Osteoprotegerin Regulates Vascular Function through Syndecan-1 and NADPH Oxidase Derived Reactive Oxygen Species.

Rhéure Alves-Lopes[*1], Karla Bianca Neves[*1], Anastasiya Strembitska[2], Adam P Harvey[1], Katie Y Harvey[1], Hiba Yusuf[1], Susan Haniford[1], Ross T Hepburn[1], Jennifer Dyet[1], Wendy Beattie[1], Delyth Graham[1], Augusto C Montezano[1].

1 Institute of Cardiovascular and Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow

2 Centre for Integrative Genomics, University of Lausanne, 1015 Lausanne, Switzerland

* Both authors contributed equally to this work

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Corresponding Author:

Augusto C Montezano, PhD

Institute of Cardiovascular and Medical Sciences,

BHF Glasgow Cardiovascular Research Centre,

University of Glasgow,

126 University Place, Glasgow, G12 8TA,

Phone: + 44 (0)141 330 8015, Fax: + 44 (0)141 330-3360,

Email: Augusto.Montezano@glasgow.ac.uk

Abstract

Osteogenic factors, such as osteoprotegerin (OPG), are protective against vascular calcification. However, OPG is also positively associated with cardiovascular damage, particularly in pulmonary hypertension, possibly through processes beyond effects on calcification. In this study, we focused on calcification-independent vascular effects of OPG through activation of syndecan-1 and NADPH oxidases 1 and 4. Isolated resistance arteries from WKY rats, exposed to exogenous OPG, studied by myography exhibited endothelial and smooth muscle dysfunction. OPG decreased NO production, eNOS activation and increased ROS production in endothelial cells. In VSMCs, OPG increased ROS production, H_2O_2 /peroxynitrite levels and activation of Rho kinase and myosin light chain. OPG vascular and redox effects were also inhibited by the syndecan-1 inhibitor synstatin. Additionally, heparinase and chondroitinase abolished OPG effects on VSMC-ROS production, confirming syndecan-1 as OPG molecular partner and suggesting that OPG binds to heparan/chondroitin sulphate chains of syndecan-1. OPG-induced ROS production was abrogated by NoxA1ds (Nox1 inhibitor) and GKT137831 (dual Nox1/Nox4 inhibitor). Tempol (SOD mimetic) inhibited vascular dysfunction induced by OPG. In addition, we studied arteries from Nox1 and Nox4 KO mice. Nox1 and Nox4 knockout abrogated OPG-induced vascular dysfunction. Vascular dysfunction elicited by OPG is mediated by a complex signalling cascade involving syndecan-1, Nox1and Nox4. Our data identify novel molecular mechanisms beyond calcification for OPG, which may underlie vascular injurious effects of osteogenic factors in conditions such as hypertension and/or diabetes.

Introduction

Despite advances in therapeutics, cardiovascular disease remains the leading cause of morbidity and mortality worldwide [1]. In cardiovascular diseases, such as hypertension and atherosclerosis, the vasculature undergoes functional, structural and mechanical changes characterised by endothelial dysfunction, increased contractility, inflammation, fibrosis and proliferation of vascular smooth muscle cells (VSMCs)[2, 3]. Increasing evidence suggests that processes associated with vascular calcification may also be involved [4]. In vascular injury VSMCs undergo a phenotypic switch to an osteoblast-like phenotype, associated with increased production of pro-osteogenic factors, followed by a decrease in the levels of anti-osteogenic factors, such as osteoprotegerin (OPG)[5]. Currently, OPG is linked to endothelial dysfunction, vascular damage and increased cardiovascular risk in diabetes and metabolic diseases[6, 7], suggesting that these osteogenic factors may influence vascular biology independently of calcification processes. In addition, a putative role for OPG in vascular dysfunction and remodelling is suggested, where studies show that OPG polymorphisms are associated with increased intima-to-media thickness and reduced forearm blood flow[8], processes characteristically associated with hypertension.

OPG is a soluble glycoprotein belonging to the tumour necrosis factor (TNF) receptor family, which classically functions as a decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL). The structure of OPG contains a death domain and a heparin-binding region, which confers signalling properties. Proteoglycans and glycosaminoglycans, such as heparin, dermatan sulphate, chondroitin sulphate and syndecan-1, bind to OPG regulating interactions with TRAIL and RANKL[9]. These interactions are also important to OPG-induced signalling, independently of TRAIL/RANKL. In human microvascular endothelial cells, OPG increases proliferation and migration through activation of ERK1/2 by interactions with integrins[10]. Syndecan-1 engages

with the extracellular matrix by its glycosaminoglycan (GAG) chains and regulates a series of signalling pathways, including integrin activation[11]. A few studies have identified syndecan-1 as a molecular partner of OPG and, by binding to syndecan-1, OPG induces angiogenesis[12] and monocyte migration[13]. Syndecan-1 is formed by an extracellular domain, composed by GAGs chains and an integrin association site, and an intracellular domain containing phosphorylation and biding sites for many other signalling proteins, such as actin, fascin, syntenin, synectin, FAK, Src, calmodulin and others[11].

