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The association between blood pressure and glucosylceramides in spontaneously hypertensive rats

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Summary

Pharmacological inhibition of glucosylceramide synthesis has been shown to improve several aspects of the metabolic syndrome, such as insulin sensitivity and lipid metabolism. In addition, hypertension, another aspect of the metabolic syndrome, is associated with increased ceramide levels. Here we investigated whether hypertension is associated with altered glucosylceramide levels and whether inhibition of glucosylceramide synthesis affects blood pressure (BP) in hypertensive rats. For this purpose we determined glucosylceramide levels in normotensive Wistar Kyoto (WKY) rats, Spontaneously Hypertensive Rats (SHR), and in SHR treated with antihypertensive drugs or the glucosylceramide synthase inhibitor AMP-DNM, and subsequently recorded BP of these SHR.

Mass spectrometric analysis revealed elevated levels of glucosylceramides (±60% increase) in SHR arterial tissue compared to WKY. In contrast, plasma glucosylceramide levels were lower in SHR (± 50% decrease). Lowering BP by means of a 4-week treatment with losartan or hydralazine, substantially reduced glucosylceramide levels in arterial tissue (± 35% decrease) of SHR whereas it tended to increase plasma levels of this glycosphingolipid. A four-week oral treatment with the glucosylceramide synthase inhibitor AMP-DNM resulted in reduced glucosylceramide levels in arterial tissue (± 30% decrease). This was, however, not associated with a change in arterial ceramide levels nor a concomitant change in BP. In addition, AMP-DNM treatment did not influence endothelial dysfunction or ceramide-mediated contractions in SHR isolated carotid arteries.

From these data we conclude that hypertension is associated with profound alterations in vascular and plasma glucosylceramide levels, but that inhibition of glucosylceramide synthesis does not influence BP in SHR.

Introduction

Sphingolipids and glycosphingolipids are essential structural components of mammalian cell membranes (for review see van Meer *et al.*[1]). The large heterogeneity in sphingolipid and glycosphingolipid structures suggests a high degree of functional complexity. Indeed, next to their structural role, these lipids are now known to possess a variety of signaling properties and have emerging pathophysiological roles in health and disease [2,3]. Over the past decades, research has revealed that these bioactive lipids, next to their regulatory function in the immune and nervous system, also have important regulatory roles in the cardiovascular system [4,5]. For instance, we have previously shown that hypertension is associated with profound alterations in sphingolipid biology. In plasma from both spontaneously hypertensive rats (SHR) and hypertensive humans, ceramide levels are increased compared to normotensive controls [6]. In addition, in the vasculature of SHR, but not of normotensive rats, pharmacological elevation of ceramide induces the release of the vasoconstrictor thromboxane A_2, via a calcium-independent phospholipase A_2 ($iPLA_2$), cycooxygenase-1 (COX-1) and thromboxane synthase (TXAS)-dependent mechanism. Interestingly, antihypertensive therapy reduces vascular ceramide levels and may prevent endogenous ceramide-induced vasoconstriction [7]. Increased ceramide levels have also been observed in diabetes [8] and obesity [9], together with hypertension the main risk factors for cardiovascular morbidity and mortality.

Glucose linkage to the sphingosine moiety of ceramide, a reaction catalyzed by the enzyme glucosylceramide synthase, gives rise to the simplest glycosphingolipid; glucosylceramide, a pivotal building block of many more complex glycosphingolipids. The role of this glycosphingolipid in the cardiovascular system has not been studied extensively. Studies with inhibitors of glucosylceramide synthase however, have revealed that glycosphingolipids exert important functions in glucose metabolism, adipocyte function and lipid homeostasis. For instance the hydrophobic iminosugar N-(5-adamantane-1-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), a well-tolerated and potent inhibitor of glucosylceramide synthase, markedly lowered circulating glucose levels, improved oral glucose tolerance, reduced HbA1c, and improved insulin sensitivity in muscle and liver of mice and rats [10]. Moreover, this inhibitor is able to improve the adipocyte function and reduce inflammation in adipose tissue of obese mice [11]. AMP-DNM also decreases plasma cholesterol and phospholipids, and increased biliary lipid secretion [12,13]. Also the chemically distinct glucosylceramide synthase inhibitor (1R,2R)-nonanoic acid [2-(2',3'-dihydro-benzo [1,4] dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-

