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DISSERTATION

Rolle der löslichen Guanylatzyklase bei der funktionellen Alterung mesenterialer Arterien der Maus

Role of soluble guanylyl cyclase in the functional aging of mesenterial arteries in mice

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Frau Cheng Zhong
aus Guizhou, Volksrepublik China

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CVDs	cardiovascular diseases
PDE	phosphodiesterase
sGC	soluble guanylyl cyclase
eNOS	endothelial nitric oxide synthases
cGMP	cyclic guanosine monophosphate
NO	nitric oxide
ROS	reactive oxygen species
PKG	protein kinase G
VSMC	vascular smooth muscle cell
ACh	acetylcholine
L-NAME	Nω-nitro-L-arginine methylester hydrochloride
PE	phenylephrine
KCl	potassium chloride
SNP	sodium nitroprusside
DMSO	dimethylsulphoxide
iNOS	inducible nitric oxide synthases
nNOS	neuronal nitric oxide synthases
PBS	phosphate-buffered saline
EC_{50}	half maximal effective concentration

Abstract

Aging of arteries is a pathophysiological factor in the development of cardiovascular diseases. It is a continuing and complex process, which ends in remodelling of all arterial wall layers and increased arterial stiffness. Endothelial dysfunction, i.e. the decreased ability to release nitric oxide (NO), is a hallmark of early vessel aging. Reduced endothelial NO-synthase (NOS) expression and activity as well as downstream signalling may be causal in this context. We hypothesize that aging not only affects endothelial cell function, but also impairs the NO signalling in the vascular smooth muscle cells (VSMC). To test the hypothesis, the second branch of mesenteric arteries from 13 weeks (juvenile) and 40 weeks (aged) mice were studied using wire myography. Contraction and relaxation were investigated using pharmacological approaches. Further, the expression of soluble guanylyl cyclase (sGC) subunits, NOS and phosphodiesterases (PDE) as well as the cyclic guanosine monophosphat (cGMP) concentration was analysed. Vessel structure was investigated by histological methods. Endothelium-dependent relaxation by acetylcholine (ACh) was impaired in aged arteries compared to juvenile one. Pre-treatment with L-NAME (NOS inhibitor) decreased the relaxation and abolished differences between groups. Expression of eNOS and nNOS at the mRNA level was similar in both groups. The NO donor SNP induced similar concentration dependent relaxations in juvenile and aged arteries. However, the follow up after a bolus application of SNP revealed slower relaxation speed in aged vessels. Pharmacological activation of sGC by bolus application of runcaciguat induced slower relaxation in the aged vessels, as well. The biggest difference between the two groups occurred 2 min after application of runcaciguat. At this time point, cGMP concentrations were significantly lower in aged vessels. Cumulatively applied sildenafil (PDE5 inhibitor) induced relaxation, which was less pronounced in aged compared to juvenile vessels. The mRNA expression of sGC subunits α_1 and α_2 and of PDE5 was lower in aged samples. Histological study did not show morphological changes between juvenile and aged arteries (mesenteric arteries and aorta). Media-Lumen-ratios did not differ between groups. The results show that NO signalling in the VSMC contributes to the impaired relaxation in aged mesenteric arteries. The functional limitations precede the structural changes in the process of aging. Lower mRNA expression of α subunits may be the reason for slower dynamics of cGMP catalysis by sGC. The limited ability of sGC to catalyse cGMP generation in a short time

range (2 min) may influence cardiovascular control and contribute to the development of cardiovascular diseases.

Zusammenfassung

Das Altern von Gefäßen ist ein pathophysiologischer Faktor bei der Entstehung kardiovaskulärer Erkrankungen. Ein Kennzeichen vaskulären Alterns ist die endotheliale Dysfunktion, die verminderte Fähigkeit des Endothels, Stickstoffmonoxid (NO) zu genieren. Hierfür können eine abnehmende Expression und Aktivität der endothelialen NO (eNOS) verantwortlich sein. Wir stellen die Hypothese auf, dass Alterungsvorgänge aber auch in der glatten Gefäßmuskulatur stattfinden und mit funktionellen Einschränkungen einhergehen. Mesenteriale Arterien (2. Abgang der A. mesenterica) von Mäusen im Alter von 13 (juvenil) und 40 Wochen (alternd) wurden in die Studie einbezogen und mittels Drahtmyographie untersucht. Die kontraktile und relaxierende Funktion der Gefäße wurde mittels pharmakologischer Stimulation bewertet. Weiterhin wurden die Expression der Untereinheiten löslichen Guanylatzyklase (sGC), der NOS, der Phosphodiesterasen (PDE) sowie die cGMP-Konzentrationen in den Gefäßen gemessen. Untersuchungen der Gefäßstruktur erfolgten mittels histologischer Methoden. Die endothelial-vermittelte Gefäßrelaxation durch Applikation von ACh war in alternden Arterien gegenüber den juvenilen vermindert. Behandlung mit dem unspezifischen Inhibitor von NOS, L-NAME, verursachte eine geringe Relaxation und die Unterschiede zwischen den Gruppen verschwanden. Expressionen von eNOS und neuronaler NOS unterschieden sich nicht im Vergleich der Gruppen. Die Konzentration-Wirkungskurven für den NO-Donor SNP waren in juvenilen und alternden Gefäßen gleich. Jedoch sind die Relaxationen auf eine Bolusgabe von SNP in den alternden Gefäßen langsamer. Das trifft auch für die pharmakologische Aktivierung der sGC durch den Aktivator Runcaciguat zu. Zwei Minuten nach Applikation des Bolus war der Unterschied in der Relaxationsgeschwindigkeit zwischen den Altersgruppen am größten und die cGMP-Konzentrationen in den alternden Gefäßen deutlich niedriger. Die Antwort auf Sildenafil (PDE5-inhibitor), in steigenden Konzentrationen kumulativ appliziert, war in alternden Gefäßen geringer. Die Untereinheiten sGC-α_1 und sGC-α_2 sowie von PDE5 waren hier weniger stark exprimiert. Die histologischen Studien zeigten keine strukturellen Veränderungen. Media-Lumen-Verhältnisse waren in beiden Gruppen gleich. Die Ergebnisse weisen darauf hin, dass Alterung mit funktionellen Veränderungen des NO-Signalweges in den glatten Gefäßmuskelzellen einhergeht. Die funktionellen Veränderungen gehen der strukturellen voraus. Die langsame Reaktion der sGC auf Stimulation mit NO als auch mit dem sGC-Aktivator sind möglicherweise auf die

verminderte Expression der α-Untereinheiten zurückzuführen. Die verminderte Fähigkeit der sGC in den alternden Gefäßen, cGMP in einem kurzen Zeitraum ausreichend zur Verfügung zu stellen, kann für die Regulation des Kreislaufs und für die Entstehung kardiovaskulärer Erkrankungen von Bedeutung sein.

1. Introduction

Cardiovascular diseases (CVDs) are a leading cause of mortality worldwide. The incidence of cardiovascular diseases and events increases with age and induces considerable cost for the social system. By 2030, around 20% population will reach or over 65 years old, among them, CVDs make up 40% mortality in US and occupy 61% medical costs [2-4]. Hypertension is the most common risk factor for CVD [5]. Particularly in industrialized and emerging countries, the incidence of arterial hypertension increases with age. Even in the middle age population, elevated arterial blood pressure (isolated systolic hypertension) is an independent risk predictor for death from cardiovascular diseases in women and men [6-8]. In the long-term run, not only coronary disease, stroke, cardiovascular death are consequences of hypertension, but many other diseases, such as heart failure, hypertensive cardiomyopathy or peripheral artery disease [5]. Even though abundant epidemiological evidences reflect age related damage in the cardiovascular system, the mechanisms behind CVDs progress are not completely known. Remarkably, the prevalence for essential arterial hypertension with unknown cause is 50% to 88% in the population of hypertensive subjects suggesting a significant lack in knowledge about the pathophysiology of hypertension [9, 10].

1.1. Vessel aging

Aging of vessels is due to several pathomechanisms, which vary in large and middle-sized arteries, small arteries, and microvessels. In large and middle-sized arteries, the media degenerates and fibrosis develops in the elderly. This goes along with loss of elastin fibers and accumulation of collagen fibers [11]. Remodelling of the arteries includes degeneration and apoptosis of the media. The pathomechanisms behind these phenomena are largely unknown. Media degeneration and arteriosclerosis develop in parallel and may share some pathways leading to vessel remodelling. Arteriosclerotic changes, which tend to increase with age, start in the intimal layer of arteries with thickening of this structure. In a second stage, phagocytosis of lipids by monocytes-derived foamy macrophages occurs and inflammatory cells appear. Later, atheromatous plaques are formed, which calcify. In the progressed stage, all arterial layers are involved [11]. All these types of remodelling go along with increased vascular stiffness, which in turn increases cardiac afterload and increases blood pressure amplitude. Thus, with aging, increased blood pressure and pulse pressure, respectively, can further stimulate

arterial remodelling [12]. Therefore, aged large arteries are characterized by increased stiffness [13]. Important pathophysiological factors in the aging are oxidative stress and inflammation, resulting in the generation of a pro-inflammatory and pro-thrombotic phenotype, with reduced elastic fibers, increased collagen, endothelial apoptosis and atherosclerosis [14-17]. Another hallmark of aged large and middle sized arteries is the endothelial dysfunction, characterized by a reduced ability to dilate. This is mainly due to an impaired NO-system. This declined endothelial vasodilation is a first risk marker of arterial aging.

Aging in small arteries and arterioles is partly similar to large arteries, with increased stiffness and reduced dilation. In addition, in the distal part of the vasculature, upstream signalling from capillaries to arterioles is important for the control of organ perfusion [18]. This upstream signalling is mainly due to hyperpolarization of endothelial cells, which propagates upstream, but may include the NO system, as well. Aging may go along with impaired signalling [19, 20]. Similar to the situation in large arteries, reactive oxygen species play an important role for impaired microvascular function in aging [21].

Organic nitrates are used in clinical work to improve reduced organ perfusion and total peripheral resistance, and to treat arterial pulmonary hypertension. However, rapid tolerance and endothelial dysfunction restrict the application of organic nitrates [22]. In the last decade, phosphodiesterase (PDE) inhibitors and soluble guanylyl cyclase (sGC) stimulators and activators are increasingly applied for treatment of CVDs [23]. Although, they are powerful in their dilatory action, their blood pressure lowering action may partly prevent improvement of organ perfusion [24]. Further research is necessary to develop appropriate therapeutic application based on these substances. Furthermore, efforts are necessary for better understanding of the pathophysiology of aging in the cardiovascular system.

1.2. Nitric oxide (NO)-sGC-cyclic guanosine monophosphate (cGMP) pathway

NO is a highly diffusible gas, which generation from L-arginine is catalyzed by NO synthases (NOS). The endothelial NOS (eNOS) is the dominant isoform in the endothelium [25]. NO has a “half-time” just several seconds. Its bioavailability in the vessel wall results from the generation rate, the diffusion in the blood and the transportation by erythrocyte haemoglobin, respectively, and from scavenging by reactive oxygen species (ROS). Important stimulators of NO production are the shear stress at

the vessel wall (endothelial luminal site) and G-protein receptor agonists such as histamine or acetylcholine (ACh) [26]. NO diffuses into the vascular smooth muscle cells (VMSC) and activates the sGC thereby increasing the generation of cGMP, which in turn stimulates the protein kinase G (PKG). The kinase influences several proteins, all of which lead to a reduction of the cytosolic calcium concentration and vasodilation. NO may also influences sGC expression, which can be interpreted as a regulatory effect on the NO-sGC-cGMP system [27].

The sGC is dimer, which consists of an alpha- and a beta-subunit. Genes of α and β-subunits are doubled. On the possible combinations of proteins, the α_1/β_1 and α_2/β_2 variants are the most abundant [28]. The protein subunits have a hem group, with an iron divalent atom (Fe^{2+}). The NO-binding prosthetic group is located in the beta subunit [29]. Oxidative stress transforms the divalent iron atom into a trivalent iron. This can result in loss of the hem group. Both events make the sGC insensible for NO [30]. Reduced NO bioavailability in aged vessels as well as reduced ability of NO to activate the sGC is due to an increase of reactive oxygen species (ROS), among them superoxide and hydrogen peroxide [14, 31, 32]. Besides, vessel aging and hypertension, respectively, seem to go along with reduction in sGC expression [33, 34].