Taken together, growing evidence suggests a dual role for OPG in the cardiovascular system. OPG can act as inhibitor of vascular calcification and may also regulate vascular biology independently of vascular calcification. There is a paucity of information on how OPG may influence vascular function. Syndecan-1[13] has been described as molecular partner of OPG. In addition, NADPH oxidase (Nox)-derived reactive oxygen species (ROS) have been involved in vascular dysfunction and injury, especially in hypertension[2, 14-17]. Taking these concepts together, this study aims to understand the vascular pleiotropic effects of OPG, and to elucidate whether OPG through interaction/activation of syndecan-1 influence vascular function and redox balance in vascular cells.

Materials and Methods

Animals

Animals were used in accordance with the United Kingdom Animals Scientific Procedures Act 1986 and the ARRIVE Guidelines and approved by the institutional ethics review committee. Animals were housed in individual cages under controlled conditions of 22-24°C and a 12-hour light/dark cycle with free access to food and water. Eighteen-week-old male Wistar-Kyoto (WKY) rats were euthanized and mesenteric arteries were isolated

for vascular studies and primary culture of VSMCs. Wildtype (WT; C57BL/6J) and Nox1[14] or Nox4[15] knockout (KO) mice were generated as previously described. Twenty-week-old male mice were euthanized, and mesenteric arteries were isolated for vascular studies and primary culture of VSMCs.

Cell culture

VSMCs from WKY rats (normotensive) and Nox1/Nox4 KO mice and respective WT mice were studied. Animals were euthanized by overdose of anaesthetic gas (isoflurane) followed by cervical dislocation. Briefly, mesenteric resistance arteries were isolated, cleaned from fat/connective tissue and VSMCs were dissociated by enzymatic digestion (2 mg/mL collagenase type 1, 0.12 mg/mL elastase, 0.36 mg/mL soybean trypsin inhibitor, and 2 mg/mL bovine serum albumin fraction V). Cells were plated in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum (FBS) and antibiotics. Rat aortic endothelial cells (RAEC) were purchased from ECACC (European Collection of Authenticated Cell Cultures) and were cultured accordingly to manufacturer's instructions. At sub confluence, cells were rendered quiescent with 0.5% FBS medium for 16h. Low-passage cells (4–7) were studied.

Wire Myography

Second-order branches of mesenteric artery without perivascular fat were isolated (2 mm in length) from WKY rats and, WT, Nox1 KO, and Nox4 KO mice and mounted on a wire myograph (DMT myograph; ADInstruments Ltd., Oxford, U.K.). Vessel segments were equilibrated in Krebs Henseleit-modified physiological salt solution (in mmol/L: 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 0.026 EDTA, and 5.5 glucose) at 37°C, continuously bubbled with 95% O2 and 5% CO2, pH 7.4. At the beginning

of each experiment, arteries were contracted with 62.5 mmol/L KCl to test for functional integrity. Arteries where pre-incubated or not with recombinant OPG (50 ng/mL) (R&D systems, Abingdon, U.K.) for 1h. Endothelium-dependent relaxation was assessed in all vessels by cumulative concentration-response curves to acetylcholine (ACh) (1 nmol/L to 10 μmol/L) where vessels were pre-contracted with phenylephrine (Phe) or U46619 at a concentration to achieve approximately 80% of maximal response (30 nmol/L). After that, endothelium-independent relaxation was assessed by the concentration-response curves to sodium nitroprusside (SNP, 1 nmol/L to 10 μmol/L) and contractile responses mediated by Phe (1 nmol/L to 10 μmol/L) were evaluated in endothelium-intact arteries. In arteries from Nox1 KO mice, vascular contraction was assessed by cumulative concentration-response curves to U46619 (0.1 nmol/L to 10 μmol/L). In some experiments, arteries were pre-incubated with the SOD mimetic tempol (1 μmol/L), the Nox1 inhibitor NoxA1ds (10 μmol/L), and the syndecan-1 inhibitor synstatin (SSNT - 10 μmol/L) prior to incubations with OPG.