ylmethyl-ethyl]-amide-L-tartaric acid (Genz-123346) lowered glucose and HbA1c levels and improved glucose tolerance in the Zucker diabetic fatty rat and diet-induced obese mice [14]. These data suggest that inhibition of glycosphingolipid synthesis has beneficial effects on several aspects of the metabolic syndrome, whereas its effect on BP, another feature of the metabolic syndrome [15], has not been investigated yet. This study was designed to investigate whether hypertension is associated with changes in vascular tissue and plasma glucosylceramide levels, and if so, whether BP lowering restores these alterations and/or whether inhibition of glucosylceramide synthesis influences BP.

Here we demonstrate that SHR possess higher glucosylceramide levels in vascular tissue when compared to their normotensive counterparts, whereas lower glucosylceramide levels were observed in plasma. BP lowering partially restores these alterations. However, inhibition of glucosylceramide synthase in SHR does not influence BP or vascular function. From these data we conclude that hypertension is associated with pronounced alterations in glucosylceramide levels, but that these changes are most likely a reflection of hypertension, and not causative for hypertension per se.

Methods

Ethics statement

The animal experiments performed in this study followed a protocol which was approved by the Animal Ethics Committee of Maastricht University and the Academic Medical Center Amsterdam, The Netherlands, and was in accordance with EU guidelines (2010/63/EU) on the care and use of laboratory animals.

Animals and drugs.

Adult six-months-old male SHR were purchased from Charles River (L'Arbresle, France). AMP-DNM was synthesized as reported previously [16]. This iminosugar was dispersed in the standard chow for this rat study, the standard chow served as control diet. In some experiments, rats were anesthetized with isoflurane and osmotic minipumps (2ML4; Alzet, California, USA) or a dummy device (PE tube equally sized to 2ML4 pumps) were subcutaneously implanted filled with losartan or hydralazine. During 4 weeks, a continuous 20 mg/kg.day treatment regimen was obtained as described previously [7]. Acetyl-β-methylcholine (methacholine; MCh) and phenylephrine (Phe) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies against glucosylceramide synthase, cyclooxygenase 1 and thromboxane synthase

were purchased from Cayman Chemical; calcium-independent phospholipase A_2 antibody from Abcam (Cambridge, UK) and Von Willebrand factor antibody from GeneTex (Irvin, CA, USA). Alexa Fluor 488-labeled and Alexa Fluor 546-labeled secondary antibodies were from Invitrogen (Carlsbad, CA, USA).

Blood pressure measurements

Tail-cuff BP measurements in trained conscious animals were performed 28 days after the initiation of the different drug treatments using the CODA^{tm} system (Kent Scientific Corporation, CT, USA). Differences in tail-cuff systolic BPs (SBP) were verified by intra-arterial measurements when rats were anesthetized with 2.5% isoflurane. For this purpose a PE-50 canula with PE-10 fused tip was inserted into the abdominal aorta via the femoral artery. The arterial pressure was recorded using LabChart data acquisition software (ADInstruments Ltd, Oxford, UK). When BP was stabilized, baseline values of BP were recorded and averaged over 10-15 minutes. Hereafter, blood plasma, organs and blood vessels were collected and processed.

Liquid chromatography - mass spectrometry on blood plasma and arterial tissue

Post-anesthesia, the thoracic region was opened and 2 ml blood was collected by abdominal aorta puncture using a 21G needle (BD Microlance 3) and collected in a pre-chilled (0°C) polypropylene blood collection tube containing PECT solution as described previously [6]. Blood plasma was prepared by centrifugation for 20 min at 1600 x g, 4°C within 10 min after collection and stored at -80°C. Furthermore, the thoracic aorta was isolated, cleaned on ice and snap-frozen in liquid nitrogen. For blood plasma samples, lipids were extracted from 33 µL blood plasma as described by Merrill *et al.*[17] and Wijesinghe *et al.*[18] with slight modifications. Briefly; to 33 µL of plasma 167 µL water, 1mL methanol and 0.5 mL chloroform were added together with an internal standard containing 500 pmol of the following; d17:1 sphingosine, sphinganine, sphingosine-1-phosphate and sphinganine-1-phosphate, and d18:1/12:0 ceramide, ceramide-1-phosphate, sphingomyelin and glucosylceramide. The mixture was sonicated and incubated at 48°C overnight. The following day, extracts were subjected to base hydrolysis for 2 hrs at 37°C using 150 µL of 1 mol/L methanolic KOH. Following base hydrolysis the extract was completely neutralized by the addition of 6 µl glacial acetic acid. This neutralization was confirmed by pH measurement. Half of the extract was dried down and brought up in reversed phase sample buffer (60%A:40%B)(A= methanol:water 60:40 with 5mM ammonium formate and 1%

