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Vascular sphingosine-1-phosphate homeostasis and signaling in preeclampsia

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1 INTRODUCTION

Pre-eclampsia (PE) is currently one of the leading pregnancy complications. It is estimated that around 2 to 8 % of pregnancies worldwide are complicated by PE and it accounts for the death of approximately 80,000 women and 500,000 fetuses per year (1–3) .This syndrome is a multisystemic disease that which compromises the function of different organs (e.g the liver, kidneys, placenta and brain). PE is characterized by the development of hypertension, proteinuria, vascular dysfunction, chronic immune system activation, renal dysfunction and intrauterine growth restriction. However, these symptoms are not unique to the disease and are also commonly observed in other cardiovascular and inflammatory disorders. This clinical scenario argues for the hypothesis that preeclampsia is to be considered as an improper vascular and inflammatory adaptation to the pregnancy.

Genome-wide screening studies have been identified candidate-genes linked with the development of the disease. Several SNPs have been found that alter the function or expression of critical receptors, enzymes and other biologically active proteins involved in the pathogenesis of preeclampsia (e.g eNOS, MTHFR, angiotensinogen, TNF, and prothrombin (4). Many of these genes interact with the maternal cardiovascular system, or with the regulation of maternal inflammatory responses (5–7) Nevertheless, many studies of allelic variants associated with risk of preeclampsia have provided inconclusive results (8).

Most likely, the interplay between maternal constitution, placental factors and inappropriate adaptive changes to pregnancy results in preeclampsia (9–12).

Nowadays, indeed, the etiology for PE is still insufficiently understood and treatment is restricted to managing the resulting symptoms mentioned above. The only known cure for PE is the termination of pregnancy which additionally increases the rate of preterm birth (13,14).

Furthermore, women who develop preeclampsia might exhibit a shared predisposition for pregnancy-related vascular problems and future development of cardiovascular disease.

Because the placenta is essential to the development and remission of preeclampsia, researchers have been focused their investigations on the association between abnormal placental vascular development and the development of the disease. In uncomplicated pregnancies the extravillous cytotrophoblasts of fetal origin invade the uterine spiral arteries of the decidua and myometrium and further differentiate to adopt an invasive phenotype. The invasive cytotrophoblast replace the endothelial layer of the maternal spiral arteries, transforming them from small, high-resistance vessels into large diameter ones and unresponsive of vasoconstrictive agents. The proper vessels remodeling is necessary to provide adequate feto-placental perfusion (15). In preeclampsia, cytotrophoblast fails to invade the myometrial spiral arterioles effectively (cytotrophoblast invasion of the spiral arteries

is limited to the superficial decidua, and the myometrial segments remain narrow). This leads to abnormal placentation with concomitant placental underperfusion and ischemia, which results in the release several placental factors into the maternal circulation causing the clinical syndrome (16–18). Impaired angiogenesis in early gestation may also contribute to the inadequate cytotrophoblast invasion seen in preeclampsia. Alterations in the angiogenic pathways such as Flt1 (also known as vascular endothelial growth factor receptor 1 (VEFGR-1) results in abnormal vessels remodeling (19). Furthermore, it has been shown that placental tissue from preeclamptic patients is characterized by ischemia, thrombosis, inflammation, oxidative stress and apoptosis (20–23).

Although the causative and pathophysiological mechanisms of PE are unclear, systemic inflammation and endothelial dysfunction are the major and closely interconnected hallmarks of PE. Considering the current scientific knowledge about PE, it becomes evident that the endothelium plays an important role in the pathophysiology of the disease. The vascular endothelium is a multifunctional organ and it is critically involved in modulating vascular tone and structure. Because of its location the endothelium forms the interface between the circulating blood and its constituents and the surrounding tissue (24). First just considered as simple and inert physical barrier, the endothelium has increasingly been recognized as a smart barrier, highly dynamic, which serves several biological functions to maintain proper vascular homeostasis (25). The endothelium can respond to physical and chemical stimuli by production of a wide range of factors. Endothelium-derived factors with vasodilatatory and antiproliferative effects include endothelium derived hyperpolarizing factor (EDHF), nitric oxide (NO) and prostacyclin (PGI_2), while endothelin-1 (ET-1), angiotensin II and reactive oxygen species (ROS) are among the vasoconstrictor mediator Endothelial cells also produce antithrombic molecules. The endothelium is able to: modulate the vascular tone, through balanced production of vasodilators and vasoconstrictors; maintain blood fluidity and coagulation, through production of factors that regulates platelet activity, the clotting cascade and the fibrinolytic system; regulation of inflammatory response, through the expression of cytokines and adhesion molecules. A perturbation of this equilibrium by mechanic, chemical or immunologic influences triggers inflammation and leads to endothelial dysfunction (26–31). Hence the endothelium is not only a target of inflammatory stimuli but is also actively involved in the regulation of the inflammatory process. Activation of the endothelium leads to three main processes: a) increased blood flow followed by vessel dilatation; b) increased vascular permeability and c) increased recruitment of leukocytes. Endothelial activation during acute inflammation starts with a quick stimulatory response that is independent of gene expression (32). This first response is mediated by binding of ligands such as histamine or thrombin to G-protein coupled receptors (GPCR) (33). A slower but prolonged response, characterized by modulation of gene expression (triggered by cytokines like TNF-α and IL-1), follows

this first one (34). This cytokines mediate the inflammatory response of endothelial cells mainly via the transcription factors NF-κB and activator protein 1 (AP1) (35). This process ultimately leads to a switch from a NO-mediating regulation of the vasculature towards activation of redox signaling (h2o2 necessary evil for cell signaling cit.). Many serum markers of endothelial activation and endothelial dysfunction are unbalanced in women with preeclampsia; these markers include von Willebrand antigen, cellular fibronectin, soluble tissue factor, soluble E-selectin, platelet derived growth factor, and endothelin (36,37). Indeed, the exposure of endothelial cells with serum taken from women, diagnosed with preeclampsia, results in endothelial dysfunction (10,38). A normal pregnancy is associated with vasodilation of the systemic vasculature with a substantial decrease in peripheral vascular resistance, while cardiac output is increased (37). There is also a decrease in arterial pressure, including systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure, with concomitant increase in total blood volume and plasma volume (39). During pregnancy occurs an enhanced activation of the Renin Angiotensin Aldosterone system (RAS) which is not only present in the kidney but also at the uteroplacental unit. Thus, angiotensin levels increase during pregnancy to maintain blood pressure and to help retain salt and water. However, healthy pregnant women show a vascular insensitivity to angiotensin II, preserving vascular function (40). Conversely, preeclamptic women show an exaggerated sensitivity to the vasopressors angiotensin II and norepinephrine (40,41). Indeed, women who develop preeclampsia have impaired endothelium-dependent vasorelaxation, which results in peripheral vasoconstriction decreased arterial compliance (42). Thus, ultimately leads in increases in blood pressure and pulse pressure prior to the onset of hypertension and proteinuria (43).

Abnormal lipid metabolism is a known causative factor for endothelial dysfunction and impaired vascular health. During early pregnancy maternal accumulation of fat depots and hyperlipidemia occur in order to satisfy energy and structural demands of the developing fetus (44). However, in late pregnancy maternal metabolism switch from the anabolic status to the catabolic one to support the fetus in his maximal growth phase. As a consequence, there is an exponential breakdown of fat depots and increased amount of free-fatty acids (FFA) released in the circulation. Moreover, increased lipoprotein lipase (LPL) activity and decreased hormone (HL) lipase activity blunt the clearance of trygliceride-rich lipoprotein from the circulation, causing high triglycerides concentration in maternal blood (45). However, in pregnancy related disorders such as PE, the lipoprotein profile is strongly impaired and fuels the vascular dysfunction. In PE, the activity of the HL is higher than in normal pregnancy, leading to remodeling of the lipoproteins particles towards a proatherogenic phenotype. PE patients show an abnormal lipid profile characterized by a decreased high-density lipoprotein

(HDL) concentration and significantly increased total cholesterol, low density lipoprotein (LDL), very low-density lipoprotein (VLDL) and triglyceride plasma concentrations (46).

Many investigations suggest the pivotal role of sphingolipid metabolism and signaling in vascular physiology. These bioactive lipids regulate vital cell functions as well as cell signaling through the formation of cell membrane microdomain lipid rafts. Sphingolipids are synthesized *de novo* in the endoplasmatic reticulum (ER) or derived from the catabolism of other sphingolipids by recycling pathway. Different sphingolipid species such as ceramide, sphingosine and sphingosine-1-phophate (S1P) can be synthesized and exert their biological function

Growing number of studies have shown that these lipid mediators, in particular S1P, are closely involved in the pathophysiology of many cardiovascular disease and therefore provide effective drugs targets for the treatments of pathological states (47).