Measurements of Oxidative Stress

RAEC and VSMCs were stimulated with OPG (50 ng/mL) for 5, 15, 30 and/or 60 minutes. In some experiments, VSMCs were pre-incubated with SSNT (10 μmol/L), NoxA1ds (10 μmol/L), a dual Nox1/Nox4 inhibitor, GKT137831 (10 μmol/L), heparinase I (1 mU/mL), or chondroitinase (1 mU/mL) for 30 minutes prior stimulations with OPG. NADPH-dependent ROS generation was measured by lucigenin enhanced chemiluminescence, with lucigenin as the electron acceptor and NADPH as the substrate. RAEC and VSMCs were homogenized in assay buffer (in mmol/L: 50 KH2PO4, 1 EGTA, and 150 sucrose, pH 7.4). The assay was performed with 100 μL of sample, 1.25 μL of lucigenin (5 μmol/L), 25 μL of NADPH (0.1 mmol/L) and assay buffer to a total volume of

250 µL. Luminescence was measured for 30 cycles of 18 seconds each by a luminometer (Orion II Microplate Luminometer, Berthold, Germany). Basal readings were obtained prior to the addition of NADPH to the assay. The reaction was started by the addition of the substrate. Basal and buffer blank values were subtracted from the NADPH-derived luminescence. ROS production was expressed as relative luminescence units (RLU)/µg protein. H_2O_2 was assessed with Amplex Red assay kit (Molecular Probes, Life Technologies). Cell lysates from all groups (100 µL) were incubated with the reaction mixture according to manufacturer specifications. A microplate reader was used for absorbance at 560 nm. H_2O_2 levels were corrected by protein concentration of each sample. Peroxynitrite ($ONOO^-$) levels were assessed by a nitrotyrosine ELISA (Abcam, UK) accordingly to the manufacturer's instructions. Obtained concentrations were normalized to protein concentration and expressed accordingly.

Immunoblotting

RAEC or VSMCs were stimulated with OPG (50 ng/mL) for 5, 15, 30 and/or 60 minutes. In some experiments, VSMCs were pre-incubated with SSNT (10 µM) or NoxA1ds (10 µM) for 30 minutes prior to stimulations with OPG. After stimulations, total protein was extracted from all groups. VSMCs were lysed in 50 mmol/L Tris-HCl (pH 7.4) lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 2 mmol/L sodium orthovanadate (Na3VO4), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin A, 1 µg/mL leupeptin and 1 µg/mL aprotinin. Total protein extract was sonicated and cleared by centrifugation at 10 000 rpm for 10 min and the pellet was discarded. Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins from homogenates (30 µg) were separated by electrophoresis on a polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-

buffered saline solution with 0.01 % Tween for 1 hour at room temperature. Membranes were then incubated with specific antibodies overnight at 4°C. Antibodies were as follows: anti-eNOS [Thr495], anti-eNOS[Ser1177] (Cell Signalling, Leiden, Netherlands); MYPT1 (Santa Cruz, Dallas, USA); and MLC2 [Thr19/Ser 19] (Cell Signalling, Leiden, Netherlands). Antibodies to total form of the signalling proteins or β-actin (Sigma, St Louis, USA) were used as internal housekeeping control. After incubation (1h) with secondary fluorescence-coupled antibodies (LI-COR, Cambridge, U.K.), signals were visualized by an infrared laser scanner (Odyssey Clx, LI-COR, Cambridge, U.K.). Protein expression levels were normalized to loading controls and expressed as percentage (%) of the control. Signals were quantified by densitometry using LI-COR Image Lite Pro software/ImageJ software.

Nitric oxide (NO) production

Production of NO was determined by using the NO fluorescent probe diacetate 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM), (Life Technologies, Molecular Probes, Paisley, UK). RAEC were loaded with DAF-FM diacetate (final concentration 5µmol/L, 30 minutes) in serum free media, kept in the dark, and maintained at 37ºC, as we previously described [18]. Briefly, cells were washed to remove excess probe. The medium was replaced and incubated for an additional 10 minutes to allow complete de-esterification of the intracellular diacetates. Cells were stimulated with OPG (50 ng/mL) for 5, 15 and 30 minutes. Cells were washed with phosphate-buffered saline (PBS) and harvested with mild trypsinization at 0.025%. Trypsin was inactivated with soybean trypsin inhibitor (0.025%) in PBS (1:1). After washing, the pellet was transferred to a black 96 well microplate (BD Falcon, Loughborough, UK). The DAF-FM fluorescence was assessed with a spectrofluorometer at excitation/emission wavelengths of 495/515 nm. Fluorescence intensity was normalized to the protein concentration and expressed as fluorescence emission/µg of

protein. Stimulation with serotonin (5-HT), a known eNOS activator, was used as a positive control, where incubations with L-NAME, an eNOS inhibitor, was used as a negative control

Rho kinase assay

VSMCs were stimulated with OPG (50 ng/mL) for 5, 15, 30, 60 minutes, in the presence or absence of SSNT (10 μM). Enzymatic activity of Rho kinase was evaluated in mesenteric arteries from all groups by using a ROCK Activity Assay Kit (Merck Millipore, UK) accordingly to the manufacturer's instructions. Obtained concentrations were normalized to protein concentration and expressed accordingly.