formic acid, B= methanol with 5mM ammonium formate and 1% formic acid). To the remaining extract, 1 mL chloroform and 2 mL water were added, and the lower phase was transferred to another tube, dried down and brought up in normal phase sample buffer (98%A:2%B). Sphingosine, sphinganine, sphingosine-1-phosphate sphinganine-1-phosphate and ceramide-1-phosphate were quantified via reversed phase HPLC ESI-MS/MS using a Discovery C18 column attached to a Shimadzu HPLC (20AD series) and subjected to mass spectrometric analysis using a 4000 Q-Trap (Applied Biosystems) as described by Wijesinghe *et al*. [18] Ceramides, sphingomyelins and monohexosyl ceramides were quantified via normal phase HPLC ESI-MS/MS using an amino column (Sigma) as described by Merrill *et al*. [17].
For aorta samples, lipids were extracted from 500 µL of a 10% homogenate of the tissue in PBS according to Merrill *et al*. [17] and Wijesinghe *et al*. [18] with slight modifications. Briefly to 500 µL of the 10% tissue homogenate 2 mL of methanol and 1 mL of chloroform was added together with an internal standard and processed as described above. The inter-day variability was less than 5% while the intraday variability was less than 7%. The accuracy has been previously verified (Wijesinghe *et al*. [18]).

Arterial preparation and isometric force recording

Carotid artery segments were isolated from the rats and mounted into a wire myograph for isometric tension measurements as described by Mulders *et al.* [19]. In brief, vessels were allowed to equilibrate and organ bath buffers were replaced every 15 min with carbogen aerated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (pH7.4; in mmol/L: 118.5 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.1 KH_2PO_4 and 5.6 glucose). Two high K^+-containing Krebs buffer contractions were performed (pH 7.4; in mmol/L: 23.2 NaCl, 100 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.1 KH_2PO_4 and 5.6 glucose) with 30 min washout in between. Then, 0.3 µmol/L phenylephrine was applied to gain a stabile contraction of >60% of the K^+-induced contraction, and 10 µmol/L of methacholine was added to assess endothelial integrity. After 30 min another high K^+-Krebs buffer contraction was performed. After 30 min wash-out, the enzyme sphingomyelinase (SMase; 0.1 U/mL) was applied to the organ baths to measure alterations in vasomotor tone for 1 hour. In other arteries, concentration-response curves for methacholine were generated in half-log concentration increments on phenylephrine-induced contractions.

Immunohistochemistry

Immunohistochemical protein staining and subsequent fluorescence intensity quantification in carotid artery segments of SHR were performed as described previously [6]. In brief, carotid artery segments were collected in ice-cold Krebs buffer directly after dissection and cleaning, rapidly submerged in OCT compound (Sakura, TissueTek). Frozen sections (5 µm thick) were cut on a Leica CM3050S cryostat and cold-air dried. Slides were fixed and incubated with blocking buffer. Slides were incubated at 4°C overnight with the primary antibody. The antibody against von Willebrand Factor (vWF) served as endothelium marker. Vessel slides were embedded in DAPI-containing mounting medium and imaged using a Nikon Eclipse TE2000-U fluorescence microscope (Plan Fluor ELWD 20x objective, Nikon DXM1200F digital camera) with NIS Elements AR 2.30 software. Quantification of fluorescence (fluorescent light units; FLU) was performed using NIS Elements.

