Inhibition of Glycosphingolipid Synthesis Ameliorates Atherosclerosis and Arterial Stiffness in Apolipoprotein E−/− Mice and Rabbits Fed a High-Fat and -Cholesterol Diet

Subroto Chatterjee, PhD; Djahida Bedja, MS; Sumita Mishra, PhD; Christine Amuzie, BS; Alberto Avolio, PhD; David A. Kass, MD, Dan Berkowitz, MBBCh; Mark Renehan, BS

Background—Glycosphingolipids, integral components of the cell membrane, have been shown to serve as messengers, transducing growth factor–initiated phenotypes. Here, we have examined whether inhibition of glycosphingolipid synthesis could ameliorate atherosclerosis and arterial stiffness in transgenic mice and rabbits.

Methods and Results—Apolipoprotein E−/− mice (12 weeks of age; n=6) were fed regular chow or a Western diet (1.25% cholesterol, 2% fat). Mice were fed 5 or 10 mg/kg of an inhibitor of glycosphingolipid synthesis, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), solubilized in vehicle (5% Tween-80 in PBS); the placebo group received vehicle only. At 20 and 36 weeks of age, serial echocardiography was performed to measure aortic intima-media thickening. Aortic pulse-wave velocity measured vascular stiffness. Feeding mice a Western diet markedly increased aortic pulse-wave velocity, intima-media thickening, oxidized low-density lipoprotein, Ca2+ deposits, and glucosylceramide and lactosylceramide synthase activity. These were dose-dependently decreased by feeding D-PDMP. In liver, D-PDMP decreased cholesterol and triglyceride levels by raising the expression of SREBP2, low-density lipoprotein receptor, HMGCo-A reductase, and the cholesterol efflux genes (eg, ABCG5, ABCG8). D-PDMP affected very-low-density lipoprotein catabolism by increasing the gene expression for lipoprotein lipase and very-low-density lipoprotein receptor. Rabbits fed a Western diet for 90 days had extensive atherosclerosis accompanied by a 17.5-fold increase in total cholesterol levels and a 3-fold increase in lactosylceramide levels. This was completely prevented by feeding D-PDMP.

Conclusions—Inhibition of glycosphingolipid synthesis ameliorates atherosclerosis and arterial stiffness in apolipoprotein E−/− mice and rabbits. Thus, inhibition of glycosphingolipid synthesis may be a novel approach to ameliorate atherosclerosis and arterial stiffness.

Key Words: atherosclerosis ■ glycosphingolipids ■ mice, knockout ■ molecular imaging

Atherosclerosis contributes to nearly one half of the mortality in the Western world and is growing in epidemic proportions in rapidly developing countries in Asia. High levels of blood cholesterol, high blood pressure, obesity, diabetes mellitus, stress, and lifestyle factors, including smoking, are among the risk factors contributing to this alarming increase in this disease. Although several therapeutic modalities, including cholesterol synthesis inhibitors (family of statins), cholesterol absorption inhibitor (ezetimibe), blood pressure–lowering drugs, platelet adhesion inhibitors, and many more, are prescribed alone or in combination, there is a need for novel approaches to mitigate the initiation and progression of atherosclerosis. However, these therapeutic modalities do not decrease atherosclerotic plaque burden. A relationship between high blood levels of cholesterol and glycosphingolipids was suggested several decades ago. Subsequently, a tighter correlation between the load of low-density lipoprotein (LDL) cholesterol and glucosylceramide/lactosylceramide shedding in urinary proximal tubular cells in patients with the homozygous form of familial hypercholesterolemia was reported. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an analog of glucosylceramide, was synthesized to inhibit glucosylceramide synthesis in patients with Gaucher disease. However, we observed that this compound could directly inhibit the activity of purified lactosylceramide synthase. D-PDMP is a low-molecular-weight compound that is well tolerated by experimental animals, for example, mice, rats, and rabbits, at up to 10

Received November 18, 2013; accepted March 10, 2014.

From the Departments of Pediatrics (S.C., S.M., C.A., M.R.), Medicine (D. Bedja, D.A.K.), and Anesthesiology and Critical Care Medicine (D. Berkowitz), Johns Hopkins University School of Medicine, Baltimore, MD; and Australian School of Advanced Medicine, Macquarie University, Sydney, Australia (D. Bedja, A.A.).

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.113.007559/-/DC1.

Correspondence to Subroto Chatterjee, PhD, Johns Hopkins University Hospital, 1383 Blalock Bldg, 600 N Wolfe St, Baltimore, MD 21287. E-mail Schatte2@jhmi.edu

© 2014 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.113.007559

2403
times the effective dose. In mice, the effective dose of D-PDMP is 10 mg/kg body weight when given orally compared with the use of 100 mg/kg of various adamanate derivatives used by other investigators. Because D-PDMP turnover time is a short ≈52 minutes in mice, it is rapidly detoxified and excreted with little or no side effects. Delivery of D-PDMP by oral gavage or intraperitoneal injection has no effect on appetite and the overall well-being of the experimental animals. We report here that feeding a Western diet to apolipoprotein E–deficient (ApoE−/−) mice and normal rabbits leads to extensive atherosclerosis, vascular wall thickness and stiffness, and increases in the arterial levels of glucosylceramide and lactosylceramide. Feeding these animals D-PDMP dose-dependently ameliorates atherosclerosis and vascular stiffness.