1.3. Structural versus functional changes in vessel aging

Vascular aging is a complex event, characterized by structural and functional changes. Arterial remodelling includes VSMC migration and proliferation, which contribute to intima wall thickening, probably as an adaptation to increased wall tension. The adaptive wall thickening is stronger in presence of hypertension and results in decreased compliance and increased stiffness, respectively [35, 36]. However, functional impairment may precede the structural changes as shown for vasoconstrictor responses [37, 38]. Enhanced vascular reactivity to constrictors may also be the result of endothelial dysfunction, which precedes vessel structural change caused by NO deficiency and impaired NO signalling [39]. Hence, age-related function and structure change in the arterial system is worth to further research, especially at early stages of aging.

1.4. Small mesenteric arteries

Small resistant arteries (≈100-300 μm) work as peripheral resistance arteries in cardiovascular system. Large arteries transfer the heart output into a continuous distal

blood flow, while peripheral resistance arteries control peripheral blood flow and determine total peripheral resistance [38]. Mesenteric arteries of second and higher order belong to small resistant arteries. They are extensively investigated. This type of vessel has been worked for decades as a model for the function of small resistance arteries and provides insights mechanisms of vessels aging [40-43]. Functional alterations related to aging are reduction in ACh responses, changed sensitivity to endothelin, and reduced ability to inhibit vasoconstrictor affects [44]. Further, small mesenteric arteries serve for investigation of vasomotion, physiology of the endothelium, the VSMC, and pathophysiological mechanisms in a wide variety of diseases [45-50].

2. Derivation of hypothesis

The NO-sGC-cGMP system is an important vasodilator system and its components are likely involved in the pathogenesis of arterial aging. While reduction of NO-bioavailability and endothelial dilatory function during aging is well described, the NO signalling in VSMC is less known. Further, it is not clear if reduction in the sGC function contributes to the early phase of vascular functional damage in aging. We hypothesize that reduced sGC subunit expression and impairment of sGC function, i.e. decreased ability to catalyse cGMP production, occurs already in the early phase of aging. To test the hypothesis, small mesenteric artery dilator function is investigated with focus on the NO-sGC-cGMP pathway during aging.

3. Materials and methods

3.1. Mice

Experiments were performed under the Guide for the Care and Use of Laboratory Animals of the State Government of Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin) and in consideration of the Tierschutzgesetz. C57BL/6 mice were provided by Charité – Universitätsmedizin, Forschungseinrichtungen für Experimentelle Medizin, Berlin, Germany. They were housed in cages with food and water ad libitum, with 12 hours light-dark cycles. The ages of animal were 13-14 weeks (juvenile) and 40-41 weeks (aged). The respective body masses were 28g to 31g (juvenile) and 34g to 41g (aged). Only male mice were included.

3.2. Wire myography

Wire myography is an in vitro technique for functional investigation of small resistance arteries independent of systemic influences such as blood flow or nervous control. Arteries are held under isometric condition. Thus, contraction and relaxation can be measured. The method allows to characterize vasoactive substances and determine half maximal effective concentration EC_{50}, which is a measure of the sensitivity of the given drug. Since vessels differ in structure and size, a standard normalization is performed to adjust the stretching force to a physiologically meaningful value. This corresponds a transmural pressure of 100 mmHg under the assumption that the vessel is fully relaxed [51, 52].

3.2.1. Vessel dissection

Animals were anaesthetized with isoflurane and sacrificed via cervical dislocation. After midline incision of the abdomen, the whole mesentery was excised and transferred into a tray with oxygenated ice-cold buffer with low calcium (in mmol L^{-1}): 146 NaCl, 4.5 KCl, 1.2 $NaH_2PO_4*2H_2O$, 1 $MgSO_4*7H_2O$, 5.5 glucose, 0.025 Na (EDTA), 5 HEPES, and 0.1 $CaCl_2*2H_2O$ (pH 7.4). Mesenteric arteries (diameter: 59 – 67 µm without pressure) were dissected and the perivascular fat was carefully removed.

3.2.2. Vessel mounting

Artery segment 2mm in length were dissected from second branch of mesenteric artery. They were mounted on two stainless wires (40 µm). Special attention was paid not to touch the artery wall and not to damage the endothelial cell layer while placing the

wires. The two wires were connected to two jaws and the artery was hold in the small vessel myograph (5 ml chamber, model 410A, DMT, Denmark). Jaws were connected to a force transducer and micrometer, respectively. To arrange optimal isometric conditions, arterial segments were gently stretched according to the procedure of Mulvany and Halpern (see chapter normalization) [53]. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 41:19–26). The experimental solution consisted of (in mmol L^{-1}): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4*7H_2O$, 6.1 glucose, 25 $NaHCO_3$, and 2.5 $CaCl_2*2H_2O$ (pH 7.4), under carbogen bubbling at 37℃.

3.2.3. Normalization

Normalization was performed after heating to 37°C and equilibration of the vessel. The arteries were stretched by use of the micrometer. The distending of the arterial segment was performed stepwise. Micrometer values (distance) and the corresponding force were converted into the internal circumference and the wall tension, respectively. The data were used to calculate an exponential curve describing the relation between the internal circumference and the wall tension. From the intersection of this exponential curve with a linear isobaric curve corresponding to 100 mmHg, normalized internal circumference was calculated [54]. To ensure that the vessels were stably equilibrated, they were incubated for another 30 min before performing the experiments (see 3.2.5).

3.2.4. Test vessel viability

The arterial segments were tested by applying K-PSS (in mmol L^{-1}): 123.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4·7H_2O$, 6.1 glucose, 25 $NaHCO_3$, 2.5 $CaCl_2·2H_2O$ (pH 7.4). Only vessels with an immediate response and stable contraction were included. Experimental protocols started after 10 min equilibration until a stable resting tension was achieved.

3.3. Protocols

All protocols were performed in vessels of juvenile and aged mice.

3.3.1. Assessment of endothelial function

1. Vessels were contracted with Phenylephrine (PE, 10-5 mol L-1, final concentration).

2. After reaching a stable contraction response, ACh was applied cumulatively (10^{-9}~10^{-6}mol L^{-1}) to the bath solution. The respectively next concentration was applied

after reaching a stable response for the current concentration. The relaxation was expressed as percent of the maximum pre-contraction level.

3.3.2. Assessment of role of NOS/NO for relaxation response to ACh

1. Vessels were incubated with Nω-nitro-L-arginine methylester hydrochloride (L-NAME, 10^{-4} mol L^{-1}, final concentration for 30 min) to inhibit endothelial NO synthase.

2. PE (10^{-5} mol L-1, final concentration) was applied for pre-contraction.

3. After reaching a stable contraction, ACh was added cumulatively (10^{-9}~10^{-6} mol L^{-1}).

3.3.3. Assessment of endothelium-independent relaxation

1. Vessels were incubated L-NAME (10^{-4} mol L^{-1}, final concentration) for 30 min to inhibit the endothelial NO production.

2. To pre-contract the vessel, a salt solution with 60 mmol L^{-1} KCl was applied (in mmol L^{-1}): 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4.7H_2O$, 11.1 glucose, 0.026 Na (EDTA), and 1.6 $CaCl_2$ $2H_2O$, (pH=7.4). In this protocol, KCl was used to induce contraction, because KCl (60 mmol L^{-1}) induces a more stable contraction than PE. This was necessary because of the long-term protocol for applying SNP to obtain the concentration-response.

3. Sodium nitroprusside (SNP, 10^{-11} to 10^{-5} mol L^{-1}) was added after reaching a stable contraction.

3.3.4. Measurement of time-response to SNP

1. Vessels were incubated with L-NAME (10^{-4} mol L^{-1}, final concentration) for 30 min to inhibit endothelial NO production.

2. Vessels were pre-contracted with PE (10^{-5} mol L^{-1}, final concentration).

3. SNP was applied as a bolus (10^{-5} mol L^{-1}) for 10 min.

3.3.5. Measurement of time-response to runcaciguat

1. Vessels were incubated with L-NAME (10^{-4} mol L^{-1}, final concentration) for 30 min to inhibit endothelial NO production.

2. Vessels were pre-contracted with PE (10^{-5} mol L^{-1}, final concentration).

3. After reaching a stable pre-contraction, sGC activator runcaciguat (10^{-5} mol L^{-1}) was applied as a bolus for 10 min.

3.3.6. Measurement of phosphodiesterase 5 (PDE5) inhibitor action

1. Vessels were pre-contracted with PE (10^{-5} mol L^{-1}, final concentration).

2. The PDE5 inhibitor sildenafil was cumulatively applied (10^{-9}~10^{-6} mol L^{-1}). The application of the respective next concentration was performed in the steady state situation of the current concentration.

3.4. Data acquisition

The wire myography chamber was connected to an interface unit for direct data streaming. The unit contains the heat control and the calibration procedure algorithm and works for man-unit interaction. The interface was connected to a bio-signal acquisition system (Bio Amp from, AD Instruments Ltd. Spechbach, Germany). Data were further processed, acquired and optically presented by the software Chart5 (ADInstruments Ltd. Spechbach, Germany).

3.5. Pharmacological agents

Drugs ACh, PE, L-NAME (all purchased from Sigma, USA) as well as SNP purchased from Carl Roth, Germany were dissolved in distilled water and stored at -20°C. Sildenafil (BioVision, USA) and runcaciguat (gently provided from Bayer AG, Research & Development, Pharmaceuticals, Germany) were solved in dimethylsulphoxide (DMSO, Thermo Scientific, USA) and vials with stock solution were stored at -20°C. The end concentration of DMSO in the experimental solution was lower than 0.1%. L-NAME was dissolved in distilled water under ultrasound and stored at -20 ℃. SNP powder was stored in dark tube at room temperature and solved shortly before the experiment. All ingredients for buffer solutions were purchased from Carl Roth, Germany and dissolved in distilled water and stored at 4°C.

3.6. cGMP ELISA measurement

The direct cGMP ELISA kit (Enzo life Science, Lausen, Switzerland) and multi-mode reader (BioTek Synergy HTX, Bad Friedrichshall, Germany) were used to measure intracellular cGMP concentration according to manufacturer's instructions. For this purpose, mesenteric arteries were dissected and pooled to reach a minimum weight of 25 mg of tissue sample. The protocol for artery preparation corresponds to that described in 3.3.5, however, without stretching arteries in the wire myograph. Vessels were pretreated with L-NAME (10^{-4} mol L^{-1}, 30min), pre-contracted with PE (10^{-5}mol L^{-1}) and

then the sGC activator (10^{-6} mol L^{-1}) was applied as a bolus. After 2 min, samples (mesenteric arteries) were shock frozen using liquid nitrogen and kept at -80℃ until use.