S1P is a bioactive lysosphingolipid mainly produced by erythrocytes and vascular endothelial cells, known to have endothelium-protective properties (48). The balance between S1P levels and its precursors (ceramide and sphingosine), within the cell, is tightly regulated and comprises what is defined as "sphingolipid rheostat" (49). Ceramide and sphingosine are associated with cell growth arrest and apoptosis. In contrast, S1P promotes cellular proliferation and survival. Indeed, the concept of "sphingolipid rheostat" has physiological as well as clinical relevance since ceramide/S1P ratio controls cell fate.

Once synthesized, intracellular S1P is likely exported out of the cell into the blood stream where it can activate cell-surface receptors (S1PRs) in an autocrine fashion or through the binding with plasma chaperones (50). Approximately 65% of circulating S1P is bound to HDL while remaining 35% associates to albumin (51). Despite the high concentration of S1P in the plasma (μM), its half-life in vivo is quite short (52). Indeed, several studies imply that S1P biosynthesis is finely regulated in order to guarantee maintenance of S1P levels in the plasma that would be otherwise rapidly turned over (53–55). Regulation of S1P homeostasis is therefore of critical importance to numerous downstream biological processes. A growing number of studies show that this complex signaling network plays a pivotal role in endothelial barrier function and vascular tone (56,57). Indeed, both alterations of circulating HDL-S1P complex and impaired S1P synthesis and metabolism have been implicated in cardiovascular diseases and metabolic disorders (58–61).

Most of studies, investigating the physiological and pathological action of S1P were carried out in animal models and adult human cohorts, while little is known about its role in human fetal development. Romanowicz et al. investigated for the first time the sphingolipid profile in control and PE umbilical cord arteries showing an association between PE and altered sphingolipid profile of the

umbilical cord artery wall. This study suggests that sphingolipid metabolism could have a role in the pathogenesis of PE.

However, the scientific knowledge concerning the potential role of circulating (HDL-associated) and intracellular S1P at the feto-placental unit is still scarce and inconsistent.

This project aims to describe S1P regulatory effect on the feto-placental endothelium in uncomplicated pregnancies and in pregnancy-related disorders characterised by endothelial dysfunction. Our goal is to elucidate the underlying cellular mechanisms of S1P turnover and signaling in PE to provide new insight into the pathogenesis of the disease. We hypothesized that neonatal HDL-S1P (nHDL) complex limits vascular inflammation at the feto-placental endothelium thereby attenuating endothelial dysfunction in PE. In addition, since the concentration and composition of cord blood lipids and lipoproteins are strongly dependent on metabolic conditions of the mother, we assume that composition of nHDL is altered in PE and thus unable to exert its beneficial properties on barrier function and vascular tone. S1P levels within the endothelium are dynamically regulated by serine palmitoyiltransferase (SPT), which is the rate-limiting enzyme of the de novo pathway of sphingolipid synthesis. The inhibition of SPT activity by the endoplasmic reticulum protein Nogo-B or Myriocin (antibiotic ISP-1) has been related to vascular dysfunction and hypertension (19), which are considered major players of adverse outcomes in PE. We therefore hypothesized that the regulation of the de novo S1P biosynthesis is altered in preeclampsia. That is why we believe in these conditions the endothelium-derived S1P does not affect vascular tone and barrier integrity anymore.

2 *MATERIALS AND METHODS*

2.1 Isolation of cord blood-derived neonatal HDL

Mixed (arterial and venous) umbilical cord blood was collected from 10 male and 10 female offspring. EDTA-plasma was isolated by centrifuging the samples for 15 minutes at 200 x g and 4 °C. The density of the plasma was adjusted to 1.24 g/ml using potassium bromide (Merck KGaA, Darmstadt, Germany). Subsequently 1.7 ml of adjusted plasma was pipetted into quick seal polyallomer tubes (Beckman Coulter, CA, USA) and the plasma was overlaid with potassium bromide solution with a density of 1.006 g/ml. The plasma was centrifuged at 100,000 rpm for 3 hours in an ultracentrifuge (Optima XE-90; Beckman Coulter, CA, USA) using a 70 Ti rotor. After centrifugation the nHDL fraction was isolated from the plastic tubes using a syringe. In order to locate the nHDL fraction a reference tube was used where the nHDL fraction was stained with DiI-dye (1-1´dioctadecyl-3-3-3´-tetramethyl indocarbocyanine perchlorate). Afterwards nHDL was desalted by gel filtration using PD10 columns (GE healthcare, Little Chalfont, UK) filled with Sephadex G-25 Medium. First the column was equilibrated with PBS (Medicago, Uppsala, Sweden) and then the isolated nHDL was filtered through the column for desalting. The desalted, purified nHDL was pooled and stored at -20°C until usage. nHDL was characterized by measuring ApoA-1, cholesterol and S1P content. ApoA-1 and cholesterol concentrations were measured spectrophotometrically with an AU680 Chemistry Analyzer (Beckman Coulter, CA, USA) according to manufacturer's instructions. S1P concentrations were determined by HPLC according to a protocol described elsewhere [97]. Briefly, nHDL samples were spiked with 25ng of an internal standard (D-erythro-sphingosine-1-phosphate) and S1P was extracted by a two-step chloroform-methanol extraction. After derivatization with 2,3-naphthalene-dicarboxaldehyde samples were measured on a Synergi 4u Fusion-RP 80A column (30 x 2.0 mm) using an Agilent 1290 HPLC (Agilent Technologies, Santa Clara, USA).

2.2 Isolation of primary human placental arterial endothelial cells (HPAEC)

Term placentae were collected after delivery with informed consent and approval of the ethical committee of the Medical University of Graz (Vote no.: 29-319 ex 16/17). HPAECs were isolated from arterial chorionic blood vessels as firstly described by Lang et al [13]. The amnion was removed and the chorionic plate was disinfected with Betaisodona solution (Mundipharma, Vienna, Austria). Next arterial chorionic vessels with a length of ~ 3 cm were resected and washed in Hank's balanced salt solution (HBSS; Gibco, Thermo Fisher Scientific, Carlsbad, USA). Then the artery was

cannulated and flushed with HBSS containing 0.1 U/ml Collagenase (Roche, Basel, Switzerland), 0.8 U/ml Dispase (Roche, Basel, Switzerland) and 200µg/ml Penicillin/Streptomycin (GE Healthcare, Little Chalfont; UK) for 6 minutes (flow rate: 2.5 ml/min). Detached ECs were collected in 10 ml fetal calf serum (HyClone, GE Healthcare, Little Chalfont; UK) and centrifuged at 900 rpm for 7 minutes. The cell pellet was resuspended in 1 ml endothelial microvascular basal medium (EBM; PAN Biotech, Aidenbach, Germany) containing EGM-MV Single Quots (Lonza, Basel, Switzerland) and 10 % pooled human serum from pregnant women. Cells were seeded into a well of a 12 well plate (Thermo Fisher Scientific, Carlsbad, USA) coated with 1 % gelatine (Thermo Fisher Scientific, Carlsbad, USA) for expansion. As soon as cells proliferated and reached a certain number, they were transferred into a 25 cm2 flask (NuncTM; Thermo Fisher Scientific, Carlsbad, USA) and the pregnant serum was substituted by 5 % FBS for further cultivation. Once HPAECs reached confluency in a 75 cm2 flask (NuncTM; Thermo Fisher Scientific, Carlsbad, USA) they were harvested and frozen in liquid nitrogen for long term storage. HPAECs were always cultured at 12 % O2, 5 % CO2, 90 % humidity and a temperature of 37 °C. ECs were subjected to immunocytochemical characterization.

2.3 Quantitative real-time PCR (qPCR) of HPAEC and placental vessels

Cells were thawed, expanded and seeded in 12 well plates coated with 1 % gelatine at a density of 100,000 cells/well. After 2 days medium was changed to serum free EBM and cells were treated with 10 ng/ml TNF-α only or with 10 ng/ml TNF-α in the presence of 800 µg/ml nHDL or 1 µM HSA-S1P (Avanti Polar Lipids, Alabama, USA) for 6 hours. Treatment was performed in triplicates. After treatment cells were washed twice in prewarmed HBSS and harvested in 350 µl RLT Lysis buffer (Quiagen, Hilden, Germany) supplemented with 1 % β-mercaptoethanol (Sigma Aldrich, St. Louis, USA). Next total RNA content was isolated using the RNeasy® Mini Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions. Therefore, the cell lysates were homogenized by vortexing them for 1 minute. Cells were then eluted from the spin column membrane in 30 µl of DEPC treated water (Ambion®; Thermo Fisher Scientific, Carlsbad, USA) for 5 minutes. As RNA yields were low the eluate was again pipetted onto the spin column membrane and eluted a second time to increase RNA concentration. RNA concentration was determined by measuring absorbance at 260 nm using a microfluidic UV/VIS spectrophotometer (QIAxpert; Quiagen, Hilden, Germany). 20 mg of snap frozen placental arteries and veins tissue was used for RNA isolation. The tissue samples were placed in a MagNA Lyser tube (Roche, Basel, Switzerland) and 600 µl of RLT Lysis