Methods

Animals and Treatments

ApoE−/− male mice 11 weeks of age (Jackson Laboratories, Bar Harbor, ME) were purchased, and baseline physiological parameters were measured before further experimentation. At 12 weeks of age, the ApoE−/− mice were started on a high-fat, high-cholesterol (HFHC) diet of 4.5 kcal/g, 20% fat, and 1.25% cholesterol (D12108C, Research Diet Inc, New Brunswick, NJ) for 20 to 36 weeks with and without treatment with D-PDMP (5 and 10 mg/kg) and compared with control mice fed only a chow diet and placebo mice fed HFHC plus vehicle. Food was rationed once a week to estimate the weekly growth rate and food intake. Physiological studies were performed at ≈12, 20, and 36 weeks. Tissues were harvested at 12, 20, and 36 weeks of age for molecular and histopathological studies. D-PDMP was purchased from Matreya LLC (Pleasant Gap, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless mentioned otherwise. Animals were subjected to anthropometric measurements (body weight, percent body fat) and physiological measurements (blood pressure; ultrasound to measure aortic intima-media thickness; and pulse-wave velocity [PWV], a measure of arterial stiffening and arteriosclerosis). A group of mice (n=5) were euthanized to obtain baseline values for aortic tissue, and blood samples were collected. The rest of the mice were divided into groups receiving placebo (treated with vehicle only, 5% Tween-80 in PBS), 5 mg/kg D-PDMP solubilized in vehicle, and 10 mg/kg D-PDMP solubilized in vehicle. Vehicle and D-PDMP were delivered daily by oral gavage. The physiological measurements were repeated at 20 and 36 weeks, and then the mice were euthanized. New Zealand White male rabbits (7 lb) were fed rabbit chow supplemented with 0.2% cholesterol and 14% coconut oil with and without 10 mg/kg D-PDMP for 90 days. At 1, 2, 3, and 4 months, 5 mL blood was drawn from the ear vein. Samples were prepared, and total cholesterol levels were measured at each time point. Lactosylceramide levels were measured at 3 months. All experimental protocols were approved by the Committee for Animal Care and Use at Johns Hopkins University.

Glycosyltransferase Assays

Aortic tissue was homogenized in Tris buffer (pH 7.8) and centrifuged at 10,000 rpm for 10 minutes. The supernatant was used as a source for enzyme. [3H]UDP-Gal (American Radiolabeled Chemicals, St. Louis, MO) served as the nucleotide sugar donor in LacCer synthase assay, and [3H] UDP-Glc served as the nucleotide sugar donor in GlcCer synthase assay. The details of these assays have been described previously. All assays were conducted in triplicate in 3 to 5 aortic samples from mice in each group.

Measurement of Atherosclerotic Lesions

Transcardiac echocardiography was performed in conscious mice with the 2100 Visualsonic ultrasound device (Toronto, ON, Canada) equipped with a 40-MHz linear transducer. The aorta was viewed in the 2-dimensional mode; the left ventricle was viewed along the parasternal long axis. The intima-media thickness was measured from the ascending aortic wall and computed as the difference between external and internal diameters. Inner ascending aortic diameter was measured from the inner edge to the inner edge and the external diameter was measured from the external edge to the external edge of the ascending aortic wall. All measurements were performed according to the guidelines set by the American Society of Echocardiography. For each mouse, 3 to 5 values for each measurement were obtained and averaged for evaluation.

Blood Pressure and PWV Measurements

Systolic, diastolic, and mean arterial blood pressures and heart rate were measured noninvasively in conscious mice with the CODA tail cuff blood pressure system (Kent Scientific Corp). Data were recorded for later analysis.

PWV measurement was performed noninvasively with the high-frequency, high-resolution Doppler spectrum analyzer. Mice were placed supine on a temperature- and ECG-controlled (Indus Instruments) plate under anesthesia with 1.5% isofluorane. Core temperature was maintained at 37°C. A 10-MHz Doppler probe was used to measure blood flow velocity signals at the thoracic and abdominal aorta sequentially. PWV was calculated by the thoracic-abdominal distance divided by the pulse transit time between flow pulses recorded at the thoracic and abdominal aortic sites. Pulse transit time was determined by the time delay between the foot of the proximal and distal aortic flow waves in reference to the R wave of the ECG. The sequential flow measurements in the aorta were taken a short time apart, and there was no detectable difference in physiological parameters (e.g., heart rate) between the 2 measurements. Heart rate was maintained at a normal physiological heart rate of ≈500 bpm. Estimated effect of mean pressure (MP, mmHg) on aortic PWV (PWVp, m/s) was calculated with the following equation (A. Avolio et al, unpublished observations):

\[ PWVp = 0.006(MP)^{0.75} - 0.0564MP + 3.7911 \]

This relationship was used to correct for changes in PWV caused by mean pressure between the treated and control groups.