3.7. mRNA expression of key enzymes

The whole mesenteric arterial tree was dissected from each mouse and stored in liquid nitrogen at -80℃. RNA-Bee-reagent (Biozol, Eching, Germany) was used to extract RNA. Samples were homogenized and separated into aqueous and organic phase. The total RNA was reverse transcribed into cDNA using High Capacity cDNA RT-Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed by Lightcycler LC480 (Roche Diagnostics, Mannheim, Germany) to detect relative amplified DNA through hydrolysis probe fluorescent detection. Housekeeping gene RPL32 was chosen to normalize mRNA expression levels, the expression of interest genes (nNOS, iNOS, eNOS, GUCY1a1, GUCY1a2, GUCY1b1, GUCY1b2, PDE3a, PDE3b, PDE5) were calculated by ΔCt method. (Primer sequences: see **Table 1**)

Table 1. List of primers

Gene	Primer/ Probe	Sequence (5'-3')
nNOS	Forward	CCCGACAGGCCAAAGAAATA
	Reverse	ACGTCCCCGCAGACATAAAT
iNOS	Forward	AGCGGCTCCATGACTCCCA
	Reverse	GGCACCCAAACACCAAGCT
eNOS	Forward	GACCCTCACCGCTACAACAT
	Reverse	ATGAGGTTGTCCTGGTGTCC
GUCY1a1	Forward	ATTTCATGCTGGACCGAGAC
	Reverse	TTCCCTTGGAAGTCCCTCTT
GUCY1a2	Forward	CTGGACTCACTAGGCGAAAG
	Reverse	GTCATGTGTATCGTCTGAGGC
GUCY1b1	Forward	AGCCCTTACACCTTCTGCAA
	Reverse	CATTGCCACACTGAGTGACC
GUCY1b2	Forward	CAGGTGTTGTGGGAGACAAG
	Reverse	TCCTAGAGGCCGTGTTTACG
PDE3a	Forward	TTCCTGGCCTCCCAAGTGT
	Reverse	CCGTGTGTAAATCCACTGTCAGA
PDE3b	Forward	ATGGCTACCGGGACATTCC
	Reverse	GGGTTGTCAAATACCAAACAGCAT
PDE5	Forward	CCGACTTCAGCTTCAGTGACTT
	Reverse	GGTCAGTGAACATCCGAATTG
RPL32	Forward	TTCATCAGGCACCAGTCAGA
	Reverse	TTGTCAATGCCTCTGGGTTT

3.8. Vessel morphometry

The second branch of the mesenteric artery as well as thoracic and abdominal aorta samples were fixed with 4% buffered formalin (SAV Liquid Production GmbH, Germany) for 2h at room temperature. Samples were moved to the refrigerator and stored for 24h at 4 ℃. After washing with 1× PBS with following composition (in mmol L^{-1}): 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4 (pH 7.4), samples were stored in 30% sucrose solution

at 4 ℃. Dehydration was performed in rising alcohol series: 70%, 80%, 96%, 99%, 99% (Leica TP 1020). To help visualization of blood vessels, which were dehydrated, 2 ml of 2% eosin was added into 96% ethanol. Later, xylol was used to remove ethanol to make samples dissolving and embedding in paraffin (Modular Tissue Embedding Center EC 350). Paraffin blocks were cooled at -20 ℃ at least 2 hours before slicing. Samples were sliced 4µm for aorta and 6µm for mesenteric arteries via microtome (Thermo Scientific Microm HM355S) and sections were transferred to microscope slides with help of wet paint brush. Slides were then dipped in hot-water bath to swell up the sections. After that, sections were put in heating oven (Memmert SFP 400), 60℃, at least 6 hours or overnight to dewax for staining. Van Gieson staining (1% acid fuchsin mixed with picric acid according to 1:10) was used to show elastic fibers in black. Samples' inner and outer diameter were calculated via Image J 1.48 (digital images: Color Camera Nikon DS-Ri2 Numerical Aperture). They served for calculation of media lumen ratio (wall area: lumen area).

3.9. Statistics

Raw data were initially stored in Excel database. The calculation of relaxation in percent was based on the difference between stable contraction tension (T) and tension at maximum relaxation for each dose (Rmax). Relaxation was expressed by R%=(T-Rmax)/T ×100. Functional data were presented as mean ± SEM. Shapiro–Wilk test was used to test normal distribution. In case of non-normal distribution, Brunner test, similar to a nonparametric two-way ANOVA, was performed to test general difference in the concentration response between two groups [55]. Mann–Whitney U test (GraphPad Prism 9.0.1) was used for post hoc comparisons. P-value <0.05 was considered significant. EC_{50} was calculated using concentration-response data using GraphPad Prism. Median, box, maximal and minimum whiskers were used to present contraction power, histological parameters, and mRNA expression.

4. Results

4.1. Aged-related endothelial dysfunction

Isolated mesenteric artery rings were pre-contracted by PE (10^{-5} mol L^{-1}) and exposed to ACh (10^{-9} to 10^{-6} mol L^{-1}) to test the endothelium-derived vasorelaxation in different age groups (**Figure 1A**). Dilatation to ACh was reduced in aged animals (**Figure 1B**). To evaluate the role of NO in the ACh-induced dilatation vessels were pre-treated with the non-specific NOS-blocker L-NAME (10^{-4} mol L^{-1}) (**Figure 1C**). Under these conditions, the relaxation to ACh weakened and did not show significant differences comparing juvenile and aged animals, suggesting a prominent role of NO for reduced relaxation in aged animals (**Figure 1D**). qPCR was performed to get closer look at eNOS and nNOS mRNA expression. The expression of NOS isoforms was similar in both groups (**Figure 1E, F**).

4.2. Age-related responses to sGC activation

To test the NO downstream signalling pathways in VSMC, SNP (10^{-11} to 10^{-5} mol L^{-1}) was cumulatively applied after pre-constriction with 60mmol L^{-1} KCl solution. In addition, vessels were pre-treated with L-NAME (10^{-4} mol L^{-1}) to block endothelial NO-production. The concentration-responses to SNP were similar in juvenile and aged vessels (**Figure 2A, B**). With the aim to investigate the dynamics of sGC function, SNP or sGC activator runcaciguat were applied as bolus for 10 minutes. Remarkably, vessel relaxation to runcaciguat bolus application was faster in juvenile compared to aged arteries within the first 2 min of application. SNP bolus induced dilatation was also faster in juvenile vessels (**Figure 3A, B, C, D**). Since sGC exerts its effect via cGMP production, cGMP concentrations were determined in the juvenile and aged vessels after stimulation with runcaciguat. The cGMP-concentration was smaller in aged compared to juvenile vessels in the time interval of 2 min after agonist application (**Figure 3F**). The mRNA expression of sGC subunits α_1 and α_2 was lower in aged vessels than in juvenile ones. No difference could be seen for the β_1-subunit (**Figure 3E**).

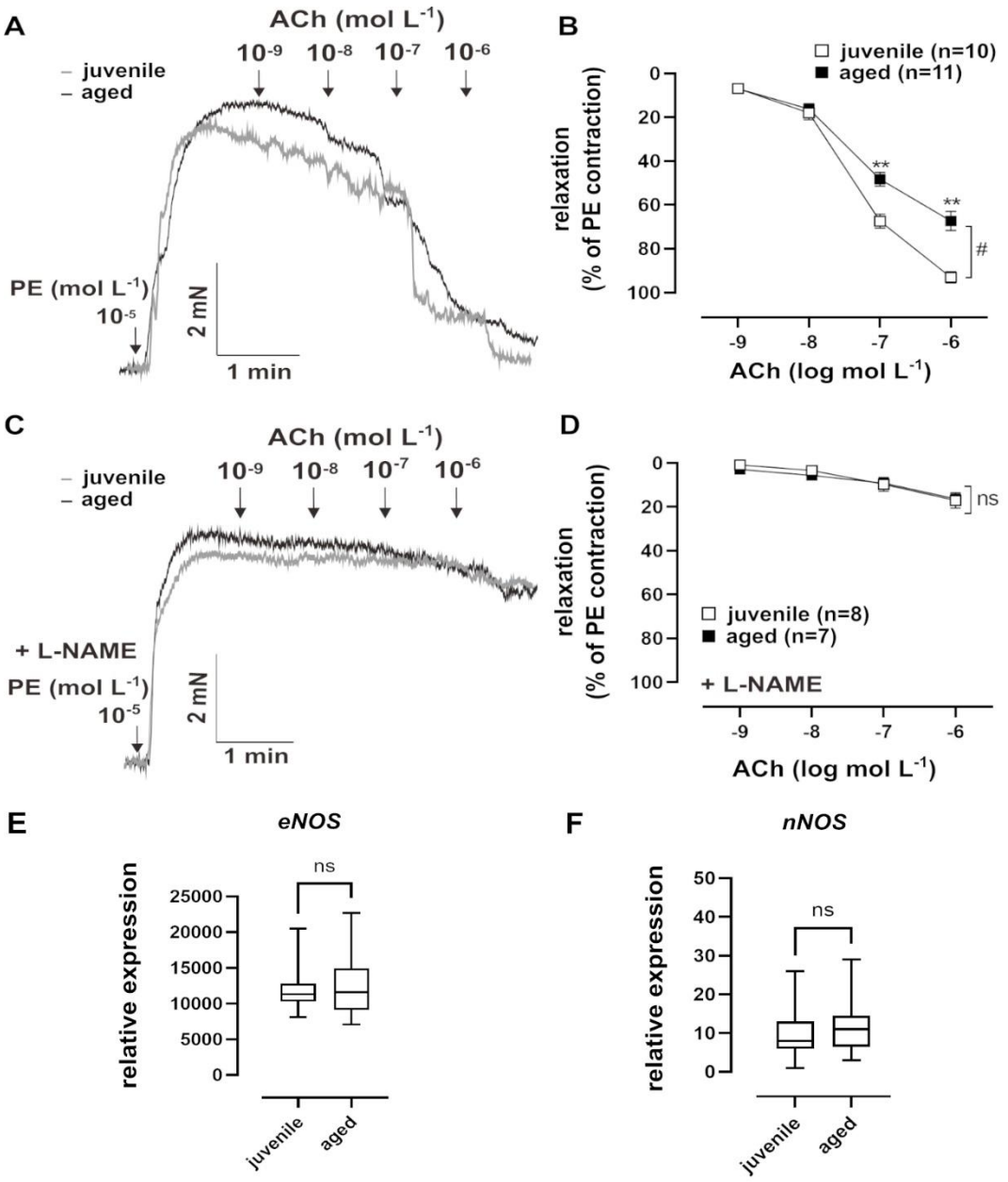

Figure 1. Aging affects endothelium-dependent relaxation.

Acetylcholine (ACh)-induced, relaxation after pre-contraction with phenylephrine (PE, 10^{-5} mol L^{-1}) was weaker in aged vessels. **A**: Original traces from a juvenile and aged vessel. **B**: Concentration-response curves (mean ± SEM) for ACh. **C**: Representative original traces for relaxation of L-NAME (10^{-4} mol L^{-1}) pre-treated vessels. **D**: Concentration-response-curves for juvenile and aged vessels after L-NAME pre-treatment (mean ± SEM). Relaxation did not differ between both groups. **E** and **F**: mRNA expression of eNOS and nNOS, respectively, in juvenile and aged vessels (juvenile: n=23; aged: n=17) (# $p < 0.05$, Brunner test, ** $p < 0.01$, Mann-Whitney test). This figure was cited from [1].

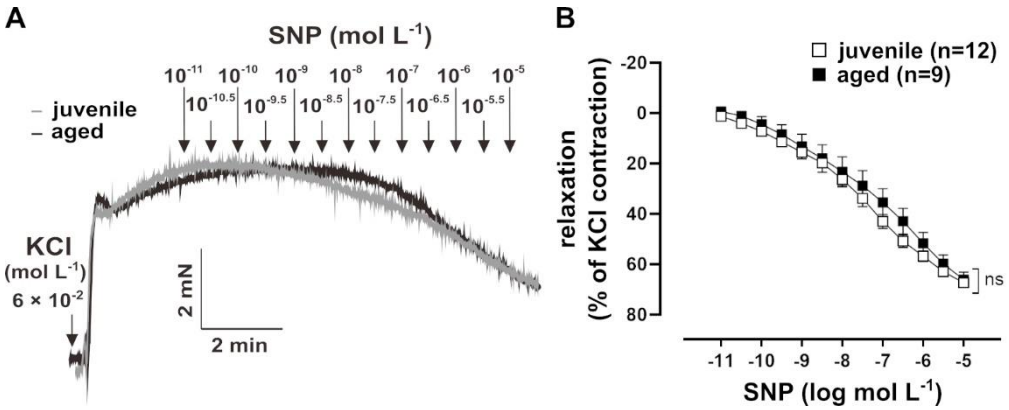

Figure 2. Age-related endothelium-independent vasorelaxation.

A: Representative original traces showing the effect of 60 mmol L^{-1} KCl and the vessel response to cumulative sodium nitroprusside application (SNP, 10^{-11}- 10^{-5} mol L^{-1}) after L-NAME (10^{-4} mol L^{-1}) pre-treatment. **B:** Concentration-response curve for SNP for juvenile and aged vessels (mean ± SEM). This figure was cited from [1].