Statistical data analysis

SBP, heart rate, organ weight, protein quantification, aortic and blood plasma sphingolipid content, and isometric tension measurements in carotid artery segments are presented as mean ±SEM with 'n' being the number of individual rats. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparisons test (95% confidence interval). Full concentration response curves were analyzed by one-way repeated measures ANOVA. All statistical analyses were performed using Prism (GraphPad Prism Software, San Diego, CA, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

Hypertension in rats is associated with alterations in plasma and arterial glucosylceramides.
Sphingolipidomic analysis by means of LC-MS/MS revealed that arterial tissue (aorta) from spontaneously hypertensive rats contained higher levels (approximately 60% increase) of glucosylceramides than arterial tissue derived from normotensive Wistar Kyoto rats (Figure 1A, basal values 41.6±3.4 pmol versus 25.2±0.8 pmol, n=3-4 (pooled aortae), $p<0.05$,). Interestingly, glucosylceramide levels were approximately 50% lower in plasma from SHR compared to plasma from WKY (Figure 1B, basal values 31.1±1.2 vs 62.5±4.1 pmol, n=8-12, $p<0.05$).

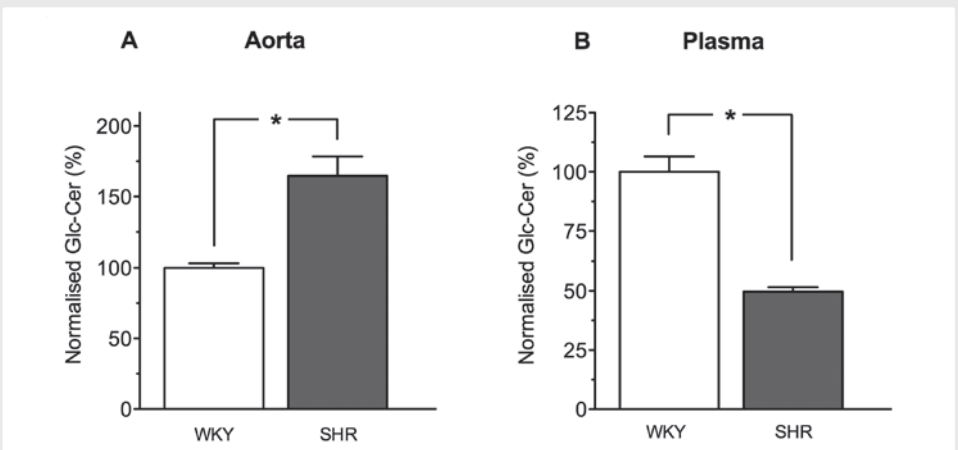

Figure 1. Normalized glucosylceramide levels in aorta **(A)** and plasma **(B)** of normotensive WKY rats versus spontaneously hypertensive rats (SHR). Data are expressed as mean ±SEM, n=3-12, * $p<0.05$

Blood pressure lowering reduces arterial glucosylceramide levels in hypertensive rats
Four-week treatment with either the angiotensin II type 1 receptor antagonist losartan or the vasodilator hydralazine reduced BP, as previously reported [7], to a similar extend (BP untreated 191±7 mmHg, versus losartan 125±5 mmHg or hydralazine 113±14 mmHg, n=4-6, $p<0.05$). This was accompanied by a concomitant decrease in arterial glucosylceramide levels (Figure 2A, 34.9% for losartan and 30.6% for hydralazine, n=7-8, $p<0.05$). Interestingly, antihypertensive treatment, in particular losartan, reached a trend towards increased plasma

glucosylceramide levels (Figure 2B, 29.8% for losartan and 13.3% for hydralazine compared to untreated SHR, n=7-8, p>0.05).