Statistical Analysis

Data are presented as mean±standard error of the mean (SEM). Statistical comparisons were made with 1-way ANOVA followed by Dunnet's *post-hoc*, Tukey's *post-hoc* or 2-tailed Student's *t* test when appropriate. *P-value*<0.05 was considered statistically significant.

Results

OPG induces vascular dysfunction through syndecan-1

Incubation of resistance arteries from control WKY rats with OPG reduced sensitivity to Ach (endothelium-dependent relaxation) (Figure 1A). Molecular mechanisms related to endothelial dysfunction were further dissected. NO production (Figure 1B) and eNOS phosphorylation at the activation site Ser1177 (Sup Figure 1) were decreased at 5 min after OPG stimulation. Syndecan-1 has been identified as a molecular partner of OPG. OPG regulation of vascular function was evaluated after inhibition of syndecan-1 by synstatin

(SSNT), which inhibits the interaction of the syndecan-1 domain responsible for activation of integrins[19]. OPG increased eNOS inactivation in a syndecan-1-dependent manner, as OPG-induced eNOS phosphorylation at the inhibitory site Thr495 was inhibited by SSNT (Figure 1C). SSNT also blocked OPG-induced endothelial dysfunction (Figure 1D). Moreover, OPG increased ROS-production in RAECs via syndecan-1(Figure 1E). To further confirm the role of oxidative stress on OPG-induced endothelial dysfunction, mesenteric arteries from WKY rats were pre-incubated with tempol, a SOD mimetic. Removal of ROS by tempol abrogated OPG-induced effects in endothelial function (Figure 1F). Treatment with SSNT or tempol did not alter responses to Ach in control arteries not exposed to OPG (Sup Figures 2A and 2B).

In VSMCs, OPG decreased sensitivity to SNP (endothelium-independent relaxation) (Figure 2A) and increased the sensitivity to contraction (Figure 2B). OPG-induced VSMC dysfunction was abrogated by the syndecan-1 inhibitor (Figure 2C), as well as OPG-induced effects in contraction (Emax) (Figure 2D). Similar effects were observed in arteries only exposed to SSNT in SNP-induced relaxation (Sup Figure 3A) and vascular contraction (Sup Figure 3B). In cultured VSMCs, OPG increased MLC Ser19 phosphorylation (Figure 2E), MYPT1 inactivation site phosphorylation (Sup Figure 4A), Rho kinase activation (Sup Figure 4B), ROS production (Figure 2F) and H2O2 levels (Sup Figure 5A) through syndecan-1. Syndecan-1 core protein on the extracellular domain contain heparin sulfate and chondroitin sulfate chains, which are possible binding sites for OPG. Treatment of VSMCs with heparinase, which removes heparin sulfate chains, and chondroitinase, which removes chondroitinase sulfate chains, blocked the effects of OPG on ROS production (Sup Figure 5B). Similar to the observations in endothelial function, treatment of resistance arteries with tempol abrogated OPG-induced decrease in VSMC relaxation (Sup Figure 6A) and increase in contraction (Sup Figure 6B).

Nox1 and Nox4 play a role in OPG-induced vascular dysfunction

We assessed Nox1 and Nox4 mRNA expression in RAECs and WKy VSMCs after exposure to OPG. Stimulation with OPG did not alter gene expression of Nox1 (Sup Figure 7A) or Nox4 (Sup Figure 7B) in RAECs, but increased Nox1 (Sup Figure 7C) and Nox4 (Sup Figure 7D) gene expression in VSMCs. Moreover, OPG-induced ROS production was inhibited by a dual Nox1/Nox4 inhibitor (Sup Figure 7E). To further confirm the role of Nox1, OPG effects on ROS production were assessed after pre-incubation with NoxA1ds. The specific Nox1 inhibition abrogated the increase in ROS generation (Figure 3A), but not H2O2 levels (Figure 3B), induced by OPG. Nox1 activation leads to increased levels of superoxide anion, which can react with NO to form peroxynitrite ($ONOO^-$). OPG increased $ONOO^-$ levels in VSMCs (Sup Figure 8A); an effect inhibited by SSNT (Sup Figure 8B) and NoxA1ds (Figure 3C). In terms of vascular dysfunction, Nox1 inhibition abrogated OPG-induced endothelial dysfunction (Figure 3D), VSMC reduced relaxation (Figure 3E) and increased contraction (Emax) (Figure 3F). NoxA1ds alone did not alter Ach- (Sup Figure 9A) or SNP-induced (Sup Figure 9B) relaxation, but increased sensitivity to Phe-induced contraction (Sup Figure 9C).