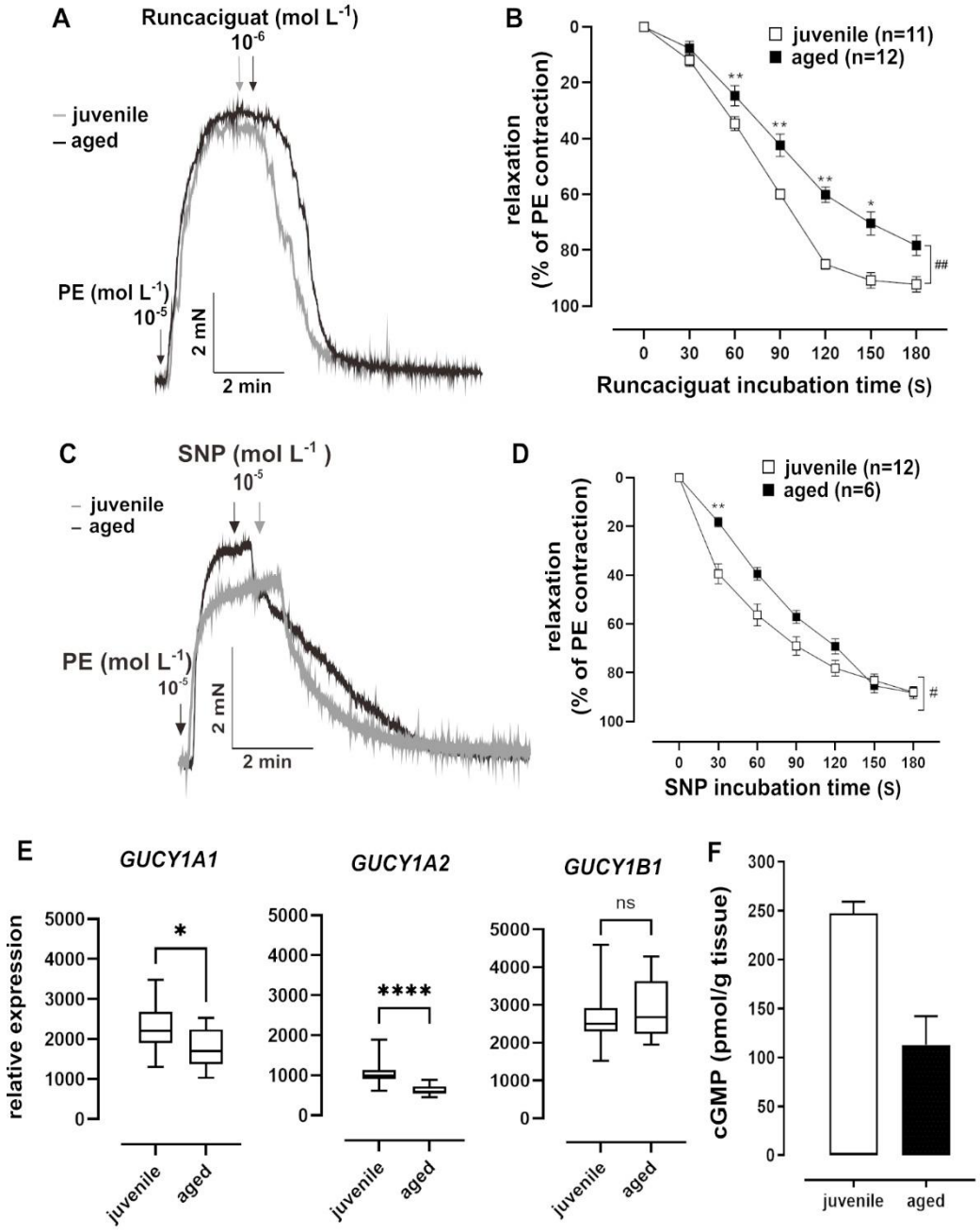

Figure 3. Dynamics of sGC activation.

A: Representative original traces for the response to runcaciguat (bolus, 10^{-6} mol L^{-1}) after L-NAME (10^{-4} mol L^{-1}) pre-treatment and phenylephrine (PE, 10^{-5} mol L^{-1})-pre-constriction. **B**: Response to runcaciguat bolus application (mean ± SEM) in juvenile and aged vessels: **C**: Representative responses to sodium nitroprusside (SNP, 10^{-5} mol L^{-1}) after L-NAME (10^{-4} mol L^{-1}) pre-treatment and phenylephrine (PE, 10^{-5} mol L^{-1}) pre-constriction. **D**: Response to SNP (mean ± SEM)**. E**: Expression of sGC subunits α_1 (GUCY1A1), α_2 (GUCY1A2), and β_1 (GUCY1B2) (juvenile: n=16; aged: n=10). **F**: cGMP concentration in mesenteric vessels after 2 min stimulation with runcaciguat (juvenile:

n=17 mice, 2 pooled samples; adult: n=9 mice, 2 pooled samples) (# $p < 0.05$, ## $p < 0.01$, Brunner test, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney test). This figure was cited from [1]

4.3. Age-related effects of PDE5 inhibition

Inhibition of the cGMP specific PDE5 using sildenafil in cumulative concentrations resulted in a concentration dependent relaxation in PE (10^{-5} mol L^{-1}) pre-contracted vessels. The relaxation was stronger in juvenile compared to aged vessels (**Figure 4A, B**). PDE3a and PDE5 mRNA-expression were lower in aged vessels, as it was for the PDE3a (**Figure 4C, E**). In contrast, the PDE3b expression was higher in aged vessels (**Figure 4D**).

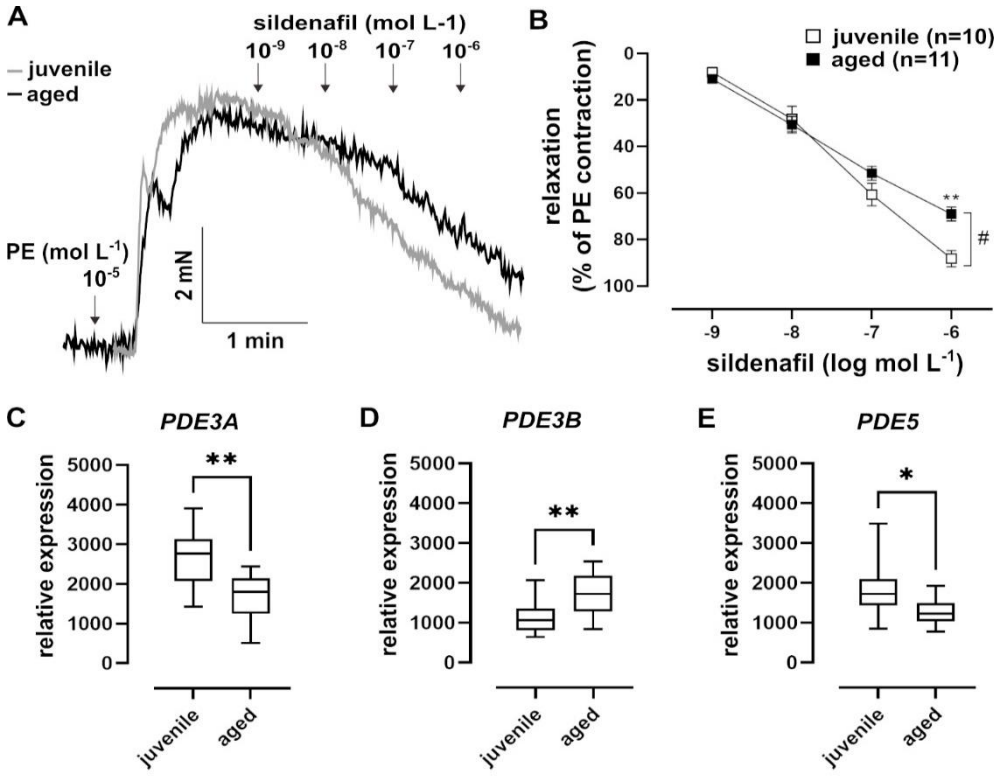

Figure 4. Age-related responses to PDE5 inhibition.

A: Representative original traces showing the response to sildenafil (10^{-9}-10^{-6} mol L^{-1}) after pre-contraction with phenylephrine (PE, 10^{-5} mol L^{-1}). **B**: Concentration response curves for juvenile and aged vessels (mean ± SEM). **C, D, E**: Expression for PDE3a, PDE3b, and PDE5, respectively, on the mRNA level (juvenile: n=16; aged: n=10) (#, $p < 0.05$, Brunner test, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney test). This figure was cited from [1].

4.4. Vessel histology

Vessel histology was investigated to check if development of vascular dysfunction during aging goes along with structural changes (remodelling). Neither resistance arteries (small mesenteric arteries) nor the aorta (large conduit artery) showed significant changes in their structure (**Figure 5A, B, C, D**). The media-to-lumen ratios remained similar in aged vessels compared to juvenile vessels. There were no signs of atherosclerosis or fibrosis (**Figure 5E**).

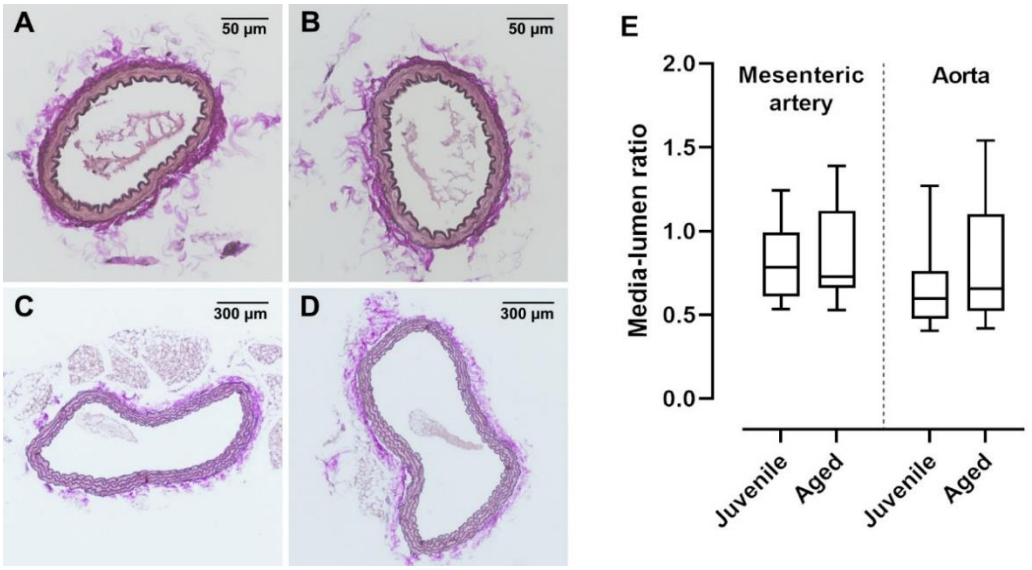

Figure 5. Histology in mouse mesenteric artery and aorta.

A, B: Van-Gieson staining of two small mesenteric arteries from juvenile and aged animals (magnification x120). **C, D**: Van-Gieson staining of two aortas from juvenile and aged animals (magnification x23). **E**: Media-lumen-ratio of aorta and small mesenteric arteries in juvenile and aged vessels (juvenile: n=26; aged: n=31 in mesenteric arteries; juvenile: n=20; aged: n=19 in aorta). This figure was cited from [1].

4.5. PE induced contraction

With the aim to investigate the ability of vessels to react to PE, we calculated the mean of all phenylephrine (PE, 10^{-5} mol L^{-1}) induced contractions for juvenile and aged vessel. PE induce contraction were normalized to the response of the vessel to potassium chloride solution (K-PSS, 123.7 mmol L^{-1}) in the beginning of the experiment The response to PE was similar in juvenile and aged vessels (118.56 ± 7.92% (juvenile, n = 37) vs. 120.61 ± 5.64 (aged, n = 31), Mann–Whitney test, $p > 0.05$) (**Figure 6. A**). After

pre-treatment with the eNOS inhibitor (L-NAME, 10^{-4} mol L^{-1}, 30 min), PE induced contractions were increased by 24% in juvenile samples (Mann–Whitney test, $p < 0.001$), but reduced 21% in aged ones (Mann–Whitney test, $p < 0.05$), (146.62 ± 6.71 (juvenile, n=18) vs. 99.71 ± 7.60 % (aged, n=13), Mann–Whitney test, $p < 0.001$) (**Figure 6. B**).