Histopathology

Masson trichrome staining was performed on 5-μm thin slices of the ascending aorta from mice 36 weeks after treatment and HFHC diet. These samples were photographed with a Nikon 80I Eclipse equipped with a Nikon DS-EI1 camera, and NIS-Elements software (Nikon, Japan) was used for image analysis.

Measurement of Triglyceride and Cholesterol Levels

The serum levels of triglycerides, LDL cholesterol, and high-density lipoprotein (HDL) cholesterol were measured with commercially available kits from Wako Chemicals (Richmond, VA). Liver levels of these lipids were quantified by high-performance thin-layer chromatography followed by densitometric scanning of charred plates.

Measurement of Oxidized LDL Levels

The serum level of oxidized LDL was measured with an ELISA assay and monoclonal antibody against human oxidized LDL (Avanti Polar Lipids, Alabaster, AL). The ApoE−/− mouse serum was plated in a 96-well microtiter plate at a 1:10 dilution in PBS containing 0.27 mmol/L EDTA and 20 μmol/L butylated hydroxytoluene overnight at 4°C. The oxidized LDL antibody was added after washing at a concentration of 5 μg/mL in a solution of 0.27 mmol/L EDTA and 0.02% sodium azide in PBS and incubated overnight at 4°C. The binding of the secondary antibody to goat anti-mouse IgM conjugated to alkaline phosphatase was quantified with the chemiluminescent substrate pNPP (New England Biolabs, Ipswich, MA). After a 4-hour incubation with the secondary antibody, the reaction
was stopped with NaOH, and the plate was read at 495 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA).

**High-Performance Liquid Chromatography Analysis of Glycosphingolipid Levels in Liver Tissue in ApoE−/− Mice**

Approximately 10 mg liver tissue was homogenized in chloroform-methanol (2:1, vol/vol), and lipids were extracted according to the Bligh and Dyer method. The total lipid extracts were dried in nitrogen and subjected to deacylation with sphingosine ceramide N-deacylation. After deacylation, lipids underwent o-phthalaldehyde derivatization and quantification of the levels of glucosylceramide and lactosylceramide by reverse-phase high-performance liquid chromatography. A C18 column was used with an isocratic organic mobile phase (methanol-water, 88:12, vol/vol) and calibrated with standard glycosphingolipids of known chemical structure and column affinity. All samples were analyzed in triplicate, and a representative quantity (n=3) of liver tissue samples was used for each treatment from control, placebo, and 5 and 10 mg/kg D-PDMP–treated ApoE−/− mice.

**Analysis of Gene Expression by Quantitative Real-Time Polymerase Chain Reaction**

A 50-mg piece of liver tissue was homogenized from each subject, and total RNA was isolated with TRizol reagent according to the manufacturer’s instruction (Invitrogen, Camarillo, CA). RNA (2 μg) was reverse transcribed with SuperScript II using random primers. Real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in an Applied Biosystems StepOne real-time PCR system with the following thermal cycling conditions: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute for denaturation, annealing, and elongation. Relative mRNA levels were calculated by the method of 2−DDCt. Data were normalized to GAPDH mRNA levels. To determine the specificity of amplification, melting curve analysis was applied to all final PCR products. All samples were performed in triplicate.

**Figure 1.** Aortic wall thickening and pulse-wave velocity (PWV) in atherosclerotic mice are ameliorated by treatment with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP). Aortic (AoR) ultrasound imaging: 2-dimensional B-mode ultrasound images of the aorta from ApoE−/− mice fed chow. Top (A–C) shows 20-week-old mice; bottom (D–F) shows 36-week-old mice. Control (A) ApoE−/− mice fed chow. ApoE−/− mice fed a high-fat, high-cholesterol (HFHC) diet plus vehicle (B) and HFHC plus 10 mg/kg (mpk) D-PDMP (C). Thirty-six-week-old control ApoE−/− mice fed mice chow (D), placebo mice fed HFHC chow (E), and HFHC-fed mice treated with 10 mg/kg D-PDMP (F). Arrows indicate marked increases in aortic wall thickening in HFHC plus vehicle–fed placebo mice (B) at 20 weeks of age. This was followed by a marked increase in Ca2+ deposits at 36 weeks of age (marked by asterisk) in this group of mice compared with control mice. This was prevented by feeding D-PDMP (F). Aortic wall thickness and aortic wall stiffness in atherosclerotic mice are ameliorated by treatment with D-PDMP (G and H). Quantification of intima-media thickness (AoIMT; G) and PWV (H) in ApoE−/− mice on a chow diet (control, 0.63±0.04 mm and 4.37±0.26 m/s), HFHC diet plus vehicle (placebo, 1.21±0.06 mm and 6.38±0.89 m/s; H), and HFHC diet plus treatment with 5 and 10 mg/kg D-PDMP (1.04±0.04 mm and 6.07±0.5 m/s; 0.73±0.04 mm/4.24±0.15 m/s, respectively). Note that both AoIMT and PWV increase continuously in placebo mice from 12 to 36 weeks and that this increase was ameliorated with treatment with D-PDMP in a dose-dependent manner. A 2-way repeated-measures ANOVA with the Bonferroni multiple-comparisons posttest was performed. *P≤0.05, **P≤0.01, ***P≤0.001; n=3 to 5.
triplicate. Primers used in the present study (Table I in the online-only Data Supplement) were synthesized by Integrated DNA Technologies (Coralville, IA). Expression suite software (Applied Biosystems) was used to analyze the data.

