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Smooth Muscle Specific Deletion of Ndst1 Leads to Decreased Vessel Luminal Area and No Change in Blood Pressure in Conscious Mice

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Abstract

Heparan sulfate proteoglycans are abundant matrix and membrane molecules. Smooth muscle specific deletion of one Heparan sulfate biosynthetic enzyme, N-deacetylase-N-sulfotransferase1 leads to decreased vascular smooth muscle cell proliferation, and vascular wall thickness. We hypothesized that this may lead to changes in blood pressure in conscious mice. Blood pressure was measured via telemetry in SM22αCre⁺Ndst1^{-/-} (n=4) and wild type (n=8) mice. Aorta and thoracodorsal artery luminal area is significantly smaller in SM22αCre⁺Ndst1^{-/-} (n=4–8, P=0.02, P=0.0002) compared to wild type (n=7) mice. Diurnal differences were observed in both cohorts for systolic, diastolic, mean arterial blood pressure, and heart rate (P<0.001 from T-Test). No significant differences were found in the above parameters between the cohorts in either light or dark times using a linear mixed model. In conclusion, deletion of N-deacetylase-N-sulfotransferase1 in smooth muscle did not influence any of the blood pressure parameters measured despite significant decrease in aorta and thoracodorsal artery luminal area.

Keywords

blood pressure; Ndst1; vascular; VSMC; Heparan Sulfate Proteoglycan

Introduction

Heparan sulfate proteoglycans (HSPG) are highly abundant molecules on the cell membrane as well as in the extracellular matrix [1]. HSPG molecules have the ability to interact with a variety of ligands, influencing the bioactivities of many substances such as lipids, chemokines and growth factors [2–3]. HSPGs regulate key steps in vasculogenesis [4] and angiogenesis through interactions with vascular endothelial growth factor A (VEGF A), transforming growth factor beta (TGFβ) or basic fibroblast growth factors (bFGF) [5–7] among others.

HSPGs are composed of long polysaccharide chains attached covalently to a core protein [8]. The synthesis of heparan sulfate chains involves a series of enzymatic reactions. Ndst1 is one of the enzymes that are important in the initial steps of HS synthesis [8–9]. Previously, we have reported that genetic ablation of N-deacetylase-N-sulfotransferase1(Ndst1) in smooth muscle resulted in smaller femoral artery comprised of a thinner vascular wall with significantly fewer vascular smooth muscle cells (VSMC) [10–11]. However, it is not clear how decreased number of vascular smooth muscle cells, and decreased medial wall thickness in macrovessels may contribute to blood pressure. Hence, the rationale of this study. The goal of the present study was to test the hypothesis that alterations in HS fine structure influenced arterial blood pressure in conscious mice.

The major methods of measuring blood pressure and related parameters are tail cuff, implanted fluid catheters, Millar solid state catheters and implanted telemetry systems [12]. The advantages of using the telemetry system over the other methods include long term simultaneous measurements of systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and heart rate (HR) in unrestrained and conscious animals [13, 14].

Significant interest persists in the ability to deliver small molecules to target HSPG in the settings of atherosclerosis, inflammation, angiogenesis and oncology [15]. Genetic mouse models have provided better understanding of the biological role of HSPG in normal development and in the pathogenesis of diseases [15–22]. Hence, these studies are important for future steps in drug discovery for designing small molecules that target HSPG as therapies to enhance or slow specific cellular processes beneficial to patients in the clinical setting.