The role of Nox1-derived ROS in OPG-induced changes in vascular function was further confirmed in resistance arteries and VSMCs from Nox1 KO mice. OPG increased ROS generation and H2O2 levels in VSMCs from WT (Sup Figures 10A and 1B; respectively) but not Nox1 KO mice (Sup Figures 10 C and 10D; respectively). Accordingly, OPG only induced vascular dysfunction in resistance arteries from WT mice and not Nox1 KO mice, including endothelial dysfunction (Figures 4A, 4B), VSMC reduced relaxation (Figures 4C, 4D) and increased contraction (Figures 4E, 4F). We also questioned the role of Nox4 in the changes in vascular function induced by OPG. Similar to Nox1, Ach relaxation (Figure 5A) and SNP relaxation (Figure 5B) were not altered in resistance arteries exposed to OPG. OPG-increased Phe-induced contraction only in arteries from WT mice (Figure 5C),

but not in arteries from Nox4 KO mice (Figure 5D). In VSMCs from Nox4 KO mice, OPG did not increase ROS (Figure 5E) or H2O2 (Figure 5F) production. MLC activation was increased by OPG at 15 mins in cells from WT mice in a syndecan-1 dependent manner (Figure 6A), while in VSMCs from Nox1 mice OPG only increased MLC phosphorylation at a later timepoint (60 mins) (Figure 6B). OPG reduced MLC activation in VSMCs from Nox4 KO mice (Figure 6C).

Discussion

OPG is an important inhibitor of vascular calcification, but association studies suggest a potential role in the regulation of vascular function[8]. Our study demonstrates that OPG, through interactions with syndecan-1, influence endothelial function and VSMC relaxation and contraction. The activation of the OPG/syndecan-1 axis leads to Nox-ROS-dependent vascular dysfunction. Our data also shows a role for NADPH oxidases, where Nox1/4 seem to be involved in OPG-induced vascular dysfunction and ROS production. Animal studies have demonstrated a protective role of OPG in vascular calcification[20, 21] and atherosclerosis[22, 23]. In humans, high serum levels of OPG have been associated with cardiovascular diseases[24, 25], including chronic kidney disease [26], and high mortality rates [27]. These observations suggest that OPG could influence other aspects of cardiovascular biology beyond vascular calcification. Epidemiological studies also demonstrate that levels of OPG ligands, such as RANKL, variably predict the incidence of cardiovascular complications[28]. Considering these observations, the role of OPG in inducing cardiovascular damage could be due to the role of OPG as a decoy receptor for TRAIL or RANKL, where high levels of OPG are a consequence of high levels of TRAIL/RANKL. However, OPG regulates a series of physiological and pathological processes, independently of interactions with TRAIL/ RANKL, such as leukocyte adhesion to endothelial cells[29]. Here we observed a direct vascular effect of OPG suggesting that the clinical correlation between OPG and endothelial

dysfunction and vascular damage may be due to direct vasoactive properties of OPG by binding to syndecan-1. SSNT, a small peptide that displaces the biding between integrins and syndecan-1, inhibited OPG-induced vascular effects. Moreover, in a study evaluating cancer growth in mice, SSNT inhibited syndecan-1-induced angiogenesis, through the disruption of syndecan-1/integrin interactions[19].

The complexity of syndecan-1 signalling is further demonstrated in lung epithelial cells where the intracellular domain of syndecan-1 participates in cell migration and focal adhesion disassembly, while the extracellular domain controls cell adhesion[30]. In monocytes, incubation with heparinase I, to remove heparin sulfate chains, and chondroitinase, to remove chondroitin sulfate chains, inhibited OPG-syndecan-1-induced chemotaxis of monocytes[13]. Similar results were observed in VSMCs, where OPG-induced ROS formation was inhibited when GAGs chains were removed, suggesting that OPG interacts with GAGs in the extracellular domain of syndecan-1. The combination between the effects of GAGs and integrins on OPG-syndecan-1 signalling highlights the multidimensional role of syndecan-1 in OPG-induced ROS generation and vascular effects. It is also important to note that the Fas receptor was recently identified as other OPG signalling effector [33] and although not explored in our study, could also participate on OPG-induced vascular effects.