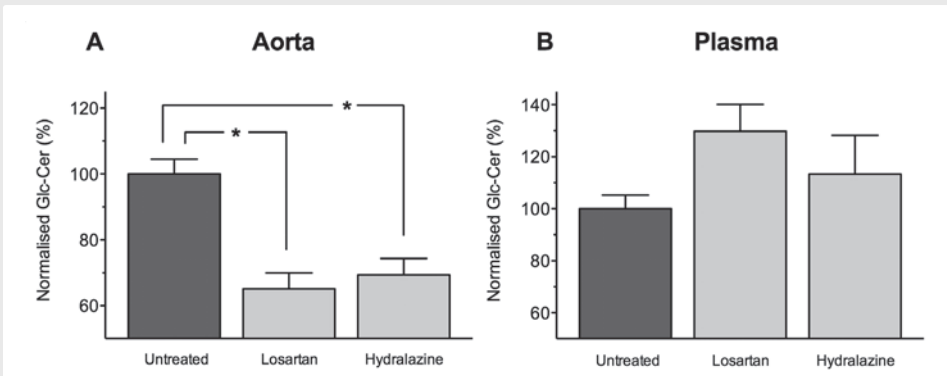

Figure 2. Normalized glucosylceramide levels in aorta (A) or plasma (B) of untreated SHR or SHR that were treated for 4 weeks with the antihypertensive drugs losartan or hydralazine. Data are expressed as mean ±SEM, n=7-8, * p<0.05

AMP-DNM reduces arterial glucosylceramide levels but has no influence on blood pressure

Four-week oral treatment with the glucosylceramide synthase inhibitor AMP-DNM resulted in significantly lowered arterial glucosylceramide levels (Figure 3A, 30.9±12.7% decrease compared to untreated SHR, n=8, p<0.05), but had no significant effect on plasma glucosylceramide levels (Figure 3B, 14.2±9.7% compared to control rats). Although the treatment with AMP-DNM resulted in a lower heart rate (Figure 4A, 409±9 versus 441±8 bpm for AMP-DNM and untreated SHR respectively, n=8, p<0.05), this was not associated with changes in BP (Figure 4B, 204±3 versus 208±5 mmHg for AMP-DNM and untreated SHR respectively, n=8, P>0.05).

Inhibition of glucosylceramide synthase has no effect on endothelial function

BP lowering significantly improves sphingolipid-associated endothelial function in the SHR as previously reported [7]. Treatment with the iminosugar AMP-DNM did not improve

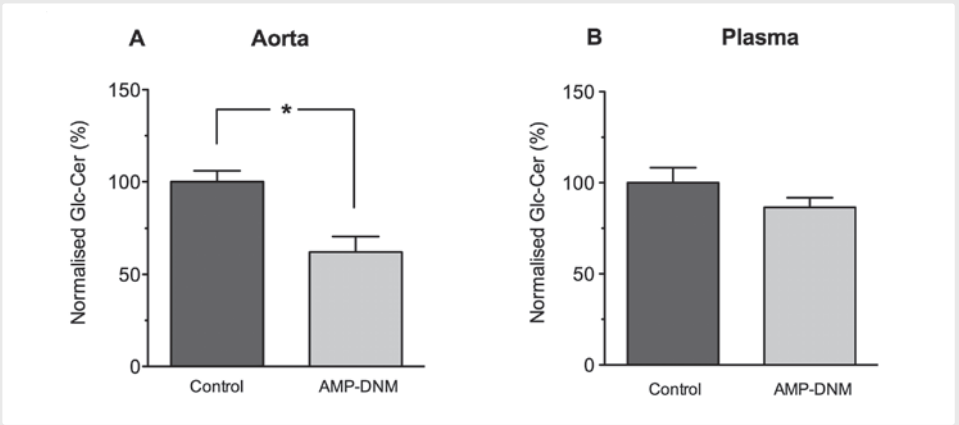

Figure 3. Normalized glucosylceramide levels in aorta (A) or plasma (B) of untreated SHR or SHR that were treated for 4 weeks with the glucosylceramide synthase inhibitor AMP-DNM.

endothelial function, as assessed by methacholine-induced vasodilation (Figure 5 A and B, n=7-8, p>0.05). In addition, treatment had no influence on sphingomyelinase-induced vasoconstriction of isolated carotid artery segments (Figure 5C, n=6-7, p>0.05),