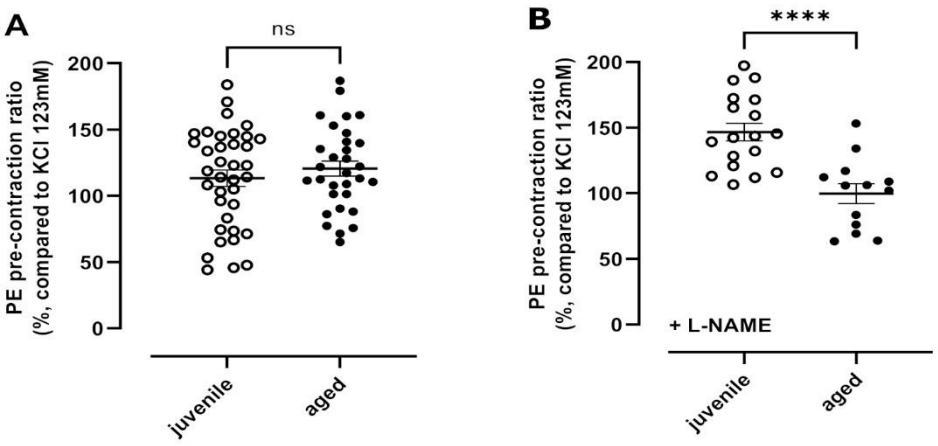

Figure 6. Age-related vessel contraction to PE.

A: Small mesenteric artery response to phenylephrine (PE, 10^{-5} mol L^{-1}) in juvenile and aged vessels. **B**: Small mesenteric artery response to PE (10^{-5} mol L^{-1}) in juvenile and aged vessels after L-NAME (10^{-4} mol L^{-1}) pre-treatment for 30 min.

5. Discussion

The results of the study show an impaired dilatory function in small mesenteric arteries during aging, which precedes the appearance of histological changes. Data suggest that the affected dilatory capacity is due to an impaired sGC function in the vascular smooth muscle cell.

5.1. Endothelium-dependent vasorelaxation during aging

The NO-sGC-cGMP-axis is the main dilator system in large and small arteries. Reduced dilatation, which can be measured by increasing the blood flow through the vessel (flow mediated dilation) is a marker of endothelial dysfunction and a predictor of cardiovascular diseases [56-58]. Aging and development of endothelial dysfunction are associated. Oxidative stress and inflammation are important pathophysiological factors in the process of vessel aging [59]. In our study, we observed a reduced ACh induced endothelium-dependent relaxation already at age of 40-41 weeks (aging animals). Inhibition of NOS-isoforms resulted in similar relaxation in juvenile and aging vessels. This finding points at an important role of the NO-sGC-cGMP-axis in the course of aging. The mRNA expression for eNOS did not differ between juvenile and aging arteries, suggesting that eNOS expression may not be responsible for reduced dilatation in our study. The finding is in agreement with that in soleus arterioles of 22-month-old Fischer 344 rats, where eNOS mRNA expression did not change with aging. However, in the cited study, the eNOS protein was enhanced in the older rat [60]. In WKY rats, aortic eNOS expression did not change significantly until an age of 63 weeks, while iNOS mRNA and protein expression increased [61]. Further, the eNOS content in abdominal aorta, iliac artery and femoral artery was not different comparing 4- and 20-months-old Fisher 344 rats, although relaxation was impaired in old animals [62]. Other studies showed reduced NO bioavailability going along with decreased eNOS expression [63]. Interestingly, enhanced eNOS mRNA expression has been seen in old human saphenous veins and in the aging brain in rats [64, 65]. These observations indicate that aging affects eNOS mRNA and protein expression differently in the animal models and in human. Posttranscriptional modification of NOS that occur with aging may be accountable for the inconsistent results concerning mRNA and protein expression. We studied all NOS subtypes. The eNOS is the dominant isoform in the endothelial cells, while nNOS is predominantly expressed in the central nervous system and contributes to neuronal

function. The expression of iNOS depends on the cytokine level and is low under physiological conditions [66]. In our study, nNOS expression was very low and iNOS was not detectable. The missing iNOS expression fits to the observation of no atherosclerosis in the mesenteric arteries at age of 40 weeks.

5.2. Impaired signalling pathway in smooth muscle cell with aging

In the process of vascular aging, endothelial cells and VSMC reorganize to adapt to the changed requirements. These are increased blood pressure, changed metabolite composition including lipids and other more. Endothelial dysfunction is a hallmark of vessel aging and goes along with decreased NO-bioavailability [67]. Remarkably, NO is not only a vasodilator, it can also protect cells from proliferation [68]. Reduced ACh induced dilatation in aged vessels our study point at decreased endothelial and/or VSMC function. Data about the impairment of VSMC function are rare for the period of aging until the 40 weeks. To clarify the mechanisms, we investigated the sGC-cGMP pathway in VSMC and tested the hypothesis of a reduced function in mouse during aging. The relaxation response of juvenile and aged vessels to the cumulatively applied NO-donor SNP was similar, i.e. concentration-response curves did not differ. Thus, sGC and downstream pathways have similar sensitivity to the natural agonist of sGC. However, a test of the dynamic behaviour of sGC activation by bolus application and follow up of vessels tone over minutes, revealed a faster response in juvenile arteries compared to age vessels. The difference was strongest 30 sec after NO application. We interpret this response as a reduced action of sGC or downstream pathways in VSMC. Treatment of arteries with the artificial sGC activator runcaciguat led to the same result, namely a faster relaxation in juvenile vessel. The maximum difference was at 2 min after bolus application. At this time point, we determined the sGC concentration in the vessel and found higher values in the juvenile group. It seems that the sGC rather than the downstream pathways are responsible for the slower response to the NO or runcaciguat. However, a changed cGMP-specific PDE activity has also to be taken into consideration. Endothelium-independent, SNP-induced relaxation is rarely examined during aging. In a study in human elderly arterioles, SNP-induced vasodilatation of gastric submucosal arterioles was significantly decreased compared with that from young patients [69]. Aging impaired the endothelium-independent vasorelaxation induced by sGC activator YC-1 in old spontaneously hypertensive rats, while relaxation was normal in old WKY rats [34]. The protein expression of α- and β-subunits did not differ between 6 weeks- and 17 month-

old WKY rats. Surprisingly, the cGMP content in aortas of WKY rats was higher in older rats after sGC stimulation with YC-1. This phenomenon has been interpreted as an adaptive mechanism compensating the reduced endothelium dependent vasodilatation in aged vessels [34].

The sCG is a protein heterodimer consisting of α and β subunits, both of them with doubled genes giving α_1, α_2, β_1, and β_2 subunits. The enzyme is abundantly expressed in kidney, lung, liver, cerebrum, heart, and muscle [70, 71]. Combinations of α_1, and α_2, respectively, with β_1 are most frequent [28, 71]. The β_1 subunit is crucial for sGC function, because β_1 deficiency leads to complete loss of function. Deficiency of α_1- or α_2- subunit reduces the sGC function [72-76]. The sGC α_1/β_1 variant is important for blood pressure modulation in mice [77]. We found reduced mRNA expression of α_1- and α_2-subunits in the aged mice and this could explain the reduced sGC function in our aging model. The β_1-mRNA did not differ comparing juvenile and aged vessels. In 17 months old rats, mRNA expression of α_1 and β_1 were lower than young aortic tissue [33], while sGC expression was increased in the aorta of aging rats in another study [34]. The results also differ when comparing rat lung, where the expression increased during aging, with the heart showing decrease of sGC expression [78]. Reduced sGC expression may contribute to the development of salt-sensitive hypertension. The sGC β_1 mRNA expression was reduced and the sGC β_2 was increased in kidneys of Dahl salt-sensitive rats. Consequently, the NO-insensitive sGC α_1/β_2 was stronger represented [79]. Altogether, the expression studies suggest an important role of sGC for vascular dilatory function in physiology and pathophysiology.

The cGMP-specific PDE5 is abundantly expressed in vascular smooth muscle cells [80, 81]. The cGMP-hydrolysing activity of PDE5 is an important factor in the control of cellular cGMP concentration [82]. In addition, PDE3 with its mixed specificity for cAMP and cGMP contributes to the modulation of cGMP levels [83]. We found a similar expression on the mRNA level for PDE3A, B, and PDE5, respectively, in juvenile mesenteric arteries. During aging, PDE5 and PDE3A expression decreased; that of PDE3B increased. The decrease in PDE5 and PDE3A expression could be an adaptive reaction to the decreased sGC activity during aging. The concentration-response to sildenafil (PDE5 inhibitor) was less in aging compared to juvenile animals. This observation supports the idea or decreased sGC function in aged animals, however, it may also be a consequence of the lower PDE5 expression. There are not many studies

about PDE5 expression during aging. In senescent human VSMC, PDE5 mRNA expression was 2.3-fold elevated [84]. PDE5 expression was increased in older human Leydig cells [85]. The few studies available do not allow conclusions concerning the effect of aging on PDE5 expression in VSMC.

5.3. Vascular morphology in aging

Our study reveals a decreased endothelial induced dilatation already at age of 40-41 weeks. Data suggest that this reduction is due to an impairment of the NO-system function with aging. The observation agrees with several other studies in animal models and in humans [86-88]. An endothelial dysfunction seems to contribute to this behaviour. However, we observed an impaired function of the NO signalling in VSMC, too. The finding points at a decreased dilatory ability in aging vessels, which is due to functional deficiencies in the endothelial as well in VSMC. At the same time, structural changes were not detectable with classical histological methods in aging small mesenteric arteries and aorta. Thus, functional impairment precedes the structural changes during aging. i.e. the transition from a “contractile to synthetic” type of arteries [35]. The remodelling of arteries in older ages is characterized by proliferation of VSMC and vessel calcification resulting in increased arterial stiffness [35]. Thickening of the intima accompanies the remodelling of the media and increases the susceptibility to atherosclerosis [89-92].

5.4. Contractile properties during aging

In this study, PE (10^{-5} M) induced similar contractile responses (in % of maximum KCl induced contraction) in juvenile and aged arteries. However, after inhibition of NOS by L-NAME vessels PE contraction was stronger in juvenile group compared to in the aged group. This indicates a reduced NO-bioavailability, which refers to endothelial dysfunction with aging, and suggest a reduced contractile ability of aged vessels to PE. Phenylephrine (PE) induces vasocontraction through selective stimulation of α_1-adrenoceptors in vascular smooth muscle cells. Receptors activate phospholipases (PLC, PLA2, PLD). In addition, calcium channels, sodium/proton and sodium/calcium exchanger are activated. Signalling modulates potassium channels [93]. The main response to PE is an increase in cytosolic calcium levels. There are differences in the expression of α_1-adrenoceptor subtypes and the efficiency in response to agonists [94]. The reduced contractile response to adrenoceptor agonists can be due to decreased receptor density, changed signalling, or alterations in the contractile apparatus [95]. Studies show a changed calcium homeostasis [96, 97]. Reduced activity of the sodium/potassium pump may

contribute to reduced contractile responses in aged animals [98]. Further, reduced L-Type calcium channel expression [99], impaired interaction of voltage-dependent Ca^{2+} channel Cav3.2 and the RyR channel contribute to age related reduction in the contractile function [100]. It has also been shown that calcium spark generation depends on Cav1.2 and Cav3.2 channels and that latter do not contribute to spark generation in old mice [100, 101]. All these structures may play a role for the decreased adrenoceptor mediated contractions in the aging animal.

6. Conclusion

Our investigation of endothelial and endothelial independent vasodilation point at a reduced function of the sGC, which may contribute to the impairment of endothelial, ACh induced relaxation in aging vessel (40-41 weeks). The data show that, signalling pathways via the sGC are less functional in a relatively early stage of aging compared to juvenile vessels. The slower relaxation response to exogenously applied NO as well as to the sGC activator runcaciguat within 2 min after application may influence cardiovascular regulation in the aging animal. In addition, we observed decreased responses to PE, suggesting an aging process for signalling pathways for PE and probably other agonists. It is worthy of note that these functional changes do not go along with structural changes in the vessels in this period of aging, supporting the finding that endothelial dysfunction precedes the vessel remodelling. Our study adds the finding of reduced sGC function to the pathophysiology of aging arteries.