buffer supplemented with 1 % β-mercaptoethanol was added to each tube. Tissue samples were homogenized using a MagNA Lyser Instrument (Roche, Basel, Switzerland) set to 6,500 rpm for 20 seconds, followed by a cooling step for 1 minute. This procedure was repeated three times. Afterwards the samples were centrifuged for 3 minutes at full speed. The supernatant was transferred into a new tube and used for further processing. After homogenization, RNA was isolated using the RNeasy® Mini Kit as described in 2.4.1 with the slight difference that the second elution step was skipped due to enough RNA yields. 250 ng of total RNA of HPAECs were reverse transcribed by using random hexamer primers (200µg/reaction; Thermo Fisher Scientific, Carlsbad, USA) and Super ScriptTM II Reverese Transcriptase (200 units/reaction; InvitrogenTM, Thermo Fisher Scientific, Carlsbad, USA) in 20 µl reaction volume. cDNA synthesis was carried out according to manufacturer's instruction for SuperScriptTM II Reverse Transcriptase. RNAse inhibitor (40 units/reaction; RNaseOUTTM, Thermo Fisher Scientific, Carlsbad, USA) was included during reverse transcription to prevent RNA degradation. cDNA synthesis from placental tissue RNA was performed as described for RNA from HPAECs with the difference that 2 µg of total RNA was used and that the reaction volume was 40 µl. Therefore, the amount of all reaction components was doubled. Quantitative real-time PCR was performed on the CFX384 cycler (BioRad Technologies, Vienna, Austria) using TaqMan® Gene Expression assays (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA). Used TaqMan® Gene Expression assays are listed in Table 2. The efficiency of all gene expression assays was determined by a five-point standard curve (Range: 12.5 ng/µl – 0.02 ng/µl cDNA). The cDNA used for the standard curve was extracted from HPAECs grown in 60 mm dishes as described in section 2.4.1 and 2.4.3. qPCR efficiency was calculated using the formula E=〖10〗^(1/-Slope). The slope was derived from a graph where the Cq values were plotted against the logarithmic cDNA concentrations. The slope of the standard curve should lie between -3.2 and -3.5 and the reproducibility of replicates (R2) should be > 0.980. According to the manufacturer the efficiency of all TaqMan gene assays used should be in the range of 90 % – 110 %. qPCR was performed in 10 µl reactions. All samples were analyzed in triplicates and cycling conditions are shown in Table 4. For each PCR-run a non-template control and a no reverse transcription control were included. The quantification cycle (Cq) was determined by using a multi-variable, non-linear regression model implemented in the CFX Manager 3.1 Software (BioRad Technologies, Vienna, Austria). Subsequently Cq values were used to calculate the relative gene expression by applying the ΔΔCq method.

2.4 Immunoblot analysis

Cells were thawed, expanded and seeded at a density of 200,000 cells/well in 6 well plates (Thermo Fisher Scientific, Carlsbad, USA) coated with 1 % gelatine. After 2 days medium was changed to serum free EBM and cells were treated with 10 ng/ml TNF-α only (Sigma Aldrich, St. Louis, USA) or with 10 ng/ml TNF-α in the presence of 800 µg/ml nHDL or 1 µM HSA-S1P for 6 hours. To perform Western blotting, medium was removed, and cells were washed twice in HBSS. Afterwards cells were scraped and collected in 50 µl RIPA lysis and extraction buffer (Sigma Aldrich, St. Louis, USA) containing protease inhibitors (Roche, Basel, Switzerland). Total protein concentration was determined by bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Carlsbad, USA) according to manufacturer's guidelines. Protein lysates were mixed 1:1 with 2 x Laemmli buffer (Sigma Aldrich, St. Louis, USA) and denatured at 96°C for 5min. 10 µg of total protein were loaded onto 4 - 20% SDS-PAGE gradient gels (BioRad Technologies, Vienna, Austria) and resolved at 120 V for 1h 10 min. Proteins were transferred to a nitrocellulose membrane (BioRad Technologies, Vienna, Austria) using the TransBlot Turbo Transfer System (BioRad Technologies, Vienna, Austria). Nonspecific binding sites were blocked for 1h with 5 % non-fat dry milk (BioRad Technologies, Vienna, Austria) in Tris-buffered saline (TBS; Gatt-Koller, Absam, Austria) + 0.1% Tween 20 (Sigma Aldrich, St. Louis, USA). Thereafter, membranes were incubated with the appropriate primary antibody overnight at 4°C. Subsequently, the membranes were washed for 30 minutes in TBS Buffer + 0.1 % Tween 20 and incubated for 1 hour with the appropriate horseradish peroxidase conjugated secondary antibody. After another wash for 30 minutes the blots were developed for 5 minutes using SuperSignal® Chemiluminescent Substrate (Thermo Fisher Scientific, Carlsbad, USA). Immunolabeling was visualized with the Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France) and band densiometry was performed using the Fusion© Software (Vilber Lourmat, Marne-la-Vallée, France). β-Actin or Hsp90 was used as reference protein. All antibodies were diluted in 5 % non-fat dry milk (BioRad Technologies, Vienna, Austria).

2.5 Reactive oxygen species (ROS) Assay

20,000 cells/well were seeded in a dark wall, clear bottom 96-well microplate (Costar®; Corning Inc., New York, USA) coated with 1 % gelatine. The next day DCFDA (Abcam, Cambrige, UK) was diluted to a final concentration of 10 µM in HBSS. Afterwards cells were washed once in pre-warmed HBSS. Then 100 µl DCFDA solution was added to each well and cells were stained for 45 minutes at 37 °C in the dark. After staining cells were washed again with pre-warmed HBSS. Subsequently

cells were treated with 0.5 and 1 µM AngII (Sigma Aldrich, St. Louis, USA) only or with 0.5 and 1 µM AngII in the presence of 800 µg/ml nHDL or 1 µM HSA-S1P for 4 hours. As a positive control, cells were treated with 200 µM tert-butyl hydrogen peroxide (TBHP; Abcam, Cambrige, UK) for 4 hours. All treatment compounds were diluted in phenol red free DMEM (Gibco, Thermo Fisher Scientific, Carlsbad, USA) without supplements. After treatment of the cells, fluorescent intensity of oxidized dichlorofluorescein (DCF) was measured immediately at a fluorescence plate reader (FLUOstar Optima; BMG Labtech, Offenburg, Germany) at Ex/Em = 485/535.

2.6 Serine Palmitoyl Transferase (SPT) activity assay

HPAEC were thawed, expanded and seeded in 60 mm dishes (Eppendorf AG, Hamburg, Germany) coated with 1 % gelatine at a density of 600,000 cells/dish. After 2 days medium was changed to serum free EBM and cells were treated either with 10 ng/ml TNF-α, 50 ng/ml TNF-α, 1 µM AngII or 5 µM AngII for 24 hours. Afterwards cells were washed once in pre-warmed HBSS. Then 200 µl of SPT reaction buffer were added to each dish. Subsequently cells were scraped, and the lysates collected on ice. SPT reaction buffer is composed of 0.1 M Hepes pH 8.3 (Sigma Aldrich, St. Louis, USA), 5 mM dithiothreitol (InvitrogenTM, Thermo Fisher Scientific, Carlsbad, USA), 2.5 mM EDTA pH 7.4 and 50 µM Pyridoxal 5' Phosphate (Sigma Aldrich, St. Louis, USA). Cell lysates were sonicated 2 x for 10 seconds (50 % amplitude, 50 % pulsation) using an ultrasonic processor (UP100H; Hielscher, Teltow, Germany). For normalization purposes, total protein content of the cell lysates was measured on an UV/VIS spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Carlsbad, USA) according to manufacturer's instructions. Thereafter 100 µl of cell lysate were mixed with 1 µl palmitoyl coenzyme A (0.2 mM; Sigma Aldrich, St. Louis, USA) and 10 µl [3H]Serine (33.3 mM, 1mCi/ml; Perkin Elmer, Boston, USA). SPT reaction was carried out in a heating block for 15 minutes at 37 °C. In order to stop the reaction 50 µl $NaBH_4$ (5 mg/ml; Sigma Aldrich, St. Louis, USA) were added to each tube for 5 minutes. Additionally $NaBH_4$ reduces the formed [3H]3-ketosphinganine to [3H]sphinganine. Next lipids were extracted by first adding 750 µl chloroform (Merck, Darmstadt, Germany) and methanol (Sigma Aldrich, St. Louis, USA) (ratio 1:2), followed by 250 µl chloroform and 250 µl NH_4OH (Merck, Darmstadt, Germany). After careful vortexing, tubes were spun down for 10 minutes at 12,000 rpm. Subsequently, the lower phase was transferred into a new tube. The tube was left open overnight under a fume hood to remove the organic solvent. The next day a thin layer chromatography (TLC) plate (Merck, Darmstadt, Germany) was dried for 10 minutes at 80 °C and a TLC tank (Camag, Mississippi, USA) was filled with TLC running buffer