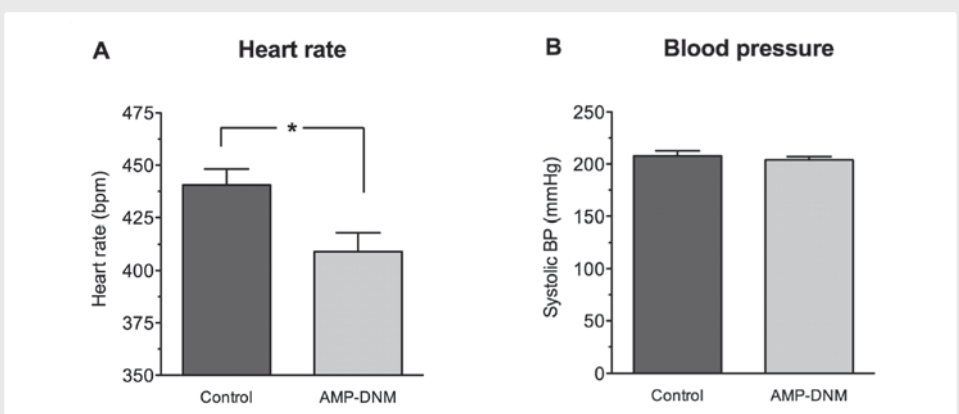

Figure 4. Heart rate (A) and systolic blood pressure (B) and in untreated SHR and SHR treated for 4 weeks with the glucosylceramide synthase inhibitor AMP-DNM. Data are expressed as mean ±SEM, n=7-8, * p<0.05

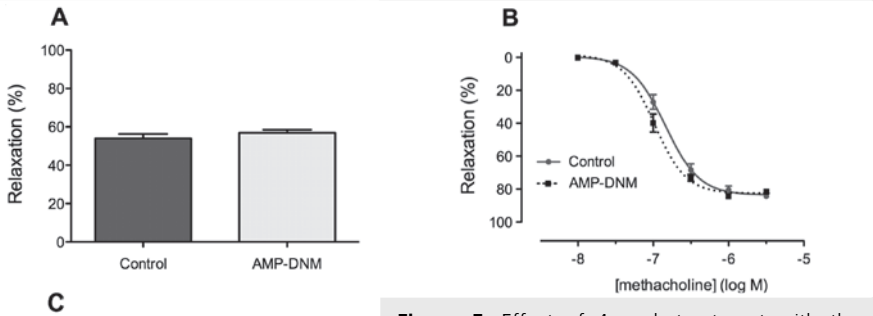

Figure 5. Effect of 4-week treatment with the glucosylceramide synthase inhibitor AMP-DNM on endothelial function as determined via methacholine-induced relaxation in phenylephrine pre-constricted isolated carotid arteries. (A) single concentration (10 mmol/L) or (B) concentration response curve to methacholine.
(C) Sphingomyelinase-induced (endothelium-dependent) vasoconstriction in treated and non-treated SHR. Data are expressed as mean ±SEM, n=6-8, p>0.05

Inhibition of glucosylceramide synthase does not influence the expression of vascular glucosylceramide synthase, iPLA$_2$, COX-1, or TXAS.

Immunohistochemical analysis of carotid artery segments revealed that 4-week treatment with AMP-DNM had no significant influence on endothelial and smooth muscle cell glucosylceramide synthase expression (Figure 6A) nor on the enzymes involved in the sphingomyelinase-induced TXA_2 synthesis being iPLA$_2$, COX-1 and TXAS (Figure 6B-D, n=6-8, p>0.05).