7. Bibliography

1. Zhong, C., M. Xu, S. Boral, H. Summer, F.B. Lichtenberger, C. Erdogan, M. Gollasch, S. Golz, P.B. Persson, J. Schleifenbaum, A. Patzak, and P.H. Khedkar, *Age Impairs Soluble Guanylyl Cyclase Function in Mouse Mesenteric Arteries.* Int J Mol Sci, 2021. **22**(21).

2. Lozano, R., M. Naghavi, K. Foreman, S. Lim, K. Shibuya, V. Aboyans, J. Abraham, T. Adair, R. Aggarwal, S.Y. Ahn, M. Alvarado, H.R. Anderson, L.M. Anderson, K.G. Andrews, C. Atkinson, L.M. Baddour, S. Barker-Collo, D.H. Bartels, M.L. Bell, E.J. Benjamin, D. Bennett, K. Bhalla, B. Bikbov, A. Bin Abdulhak, G. Birbeck, F. Blyth, I. Bolliger, S. Boufous, C. Bucello, M. Burch, P. Burney, J. Carapetis, H. Chen, D. Chou, S.S. Chugh, L.E. Coffeng, S.D. Colan, S. Colquhoun, K.E. Colson, J. Condon, M.D. Connor, L.T. Cooper, M. Corriere, M. Cortinovis, K.C. de Vaccaro, W. Couser, B.C. Cowie, M.H. Criqui, M. Cross, K.C. Dabhadkar, N. Dahodwala, D. De Leo, L. Degenhardt, A. Delossantos, J. Denenberg, D.C. Des Jarlais, S.D. Dharmaratne, E.R. Dorsey, T. Driscoll, H. Duber, B. Ebel, P.J. Erwin, P. Espindola, M. Ezzati, V. Feigin, A.D. Flaxman, M.H. Forouzanfar, F.G. Fowkes, R. Franklin, M. Fransen, M.K. Freeman, S.E. Gabriel, E. Gakidou, F. Gaspari, R.F. Gillum, D. Gonzalez-Medina, Y.A. Halasa, D. Haring, J.E. Harrison, R. Havmoeller, R.J. Hay, B. Hoen, P.J. Hotez, D. Hoy, K.H. Jacobsen, S.L. James, R. Jasrasaria, S. Jayaraman, N. Johns, G. Karthikeyan, N. Kassebaum, A. Keren, J.P. Khoo, L.M. Knowlton, O. Kobusingye, A. Koranteng, R. Krishnamurthi, M. Lipnick, S.E. Lipshultz, S.L. Ohno, J. Mabweijano, M.F. MacIntyre, L. Mallinger, L. March, G.B. Marks, R. Marks, A. Matsumori, R. Matzopoulos, B.M. Mayosi, J.H. McAnulty, M.M. McDermott, J. McGrath, G.A. Mensah, T.R. Merriman, C. Michaud, M. Miller, T.R. Miller, C. Mock, A.O. Mocumbi, A.A. Mokdad, A. Moran, K. Mulholland, M.N. Nair, L. Naldi, K.M. Narayan, K. Nasseri, P. Norman, M. O'Donnell, S.B. Omer, K. Ortblad, R. Osborne, D. Ozgediz, B. Pahari, J.D. Pandian, A.P. Rivero, R.P. Padilla, F. Perez-Ruiz, N. Perico, D. Phillips, K. Pierce, C.A. Pope, 3rd, E. Porrini, F. Pourmalek, M. Raju, D. Ranganathan, J.T. Rehm, D.B. Rein, G. Remuzzi, F.P. Rivara, T. Roberts, F.R. De Leon, L.C. Rosenfeld, L. Rushton, R.L. Sacco, J.A. Salomon, U. Sampson, E. Sanman, D.C. Schwebel, M. Segui-Gomez, D.S. Shepard, D. Singh, J. Singleton, K. Sliwa, E. Smith, A. Steer, J.A. Taylor, B. Thomas, I.M. Tleyjeh, J.A. Towbin, T. Truelsen, E.A. Undurraga, N. Venketasubramanian, L. Vijayakumar, T. Vos, G.R. Wagner, M. Wang, W. Wang, K. Watt, M.A. Weinstock, R. Weintraub, J.D. Wilkinson, A.D. Woolf, S. Wulf, P.H. Yeh, P. Yip, A.

Zabetian, Z.J. Zheng, A.D. Lopez, C.J. Murray, M.A. AlMazroa and Z.A. Memish, *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2095-128.

3. North, B.J. and D.A. Sinclair, *The intersection between aging and cardiovascular disease.* Circ Res, 2012. **110**(8): p. 1097-108.

4. Heidenreich, P.A., J.G. Trogdon, O.A. Khavjou, J. Butler, K. Dracup, M.D. Ezekowitz, E.A. Finkelstein, Y. Hong, S.C. Johnston, A. Khera, D.M. Lloyd-Jones, S.A. Nelson, G. Nichol, D. Orenstein, P.W. Wilson, Y.J. Woo, C. American Heart Association Advocacy Coordinating, C. Stroke, R. Council on Cardiovascular, Intervention, C. Council on Clinical, E. Council on, Prevention, A. Council on, Thrombosis, B. Vascular, C. Council on, C. Critical, Perioperative, Resuscitation, N. Council on Cardiovascular, D. Council on the Kidney in Cardiovascular, S. Council on Cardiovascular, Anesthesia, C. Interdisciplinary Council on Quality of, and R. Outcomes, *Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association.* Circulation, 2011. **123**(8): p. 933-44.

5. Fuchs, F.D. and P.K. Whelton, *High Blood Pressure and Cardiovascular Disease.* Hypertension, 2020. **75**(2): p. 285-292.

6. Safar, M.E., B.I. Levy, and H. Struijker-Boudier, *Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases.* Circulation, 2003. **107**(22): p. 2864-9.

7. Mitchell, G.F., Y. Lacourciere, J.P. Ouellet, J.L. Izzo, Jr., J. Neutel, L.J. Kerwin, A.J. Block, and M.A. Pfeffer, *Determinants of elevated pulse pressure in middle-aged and older subjects with uncomplicated systolic hypertension: the role of proximal aortic diameter and the aortic pressure-flow relationship.* Circulation, 2003. **108**(13): p. 1592-8.

8. Antikainen, R., P. Jousilahti, and J. Tuomilehto, *Systolic blood pressure, isolated systolic hypertension and risk of coronary heart disease, strokes, cardiovascular disease and all-cause mortality in the middle-aged population.* J Hypertens, 1998. **16**(5): p. 577-83.

9. Berglund, G., O. Andersson, and L. Wilhelmsen, *Prevalence of primary and secondary hypertension: studies in a random population sample.* Br Med J, 1976. **2**(6035): p. 554-6.

10. Kotliar, C., S. Obregon, M. Koretzky, F. Botto, A. Di Leva, M. Boscaro, A. Ali, and K.C. Ferdinand, *Improved identification of secondary hypertension: use of a systematic protocol.* Ann Transl Med, 2018. **6**(15): p. 293.
11. Sawabe, M., *Vascular aging: from molecular mechanism to clinical significance.* Geriatr Gerontol Int, 2010. **10 Suppl 1**: p. S213-20.
12. Laurent, S., M. Briet, and P. Boutouyrie, *Large and small artery cross-talk and recent morbidity-mortality trials in hypertension.* Hypertension, 2009. **54**(2): p. 388-92.
13. McEniery, C.M., I.B. Wilkinson, and A.P. Avolio, *Age, hypertension and arterial function.* Clin Exp Pharmacol Physiol, 2007. **34**(7): p. 665-71.
14. El Assar, M., J. Angulo, and L. Rodriguez-Manas, *Oxidative stress and vascular inflammation in aging.* Free Radic Biol Med, 2013. **65**: p. 380-401.
15. Donato, A.J., I. Eskurza, A.E. Silver, A.S. Levy, G.L. Pierce, P.E. Gates, and D.R. Seals, *Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB.* Circ Res, 2007. **100**(11): p. 1659-66.
16. Fritze, O., B. Romero, M. Schleicher, M.P. Jacob, D.Y. Oh, B. Starcher, K. Schenke-Layland, J. Bujan, and U.A. Stock, *Age-related changes in the elastic tissue of the human aorta.* J Vasc Res, 2012. **49**(1): p. 77-86.
17. Incalza, M.A., R. D'Oria, A. Natalicchio, S. Perrini, L. Laviola, and F. Giorgino, *Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases.* Vascul Pharmacol, 2018. **100**: p. 1-19.
18. Socha, M.J. and S.S. Segal, *Microvascular mechanisms limiting skeletal muscle blood flow with advancing age.* J Appl Physiol (1985), 2018. **125**(6): p. 1851-1859.
19. Mughal, A., A.M. Sackheim, M. Sancho, T.A. Longden, S. Russell, W. Lockette, M.T. Nelson, and K. Freeman, *Impaired capillary-to-arteriolar electrical signaling after traumatic brain injury.* J Cereb Blood Flow Metab, 2021. **41**(6): p. 1313-1327.
20. Harraz, O.F., T.A. Longden, F. Dabertrand, D. Hill-Eubanks, and M.T. Nelson, *Endothelial GqPCR activity controls capillary electrical signaling and brain blood flow through PIP2 depletion.* Proc Natl Acad Sci U S A, 2018. **115**(15): p. E3569-E3577.
21. Li, Y. and P.J. Pagano, *Microvascular NADPH oxidase in health and disease.* Free Radic Biol Med, 2017. **109**: p. 33-47.
22. Daiber, A. and T. Munzel, *Organic Nitrate Therapy, Nitrate Tolerance, and Nitrate-Induced Endothelial Dysfunction: Emphasis on Redox Biology and Oxidative Stress.* Antioxid Redox Signal, 2015. **23**(11): p. 899-942.

23. Sandner, P., D.P. Zimmer, G.T. Milne, M. Follmann, A. Hobbs, and J.P. Stasch, *Soluble Guanylate Cyclase Stimulators and Activators.* Handb Exp Pharmacol, 2021. **264**: p. 355-394.
24. Dautzenberg, M., A. Kahnert, J.P. Stasch, and A. Just, *Role of soluble guanylate cyclase in renal hemodynamics and autoregulation in the rat.* Am J Physiol Renal Physiol, 2014. **307**(9): p. F1003-12.
25. Forstermann, U., E.I. Closs, J.S. Pollock, M. Nakane, P. Schwarz, I. Gath, and H. Kleinert, *Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions.* Hypertension, 1994. **23**(6 Pt 2): p. 1121-31.
26. Torreilles, J., *Nitric oxide: one of the more conserved and widespread signaling molecules.* Front Biosci, 2001. **6**: p. D1161-72.
27. Filippov, G., D.B. Bloch, and K.D. Bloch, *Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells.* J Clin Invest, 1997. **100**(4): p. 942-8.
28. Montfort, W.R., J.A. Wales, and A. Weichsel, *Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor.* Antioxid Redox Signal, 2017. **26**(3): p. 107-121.
29. Childers, K.C. and E.D. Garcin, *Structure/function of the soluble guanylyl cyclase catalytic domain.* Nitric Oxide, 2018. **77**: p. 53-64.
30. Evgenov, O.V., P. Pacher, P.M. Schmidt, G. Hasko, H.H. Schmidt, and J.P. Stasch, *NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential.* Nat Rev Drug Discov, 2006. **5**(9): p. 755-68.
31. Schmidt, H.H., P.M. Schmidt, and J.P. Stasch, *NO- and haem-independent soluble guanylate cyclase activators.* Handb Exp Pharmacol, 2009(191): p. 309-39.
32. Zhao, X.X., H. Cho, S. Lee, J.S. Woo, M.Y. Song, X.W. Cheng, K.H. Lee, and W. Kim, *BAY60-2770 attenuates doxorubicin-induced cardiotoxicity by decreased oxidative stress and enhanced autophagy.* Chem Biol Interact, 2020. **328**: p. 109190.
33. Kloss, S., A. Bouloumie, and A. Mulsch, *Aging and chronic hypertension decrease expression of rat aortic soluble guanylyl cyclase.* Hypertension, 2000. **35**(1 Pt 1): p. 43-7.
34. Ruetten, H., U. Zabel, W. Linz, and H.H. Schmidt, *Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats.* Circ Res, 1999. **85**(6): p. 534-41.