Materials and Methods

Generation of Ndst1 deficient mouse models

Ndst1^{flox/flox} mice (gift from Dr. Esko, [23]) were mated with male SM22 α -cre mice (gift from Dr. M. Parmacek) [24–25]. F1 SM22 α cre⁺Ndst1^{wt/flox} males were mated with Ndst1^{flox/flox} females to generate mice with smooth muscle specific deletion of Ndst1 (SM22 α cre⁺Ndst1^{-/-}). All the mice including the control mice used for this study were of the C57BL6 strain. The genotype of wild type (WT) control mice was Cre⁻Ndst1^{wt}. The following primers were used to genotype the Ndst1^{flox}, Ndst1^{wt} and Ndst1^{-/-} alleles: Ndst1 (1-10R) 5'-ccaggcgctcaggcctcctg-3', Ndst1 (1-16R) 5'-catcctctgaggtgaccgc3', Ndst1 (1-17F) 5'-tcccacatggcgagactgaggttc-3' [26]. Ndst1^{wt} and Ndst1^{flox} alleles were identified by primer pair 1-10R and 1-17F while Ndst1^{-/-} allele was genotyped using 1-16R and 1-17F. Mice harboring smooth muscle specific deletion of the Ndst1 allele were also genotyped for Cre using primers: 5'-ccaattactgaccgtacacc-3'; 5'-gttcactatccaggttacgg-3' [26].

Telemetry

All surgical procedures were according to the University of Minnesota IACUC policy and best practice for small animal surgery. WT and SM22 α Cre⁺Ndst1^{-/-} male mice of 16–18 wk of age were surgically implanted with a telemetric probe and data were collected for 48 hours.

DBP, SBP, MAP, PP and HR were measured in WT (n=8) and SM22 α Cre⁺Ndst1^{-/-} (n=4) male mice using implantable telemetric transmitters (DSI, Minneapolis, MN). The transmitters were implanted according to the manufacturer's instructions. Briefly, telemetric probe was placed in the aortic arch of an anesthetized mouse via a catheter through the left carotid artery. Mice were allowed to recover for seven days before any measurements were

made. Post-operative recovery was carefully monitored. Out of fifteen mice, three did not survive the implantation due to post-surgical complications. The mice were then synchronized to a light/dark cycle of 12/12 h with lights on at 5:30AM and data were collected for 48 hours. The location of the catheter tip was verified upon explantation.

Luminal Area Measurements

Thoracic Aortas—Mice were sacrificed using CO₂ asphyxia. The thorax was exposed and the aortic arch was located. From the arch, the first 4 mm of the vessel was embedded in paraffin and 7µm sections were cut using a microtome. At least 4 sections from each animal were measured. The sections were then stained with Hematoxylin and Eosin (H&E). Area measurements were done by a blinded observer and pictures were taken using the Zeiss Axio Imager M1 Upright Microscope. All measurements were performed using Axiovision LE 4.7 software. The internal elastic lamina (IEL) was considered as the boundary between the lumen and the muscle layer.

Thoracodorsal Arteries—Mice were sacrificed using CO₂ asphyxia, the thoracodorsal artery (TDA) was isolated and placed in Krebs-HEPES containing (in mM) NaCl 118.4, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 4, KH₂PO₄ 1.2, CaCl₂ 2, Hepes 10, glucose 6 and supplemented with 1% BSA. Arteries were free of surrounding tissue, were placed in an arteriograph (Danish MyoTechnology, DMT) where they were cannulated at both ends with glass micropipettes and secured with 10–0 nylon monofilament suture. Arteries were perfused with Krebs-HEPES supplemented with 1% BSA and superfused with a calcium free Krebs-HEPES containing ethylenbis-(oxyethylenenitrolo) tetra-acetic acid (EGTA, 2 mmol/L) and sodium nitroprussiate (10 µmol/L). The arteriograph was placed on an Olympus IX-71 microscope, the TDAs were visualized with a 20× objective and were subjected to a gradient of pressure from 10 to 140 mmHg with a 5 minute stabilization period for each pressure to measure the passive diameter using the Slidebook software. The intraluminal pressure was increased from 10 mmHg to 140 mmHg by steps of 20 mmHg and the lumen diameter was measured in µm using the DMT vessel acquisition suite. The lumen area was calculated in µm², for each pressure step, using the lumen diameter values according to the formula: $\text{lumen area} = \pi \times \text{lumen diameter}^2 / 4$.

Statistics

Student T-test: Statistical significance between the two cohorts was done by Student T-test and a P value of less than 0.05 was considered significant.