**Western Blot Analysis**

Approximately 90 to 100 mg frozen liver tissue from each animal was homogenized in 1 mL buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) containing protease inhibitor cocktail (Roche). After protein quantitation with the Bradford protein assay reagent (PIERCE), equal amounts of homogenate proteins were resolved by SDS-PAGE. Respective proteins were detected by immunoblotting. Immunoreactive bands of predicted molecular mass were visualized with an ECL plus kit (GE Healthcare Life Sciences) and quantified with the KODAK Molecular Imaging Software (Kodak, New Haven, CT). GAPDH was used as a loading control. The data represent the mean±SD of 3 independent experiments.

**Statistical Analysis**

All values are expressed as dot plots alongside mean±SEM. Comparison between groups was performed with the Kruskal-Wallis test and the Dunn multiple-comparison posttest. For repeated measures in mice and rabbits, a 2-way repeated-measures ANOVA was performed with a Bonferroni multiple-comparison test for comparisons between groups. GraphPad PRISM and Excel statistical software were used.

**Results**

**Aortic Wall Thickness, Vascular Stiffness, and Remodeling With and Without Treatment**

Ultrasound studies revealed that, compared with control mouse aorta (Figure 1A), feeding a Western diet to mice from 12 to 20 weeks of age contributed to a marked increase in aortic wall thickening (indicated by arrows in Figure 1B) in placebo mice. This was not observed in mice fed an HFHC diet plus 10 mg/kg D-PDMP (Figure 1C). At 36 weeks of age, the control mice show some Ca²⁺ deposit, perhaps as a result of aging (Figure 1D). However, extensive Ca²⁺ deposits were noted in placebo mice (Figure 1E), which were prevented by treatment with 10 mg/kg D-PDMP (Figure 1F).

Further quantitative analysis revealed that aortic intima thickening progressed rapidly in placebo mice fed an HFHC diet from 12 to 36 weeks of age compared with control mice. However, D-PDMP exerted a dose-dependent decrease in aortic intima thickening. In fact, the use of 10 mg/kg D-PDMP maintained the aortic intima-media thickening in HFHC-fed mice comparable to that in control mice (Figure 1G). Likewise, PWV measurement revealed a similar pattern, that is, suggesting that the thickening of the aortic wall was associated with arterial stiffening. This was lowered to control levels on treatment with 5 and 10 mg/kg D-PDMP in a dose-dependent manner (Figure 1H). Because blood pressure did not change significantly with treatment, the increase in arterial stiffness is largely independent of blood pressure.

Body weights for the baseline (29.4±0.93 g), control (31.1±1.61 g), and placebo (34.5±0.88 g) groups were not significantly different. At 36 weeks, the mice fed 5 and 10 mg/kg D-PDMP plus an HFHC diet showed a dose-dependent increase in body weight (36.5±1.77 and 41.0±0.55 g) compared with the other groups. The main reason was an increase in bone mass density and muscle mass. These mice were also physically active and less aggressive than the placebo group on an HFHC diet plus vehicle only. The latter instead showed an increase in percent body fat and were fragile. This suggests that HCHF may have an association with bone loss and calcified aortic wall, shown in Figure 1E, and no association with obesity.

**Assessment of Atherosclerosis**

Masson trichrome staining of 5-μm thin slices of the aorta in control mice revealed normal aortic wall thickness with no fibrosis (Figure 2A). In contrast, 36-week-old placebo mice...
fed an HFHC diet alone and a daily dose of vehicle exhibited narrowed lumen volume, occupied largely by plaques loaded with cholesterol esters (needle-like white crystals), amorphous Ca²⁺ deposits within the plaque, and extensive fibrosis (Figure 2B).

Treatment with 5 mg/kg D-PDMP significantly improved lumen volume; cholesterol esters were expunged from the plaque (Figure 2C) compared with placebo (Figure 2B), with reduced fibrosis and elastin fragmentation. The most unexpected result was the observation that treatment with 10 mg/kg D-PDMP completely prevented atherosclerosis in these mice at 36 weeks of age (Figure 2D). Notably, coronary artery disease, the principal cause of morbidity and mortality resulting from atherosclerosis, was drastically prevented by the treatment. A clear lumen was observed at 36 weeks for control mice, whereas a narrowed lumen volume was observed for placebo mice fed an HFHC diet (Figure 2E). This was mitigated by treatment with 5 and 10 mg/kg D-PDMP (Figure 2E). Histopathological analysis of the left coronary artery and quantification of lumen area revealed that D-PDMP reduces obstruction and plaque deposition in ApoE⁻/⁻ mice (Figure 1 in the online-only Data Supplement).