(CHCl3 : Methanol : NH4OH in ration 65:25:2) at least 30 minutes before running the TLC plate. Afterwards the dried samples were resuspended in 40 µl CHCl3 and loaded onto the TLC plate drop by drop (1µl) at a minimum distance of 1.5 cm. Sphinganine (Avanti Polar Lipids, Alabama, USA) and C18:0 ceramide (Avanti Polar Lipids, Alabama, USA) were included as standards. The TLC plate ran for 1 hour 30 minutes and dried for 1 hour under the fume hood. To visualize the separated lipids, the TLC plate was placed for 1 hour in a chamber which was saturated with iodine vapour. Spots containing the [3H]Sphinganine fraction of the samples were identified with the help of the sphinganine standard and marked with a pencil. To allow the Iodine to evaporate from the TLC plate, it was placed under the fume hood overnight. The following day the marked sphinganine spots were cut out and placed in a scintillation vial containing 20 ml scintillation cocktail (Ultima GoldTM, Perkin Elmer, Boston, USA). The scintillation vials were placed at 4 °C overnight to reduce unspecific signals and then [3H] Sphinganine was detected using a β-Counter (Tri-Carb 2800TR, Perkin Elmer, Boston, USA). Each sample was counted for 5 minutes and resulting CPM values were quantified using a standard curve of [3H] Serine. Finally, the calculated values were normalized to the total protein content.

2.7 Sphingolipid analysis by LC-MS/MS

Placental arteries homogenates from normotensive and preeclamptic patients were used for quantification of sphingolipids by LC-MS/MS. The levels of ceramide (Cer) species, sphingosine (Sph) and S1P were analyzed by the Lipidomics Analytical Core at the Medical University of South Carolina.

2.8 Placental arteries immunostaining

Isolated the vessels were incubated in calcium-free Krebs for at least 30 minutes to allow vessel vasodilation. Then the arteries were fixed with 4% PFA and left overnight at 4 °C. PFA-fixed arteries were OCT-embedded. For immunofluorescence, frozen placental artery sections were stained for Nogo-B (1:200, R&D), S1PR1 (1:200, R&D), CD31(1:200, Invitrogen) and αSMA (1:200, BDbioscience) overnight at 4 °C, and were then stained with Cy5-labeled anti-goat antibody (#A21436, Invitrogen, 1:500) Alexa 488 anti-rabbit (#016-540-084, Jackson ImmunoResearch, 1:200) and Alexa 568 anti-mouse in PBS for 1 h. Nuclei were stained with DAPI. Confocal

immunofluorescence images of the tissues were captured on an Olympus Fluoview confocal microscope.

2.9 Statistical analysis

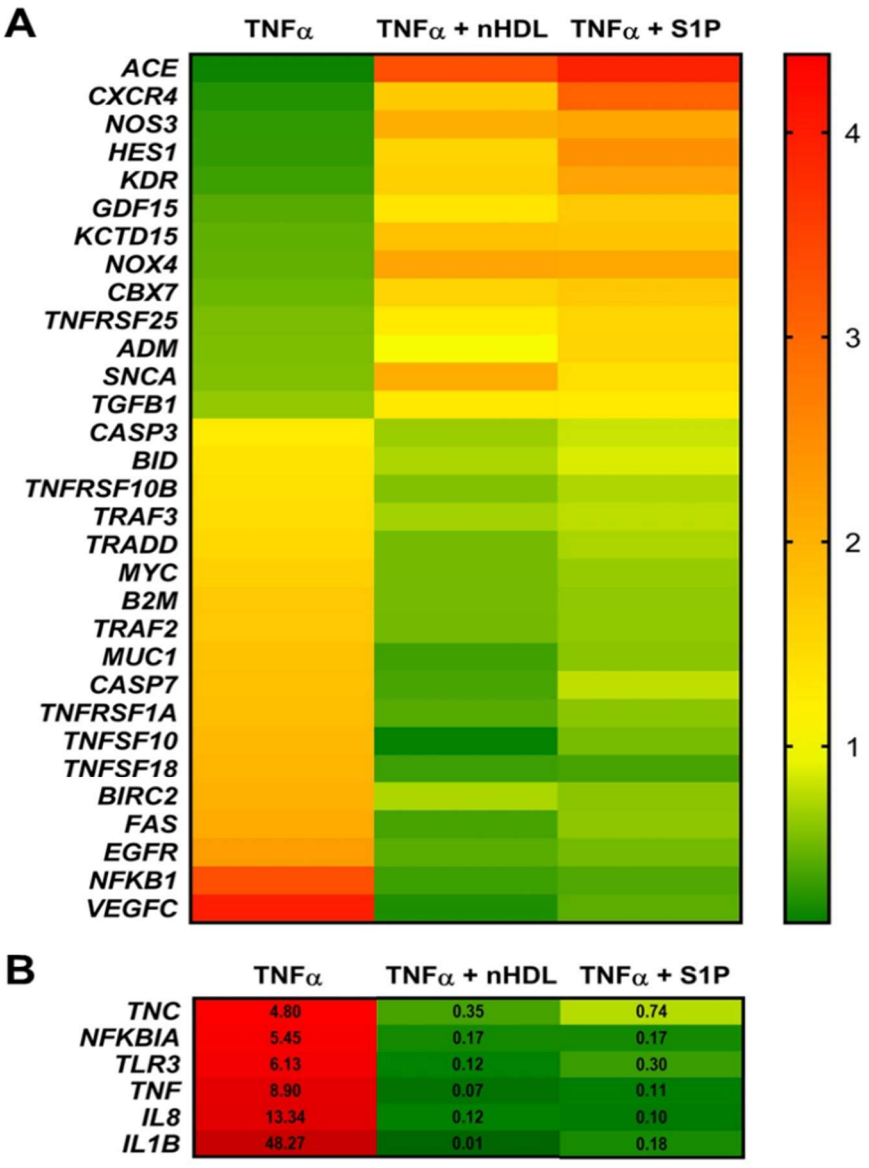
All statistical analyses were performed using Graph Pad Prism 7 Software (GraphPad Software Inc., San Diego, USA). Values are presented as Mean ± SEM unless stated otherwise in the figure legend. For PrimePCR data differences in experimental groups were evaluated by multiple Students' t-test using the Holm-Sidack method to correct for multiple comparison. The ROS assay and remaining qPCR data were analyzed by one-way ANOVA including Tukey post-hoc analysis for multiple comparison. $p < 0.05$ was considered significant.

3 RESULTS

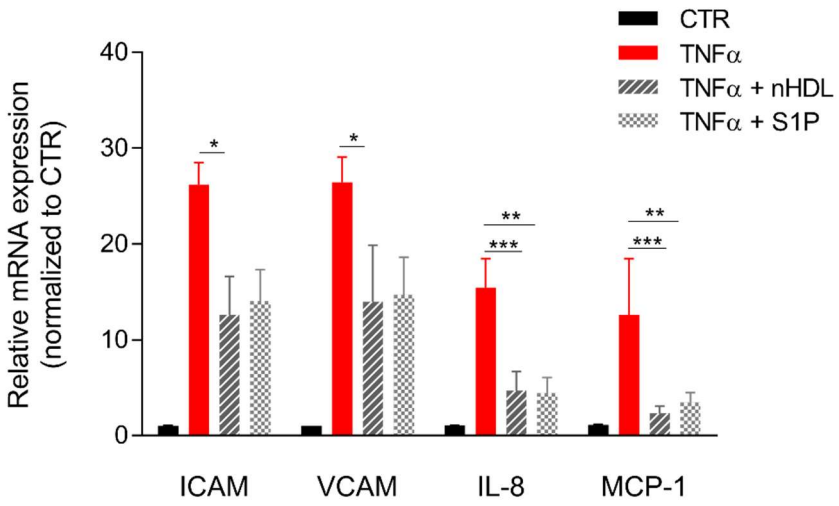
3.1 nHDL-S1P lowers the HPAECs inflammatory response by NF-κB signaling inhibition.