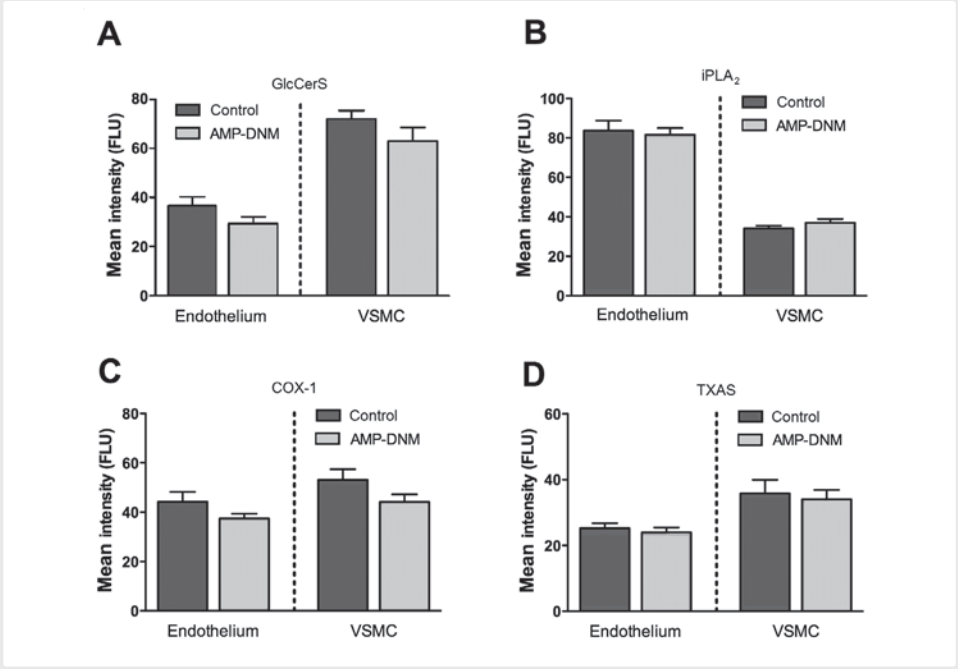

Figure 6. Effect of 4-week treatment with the glucosylceramide synthase inhibitor AMP-DNM on the expression of (A) glucosylceramide synthase, (B) calcium-independent phospholipase A_2 ($iPLA_2$), (C) cyclooxygenase (COX)-1 and (D) thromboxane synthase (TXAS) in endothelium or smooth muscle cell layer in carotid arteries. Data are obtained from quantification (fluorescence) immunohistochemical detection of the individual proteins. Von Willebrand factor was used as endothelial marker. Data are expressed as mean ±SEM, n=6-8, $p>0.05$

Discussion

In this study we demonstrate that hypertension is associated with pronounced divergent changes in vascular (increased) and plasma (decreased) glucosylceramide levels. These changes are partially restored by antihypertensive therapy. However, inhibition of glucosylceramide synthesis has no influence on BP in the SHR after 4 weeks treatment, suggesting that glucosylceramide does not play a major role in the regulation of BP in this setting.

Pharmacological inhibition of glycosphingolipid biosynthesis has been shown to improve glucose tolerance, insulin responsiveness [10,14] and adipocyte function [11] and to reduce atherosclerosis [20] and hepatic steatosis [13], which are all hallmarks of the metabolic syndrome. However, the levels of glucosylceramide in a context of hypertension and a possible effect of glucosylceramide synthase inhibitors on BP in hypertension, being another important feature of the metabolic syndrome, have not been investigated yet. Aforementioned, and the fact that we have demonstrated previously that hypertension is associated with profound alterations in sphingolipid biology [6], prompted us to perform this study in spontaneously hypertensive rats and normotensive WKY rats.

Mass spectrometric analysis revealed that vascular tissue (aorta) of SHR contains substantially higher levels (60% increase) of glucosylceramide compared to aorta from normotensive rats. Although the mass spectrometric method used does not allow us to discriminate between glucosylceramide and galactosylceramide, control experiments in this study have shown that the majority of monohexosylceramide, is indeed glucosylceramide (data not shown). The cause of this increase in glucosylceramide levels is not likely due to an increased expression of glucosylceramide synthase in the vasculature, since immunohistochemical analysis demonstrated equal expression levels of this enzyme in the endothelium and smooth muscle cell layer of carotid arteries of SHR and WKY rats. Interestingly, plasma glucosylceramide levels in SHR proved to be 50% lower when compared to plasma from WKY rats. It might be possible that the cause of this discrepancy between arterial and plasma glucosylceramide levels is a reduced export of glucosylceramide from the endothelium to plasma, however, to the best of our knowledge, no plasmalemmal transporter for glucosylceramide that might be responsible for this phenomenon has been identified yet. We have previously shown that the increased vascular ceramide levels observed in SHR, are sensitive to antihypertensive therapy with the angiotensin II type 1 receptor antagonist losartan and the vasodilator hydralazine [7]. Here we show that also the increased arterial glucosylceramide levels in SHR are sensitive to antihypertensive treatment and are decreased by more than 30% after 4 weeks of treatment

with either compound. Although antihypertensive treatment tended to increase plasma glucosylceramide levels, this failed to reach statistical significance with the given number of experiments. These indications are in line with our previous study that showed clear reductions of ceramide in arterial tissue but not, or only marginally, in plasma after antihypertensive treatment [7]. Maybe the effect of antihypertensive drugs on plasma sphingolipids will become more apparent after prolonged treatment. Although the effect of antihypertensive drugs on glucosylceramide levels in arterial tissue clearly demonstrates BP dependency, it does not allow us to draw conclusions on causality.