**Treatment With D-PDMP Dose-Dependently Decreases the Activity of Glycosyltransferases in the Aorta in ApoE⁻/⁻ Mice**

High activity of glucosylceramide synthase and lactosylceramide synthase in the aorta in ApoE⁻/⁻ mice was observed in 20-week-old mice fed an HCHF diet (Figure 3A and 3B). When these mice were given a daily supply of D-PDMP by oral gavage during this period (at 20 weeks of age), it dose-dependently decreased the activity of these enzymes. We noted a 10-fold increase in the activity of LacCer synthase in 36-week-old ApoE⁻/⁻ mice fed a Western diet (Figure 3C). However, mice up to 36 weeks of age did not exhibit any further increase in the activity of GlcCer synthase compared with 20-week-old mice (Figure 3D). These observations notwithstanding, some of this increase may be attributed to the effects of aging. D-PDMP dose-dependently decreased the activity of both GlcCer synthase and LacCer synthase in 36-week-old mice.
mice, as indicated by the mass of LacCer (Figure 3E) and GlcCer (Figure 3F).

Treatment With D-PDMP Ameliorates Hyperlipidemia in ApoE−/− Mice

Feeding an HFHC diet markedly increased the serum level of oxidized LDL measured with an ELISA assay. In contrast, feeding the glycosyltransferase inhibitor dose-dependently decreased the serum levels of oxidized LDL to below baseline levels in the ApoE−/− mice serum (Figure 4A). Similarly, the serum levels of HDL cholesterol (Figure 4B) and triglycerides (Figure 4C) were increased in serum from mice fed a Western diet. This was also decreased to baseline values in mice fed D-PDMP. Interesting, although feeding the Western diet decreased the serum levels of HDL cholesterol (Figure 4D), treatment with D-PDMP raised HDL cholesterol level in the serum. Thus, D-PDMP may be cardioprotective.

Effect of D-PDMP on the Expression of Genes Involved in LDL Metabolism

As shown in Figure 5A, treatment with 10 mg/kg D-PDMP increased the mRNA levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and sterol regulatory element binding transcription factor 2 up to ≥5.2-fold (P<0.0001) and 3.6-fold (P<0.0001), respectively. The mRNA level of LDL receptor was also elevated by D-PDMP respectively.

Effect of D-PDMP on the Expression of Transporter Genes Involved in Cholesterol Efflux, Bile Synthesis, and Triglyceride Metabolism

Real-time PCR showed that the expression level of the CD36, SRB-1, and ABCA1 genes were significantly upregulated by D-PDMP treatment (Figure 5B). These transporters play a major role in cholesterol efflux from peripheral tissues. As shown in Figure 5C, treatment with D-PDMP dose-dependently increased the mRNA levels of lipoprotein lipase and very-low-density lipoprotein (VLDL) receptor to ≥4.8-fold (P<0.0001) and 2.4-fold (P<0.0001) in liver samples of drug-treated mouse. The level of Cyp7A1 was also significantly upregulated by drug treatment (Figure 5D), indicating cholesterol catabolism and bile acid synthesis on drug treatment.

Effect of D-PDMP on the Expression of Proteins Involved in LDL Metabolism

Western immunoblot assays revealed that placebo mice liver tissue had significantly lower expression of SREBP2 protein and LDL receptor protein mass (Figure 6A and 6B). In contrast, treatment with D-PDMP markedly increased the expression of these proteins. These findings are in tandem with the gene expression data above, suggesting that drug treatment prevented atherosclerosis by increasing cholesterol metabolism via the LDL receptor pathway.

Treatment With D-PDMP Ameliorates Atherosclerosis in Normal Rabbits Fed a Western Diet

Treatment with the glycosphingolipid glycosyltransferase inhibitor D-PDMP prevented atherosclerosis in New Zealand White rabbits. Rabbits fed an HFHC diet showed a marked increase in aortic lactosylceramide levels (Figure 7A), accompanied by extensive atherosclerosis, and a 17-fold increase in aortic lactosylceramide levels (Figure 7A). In contrast to rabbits fed an HFHC diet alone, rabbits treated with D-PDMP showed a prevention of atherosclerosis as measured by lactosylceramide and cholesterol levels, which were similar to levels in controls (Figure 7).