We investigated whether nHDL-S1P complex is capable of counteracting effects of TNFα-induced inflammatory response on the endothelium. TNF-α induces the expression of genes belonging to the TNF superfamily like TNFSF10 and TNFSF18 and the TNF receptor superfamily including TNFRSF1A and TNFRSF10 whereas the expression of TNFRSF25 was downregulated. TNFSF10 and TNFRSF10B are members of the TRAIL pathway which induces TNF-α mediated apoptosis [116]. Apart from these two genes CASP3, CASP7, FAS, MYC and BID, which are also associated with apoptosis, were slightly upregulated. As TNF-α exerts its pro-inflammatory effects through the NF-κB signaling pathway it is not surprising that genes like TRADD, TRAF2, TRAF3, BRIC2, NFKB1 and NFKBIA are upregulated upon TNF-α stimulation. Although NFKBIA is a negative regulator of NF-κB signaling upregulation of this gene is part of a negative feedback regulatory mechanism for terminating TNF-α induced NF-κB response [117]. The most pronounced increase in gene expression upon TNF-α treatment could be observed for the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α itself which was to be expected as TNF-α is potent inducer of inflammation. nHDL as well as S1P successfully counteracted the effects of TNF-α on mRNA expression of all inflammation related genes shown (Figure 1A-B). To further clarify the role of nHDL-S1P in TNF-α induced inflammation, the influence on mRNA expression of the inflammatory mediators ICAM1, VCAM1, IL-8 and MCP1 in HPAECs was elucidated (Figure 1C). ICAM1 and VCAM1 are intracellular adhesion molecules which control the firm adhesion of leukocytes to the endothelium and therefore are important mediators of inflammation in ECs [118]. IL-8 and MCP1 act as chemotactic cytokines during inflammation for neutrophils [119] and monocytes [120] respectively. mRNA expression of all these genes was increased upon TNF-α stimulation in HPAECs. The most pronounced effect could be observed for VCAM1 with a nearly 30-fold increase of mRNA followed by ICAM1 (28 fold), IL-8 (21 fold) and MCP1 (12 fold). When HPAECs were treated with TNF-α in the presence of 800µg/ml nHDL this inflammatory response was significantly attenuated. Expression of MCP1 dropped by 80 % followed by IL-8 by ~ 65 % compared to controls. The expression of the adhesion molecules ICAM1 and VCAM1 was also reduced by ~50 % and ~ 40 % respectively. Co-incubation of HPAECs with TNF-α and S1P reduces the inflammatory response although the effects were less pronounced suggesting there is a carrier specificity for the beneficial effect of the lipid. It is well known that TNF-α signals by the NF-κB pathway to promote the inflammatory response. NF-κB controls the general pro-inflammatory response on ECs by controlling the expression of adhesions molecules and cytokine production e.g., IL-8 and MCP1 [121–124]. To

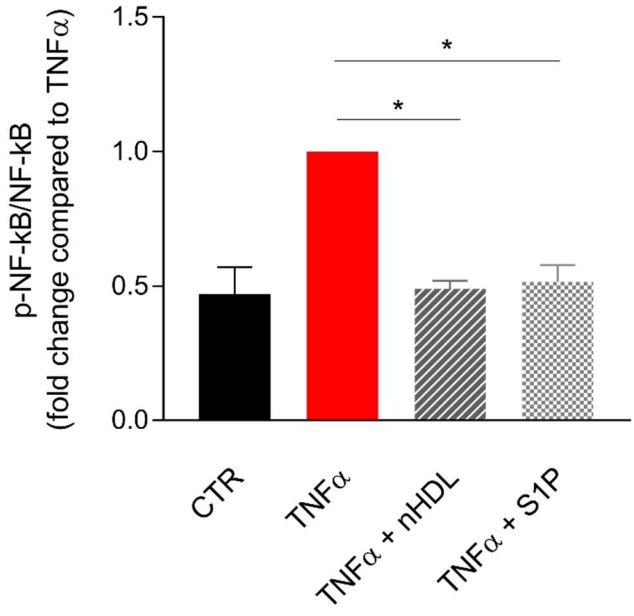
check whether nHDL and S1P are able to influence this signaling pathway, the phosphorylation of NF-κB subunit p65 at Ser536 was examined. This phosphorylation leads to enhanced transactivation of NF-κB corroborated by increased expression of regulated genes [125]. TNF-α induces the phosphorylation of p65 at Ser536. In the presence of nHDL the phosphorylation of p65 at Ser536 dropped by 50% to basal levels as seen under control conditions (Figure 1D). Similar suppression of p-NF-κB was obtained upon TNF-α and S1P treatment of HPAECs. The alleviative effect of nHDL and S1P on the transactivation of the NF-κB pathway was already observed after 2 hours (data not shown) and persisted at least for 6 hours after induction of inflammation with TNF-α. These findings implicate that nHDL and S1P exert their anti-inflammatory actions in HPAECs by inhibiting NF-κB signaling and correlate with observed reduction of mRNA expression for selected inflammatory markers. Furthermore, 15 of the 24 genes in Figure 1A that were found to be upregulated upon TNF-α stimulation are also regulated by NF-κB possibly explaining how nHDL and S1P affect their transcription.


A
TNFα
TNFα + nHDL
TNFα + S1P
ACE
CXCR4
NOS3
HES1
KDR
GDF15
KCTD15
NOX4
CBX7
TNFRSF25
ADM
SNCA
TGFB1
CASP3
BID
TNFRSF10B
TRAF3
TRADD
MYC
B2M
TRAF2
MUC1
CASP7
TNFRSF1A
TNFSF10
TNFSF18
BIRC2
FAS
EGFR
NFKB1
VEGFC
4
3
2
1
B
TNFα
TNFα + nHDL
TNFα + S1P
TNC 4.80 0.35 0.74
NFKBIA 5.45 0.17 0.17
TLR3 6.13 0.12 0.30
TNF 8.90 0.07 0.11
IL8 13.34 0.12 0.10
IL1B 48.27 0.01 0.18

C


CTR
TNFα
TNFα + nHDL
TNFα + S1P
Relative mRNA expression (normalized to CTR)
40
30
20
10
0
*
*
**
***
**
***
ICAM
VCAM
IL-8
MCP-1

D


p-NF-kB/NF-kB (fold change compared to TNFα)
1.5
1.0
0.5
0.0
*
*
CTR
TNFα
TNFα + nHDL
TNFα + S1P

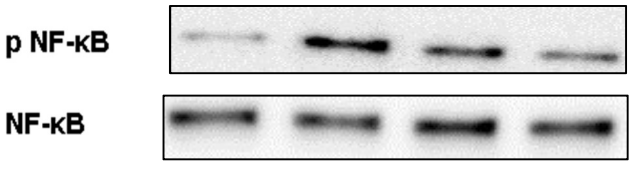

p NF-κB
NF-κB

Figure 1. nHDL-S1P complex limits vascular inflammation at the feto-placental endothelium. HPAECs were seeded at a density of 100,000 cells/well in 12 well plates and treated with 10 ng/ml TNF-α only or with TNF-α in the presence of 800 µg/ml nHDL or 1 µM HSA-S1P for 6 h. (A) After total RNA isolation and reverse transcription, expression of 128 genes related to preeclampsia and TNF-induced apoptosis was examined. Genes which are significantly regulated as determined by multiple t-testing are depicted in the heatmap. The heatmap shows the fold change mRNA expression. (B) Genes where the fold change values exceed the scale shown in A are depicted here. The numbers represent the respective fold change values. (C) Total RNA was reverse transcribed and analyzed by RT-PCR using TaqMan probes. Differences in mRNA expression are calculated using the ΔΔ-Cq method. Data are presented as fold change (mean ± SEM; n = 4) compared to control condition. * = $p<0.05$; ** = $p<0.01$; *** = $p < 0.001$. (D) The phosphorylation state of NF-kB at Ser536 was examined by Western blot analysis in whole cell lysates. After protein separation and blotting, membranes were incubated overnight at 4 °C with the primary antibody. Data were normalized to TNF-α and are shown as fold change (mean ± SEM; n = 3). * $p < 0.05$.

3.2 nHDL-S1P protects HPAECs from AngII-induced oxidative stress

Increases production of ROS plays a crucial role in the progression of endothelial dysfunction and hypertension [36]. A major source of ROS in endothelial cells is the NADPH oxidase complex whose activity is stimulated by AngII [128]. In this set of experiments, we investigated whether nHDL-associated S1P is capable to influence AngII-induced ROS formation in HPAECs. Treatment of HPAECs with AngII increased intracellular ROS production by ~ 60 % (0.5 µM AngII) and ~ 85 % (1 µM AngII) compared to control conditions. In the presence of nHDL ROS production dropped by 75 % (0.5 µM AngII) and 80 % (1 µM AngII) when compared to ROS levels caused by AngII. nHDL works as effective inhibitor of oxidative stress because ROS production (Figure 2A). S1P conjugated with albumin is less effective in preventing ROS production in HPAECs under hypertensive conditions. Immunoblot analysis also suggest an effect of nHDL and S1P on the protein expression of NADPH oxidase 1 (NOX1) in HPAECs. As shown in Figure 2B AngII treatment markedly increased NOX1 levels whereas nHDL and S1P reduced the induction of NOX1 expression upon AngII treatment.