Treatment of SHR with the iminosugar AMP-DNM reduced glucosylceramide levels in arterial tissue to almost the same extend as losartan and hydralazine (±30%). This was, however, not associated with a concomitant reduction in BP. The latter suggests that glycosphingolipids are most likely not involved in BP regulation, and may be dysregulated in SHR as a consequence of high BP.

Table 1 General body parameters, blood metabolites, and sphingolipid presence of untreated SHR and AMP-DNM treated SHR

	Untreated (n=8)	AMP-DNM (n=8)
Weight change (g)	18 ± 2	-19 ± 4 *
Rel. heart weight (g)	3.60 ± 0.07	3.70 ± 0.15
Rel. kidney weight (g)	3.73 ± 0.06	3.61 ± 0.06
Total cholesterol (mmol/L)	1.38 ± 0.04	1.17 ± 0.05 *
Triglycerides (mmol/L)	0.13 ± 0.02	0.12 ± 0.01
Fasting glucose (mmol/L)	3.69 ± 0.05	3.82 ± 0.10
Ceramide plasma (µmol/L)	3.94 ± 0.10	3.32 ± 0.14 *
Ceramide aorta (nmol/L)	42.43 ± 3.56	34.27 ± 4.67
Glc-Ceramide plasma (µmol/L)	0.30 ± 0.02	0.26 ± 0.02
Glc-Ceramide aorta (nmol/L)	1.61 ± 0.13	1.15 ± 0.13 *

Data expressed as mean ±SEM, * p< 0.05.

Interestingly, also here we did not observe a clear effect of the drug on plasma glucosylceramide levels. Whether this is due to the already very low glucosylceramide plasma levels observed in SHR remains elusive, but in other rodent models, as exemplified by LDL receptor deficient mice and ApoE3*Leiden mice fed on a western diet, iminosugar treatment

markedly lower plasma glucosylceramide levels [20]. A possible explanation for our results forms inhibition of the activity of the non-lysosomal glucocerebrosidase, GBA2 by AMP-DNM [21-23]. GBA2 is extremely sensitive to inhibition by the iminosugar (IC_{50} of 1 nmol/L) [16], thus perhaps plasma GlcCer levels are less affected due to an equal stoichiometry between both GlcCer synthesis and breakdown within the given time-frame. In contrast to plasma GlcCer levels, AMP-DNM treatment of SHR rats did result in significant plasma cholesterol lowering, in parallel with the latter study [20], and overall reduction in body weight (Table 1 and [20]). In the present study, AMP-DNM treatment induced a small reduction in plasma ceramide levels. Theoretically, this lowering of ceramide levels may translate in a reduced BP, since we have previously shown that ceramide, at least in hypertensive rats, increases vascular tone by stimulating the release of thromboxane A_2 [6]. Most likely because the effects of AMP-DNM were insignificant on especially vascular ceramide levels this was not associated with a reduction in BP. In line with this, the four-week treatment with AMP-DNM had no influence on endothelial function, as demonstrated by unaltered responses to the endothelium-dependent vasodilator methacholine. Pharmacological BP lowering by losartan clearly reduces sphingomyelinase-induced vasoconstriction in isolated carotid arteries [7]. AMP-DNM, however, was not effective in this respect. Accordingly, and in contrast to losartan, AMP-DNM had no influence on the expression of enzymes involved in thromboxane A_2 synthesis.

In conclusion, we have demonstrated that hypertension is associated with pronounced alterations in glucosylceramide levels and that these changes can be partially restored by BP lowering. In contrast, lowering glucosylceramide levels in SHR has no influence on BP, suggesting that glucosylceramide has no major role in BP regulation per se.

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