Linear mixed-effects models were fitted to investigate the SM22αCre⁺Ndst1^{-/-} deletion effect on each of the five response variables, SBP, DBP, MAP, PP, and HR. Each of the 5 response variables were measured every 15 seconds for two days. To reduce data dimension, we used 10-minute average values; we checked that the results were not sensitive to this choice. The 3 covariates are: 1) day effects with day=1 or 2, referring to one of the two days; 2) diurnal effects with dark =1 for dark time, =0 for light time, where we considered 5:30am to 5:30pm as light time, and 5:30pm to 5:30am as dark time; 3) SM22αCre⁺Ndst1^{-/-} deletion effect with group=0 or 1 for the WT or SM22αCre⁺Ndst1^{-/-}. To account for possible correlations among the repeated blood pressure measurements on the same mouse, we used a random intercept term to represent the mouse-specific effect, and also used a first-order *autoregressive*, i.e. AR(1), correlation structure for the residual error (i.e. the correlation between any two residual errors on a mouse decreases by ρ times as 1 time lag increases). Specifically, our model on mouse has the following form:

$$\begin{aligned}
 Y_{ij} &= b_{0i} + b_1 \text{day}_i + b_2 \text{dark}_i + b_3 \text{group}_i + b_4 \text{dark}_i * \text{group}_i + \varepsilon_{ij} \\
 b_{0i} &\sim N(0, \sigma_0^2), \varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2) \\
 \text{cov}(\varepsilon_{ij}, \varepsilon_{i'j'}) &= \sigma_\varepsilon^2 \delta^{ij-j'}
 \end{aligned}$$

In the model, within a day, b_3 detects the possible blood pressure difference of SM22 α Cre⁺Ndst1^{-/-} versus WT in light times, and $b_3 + b_4$ detects the difference between SM22 α Cre⁺Ndst1^{-/-} versus WT in dark times.

Results

Body weight was significantly different between WT (27.37 ± 0.60 g) and SM22 α Cre⁺Ndst1^{-/-} (24.82 ± 0.12 g*, *P=0.005). Area measurements revealed that SM22 α Cre⁺Ndst1^{-/-} have a significantly smaller luminal area in both thoracic aorta (P=0.02) and thoracodorsal artery (P=0.0002) (Table 1).

Using student's T-test, SBP (P<0.001), DBP (P<0.001), MAP (P<0.001) and HR (P<0.001) were significantly different between dark and light times in both the cohorts (Table 2).

Using a mixed-random linear intercept model, and adjusting for light and dark times, we did not observe any significant SM22 α Cre⁺Ndst1^{-/-} deletion effect in all five parameters measured (Figures 1 and 2).

Discussion

Increased blood pressure is an established risk factor for cardiovascular diseases [27–28]. Previous reports from our laboratory demonstrated that deletion of Ndst1, a HSPG synthetic enzyme, results in a reduction in vascular wall area and a decreased number of VSMC [10]. In this study, we hypothesized that the smaller vessels in SM22 α Cre⁺Ndst1^{-/-} mice would alter blood pressure. Telemetry, an established method for measuring blood pressure in conscious and unrestrained mice [12] was employed to test this hypothesis.

We have previously shown that the SM22 α Cre⁺Ndst1^{-/-} mice exhibited a significant decrease in transcript abundance of Ndst1 in smooth muscle cells [10]. In parallel, we demonstrated a significant loss of N- and 2-O- sulfated disaccharides as assessed by HPLC [10].

Our results indicate an increase in SBP, DBP, MAP, and HR during dark times in both cohorts. This is in concordance with previous studies [10, 29]. The diurnal difference that we observed indicates and further confirms that telemetry is a reliable method for discerning blood pressure and related parameters in mice.