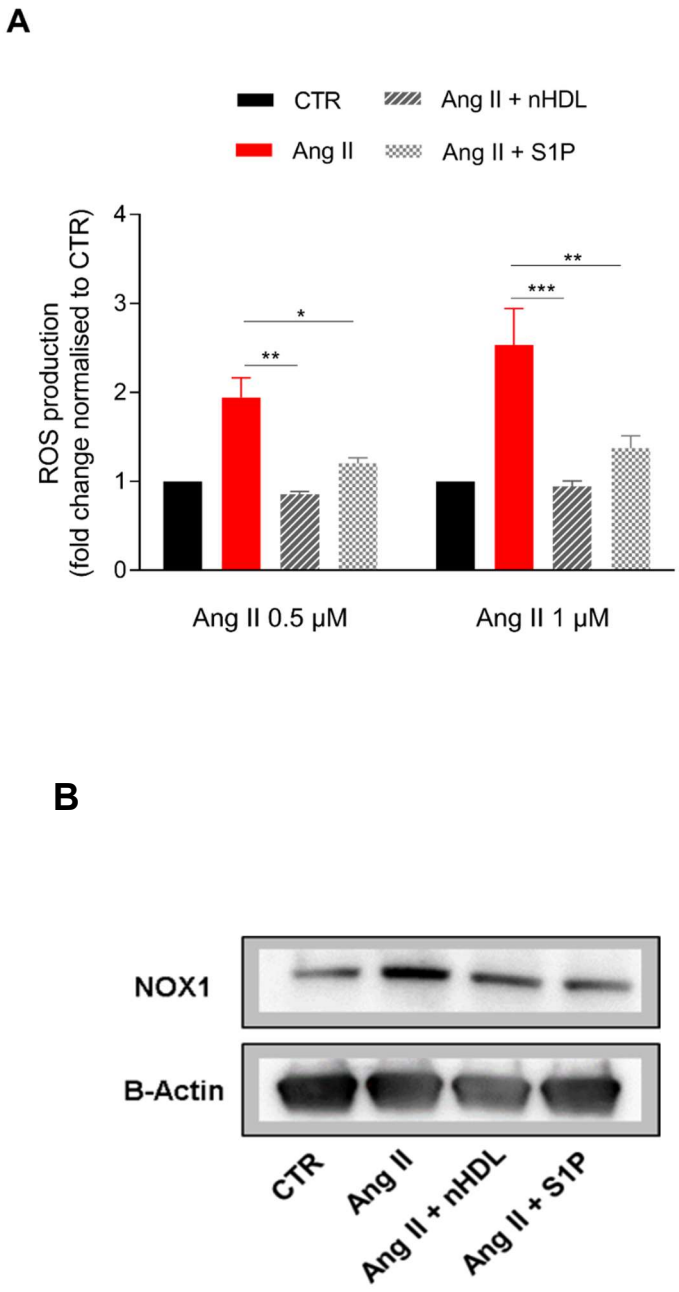


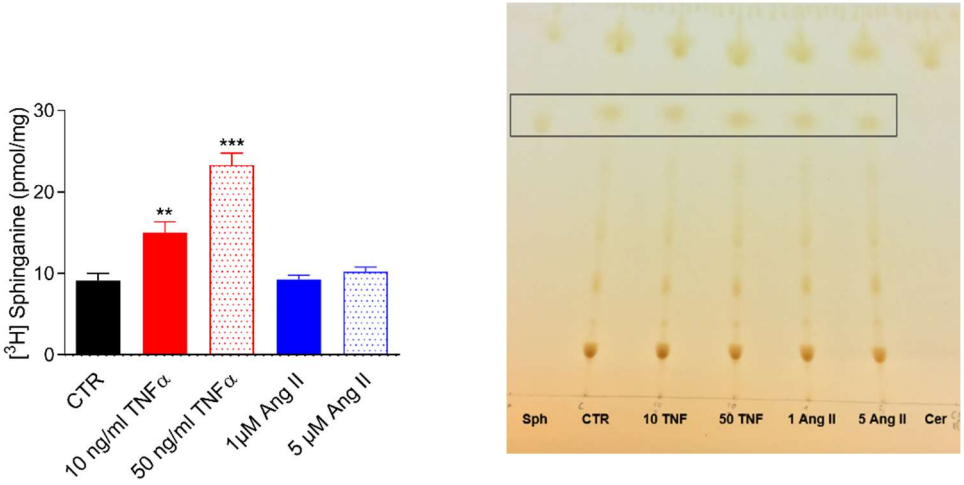
Figure 2. nHDL-S1P prevents the AngII-induced ROS generation in HPAECs. (A) HPAECs were seeded at a density of 20,000 cells in a 96 well plate and incubated with Dichlorofluorescin diacetate (DCFDA) for 45 minutes. After one washing step HPAECs were treated with 0.5 and 1 µM Angiotensin II only or with 0.5 and 1 µM AngII in the presence of 800 µg/ml nHDL or 1 µM HSA-S1P for 4 hours. Intracellular oxidation of DCFDA into dichlorofluorescein was detected by fluorescence spectroscopy (Ex/Em: 295/529). Tert-butyl hydrogen peroxide (200µM) was used as a positive control. The bar chart shows the generation of ROS as fold change (mean ± SEM; n = 3) compared to control. * $p < 0.05$; ** $p < 0.01$ (B) HPAECs were seeded at a density of 200,000 cells/well in 6 well plates and treated with 1 µM AngII only or with AngII in the presence of 800 µg/ml nHDL or 1 µM S1P for 6 h. Protein expression of NOX1 was analyzed by Western blot in whole cell lysates. After protein separation and blotting, membranes were incubated overnight at 4 °C with the primary antibody. β-Actin served as internal loading control.

3.3 Preeclampsia alters sphingolipid metabolism

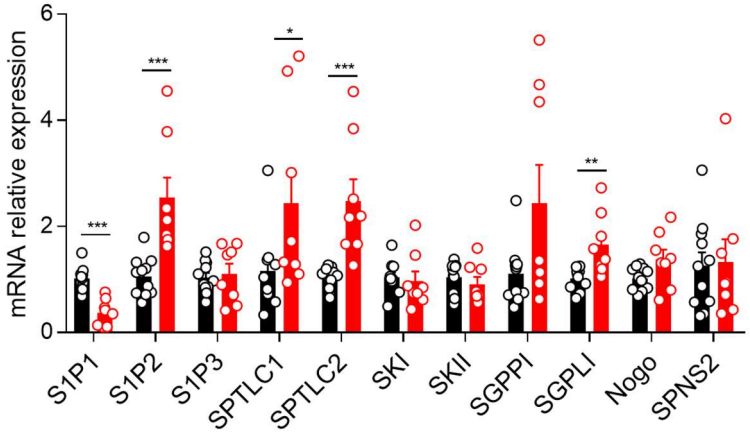
It has been shown that cultured endothelial cells which undergo through a pro-inflammatory challenge, exhibit increase sphingolipids biosynthesis to counteract the endothelial inflammatory response. Along with the previous findings, we demonstrate that the STP, which is the first and rate-limiting enzyme of the sphingolipid biosynthesis, is significantly activated by TNF-α stimulation, in HPAECs (Figure 3A).

Sphingolipids metabolism plays a role in cardiovascular disease and metabolic disorders. To test if also preeclampsia affects SL metabolism in pregnancy, placental chorionic arterial vessels were isolated and analyzed for SL content and mRNA expression of the crucial enzymes involved in the pathway. When comparing the mRNA expression of S1P receptors in vessels there is a lower expression of S1P1 preeclamptic vessels in favor of and increased expression of S1P2. Moreover SPTLC, SGPPI and SGPLI are increased in preeclampsia. This evidence suggests that the SL metabolism in PE is likely associated with S1P degradation. To better understand role of SL metabolism in PE we determined the ceramides and sphingomyelin (SM) profile of control and PE placental arteries. LC-MS/MS analysis show a decrease content of C20-Cer in PE tissue compared to control. Conversely, the dihydrosphingosine (dhSph) levels are increased in placental arteries of preeclamptic women (Figure 3C). However, with respect to SM the data show an overall higher content in preeclamptic tissue compared control with a significant difference in the levels of C16-SM, C18-SM and C24-SM (Figure 3D). In agreement with the hypothesis of an impaired S1P-S1PR1 signaling in PE, our preliminary immunostaining data show a decreased expression of S1PR1 in the endothelium of preeclamptic arteries (Figure 3E).