In the vasculature, ROS are important regulators of physiological functions through modulation of redox status of signalling proteins, such as mitogen-activated protein kinases, Rho kinases, transcription factors, protein tyrosine phosphatases, integrins and other proteins that are important to development of cardiovascular diseases, such as hypertension [2, 34]. Not only ROS formation, but also NADPH oxidase activation, is important in OPG/syndecan-1-induced vascular dysfunction. ROS play a role in regulating vascular tone, with endothelial-derived H_2O_2 acting as an endothelial-derived hyperpolarizing factor (EDHF) inducing vasorelaxation[35]. In OPG-stimulated vascular cells, we observed a shift from physiological

ROS production to oxidative stress characterized by increased ROS generation in VSMCs and endothelial cells, leading to reduced levels of NO and increased levels of $ONOO^-$. Nox1 seems to participate in OPG deleterious effects in vascular function and ROS production as inhibition or genetic deletion of Nox1 lead to abrogation of OPG injurious actions. We have described the pathological role of Nox1 in vascular injury and dysfunction in aged SHRSP hypertensive rats [36], human pulmonary hypertension [37] and in Ang II-induced renal damage in TTRhRen mice[17]. In other disease states associated with vascular dysfunction, such as congenital generalized lipodystrophy, Nox1 expression and ROS production are increased and associated with endothelial dysfunction [38].

Simultaneous inhibition of Nox1 and Nox4 ameliorated cardiac damage, improved vascular contraction and portal pressure [39]. Moreover, GKT137831 prevented retinal vascular permeability and inflammation in diabetic SHR rats [40]. Dual inhibition of Nox1 and Nox4, decreased ROS generation induced by OPG, suggesting a potential role of Nox4 in OPG vascular effects. Here, we observed a relationship between OPG and Nox4, where Nox4 knockout ameliorated OPG effects in the vasculature. The exact role of Nox4 in the cardiovascular system is still not fully understood. Nox4 protective effects in the vasculature has been previously described, where Nox4 is associated with anti-atherosclerotic actions [41] and Nox4-derived H_2O_2 is important to shear stress-induced eNOS activation [42]. Nox4 activity is constitutive [43], where hyperactivation may lead to injurious ROS levels overcoming the protective actions of Nox4- H_2O_2. In renal endothelial cells from rats with polycystic kidney disease, Nox4 expression was increased and correlated with decreased expression of eNOS [44]. Additionally, Nox4 expression, increased in the endoplasmic reticulum (ER), was associated with HIV-induced endothelial dysfunction [45]. Organelle sublocation is an important aspect that will influence redox signalling. We previously demonstrated that the upregulation of Nox1 in the plasma membrane and Nox4 in the ER are

important to protein hyperoxidation, redox signalling and vascular dysfunction in hypertensive rats [46]. As we observed that syndecan-1 contributes to OPG signalling and ROS production via its integrin domain and shedding of its GAGs, it is possible that multiple mechanisms of Nox activation, and other ROS sources not assessed in our study, may occur.

OPG has been associated with pulmonary arterial hypertension due to its effects on pulmonary VSMC growth and migration, contributing to vascular remodelling [47] and treatment with antibodies targeting OPG attenuate such injurious effects in the pulmonary circulation[33]. In pre-eclampsia, OPG concentration is increased in plasma and is associated with increased blood pressure in offspring [48]. In conclusion, OPG has direct vascular effects, mediated by the proteoglycan syndecan-1, where such processes are induced by Nox1/4-dependent oxidative stress. We identify novel functions of OPG that may affect vascular function independently of effects on calcification, which in turn may contribute to vascular dysfunction and injury in cardiovascular diseases, where regulation of OPG is impaired.

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Disclosures

None

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Figure Legends

Figure 1 – OPG induces endothelial dysfunction through syndecan-1. (A) Ach cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats assessed by wire myography. (B) NO production assessed by DAF in RAECs after OPG stimulation, where L-NAME (L-NE) (10 μM) was used as a negative control and 5-HT (0.1 μM) as a positive control. (C) eNOS inactivation (phosphorylation of Thr495) in RAECs in the presence and absence of syndecan-1 inhibitor synstatin (SSNT - 10μM). (D) Ach cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats after incubation with SSNT. (E) ROS generation measured in RAECS stimulated with OPG in the presence/absence of SSNT. (F) Ach cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats after incubation with tempol. Curves are expressed by the mean ± SEM (n=3-13). * $p<0.05$ control (C) vs. OPG; † <0.05 OPG vs. OPG + SSNT or tempol after 1-way ANOVA followed by Bonferroni's post-hoc test. Data in bar graphs are expressed as mean ± SEM (n=3-11); * $p<0.05$ control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT or tempol after 1-way ANOVA followed by Dunnett's (A) or Tukey's (C, E) post-hoc test.