35. Tesauro, M., A. Mauriello, V. Rovella, M. Annicchiarico-Petruzzelli, C. Cardillo, G. Melino, and N. Di Daniele, *Arterial ageing: from endothelial dysfunction to vascular calcification.* J Intern Med, 2017. **281**(5): p. 471-482.

36. Folkow, B.J.H.d., *Structural, myogenic, humoral and nervous factors controlling peripheral resistance.* 1956. **163**.

37. Brinkmann, O., K. Schmerbach, U.J. Tietge, T. Dietrich, H. Guski, D. Linz, H. Kuhn, A. Patzak, and K. Wilfert, *Changes in arterial function in a mouse model of human familial hypercholesterolaemia.* Acta Physiol (Oxf), 2014. **211**(1): p. 61-72.

38. Moore, A., A.A. Mangoni, D. Lyons, and S.H. Jackson, *The cardiovascular system.* Br J Clin Pharmacol, 2003. **56**(3): p. 254-60.

39. Davignon, J. and P. Ganz, *Role of endothelial dysfunction in atherosclerosis.* Circulation, 2004. **109**(23 Suppl 1): p. III27-32.

40. Nyvad, J., A. Mazur, D.D. Postnov, M.S. Straarup, A.M. Soendergaard, C. Staehr, E. Brondum, C. Aalkjaer, and V.V. Matchkov, *Intravital investigation of rat mesenteric small artery tone and blood flow.* J Physiol, 2017. **595**(15): p. 5037-5053.

41. Blankesteijn, W.M., N.J. Raat, P.H. Willems, and T. Thien, *beta-Adrenergic relaxation in mesenteric resistance arteries of spontaneously hypertensive and Wistar-Kyoto rats: the role of precontraction and intracellular Ca2+.* J Cardiovasc Pharmacol, 1996. **27**(1): p. 27-32.

42. Deng, L.Y. and E.L. Schiffrin, *Morphological and functional alterations of mesenteric small resistance arteries in early renal hypertension in rats.* Am J Physiol, 1991. **261**(4 Pt 2): p. H1171-7.

43. Lariviere, R., J. St-Louis, and E.L. Schiffrin, *Vascular vasopressin receptors in renal hypertensive rats.* Am J Physiol, 1988. **255**(4 Pt 2): p. H693-8.

44. Luscher, T.F., Y. Dohi, and M. Tschudi, *Endothelium-dependent regulation of resistance arteries: alterations with aging and hypertension.* J Cardiovasc Pharmacol, 1992. **19 Suppl 5**: p. S34-42.

45. Gratton, R.J., R.E. Gandley, J.F. McCarthy, W.K. Michaluk, B.K. Slinker, and M.K. McLaughlin, *Contribution of vasomotion to vascular resistance: a comparison of arteries from virgin and pregnant rats.* J Appl Physiol (1985), 1998. **85**(6): p. 2255-60.

46. Rummery, N.M. and C.E. Hill, *Vascular gap junctions and implications for hypertension.* Clin Exp Pharmacol Physiol, 2004. **31**(10): p. 659-67.

47. Jensen, L.J. and N.H. Holstein-Rathlou, *Is there a role for T-type Ca2+ channels in regulation of vasomotor tone in mesenteric arterioles?* Can J Physiol Pharmacol, 2009. **87**(1): p. 8-20.

48. Dunn, S.M., R. Hilgers, and K.C. Das, *Decreased EDHF-mediated relaxation is a major mechanism in endothelial dysfunction in resistance arteries in aged mice on prolonged high-fat sucrose diet.* Physiol Rep, 2017. **5**(23).

49. Jiang, S., Y. Shui, Y. Cui, C. Tang, X. Wang, X. Qiu, W. Hu, L. Fei, Y. Li, S. Zhang, L. Zhao, N. Xu, F. Dong, X. Ren, R. Liu, P.B. Persson, A. Patzak, E.Y. Lai, Q. Wei, and Z. Zheng, *Gut microbiota dependent trimethylamine N-oxide aggravates angiotensin II-induced hypertension.* Redox Biol, 2021. **46**: p. 102115.

50. Martinka, P., E.Y. Lai, M. Fahling, V. Jankowski, J. Jankowski, R. Schubert, M. Gaestel, A.E. Persson, P.B. Persson, and A. Patzak, *Adenosine increases calcium sensitivity via receptor-independent activation of the p38/MK2 pathway in mesenteric arteries.* Acta Physiol (Oxf), 2008. **193**(1): p. 37-46.

51. Mulvany, M.J. and W.J.C.r. Halpern, *Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats.* 1977. **41**(1): p. 19-26.

52. del Campo, L. and M. Ferrer, *Wire Myography to Study Vascular Tone and Vascular Structure of Isolated Mouse Arteries.* Methods Mol Biol, 2015. **1339**: p. 255-76.

53. Mulvany, M.J. and W. Halpern, *Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats.* Circ Res, 1977. **41**(1): p. 19-26.

54. *Principles Of The Normalization Procedure; Standard Start; Principles Of The Standard Start Procedure.* DMT 320A User Manual: p. 11.

55. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, (Vienna, Austria: 2014): p. Available online: http://www.r-project.org/.

56. Raitakari, O.T. and D.S. Celermajer, *Flow-mediated dilatation.* Br J Clin Pharmacol, 2000. **50**(5): p. 397-404.

57. Widlansky, M.E., N. Gokce, J.F. Keaney, Jr., and J.A. Vita, *The clinical implications of endothelial dysfunction.* J Am Coll Cardiol, 2003. **42**(7): p. 1149-60.

58. Vanhoutte, P.M., Y. Zhao, A. Xu, and S.W. Leung, *Thirty Years of Saying NO: Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator.* Circ Res, 2016. **119**(2): p. 375-96.

59. El Assar, M., J. Angulo, S. Vallejo, C. Peiro, C.F. Sanchez-Ferrer, and L. Rodriguez-Manas, *Mechanisms involved in the aging-induced vascular dysfunction.* Front Physiol, 2012. **3**: p. 132.

60. Spier, S.A., M.D. Delp, C.J. Meininger, A.J. Donato, M.W. Ramsey, and J.M. Muller-Delp, *Effects of ageing and exercise training on endothelium-dependent vasodilatation and structure of rat skeletal muscle arterioles.* J Physiol, 2004. **556**(Pt 3): p. 947-58.

61. Chou, T.C., M.H. Yen, C.Y. Li, and Y.A. Ding, *Alterations of nitric oxide synthase expression with aging and hypertension in rats.* Hypertension, 1998. **31**(2): p. 643-8.

62. Luttrell, M., H. Kim, S.Y. Shin, D. Holly, M.P. Massett, and C.R. Woodman, *Heterogeneous effect of aging on vasorelaxation responses in large and small arteries.* Physiol Rep, 2020. **8**(1): p. e14341.

63. van der Loo, B., R. Labugger, J.N. Skepper, M. Bachschmid, J. Kilo, J.M. Powell, M. Palacios-Callender, J.D. Erusalimsky, T. Quaschning, T. Malinski, D. Gygi, V. Ullrich, and T.F. Luscher, *Enhanced peroxynitrite formation is associated with vascular aging.* J Exp Med, 2000. **192**(12): p. 1731-44.

64. Lu, H., L. Sun, W. Chen, Y. Zhou, K. Liu, J. Chen, Z. Zhang, C. Zhang, and H. Tian, *Sirtuin 3 Therapy Attenuates Aging Expression, Oxidative Stress Parameters, and Neointimal Hyperplasia Formation in Vein Grafts.* Ann Vasc Surg, 2020. **64**: p. 303-317.

65. Strosznajder, J.B., H. Jesko, A. Zambrzycka, A. Eckert, and M. Chalimoniuk, *Age-related alteration of activity and gene expression of endothelial nitric oxide synthase in different parts of the brain in rats.* Neurosci Lett, 2004. **370**(2-3): p. 175-9.

66. Sessa, W.C., *The nitric oxide synthase family of proteins.* J Vasc Res, 1994. **31**(3): p. 131-43.

67. Donato, A.J., D.R. Machin, and L.A. Lesniewski, *Mechanisms of Dysfunction in the Aging Vasculature and Role in Age-Related Disease.* Circ Res, 2018. **123**(7): p. 825-848.

68. Humphrey, J.D., *Vascular adaptation and mechanical homeostasis at tissue, cellular, and sub-cellular levels.* Cell Biochem Biophys, 2008. **50**(2): p. 53-78.

69. Liu, L., Y. Liu, J. Cui, H. Liu, Y.B. Liu, W.L. Qiao, H. Sun, and C.D. Yan, *Oxidative stress induces gastric submucosal arteriolar dysfunction in the elderly.* World J Gastroenterol, 2013. **19**(48): p. 9439-46.

70. Nakane, M., K. Arai, S. Saheki, T. Kuno, W. Buechler, and F. Murad, *Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung.* J Biol Chem, 1990. **265**(28): p. 16841-5.

71. Andreopoulos, S. and A. Papapetropoulos, *Molecular aspects of soluble guanylyl cyclase regulation.* Gen Pharmacol, 2000. **34**(3): p. 147-57.

72. Buechler, W.A., M. Nakane, and F. Murad, *Expression of soluble guanylate cyclase activity requires both enzyme subunits.* Biochem Biophys Res Commun, 1991. **174**(1): p. 351-7.

73. Buys, E.S., M.J. Raher, A. Kirby, M. Shahid, D.M. Baron, S.R. Hayton, L.T. Tainsh, P.Y. Sips, K.M. Rauwerdink, Q. Yan, R.E. Tainsh, H.R. Shakartzi, C. Stevens, K. Decaluwe, G. Rodrigues-Machado Mda, R. Malhotra, J. Van de Voorde, T. Wang, P. Brouckaert, M.J. Daly, and K.D. Bloch, *Genetic modifiers of hypertension in soluble guanylate cyclase alpha1-deficient mice.* J Clin Invest, 2012. **122**(6): p. 2316-25.

74. Friebe, A., E. Mergia, O. Dangel, A. Lange, and D. Koesling, *Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7699-704.

75. Groneberg, D., P. Konig, A. Wirth, S. Offermanns, D. Koesling, and A. Friebe, *Smooth muscle-specific deletion of nitric oxide-sensitive guanylyl cyclase is sufficient to induce hypertension in mice.* Circulation, 2010. **121**(3): p. 401-9.

76. Mergia, E., M. Thieme, H. Hoch, G. Daniil, L. Hering, M. Yakoub, C.R. Scherbaum, L.C. Rump, D. Koesling, and J. Stegbauer, *Impact of the NO-Sensitive Guanylyl Cyclase 1 and 2 on Renal Blood Flow and Systemic Blood Pressure in Mice.* Int J Mol Sci, 2018. **19**(4).

77. Mergia, E., A. Friebe, O. Dangel, M. Russwurm, and D. Koesling, *Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system.* J Clin Invest, 2006. **116**(6): p. 1731-7.

78. Bloch, K.D., G. Filippov, L.S. Sanchez, M. Nakane, and S.M. de la Monte, *Pulmonary soluble guanylate cyclase, a nitric oxide receptor, is increased during the perinatal period.* Am J Physiol, 1997. **272**(3 Pt 1): p. L400-6.

79. Gupta, G., M. Azam, L. Yang, and R.S. Danziger, *The beta2 subunit inhibits stimulation of the alpha1/beta1 form of soluble guanylyl cyclase by nitric oxide. Potential relevance to regulation of blood pressure.* J Clin Invest, 1997. **100**(6): p. 1488-92.

80. Bender, A.T. and J.A. Beavo, *Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use.* Pharmacol Rev, 2006. **58**(3): p. 488-520.

81. Liu, H., V. Manganiello, N. Waleh, and R.I. Clyman, *Expression, activity, and function of phosphodiesterases in the mature and immature ductus arteriosus.* Pediatr Res, 2008. **64**(5): p. 477-81.