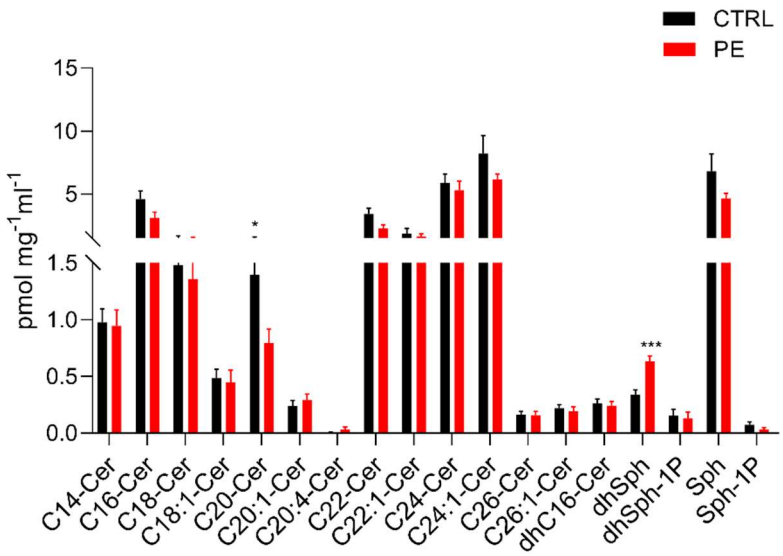
A


[3H] Sphinganine (pmol/mg)
30
20
10
0
**
***
CTR
10 ng/ml TNFα
50 ng/ml TNFα
1μM Ang II
5 μM Ang II
Sph
CTR
10 TNF
50 TNF
1 Ang II
5 Ang II
Cer

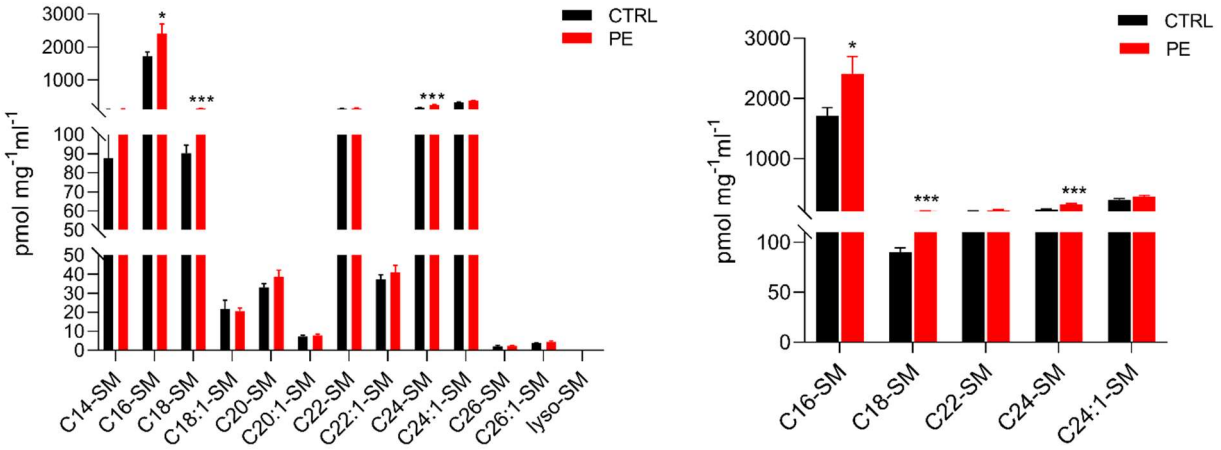
B


mRNA relative expression
6
4
2
0
***
***
*
***
**
S1P1
S1P2
S1P3
SPTLC1
SPTLC2
SKI
SKII
SGPPI
SGPLI
Nogo
SPNS2

C


CTRL
PE
pmol mg-1ml-1
15
10
5
1.5
1.0
0.5
0.0
*
***
C14-Cer
C16-Cer
C18-Cer
C18:1-Cer
C20-Cer
C20:1-Cer
C20:4-Cer
C22-Cer
C22:1-Cer
C24-Cer
C24:1-Cer
C26-Cer
C26:1-Cer
dhC16-Cer
dhSph
dhSph-1P
Sph
Sph-1P

D


CTRL
PE
pmol mg-1ml-1
3000
2000
1000
100
90
80
70
60
50
50
40
30
20
10
0
*
***
***
C14-SM
C16-SM
C18-SM
C18:1-SM
C20-SM
C20:1-SM
C22-SM
C22:1-SM
C24-SM
C24:1-SM
C26-SM
C26:1-SM
lyso-SM
CTRL
PE
pmol mg-1ml-1
3000
2000
1000
100
50
0
*
***
***
C16-SM
C18-SM
C22-SM
C24-SM
C24:1-SM

E

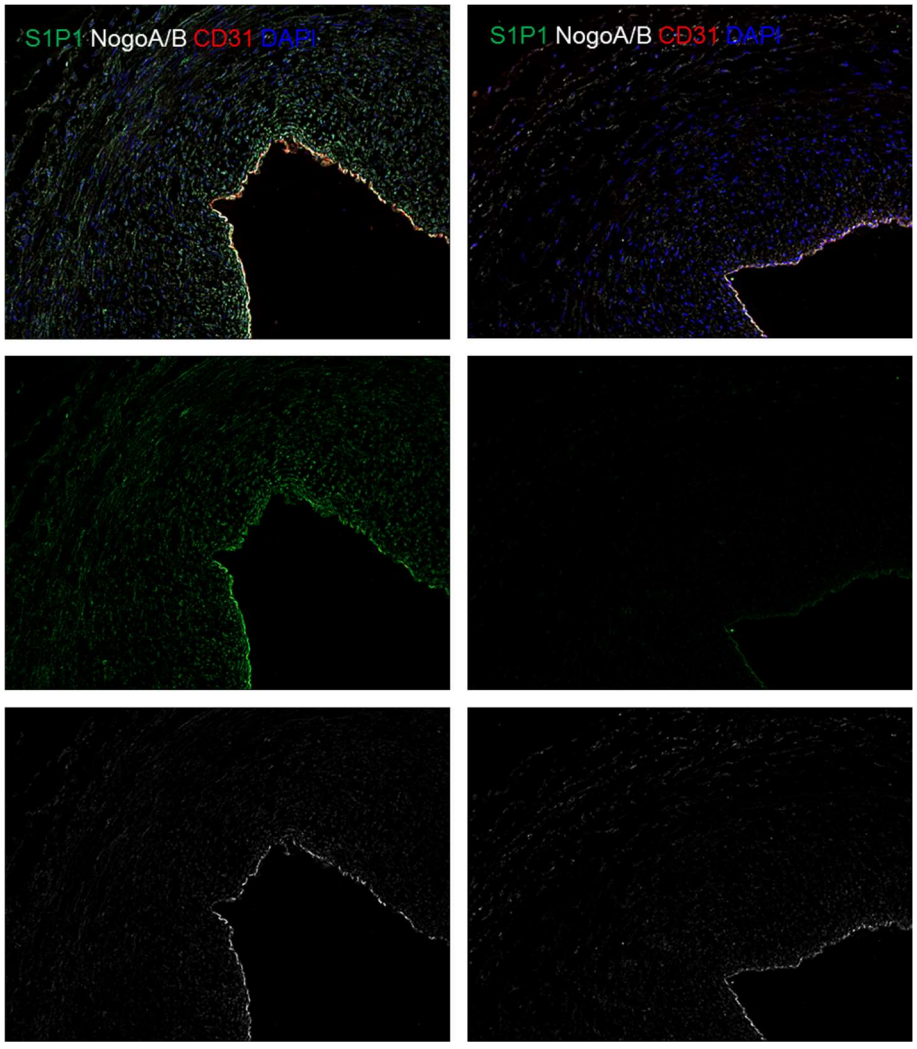

S1P1 NogoA/B CD31 DAPI
S1P1 NogoA/B CD31 DAPI

Figure 3. Sphyngolipid metabolism is dysregulated in preeclampsia. (A) SPT activity was evaluated in HPAEC by measuring [^{3}H] Sphinganine radioactivity. For this purpose, sphinganine band was cut out and analyzed by a β-counter. Reading values were quantified using a standard curve of [^{3}H] Serine. Bar chart shows the results of 4 independent experiments as mean values ± SEM. Placental chorionic arteries from healthy (n=10) and preeclamptic (n=8) subjects, were snap frozen after isolation and processed for mRNA analysis by qRT-PCR (B) and for ceramides (C) or sphingomyelin (D) quantification by LC/MS/MS. (E) Immunofluorescent staining of S1PR1 (green), CD31(red) in control and PE arteries. The nuclei were counterstained with DAPI (blue).

4 DISCUSSION

Preeclamptic pregnancies are characterized by maternal hypertension and systemic inflammation. These features are strongly depended on the functionality of the endothelium. The bioactive mediator S1P is known to have a major impact on the endothelium physiology. Therefore, the understanding of the role of sphingosine-1-phosphate (S1P) on the placental endothelium, in preeclamptic conditions, might shed a light on the pathophysiology of the disease.