Discussion

The following major findings emerged from the present study. D-PDMP, an inhibitor of glucosylceramide synthase and lactosylceramide synthase, dramatically and dose-dependently ameliorated atherosclerosis in ApoE−/− mice and normal rabbits fed a Western diet. In mice, this was accompanied by complete reversal of aortic intima-media thickening and PWV, an index of vascular stiffness. Our mechanistic studies revealed that D-PDMP decreased serum levels of cholesterol by regulating the expression of genes implicated in the biosynthesis, egress, and conversion of cholesterol to bile acids. Triglyceride levels were also dramatically reduced in the D-PDMP–treated mice as indicated by an increase in VLDL receptor and lipoprotein lipase gene expression.
A close association between glycosphingolipids and atherosclerosis was suggested by us and others. However, few attempts have been made to examine the effects of inhibiting/depleting the level of glucosylceramides in atherosclerosis in experimental animal models. Recently, Bietrix et al. used an iminosugar, N-(5-adamatane-1-yl-methoxy-pentyl)-Deoxynojirimycin (AMP-dNM), an inhibitor of glucosylceramide synthase, which markedly reduced plasma cholesterol and inhibited atherosclerosis development in ApoE*3 Leiden and LDL receptor–deficient mice. Additional studies have made use of this inhibitor in enhancing insulin responsiveness in a rat model of diabetes mellitus and in liver steatosis. Another inhibitor of GlcCer synthase, Zavesca (1,5-butylinimino, 1,5-dideoxy-D-glucitol and Genz-112638/miglustat, is already in use for substrate inhibition therapy in patients with Gaucher disease. D-PDMP inhibits both GlcCer synthase and LacCer synthase activity in cultured mammalian cells and can directly inhibit the activity of pure LacCer synthase. Our in vivo studies have shown its efficacy in mitigating vascular endothelial growth factor/fibroblast growth factor–induced angiogenesis and restenosis in rabbits after balloon angioplasty and lowering renal tumor volume by 50%. Because arteriosclerosis is a multifactorial disease, we hypothesized that a compound such as D-PDMP could be efficacious in lowering the glucosylceramide load and the phenotypes above.

An anticipated observation in this study was the remarkable filling of the lumen with atherosclerotic plaques in ApoE−/− mice fed an HFHC diet, which progressively worsened from 20 to 36 weeks of age. This was accompanied by extensive arterial wall thickening and an associated increase in the arterial wall stiffness measured by ultrasound. Consequently, PWV also increased. This was ameliorated on treatment with D-PDMP. An unexpected observation was that, from week 12 to 20, there were no visible Ca2+ deposits in the arterial wall. However, at 36 weeks of age, even mice fed chow alone had a few Ca2+ deposits. Mice fed an HFHC diet had extensive Ca2+ deposits, which were prevented by treatment with D-PDMP. These observations were made with the use of 5 and 10 mg/kg D-PDMP in a dose-dependent manner. Not only are these drug concentrations 10-fold lower than in a previous report, but also these mice did not accumulate subcutaneous fat. There was also a higher bone density compared with the placebo mice over the 36-week study, and no overt obesity was observed. As expected, D-PDMP treatment decreased the activity of not only GlcCer synthase but also LacCer synthase in the liver tissue. D-PDMP also decreased the level of serum cholesterol and triglycerides by recruiting multiple genes/pathways in lipid metabolism. For example, we noted that D-PDMP increased LDL receptor and SREBP2 gene expression and increased HMG-CoA reductase gene expression. These findings are suggestive of inhibition of cholesterol biosynthesis and increased LDL uptake. It also increased.
the expression of genes responsible for cholesterol efflux
by increasing the expression of ABCA1 and increasing the
expression of ABCG5 and ABCG8 proteins responsible for
pumping cholesterol from liver and intestine for excretion. A
previous study showed that glucosylceramide accumulation,
in particular lactosylceramide, in cultured cells can inhibit
the efflux of cholesterol via the ABCA1/apolipoprotein A1
pathway. In contrast, D-PDMP was found to serve as a cho-
lesterol efflux accelerator by inducing the expression of these
genes. Our findings are in agreement with and expand these
observations to an animal model of atherosclerosis.

A 7-hydroxylase enzyme, coded by the gene Cyp7A1, can
convert cholesterol to bile acid. The expression of this gene
was increased in mice treated with D-PDMP. This gene plays
a pivotal role in the expression of an enzyme required for the
conversion of cholesterol to bile acids. In a previous study, the
iminosugar N sugar AMP-DNM was also shown to increase the
expression of this gene in liver tissue in ApoE*3 Leiden mice
and to facilitate the lowering of cholesterol load by conversion
to bile acids and their subsequent excretion. Moreover, in our
study, HDL cholesterol was increased ≈2-fold in the serum
of D-PDMP-treated mice. Thus, a combination of genes
implicated in the lipid and lipoprotein metabolic pathways
contributed to the complete reversal of a marked increase in
cholesterol level and atherosclerosis in the ApoE−/− mice fed
an HFHC diet.
Previous studies have shown that an enzyme lipoprotein lipase plays a crucial role in linking VLDL, the major triglyceride carrying lipoprotein, to VLDL receptors.\textsuperscript{28} We observed that the liver tissue in D-PDMP–treated mice had increased expression of the gene required for the expression of lipoprotein lipase-1 and VLDL receptors. Such observations could explain why treatment with D-PDMP could reduce the markedly increased serum levels of triglycerides in ApoE\textsuperscript{−/−} mice fed an HFHC diet for 36 weeks (Figure 4C).

