly increased in plasma of Gaucher disease patients (Hollak, 1994) (chapter VI). It has been earlier described that chitotriosidase is expressed in macrophages in atherosclerotic lesions in man (Boot, 1999) and there are reports suggesting that plasma chitotriosidase levels correlate with atherosclerosis (Artieda, 2007). In our investigation on chitotriosidase we used, besides APOE3*Leiden and LDLR(-/-) mice, also APOE(-/-) mice, another established model for atherosclerosis. We detected that chitotriosidase is expressed by macrophages in atherosclerotic lesions in the aortic sinus of all mouse models. The plasma levels in atherosclerotic mice were higher than in wild-type animals. However, we also noted that plasma chitotriosidase activity did not always correlate well with the degree of atherosclerosis at the aortic sinus. Differences were found between models and pharmacological manipulations applied. For example, we observed a nice positive correlation between plasma chitotriosidase activity and lesion area in APOE3* Leiden mice. APOE(-/-) mice showed higher chitotriosidase levels than wild-type animals, and the enzyme levels were higher when animals were switched from a normal chow to a western type diet. However, when the animals where treated with angiotensin II, a peptide able to promote atherosclerosis, chitotriosidase levels were reduced, sharply contrasting with lesion size at the aortic sinus. In the case of LDLR(-/-) mice plasma chitotriosidase levels were again higher than in normal animals, but no strict correlations were noted between lesions at the aortic sinus and enzyme levels. There may be several reasons for the observed lack in correlation between plasma chitotriosidase activity and observed lesion size at the aortic sinus in various mouse models. First of all, it has to be considered that the chitotriosidase assay does not reflect exclusively chitotriosidase but also the other chitinase AMCase activity. This chitinase, virtually absent in human plasma, is rather abundant in mouse plasma and its activity could have masked possible chitotriosidase activity changes. Moreover, significant differences exist in the promoter region of the chitotriosidase gene in mouse and man (Boot, 2005). Additionally, in mice chitotriosidase is rapidly cleared from the circulation (van Eijk, 2005). These differences may explain why other researchers observed a strong association between plasma chitotriosidase and atherosclerosis in man, but we did not so in our investigation of mice.
AMP-DNM and body cholesterol homeostasis

AMP-DNM treatment positively influences the total body cholesterol homeostasis in mice. By studying the different steps of cholesterol homeostasis, we tried to determine how AMP-DNM causes its beneficial effects. We first analyzed whether AMP-DNM is able to reduce cholesterol absorption by the intestine. Ezetimibe, a hydrophobic small compound, is a well-documented inhibitor of cholesterol absorption by binding to the transporter NPC1L1. To determine whether AMP-DNM exerts an ezetimibe-like effect, we used the fecal dual-isotope ratio method to determine the rate of cholesterol absorption in APOE*3 Leiden mice (chapter II). No effect on intestinal cholesterol uptake was found with this method. Expression of genes involved in cholesterol absorption in the intestine, like NPC1L1, ABCG5/G8, SR-BI, ABCA1 was not modified by AMP-DNM treatment (chapter II). All these data suggest that AMP-DNM is not inhibiting cholesterol absorption in the intestine. A similar conclusion was drawn from the study described in chapter VII. In C57Bl/6 mice the treatment with AMP-DNM and Ezetimibe resulted in additive effects regarding fecal neutral sterol excretion. A two-fold higher fecal neutral sterol loss was observed in animals receiving both compounds compared to that in mice treated with only one of the two compounds.

Within the laboratory various studies were conducted to study the effect of AMP-DNM on different steps of cholesterol homeostasis. It was found that AMP-DNM does not influence chylomicron formation and clearance, does not influence LPL activity and maturation of VLDL to LDL (Bietrix et al. unpublished data). Moreover, no indications were obtained for an effect of AMP-DNM on in vitro HDL-mediated efflux of cholesterol from cultured cells. Interestingly, VLDL production was reduced in APOE*3 Leiden mice treated with AMP-DNM.

In mice treated with AMP-DNM, expression of various genes involved in cholesterol synthesis are up-regulated in the liver, particularly SREBP2 and its main target gene HMGCoA reductase (chapter II). Clearly, the animals loose cholesterol upon AMP-DNM treatment and this is compensated (in part) by increased hepatic cholesterol synthesis. We discovered two different pathways by which AMP-DNM induces loss of cholesterol: an increase of biliary cholesterol secretion and a concomitant increase of trans-intestinal cholesterol excretion.

AMP-DNM clearly stimulates bile flow and biliary cholesterol excretion (chapters II, III and Bijl, 2009). This phenomenon was observed in normal C57Bl/6 mice (Bijl, 2009) as well as APOE*3 Leiden and LDLR(-/-) mice (this thesis). It is of interest to speculate about the cause for this phenomenon. Since bile salt formation is considered to be the driving force for bile cholesterol secretion, increased bile salt production could explain the phenomenon. However, we
were unable to demonstrate increased bile salt production in AMP-DNM treated mice, where fecal bile salt loss is actually slightly reduced (Bietrix, unpublished data). There must be another mechanism triggered by AMP-DNM that promotes biliary cholesterol excretion. In this connection it is relevant to keep in mind our finding that L-ido-AMP-DNM does not promote biliary cholesterol excretion like AMP-DNM, suggesting some role for lysosomal glucocerebrosidase GBA1 (see above). It might be speculated that failure of lysosomal glucosylceramide degradation somehow triggers hepatocytes to preferentially re-direct cholesterol to and across the apical membrane, rather than the regular incorporation into VLDL. In this respect, the recent findings on the relation between cholesterol metabolism and sortilin-1 (SORT1), parallel our findings with AMP-DNM. SORT1 is a protein involved in sorting and binding ligands both in the Golgi apparatus and at the plasma membrane and traffics them to the lysosome. Genome-wide association studies have identified a genetic variant in the SORT1 gene associated with reduced risk of myocardial infarction, reduced plasma levels of LDL cholesterol, and increased expression of SORT1 in liver (Teslovich, 2010; Kathiresan, 2009). In mice, increased hepatic expression of SORT1, is associated with both reduced hepatic APOB production and increased lysosomal LDL catabolism, consistent with SORT1’s role as a lysosomal trafficking protein (Strong, 2012). In mice treated with AMP-DNM hepatic gene expression of SORT1 was found up-regulated (manuscript in preparation).