Our results suggest that smooth muscle specific deletion of Ndst1 decreases luminal area in both small vessel, (TDA) and conduit or large vessels (aorta). However, there were no significant differences in any of the five parameters (SBP, DBP, PP, MAP, and HR) between SM22 α Cre⁺Ndst1^{-/-} and WT in either dark or light times. This is in concordance with a previous report from our laboratory which indicated that left ventricular end-diastolic pressure and ejection fraction did not differ in anaesthetized WT or SM22 α Cre⁺Ndst1^{-/-} mice [10]. This data indicates that although deletion of Ndst1 in smooth muscle of mice results to fewer VSMC's and a smaller luminal area, blood pressure still remains unchanged.

A previous study in mice lacking Ndst1, Ndst3 or both, indicates that these two enzymes probably are regulating a common pathway [30]. Deletion of both enzymes show a more

extreme modification in HS sulfation compared to a deletion of either one although deletion of either enzyme still results in modified HS chains [30]. Previous work from our lab has shown that Ndst3 expression in VSMC is undetectable and is unchanged in response to deletion of Ndst1[10]. Thus it seems unlikely that Ndst3 is compensating for the loss of Ndst1 in SM22 α Cre⁺Ndst1^{-/-}.

It is possible that the elasticity/compliance properties of the vessels have been altered to compensate and restore blood pressure in these mice. Studies are underway in our laboratory to test compliance in both small and large vessels. It is also possible that circulating neurohormones or growth factors that affect blood pressure are altered in this murine model. In conjunction, because HSPG serve as co-receptors to a variety of hormones and growth factors, the activities of the receptors that these compounds bind to could also have been altered in this murine model.

In summary, this study suggests that deletion of Ndst1 in smooth muscle does not affect SBP, DBP or HR.

Significant interest lies in the ability to detect genetic mutations that may deem an individual or a population pre-disposed for arterial stiffness, hypertension and other cardiovascular diseases. As such, studies like ours are essential in our understanding of genes, mutations and their influences in cardiovascular health.

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Figure 1. Systolic pressure in WT (n=8) and SM22aCre⁺Ndst1^{-/-} (n=4) was measured using Telemetry. Light period was set from 5:30 AM to 5:30PM while dark period was set from 5:30PM to 5:30AM. SBP was not significantly different between the cohorts as assessed by the linear intercept model.

Figure 2.

Diastolic pressure in WT (n=8) and SM22 α Cre⁺Ndst1^{-/-} (n=4) was measured using telemetry. Light period was set from 5:30 AM to 5:30PM while dark period was set from 5:30PM to 5:30AM. DBP was not significantly different between the cohorts as assessed by the linear intercept model.

Table 1

Luminal area of WT and SM22 α Cre⁺Ndst1^{-/-} on thoracic aorta and thoracodorsal artery (TDA).

Genotypes	Luminal Area (μm^2)	
	Aorta	TDA
WT	199507 $\pm$ 19952 (n=7)	16211 $\pm$ 1060 (n=7)
SM22 α Cre ⁺ Ndst1 ^{-/-}	125382 $\pm$ 14160 (n=4)	11056 $\pm$ 423 (n=8)
P Value	0.02	0.0002

Vales are mean $\pm$ SE. SM22 α Cre⁺Ndst1^{-/-} luminal area on aorta and TDA are both significantly smaller compared to WT.

Table 2

Pressure, pulse pressure and heart rate were measured in WT and SM22aCre⁺Ndst1^{-/-} using Telemetry.

Parameters	WT (n=8)			SM22aCre ⁺ Ndst1 ^{-/-} (n=4)		
	Light (12h)	Dark(12h)	P-value	Light(12h)	Dark(12h)	P-value
SBP	115±3.5	128±2.5	<.001	114±3.4	126±3.7	<.001
DBP	92±1.8	103±2.4	<.001	89±4.6	99±5.1	<.001
MAP	104±2.6	116±2.1	<.001	102±3.6	113±4.1	<.001
PP	23±2.4	24±2.5	0.200	25±3.5	27±3.6	0.284
HR	544±18.1	594±17.2	<.001	559±11.0	603±14.7	<.001

Values presented are mean ± SE. P<0.001 (as assessed by student's T-Test) on SBP,DBP,MAP, and HR (between Light [5:30 am to 5:30 pm] and Dark times [5:30 pm to 5:30 am]).