We have previously shown that oxidized LDL can dose-dependently increase the activity of LacCer synthase in cultured human arterial smooth muscle cells by phosphorylating

![Figure 7. Inhibiting glycosphingolipid synthesis inhibits atherosclerosis in rabbits. New Zealand White rabbits (n=3) were fed a diet with and without cholesterol (2%) and fat (14%) and with and without D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP; 10 mg/kg [mpk]) for 3 months. A marked increase in lactosylceramide levels in the aorta tissue was observed (A), accompanied by extensive atherosclerosis, and a marked increase (17-fold) in serum cholesterol levels (B) was found in rabbits fed the hyperlipidemic diet compared with the control and drug-treated rabbits at 3 months. This was prevented by treatment with D-PDMP. Statistical analysis: In A, a nonparametric 1-way ANOVA with the Kruskal-Wallis test and Dunn multiple-comparison posttest was performed. In B, a 2-way repeated-measures ANOVA was performed with Bonferroni multiple-comparison posttest. *$\text{P} \leq 0.05$, ****$\text{P} \leq 0.0001$; n=3.

![Figure 8. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) inhibits oxidized low-density lipoprotein (ox-LDL)–induced LacCer synthase (LCS) activity and atherosclerosis development. Ox-LDL activates LCS to produce large quantities of LacCer, which stimulates the activity of NADPH oxidase. Concomitantly, there is an increase in the generation of superoxide radicals (reactive oxygen species [ROS]). ROS mediates p44MAPK phosphorylation, thereby stimulating c-fos expression, promoting cell proliferation of vascular smooth muscle cells further to angiogenesis and generation of atherosclerosis. D-PDMP, a potent inhibitor of LCS, impaired ox-LDL–mediated induction of LCS activity, thereby inhibiting the above pathway. HDL indicates high-density lipoprotein; LCS, lactosylceramide synthase; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.]
the serine, threonine, and tyrosine residues in this enzyme. This was mitigated by pretreatment of cells with D-PDMP. The activation of this enzyme generated LacCer, which, in turn, activated a signaling cascade involving reactive oxygen species generation, p44/42MAPK activation, nuclear translocation factor c-Fos, and cyclin A to induce cell proliferation. In the present study, we show that feeding a Western diet significantly increases the level of oxidized LDL in the serum of ApoE−/− mice. This may have contributed to the activation of LacCer synthase in the arterial wall, thus contributing to an increase in the levels of LacCer. In contrast, in mice fed a Western diet plus D-PDMP, the level of oxidized LDL was decreased to basal levels (Figure 4A). This reduction in the LacCer level might have contributed to the resumption of cholesterol efflux at the normal level (Figure 8). An important observation made in our present study is that fibrosis contributes greatly to arterial wall thickness in our Western diet–fed model of atherosclerosis, and this also was prevented in mice fed D-PDMP.

In summary, we conclude that there is a tight relationship between glycosphingolipid metabolism and lipoprotein metabolism, which heavily affects aortic intima media thickening via fibrosis and lipid deposition. Such multigeneic changes bring about arteriosclerosis and its pathological sequelae. We demonstrate here that several genes responsible for the biosynthesis of cholesterol, its efflux, its absorption, bile acid production, and bile acid excretion were improved with treatment with a glycosphingolipid glycosyltransferase inhibitor. Additionally, the genes responsible for triglyceride metabolism were also improved after treatment, resulting in decreased triglyceride levels in ApoE−/− mice. An important observation was the increased expression of genes responsible for apoA1, the major protein in HDL, thus contributing to an increase in the plasma levels of HDL in treated mice.

Limitations

The glycosphingolipid inhibitor used in this study inhibits the synthesis of glucosylceramide synthase and lactosylceramide synthase; therefore, is not specific for a particular enzyme in the biosynthetic pathway. However, this compound is not toxic to animals when given for the duration of this study (6 months). Clearly, further studies are required using relatively more specific inhibitors in the near future. PWV depends on the serine, threonine, and tyrosine residues in this enzyme. Increased PWV may be a consequence of localized and systemic inflammation, which are components of atherosclerosis. In this study, we were able to obtain PWV-pressure relationships by altering pressure measurements, and when there was a change, the PWV values were adjusted for pressure differences using relationships obtained in mice from other studies. Results showed that pressure adjustments did not produce any major alterations; hence, this is not considered to be a significant limitation in these studies.

Acknowledgments

We thank Sara Kimiko Suzuki, Maya Hernandez, Fraulein Li, Bryan Brensinger, and Jennifer Hou for assistance during various phases of this research.

Sources of Funding

This study was funded by National Institutes of Health grants P01HL107153 and 3P01HL107153-03S1 (Dr Chatterjee).

Disclosures

None.