Supplemental Figure 1 – OPG decreases eNOS activation in RAECs. eNOS activation (phosphorylation of Ser1177) in RAECs after stimulation with OPG (50 ng/mL). Data in bar graphs are expressed as mean ± SEM (n=6); * $p<0.05$ control (C) vs. OPG stimulated cells after 1-way ANOVA followed by Dunnett's post-hoc test.

Supplemental Figure 2 – Effects of SSNT and tempol in endothelial-dependent relaxation. Ach cumulative concentration-response curve to Ach in the presence or absence of SSNT (10μM) (A) or tempol (10μM) (B) in resistance arteries obtained from WKY rats assessed by wire myography. Curves are expressed by the mean ± SEM (n=3-13).

Figure 2 – OPG induces VSMC dysfunction through syndecan-1. (A) SNP cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats assessed by wire myography. (B) Phe cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats assessed by wire myography. (C) SNP and (D) Phe cumulative concentration-response curve to OPG (50 ng/mL) in the presence or absence of SSNT (10μM) resistance arteries obtained from WKY rats assessed by wire myography. (E) MLC activation (phosphorylation of Ser19) in WKY VSMCs in the presence and absence of SSNT (10μM). (F) ROS generation measured in WKY VSMCs stimulated with OPG in the presence/absence of SSNT. Curves are expressed by the mean ± SEM (n=3-13). * $p<0.05$ control (C) vs. OPG; † <0.05 OPG vs. OPG + SSNT after 1-way ANOVA followed by Bonferroni's post-hoc test. Data in bar graphs are expressed as mean ± SEM (n=4-6); * $p<0.05$ control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT after 1-way ANOVA followed by Tukey's post-hoc test.

Supplemental Figure 3 – Effects of SSNT in VSMC relaxation and contraction. (A) SNP cumulative concentration-response curve in the presence or absence of SSNT (10μM) in resistance arteries obtained from WKY rats assessed by wire myography. (B) Phe cumulative concentration-response curve in the presence or absence of SSNT (10μM) in resistance arteries obtained from WKY rats assessed by wire myography. Curves are expressed by the mean ± SEM (n=3-13). * $p<0.05$ control (C) vs. SSNT after 1-way ANOVA followed by Bonferroni's post-hoc test.

Supplemental Figure 4 – OPG/syndecan-1 regulation of contractile signalling in VSMCs from WKY rats. (A) MYPT1 inactivation (phosphorylation of Thr853) in VSMCs in the presence and absence of SSNT (10μM). (B) Rho kinase activation in VSMCs in the presence and absence of SSNT (10μM). Data in bar graphs are expressed as mean ± SEM

(n=7-4); * p<0.05 control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT after 1-way ANOVA followed by Tukey's post-hoc test.

Supplemental Figure 5 – Role of syndecan-1 in OPG-induced ROS generation in VSMCs from WKY rats. (A) H2O2 levels measured in VSMCs stimulated with OPG in the presence/absence of SSNT. (B) ROS generation measured in VSMCs stimulated with OPG in the presence/absence of heparinase or chondroitinase (1 mU/mL). Data in bar graphs are expressed as mean ± SEM (n=5-14); * p<0.05 control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT, heparinase (hepar) or chondroitinase (chond) after 1-way ANOVA followed by Tukey's post-hoc test.

Supplemental Figure 6 – Effects of tempol in OPG regulation of VSMC relaxation and contraction. (A) SNP cumulative concentration-response curve in the presence or absence of tempol (10μM) in resistance arteries exposed or not to OPG (50 ng/mL) obtained from WKY rats assessed by wire myography. (B) Phe cumulative concentration-response curve in the presence or absence of tempol (10μM) in resistance arteries exposed or not to OPG (50 ng/mL) obtained from WKY rats assessed by wire myography. Curves are expressed by the mean ± SEM (n=3-13). * p<0.05 control (C) vs. tempol after 1-way ANOVA followed by Bonferroni's post-hoc test.

Supplemental Figure 7 – OPG regulation of Nox1 and Nox4 expression in vascular cells. (A) Nox1 gene expression and (B) Nox4 gene expression in RAECs after stimulation with OPG (50 ng/mL) assessed by RT-PCR. (C) Nox1 gene expression and (D) Nox4 gene expression in VSMCs from WKY rats after stimulation with OPG (50 ng/mL) assessed by RT-PCR. (E) ROS generation measured in VSMCs stimulated with OPG (50 ng/mL) in the presence/absence of GKT137831 (GKT - 10μM). Data in bar graphs are expressed as mean ±

SEM (n=5-20); * p<0.05 control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + GKT after student's t-test (A-D) or 1-way ANOVA followed by Tukey's (E) post-hoc test.