Several studies have been shown that S1P is involved in the regulation of endothelial inflammation (62–65). Our results highlight the ability of the neonatal HDL (nHDL) and S1P to suppress the inflammatory response of the endothelium by reducing the mRNA expression of inflammatory markers. Additionally, we could show that nHDL and S1P interfere with NF-κB signaling thereby limit the vascular inflammation. TNF-α was used as it is well known that levels of this cytokine are elevated in placentas of preeclamptic women as well as in their systemic circulations (66–68). Furthermore, TNF-α plays a key role in the exaggerated inflammatory response prevalent in PE, it is involved in the abnormal apoptotic and necrotic processes in trophoblasts during PE and promotes endothelial dysfunction (69–71). In this study we used TNF-α at a concentration of 10 ng/ml to induce the expression of the adhesion molecules VCAM1 and ICAM1 and the cytokines MCP1 and IL-8 in HPAECs as these cells do not express these inflammation related proteins under basal conditions. We could show that nHDL and S1P present anti-inflammatory properties by reducing TNF-α induced mRNA expression of VCAM1 and ICAM1 as well as of IL-8 and MCP1. Furthermore, we provided evidence that the downregulation is achieved by antagonizing the TNF-α induced activation of NF-κB which has been shown by others (63). However, as Ruiz et al already reported there is a discrepancy between mRNA expression and protein products when it comes to the evaluation of adhesion molecules (62). Furthermore, although IL-8 and MCP1 mRNA levels were not measured, this study also failed to show that S1P reduces IL-8 and MCP1 protein levels under inflammatory conditions (62). One possible explanation to this discrepancy might be that remaining amount of mRNA present is still enough to induce a pronounced inflammatory response. Furthermore, we have to consider that the dynamic of the inflammation in our specific experimental model, might be strongly time dependent. Therefore, extensive kinetic studies with multiple time points would likely draw a more complete picture of the consequences of nHDL and S1P administration in inflammation. Oxygen balance disruption with concomitant increased ROS production is also a major hallmark feature of endothelial dysfunction, which occurs in preeclampsia.

Angiotensin (AngII) is a potent vasoconstrictor that induces endothelial dysfunction and hypertension. The diverse actions of Ang II are mediated via Ang II type 1 and Ang II type 2 receptors, which couple to various signaling molecules, including NADPH oxidase (Nox), which generates reactive oxygen species (ROS). Additionally, in PE formation of autoantibodies against the angiotensin receptor 1 was reported which leads to increased AngII sensitivity (72,73).

Previous studies showed a dose dependent activation of NOX in the range of 0.01 to 1 μM AngII (74,75). Based on these findings we chose to use 0.5 and 1 μM AngII to ensure sufficient induction of NOX. Although, neither TNF-α nor AngII do fully represent the complex pathology of PE, both represent key molecules especially if one is investigating endothelial inflammation and dysfunction in the context of PE like we aimed to do.

Our work shows that nHDL is very efficient in reducing ROS formation in HPAECs thereby protecting cells from oxidative stress which is in line with observations by other groups (76–78). The exact mechanism how nHDL is affecting the regulation of intracellular ROS metabolism in non-phagocytosing cells are still poorly understood. Some speculations have been done in this regard. One possible explanation is that HDL disrupts the formation of lipid rafts on cell membranes which are important for the assembly and functionality of NOX thereby preventing the production of ROS. However, the molecular mechanism by which HDL interferes with intracellular ROS production are also incompletely understood. NOX1 is stimulated by AngII via the angiotensin receptor type 1 (ATR1). Specifically, PKC phosphorylates the NOX subunit p47phox, which causes translocation of p47phox to the cell membrane to participate in the formation of active NOX complexes (79–81). Furthermore, the assembly of the NOX complex requires the small GTPase Rac1. It has been reported that this signaling cascade leading to the activation of NADPH oxidase, is blunted in presence of HDL. Therefore, most likely HDL decreases intracellular ROS levels by interfering with the NADPH oxidase signaling. Tölle et al could also demonstrated that S1P alone is able to prevent NADPH oxidase-induced ROS production in the same way as HDL. In our experiments we got similar results showing decreased ROS levels in the presence of nHDL and a less pronounced but still significant effect with S1P conjugated with serum albumin. As S1P is associated with HDL it is likely that the effect of HDL on ROS production is partially mediated by S1P which was shown in experiments where removal of S1P from HDL particles decreased the capacity of HDL to prevent ROS production. These results highlight the importance of the S1P carrier. Indeed, it has been shown that HDL improves S1P signaling by retaining S1PR on the cell surface and by boosting S1PR recycling (82). Additionally, interactions of HDL and SR-BI facilitate the transfer of HDL bound S1P to its receptors by providing spatial proximity (83) .

We demonstrate that nHDL and S1P rescue the AngII induced upregulation of NOX1 in HPAECs which possibly provides partial insight of the mechanism by which nHDL and S1P protect the endothelium from oxidative stress. Overall our data suggest that nHDL and S1P prevent AngII-induced endothelial oxidative stress by modulating NADPH oxidase complex activation. Furthermore, our gene expression panel shows that nHDL and S1P reverse the TNF-α induced downregulation of NOX4. Conversely to all other NOX isoforms, NOX4 is described as endothelium protective mediator which is able to increase NO bioavailability and suppress cell death pathways. These findings are corroborating the hypothesis that nHDL and S1P work to maintain the functionality of the endothelium.

However, S1P effects are strongly dependent on which receptor, expressed on the cell surface, transduce the signaling cascade. Although the anti-inflammatory S1PR1 is the most abundantly expressed, the pro-inflammatory S1PR2 is also present on ECs. To investigate whether pathological conditions alter the relative expression of S1PRs would help to improve the understanding of the effects of S1P in HPAECs. Studies in HUVECs already showed that TNF-α administration increases expression of S1PR1 and even more pronounced that of S1PR2. Our results show that PE placental arteries have reduced expression of S1PR1 and increased level of S1PR2 compared to normotensive placental arteries. These data go ahead with previous findings which underlines the role of S1PR2 as a regulator of vascular inflammation and atherosclerosis. Contrary, cell surface S1PR1 loss is associated with defective endothelial barrier and vascular injury. Additionally, immunostaining of placental arteries shows a reduction of S1PR1 expression in PE, which suggest the presence of a proinflammatory state in the placenta vasculature.

The role of S1P is extremely complex and it dependent not only on the relative expression of the S1PRs but also on S1P intracellular and extracellular concentration. Increased de novo synthesis of sphingolipids in inflamed endothelium has been described as a protective mechanism to limit the vasculature damage in pathological condition. In agreement with previous studies we found that TNF-α induces the enzymatic activity of SPT, the rate limiting enzyme in sphingolipid synthesis, in HPAECs. Gene expression analysis of placental arteries supports these results. However, our data suggest that an increased SPT activity does not translate in increased production of S1P. Indeed, we noticed that in PE arteries the mRNA expression of SGPPI (sphingosine-1-phosphate phosphatase) and SGPLI (sphingosine-1-phosphate lyase) is increased. This suggest that the overall increase in SL production in PE points toward S1P degradation in favor of other SL species. Furthermore, Melland-Smith et al reported lower levels of circulating S1P in women suffering from preeclampsia compared to normotensive women and increased SPT activity in placental tissue which supports our own findings (84). Therefore, increased ceramide levels in combination with decreased S1P levels leads

to disruption of the so-called sphingolipid rheostat, which may account for observed adverse outcomes in preeclampsia. However, is becoming more and more important the concept that not all the ceramides species are associated with cell toxicity, but accumulation of specific species or other sphingolipids such as sphingomyelin, might contribute to pathology of the diseases. Therefore, we characterized the metabolic profile of ceramide (Cer), sphingomyelin (SM) and S1P in placental chorionic arteries from normotensive and preeclamptic subjects. Mass spectrometry-based lipidomics show that the major Cer species in the both control and PE groups were C16, C22:0, C22:1, C:24 and C24:1. Previous studies showed that dominant Cer species were C24:0 and C24:1 in human serum and plasma. Interestingly, there is a general tendency in decreased Cer production in PE, with a significant reduction of the level of C20. On the other hand, there is an accumulation of dhSph in PE vessels. Raised dhSph level have been associated with CAD, neurodegenerative disease and diabetes (85). Charkiewicz et al also reported elevated dhSph level in plasma of preeclamptic patients (86). Furthermore, we observed that the levels of SM species with saturated acyl chains (C16:0, C18:0, and C24:0) were high in PE arteries compared to control. Additionally, SM serum and plasma concentration positively correlate with cardiovascular disease.

In conclusion we could show that nHDL associated S1P shows anti-inflammatory properties that protect the feto-placental endothelium. Furthermore, we provided evidence that nHDL is very effective in preventing ROS formation thereby protecting the endothelium from oxidative stress prevalent in endothelial dysfunction. These effects are likely mediate by S1P. However, further experiments based on the pharmacological inhibition of the pathway are needed to corroborate this hypothesis. Nonetheless, we have demonstrated that there is a carrier specificity in the S1P-mediated biological effects. We also emphasized the role of the SL homeostasis in pregnancy-related disorders associated with vascular dysfunction such as PE. This study revealed that in preeclamptic condition S1P metabolism and signaling is perturbed. This ultimately interferes with the protective properties mediated by S1P on placenta vasculature, thereby contributing to adverse outcomes in preeclampsia.

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