References

5. Gong N, Wei H, Chowdhury SH, Chatterjee S. Lactosylceramide recruits PKCε and phospholipase A2 to stimulate PECAM-1 expression in human monocytes and adhesion to endothelial cells. Proc Natl Acad Sci U S A. 2004;101:6490–6495.
Current strategies for the treatment and prevention of atherosclerosis and related conditions are targeted mainly at lowering blood levels of low-density lipoprotein cholesterol by the use of statins, a family of compounds that inhibit cholesterol biosynthesis. Zetia (ezetimibe) is also prescribed to block intestinal uptake of dietary cholesterol. However, for the treatment of residual risk for atherosclerosis, much effort has been exerted with minimal gain. Glycosphingolipids are associated with lipoproteins with blood levels tied to cholesterol. We report here that blocking the biosynthesis of glycosphingolipids in a transgenic mouse model (apolipoprotein E–deficient) fed a Western diet (high cholesterol and high fat) can prevent atherosclerosis and vastly improve arterial elasticity. In particular, treatment with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol increased the expression of protein ABCG5/8 responsible for pumping cholesterol from liver and intestine into the blood circulation for removal. Increased levels of high-density lipoprotein cholesterol, apolipoprotein A1 gene, and scavenger receptor-1 expression responsible for reverse transport of cholesterol from macrophage/peripheral tissue to the liver occurred in treated mice. Here too treatment improved the expression of the Cyp7A1 gene responsible for the conversion of cholesterol to bile acid and its subsequent excretion. Treatment also improved the expression of the lipoprotein lipase gene, which binds to very-low-density lipoprotein (triglyceride-rich lipoproteins) and to very-low-density lipoprotein receptors facilitating triglyceride metabolism. Thus, treatment with a glycosphingolipid glycosyltransferase inhibitor provides a novel strategy to prevent atherosclerosis by upregulating/downregulating multiple genes in cholesterol and fat metabolism. This compound could be useful in patients who do not respond favorably to statin therapy.

CLINICAL PERSPECTIVE


Inhibition of Glycosphingolipid Synthesis Ameliorates Atherosclerosis and Arterial Stiffness in Apolipoprotein E−/− Mice and Rabbits Fed a High-Fat and -Cholesterol Diet

Subroto Chatterjee, Djahida Bedja, Sumita Mishra, Christine Amuzie, Alberto Avolio, David A. Kass, Dan Berkowitz and Mark Renehan

_Circulation_. 2014;129:2403-2413; originally published online April 7, 2014;
doi: 10.1161/CIRCULATIONAHA.113.007559

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/129/23/2403

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/04/07/CIRCULATIONAHA.113.007559.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/
**Supplemental Table 1.** Primers used in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR</td>
<td>CAGACTGCAAGGACAAGTCA</td>
<td>GAG CCA TCT GCA CAC TGG AA</td>
</tr>
<tr>
<td>VLDLR</td>
<td>CCACAGCAGTATCAGAAGTC</td>
<td>GCCATCACTAAGAGC AAGAG</td>
</tr>
<tr>
<td>LPL</td>
<td>AGTGTTTGTGAATGCCATGA CA</td>
<td>CGGATGCTTTCTTCTTGTGTTT</td>
</tr>
<tr>
<td>APOA1</td>
<td>GTGGGCTCTGGTCTTCCCTGAC</td>
<td>ACGGTGAACCCAGAGTGTC</td>
</tr>
<tr>
<td>CD36</td>
<td>GCC AAG CTA TTG CGA CAT GA</td>
<td>AGA ATC TCA ATG TCC GAG ACT</td>
</tr>
<tr>
<td>SREBP1</td>
<td>CCG TGG GCT GAG GAA GGA</td>
<td>TGT GTA CTT GCC CAT GGC A</td>
</tr>
<tr>
<td>SREBP2</td>
<td>CATCCCTTGGGCAGAAGTT</td>
<td>TCCTTGGCTGCTGACTTTGATC</td>
</tr>
<tr>
<td>Abca1</td>
<td>AACAGTTTTGTGCCCTTTTG</td>
<td>AGTTCCAGGCTGGGGGTACTT</td>
</tr>
<tr>
<td>HMGR</td>
<td>TCTGGCAGTCAGTGGGAACATT</td>
<td>CCTCGTCTTCTGATCAAATTT</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>CAGTGAAGGTCTGCTGGAG</td>
<td>CGCAATGAAGAAGGTGACAA</td>
</tr>
<tr>
<td>SR-BI</td>
<td>TCCCTCATCAAGCAGCAGGT</td>
<td>TTCCACATCCCCAAGGACA</td>
</tr>
</tbody>
</table>
**Supplemental Figure 1**. Histopathological analysis showing Masson’s Trichrome stained left coronary artery (LCA) of control ApoE-/- mouse on a chow diet (A), Apo E-/- on high fat high cholesterol (HFHC) diet plus vehicle (Placebo) (B), Apo E-/- on HFHC diet plus treatment with 5 (C) and 10mpk of D-DPMP (D) respectively. Quantification of the cross sectional area of LCA (E). The placebo mice (E) show a significant increase in the cross sectional area (CSA), loss of smooth muscle cells, perivascular fibrosis, damaged wall, thrombosis and occlusion as compared to control and treated mice. The 5 and 10 mpk of D-DPMP treated mice show a dose dependent decline in cross sectional area and not significantly different to control mice.