Figure 3 – Role of Nox1 in OPG-induced oxidative stress and vascular dysfunction in WKY rats. ROS generation (A), H2O2 levels (B) and $ONOO^-$ levels (C) measured in VSMCs stimulated with OPG (50 ng/mL) in the presence/absence of the Nox1 inhibitor (NoxA1ds - 10μM). Ach (D) and SNP (E) cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats assessed by wire myography. (F) Phe cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WKY rats assessed by wire myography. Data in bar graphs are expressed as mean ± SEM (n=16-26); * p<0.05 control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + NoxA1ds after 1-way ANOVA followed by Tukey's post-hoc test. Curves are expressed by the mean ± SEM (n=3-13). * p<0.05 control (C) vs. OPG; † <0.05 OPG vs. OPG + NoxA1ds after 1-way ANOVA followed by Bonferroni's post-hoc test.

Supplemental Figure 8 – Role of Nox1 in OPG-induced peroxynitrite in WKY rats. $ONOO^-$ levels were measured in VSMCs from WKY rats after OPG stimulation (50 ng/mL) (A) and after pre-incubation with SSNT (10μM) (B). Data in bar graphs are expressed as mean ± SEM (n=9-12); * p<0.05 control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT after 1-way ANOVA followed by Dunnet's (A) or Tukey's (B) post-hoc test.

Supplemental Figure 9 – Effects of NoxA1ds in vascular function of resistance arteries from WKY rats. (A) Ach and (B) SNP cumulative concentration-response curve in the presence or absence of NoxA1ds (10μM) in resistance arteries obtained from WKY rats assessed by wire myography. (B) Phe cumulative concentration-response curve in the presence or absence of NoxA1ds (10μM) in resistance arteries obtained from WKY rats

assessed by wire myography. Curves are expressed by the mean ± SEM (n=11-13). * p<0.05 control (C) vs. NoxA1ds after 1-way ANOVA followed by Bonferroni's post-hoc test.

Figure 4 – Role of Nox1 in OPG-induced vascular dysfunction. Ach cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WT (A) and Nox1 KO (B) mice assessed by wire myography. SNP cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WT (C) and Nox1 KO (D) mice assessed by wire myography. U46619 cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WT (E) and Nox1 KO (F) assessed by wire myography. Curves are expressed by the mean ± SEM (n=5-20). * p<0.05 WT vs. OPG after 1-way ANOVA followed by Bonferroni's post-hoc test.

Supplemental Figure 10 – Role of Nox1 in OPG-induced ROS and H2O2 production in VSMCs. ROS generation (A) and H2O2 levels (B) measured in VSMCs from WT mice stimulated with OPG (50 ng/mL). (C) ROS production and (D) H2O2 levels measured in VSMCs from Nox1 KO mice stimulated with OPG (50 ng/mL). Data in bar graphs are expressed as mean ± SEM (n=5-15); * p<0.05 control (C) vs. OPG stimulated cells after student's t-test.

Figure 5 – Role of Nox4 in OPG-induced vascular dysfunction and oxidative stress. Ach (A) and SNP (B) cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from Nox4 KO mice assessed by wire myography. Phe cumulative concentration-response curve to OPG (50 ng/mL) in resistance arteries obtained from WT (C) and Nox4 KO (D) assessed by wire myography. Curves are expressed by the mean ± SEM (n=5-20). * p<0.05 WT vs. OPG after 1-way ANOVA followed by Bonferroni's post-hoc test. (E) ROS production and (F) H2O2 levels measured in VSMCs from Nox1 KO mice stimulated with OPG (50 ng/mL). Data in bar graphs are expressed as mean ± SEM (n=11).

Figure 6 – Role of OPG/syndecan-1/Nox1/4 axis in the regulation of MLC activation in VSMCs. (A) MLC activation (phosphorylation of Ser19) in VSMCs from WT mice in the presence and absence of SSNT (10μM). MLC activation (phosphorylation of Ser19) in VSMCs from Nox1 KO (B) and Nox4 KO (C) mice. Data in bar graphs are expressed as mean ± SEM (n=5-6); * $p<0.05$ control (C) vs. OPG stimulated cells; † <0.05 OPG vs. OPG + SSNT after 1-way ANOVA followed by Tukey's (A) or Dunnet's (B, C) post-hoc test.