The other pathway resulting in loss of cholesterol that is stimulated by AMP-DNM is the so-called trans-intestinal cholesterol excretion (TICE), the alternative cholesterol secretion route which takes place at the intestinal level (van der Velden, 2008). In the intestine of C57Bl/6 mice treated with AMP-DNM, an increased TICE was demonstrable (chapter VII). Two genes had emerged as potential factors associated with TICE. These genes encode for the proteins Scarb2 (also referred as lysosomal integral membrane protein 2 (LIMP2)) and Rab9 (Vrins, 2009). Intriguingly, LIMP2 is the intracellular transporter of GBA1 (Reczek, 2007). Whether this is sheer coincidence or points to some mechanistic link between TICE and glucosylceramide metabolism is unclear at the moment. Of interest, the expression of LIMP2, and Rab9, in the intestine seems to be unaffected by AMP-DNM treatment that concomitantly increases TICE. Apparently, AMP-DNM activates TICE via different mechanisms. Clearly follow-up investigations are required to elucidate the possible role of glycosphingolipids in TICE.
AMP-DNM, steatohepatitis and glucose homeostasis

Atherosclerosis is the cardiovascular manifestation of the metabolic syndrome whereas non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the same syndrome (Kim and Younossi, 2008). NAFLD is characterised by fat accumulation and can progress to non-alcoholic steatohepatitis (NASH) associated with severe inflammation and different degrees of fibrosis. In a previous study, treatment with AMP-DNM of ob/ob mice clearly improved hepatic insulin sensitivity and prevented NAFLD by down regulation of hepatic fatty acid synthesis and inflammation (Bijl, 2009). In this thesis we tested the ability of AMP-DNM to correct liver steatosis and even non-alcoholic-steatohepatitis (NASH) when it already had developed in LDLR(-/-) mice (chapter VIII). After 6 weeks of AMP-DNM treatment, despite the maintenance of the animals on a high fat-high cholesterol diet, the inflammatory and fibrotic status of the liver were profoundly improved indicating a clear beneficial effect of AMP-DNM on NASH (chapter VIII). Mice treated with AMP-DNM showed an improvement of the HOMA index and a concomitant decrease of hepatic lipogenesis and increased beta-oxidation. These effects on lipid homeostasis were earlier observed in ob/ob mice treated with AMP-DNM (Langeveld, 2012). Also other iminosugars inhibiting GCS have been reported to stimulate fatty acid oxidation (Tsuduki, 2009; Kobayashi, 2010). An entirely different class GCS inhibitors, ceramide analogues like GENZ-123346, have also been found to prevent hepatosteatosis (Zhao, 2009). This study proved for the first time that AMP-DNM treatment not only prevents fat accumulation in the liver, but also significantly corrects pre-existing NASH, without intervention on the diet. In fact, treated mice showed an almost complete correction in steatotic and fibrotic status, presenting an hepatic phenotype similar to healthy control animals. These findings suggest an important potential therapeutic value of AMP-DNM for development in humans.

Conclusions and future perspectives

This thesis deals with the potent beneficial effects of the iminosugar AMP-DNM on cholesterol homeostasis, atherosclerosis, NASH and diabetes, all major features of the metabolic syndrome. AMP-DNM is a potent inhibitor of the enzyme glucosylceramide synthase, and as such effectively reducing glycosphingolipid levels in the whole body. In different mouse models and exposed to different diets and experimental conditions, AMP-DNM treatment was consistently able to improve cholesterol and glucose homeostasis, to decrease lipogenesis and increase fat-oxidation. Considering all the pleiotropic effects of AMP-DMN, it has to be questioned whether its
ability to inhibit glucosylceramide synthase explains all of its beneficial actions. A study by Wennekes and colleagues comparing AMP-DNM with L-ido-AMP-DNM, being equally potent GCS inhibitors, revealed that AMP-DNM had more potent beneficial effect on overall glucose homeostasis compared to its L-ido analogue. This was ascribed to the co-inhibition by AMP-DNM, but not L-ido-AMP-DNM of carbohydrate assimilation from food. In this thesis we speculate that the stronger effect of AMP-DNM on cholesterol homeostasis and ultimately on atherosclerosis development could partly be explained by co-inhibition of GBA1 by AMP-DNM. Recently a highly specific irreversible inhibitor of GBA1 has been generated named MDW933 (Witte, 2011). Treatment of atherogenic/hyperlipidemic mice with MDW933 in the absence and presence of a specific GCS inhibitor would allow dissect the contributions of inhibition of both enzymes to the observed increased bile flow and reduction in VLDL secretion. From all the data collected during this thesis study, it seems that AMP-DNM exerts is pleiotropic effects not via an unique target, but more likely the synergistic action of multiple targets. A complete elucidation of the mechanism of action of AMP-DNM will be required to fully exploit the therapeutic of this or other related hydrofobic iminosugars.
Reference List