82. Rybalkin, S.D., C. Yan, K.E. Bornfeldt, and J.A. Beavo, *Cyclic GMP phosphodiesterases and regulation of smooth muscle function.* Circ Res, 2003. **93**(4): p. 280-91.

83. Kukreja, R.C., F.N. Salloum, and A. Das, *Cyclic guanosine monophosphate signaling and phosphodiesterase-5 inhibitors in cardioprotection.* J Am Coll Cardiol, 2012. **59**(22): p. 1921-7.

84. Bautista Nino, P.K., M. Durik, A.H. Danser, R. de Vries, U.M. Musterd-Bhaggoe, M.E. Meima, M. Kavousi, M. Ghanbari, J.H. Hoeijmakers, C.J. O'Donnell, N. Franceschini, G.M. Janssen, J.G. De Mey, Y. Liu, C.M. Shanahan, O.H. Franco, A. Dehghan, and A.J. Roks, *Phosphodiesterase 1 regulation is a key mechanism in vascular aging.* Clin Sci (Lond), 2015. **129**(12): p. 1061-75.

85. Aversa, A., Y. Duca, R.A. Condorelli, A.E. Calogero, and S. La Vignera, *Androgen Deficiency and Phosphodiesterase Type 5 Expression Changes in Aging Male: Therapeutic Implications.* Front Endocrinol (Lausanne), 2019. **10**: p. 225.

86. Gerhard, M., M.A. Roddy, S.J. Creager, and M.A. Creager, *Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans.* Hypertension, 1996. **27**(4): p. 849-53.

87. Rodriguez-Martinez, M.A., M.J. Alonso, J. Redondo, M. Salaices, and J. Marin, *Role of lipid peroxidation and the glutathione-dependent antioxidant system in the impairment of endothelium-dependent relaxations with age.* Br J Pharmacol, 1998. **123**(1): p. 113-21.

88. Fujii, K., S. Ohmori, M. Tominaga, I. Abe, Y. Takata, Y. Ohya, K. Kobayashi, and M. Fujishima, *Age-related changes in endothelium-dependent hyperpolarization in the rat mesenteric artery.* Am J Physiol, 1993. **265**(2 Pt 2): p. H509-16.

89. Virmani, R., F.D. Kolodgie, A.P. Burke, A. Farb, and S.M. Schwartz, *Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions.* Arterioscler Thromb Vasc Biol, 2000. **20**(5): p. 1262-75.

90. Movat, H.Z., R.H. More, and M.D. Haust, *The diffuse intimal thickening of the human aorta with aging.* Am J Pathol, 1958. **34**(6): p. 1023-31.

91. Stary, H.C., *Macrophages, macrophage foam cells, and eccentric intimal thickening in the coronary arteries of young children.* Atherosclerosis, 1987. **64**(2-3): p. 91-108.

92. Sima, A.V., C.S. Stancu, and M. Simionescu, *Vascular endothelium in atherosclerosis.* Cell Tissue Res, 2009. **335**(1): p. 191-203.

93. Graham, R.M., D.M. Perez, J. Hwa, and M.T. Piascik, *alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling.* Circ Res, 1996. **78**(5): p. 737-49.
94. Gericke, A., P. Martinka, I. Nazarenko, P.B. Persson, and A. Patzak, *Impact of alpha1-adrenoceptor expression on contractile properties of vascular smooth muscle cells.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(3): p. R1215-21.
95. Marin, J. and M.A. Rodriguez-Martinez, *Age-related changes in vascular responses.* Exp Gerontol, 1999. **34**(4): p. 503-12.
96. Cassie, N.J., M.A. Cross, E.L. Phelan, and J.A. Millar, *Sensitivity to Ca2+ and the effects of a calcium channel antagonist in resistance vessels from two strains of genetically hypertensive rat.* J Cardiovasc Pharmacol, 1990. **16 Suppl 7**: p. S12-3.
97. Wanstall, J.C. and S.R. O'Donnell, *Age influences responses of rat isolated aorta and pulmonary artery to the calcium channel agonist, Bay K 8664, and to potassium and calcium.* J Cardiovasc Pharmacol, 1989. **13**(5): p. 709-14.
98. Ponte, A., C.F. Sanchez-Ferrer, C. Hernandez, M.J. Alonso, and J. Marin, *Effect of ageing and hypertension on endothelial modulation of ouabain-induced contraction and sodium pump activity in the rat aorta.* J Hypertens, 1996. **14**(6): p. 705-12.
99. Fukuda, T., T. Kuroda, M. Kono, T. Miyamoto, M. Tanaka, and T. Matsui, *Attenuation of L-type Ca(2)(+) channel expression and vasomotor response in the aorta with age in both Wistar-Kyoto and spontaneously hypertensive rats.* PLoS One, 2014. **9**(2): p. e88975.
100. Fan, G., M. Kassmann, Y. Cui, C. Matthaeus, S. Kunz, C. Zhong, S. Zhu, Y. Xie, D. Tsvetkov, O. Daumke, Y. Huang, and M. Gollasch, *Age attenuates the T-type CaV 3.2-RyR axis in vascular smooth muscle.* Aging Cell, 2020. **19**(4): p. e13134.
101. Mikkelsen, M.F., K. Bjorling, and L.J. Jensen, *Age-dependent impact of CaV 3.2 T-type calcium channel deletion on myogenic tone and flow-mediated vasodilatation in small arteries.* J Physiol, 2016. **594**(20): p. 5881-5898.

8. Statutory declaration

I, Cheng Zhong, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic ‘Role of soluble guanylyl cyclase in the functional aging of mesenterial arteries in mice [German title: Rolle der löslichen Guanylatzyklase bei der funktionellen Alterung mesenterialer Arterien der Maus]’, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

Date: Signature

9. Declaration of my own contribution to the publications

Cheng Zhong contributed the following to the below listed publication:

Publication: **C Zhong,** M Xu, S Boral, H Summer, F-B Lichtenberger, C Erdoğan, M Gollasch, S Golz, PB Persson, J Schleifenbaum, A Patzak, PH Khedkar. Age Impairs Soluble Guanylyl Cyclase Function in Mouse Mesenteric Arteries. ***Int J Mol Sci*****.** 2021 Oct 22;22(21):11412.

Contribution: I designed the study together with my supervisor and co-supervisor. I performed all of the functional measurements in the mesenteric arteries. This includes animal handling, dissection of all the small mesenteric arteries, measurement of the force using wire myography, creation of protocols (in co-operation with the co-supervisor), and performing of all the experimental protocols. Further, I prepared all vessels for histological analysis, and for measurement of cGMP concentrations as well as for qPCR analysis in the small mesenteric arteries. I performed the pharmacological experiments to this end.

I embedded the vessels in paraffin, sliced them and prepared them for histological staining. I analyzed all functional data and analyzed the histological slices.

I drafted the manuscript text and created the tables. The contents of work described in the chapter of the publication, *2.1 Vessel activity and pre-contraction*, *2.2 Aging impairs endothelium-dependent relaxation, 2.3 Endothelium-independent relaxation, and 2.4 Vessel histology is similar between juvenile and aged mice,* were all done independently by me. I prepared the vessels for analysis of chapter *2.5 Differential expression of mRNA of sGC subunits and PDE5*. I created figures 1 to 5 of the manuscript based on my own analyses. I revised the manuscript according to the reviewer's suggestion together with the co-authors.

Signature, date and stamp of first supervising university professor / lecturer

Signature of doctoral candidate

10. Print copy of selected publication

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C Zhong, M Xu, S Boral, H Summer, F-B Lichtenberger, C Erdoğan, M Gollasch, S Golz, PB Persson, J Schleifenbaum, A Patzak, PH Khedkar. Age Impairs Soluble Guanylyl Cyclase Function in Mouse Mesenteric Arteries. ***Int J Mol Sci***. 2021 Oct 22;22(21):11412.

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1	NATURE MEDICINE	114,401	53.440	0.184050
2	CELL	320,407	41.582	0.526960
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4	Annual Review of Biochemistry	24,394	23.643	0.021450
5	Signal Transduction and Targeted Therapy	3,848	18.187	0.005730
6	MOLECULAR CELL	86,299	17.970	0.161840
7	TRENDS IN MICROBIOLOGY	17,553	17.079	0.022820
8	NUCLEIC ACIDS RESEARCH	248,139	16.971	0.387070
9	MOLECULAR BIOLOGY AND EVOLUTION	61,557	16.240	0.082270
10	PROGRESS IN LIPID RESEARCH	7,328	16.195	0.004530
11	MOLECULAR PSYCHIATRY	28,622	15.992	0.046220
12	CELL DEATH AND DIFFERENTIATION	27,701	15.828	0.028730
13	NATURE STRUCTURAL & MOLECULAR BIOLOGY	32,038	15.369	0.051210
14	Nature Chemical Biology	27,428	15.040	0.047880
15	MOLECULAR ASPECTS OF MEDICINE	8,136	14.235	0.006640
16	TRENDS IN BIOCHEMICAL SCIENCES	22,003	13.807	0.025760
17	NATURAL PRODUCT REPORTS	13,293	13.423	0.011160
18	Molecular Plant	15,778	13.164	0.026860
19	Advances in Carbohydrate Chemistry and Biochemistry	752	12.200	0.000200
20	TRENDS IN MOLECULAR MEDICINE	13,213	11.951	0.014720

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	Redox Biology	15,982	11.799	0.024930
22	EMBO JOURNAL	76,189	11.598	0.055000
23	MATRIX BIOLOGY	8,972	11.583	0.011010
24	Molecular Systems Biology	10,149	11.429	0.016300
25	PLANT CELL	64,794	11.277	0.036260
26	CURRENT BIOLOGY	78,289	10.834	0.116100
27	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	7,025	10.680	0.007000
28	Cell Systems	5,813	10.304	0.035330
29	ONCOGENE	77,576	9.867	0.059180
30	CELLULAR AND MOLECULAR LIFE SCIENCES	34,003	9.261	0.033790
31	GENOME RESEARCH	47,141	9.043	0.064690
32	CURRENT OPINION IN CHEMICAL BIOLOGY	12,240	8.822	0.014190
33	EMBO REPORTS	19,502	8.807	0.027490
34	EXPERIMENTAL AND MOLECULAR MEDICINE	8,780	8.718	0.013260
35	ANTIOXIDANTS & REDOX SIGNALING	26,971	8.401	0.016700
36	CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY	4,576	8.250	0.005370
37	Science Signaling	15,954	8.192	0.023910
38	Cell Chemical Biology	5,236	8.116	0.018050
39	PLOS BIOLOGY	39,598	8.029	0.059920
40	Essays in Biochemistry	3,629	8.000	0.006450
41	BIOINORGANIC CHEMISTRY AND APPLICATIONS	1,406	7.778	0.000890

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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
42	Acta Crystallographica Section D-Structural Biology	23,670	7.652	0.020190
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44	FREE RADICAL BIOLOGY AND MEDICINE	52,714	7.376	0.034180
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47	Cell and Bioscience	3,184	7.133	0.004320
48	Genes & Diseases	1,850	7.103	0.003170
49	Molecular Ecology Resources	13,390	7.090	0.016690
50	Journal of Integrative Plant Biology	6,749	7.061	0.006430
51	BIOMACROMOLECULES	45,724	6.988	0.026020
52	INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES	79,246	6.953	0.073720
53	AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY	15,280	6.914	0.015050
54	International Review of Cell and Molecular Biology	3,057	6.813	0.004320
55	CURRENT OPINION IN STRUCTURAL BIOLOGY	12,448	6.809	0.018970
56	PROTEIN SCIENCE	16,581	6.725	0.021220
57	International Journal of Biological Sciences	10,778	6.580	0.010540
58	Open Biology	4,059	6.411	0.010280
59	MOLECULAR MEDICINE	6,239	6.354	0.004460
60	Antioxidants	9,076	6.312	0.009480
61	JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B-BIOLOGY	17,015	6.252	0.012740

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64	BIOFACTORS	5,004	6.113	0.002810
65	Biomedicines	2,391	6.081	0.003650
66	JOURNAL OF NUTRITIONAL BIOCHEMISTRY	14,446	6.048	0.010580
67	INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES	139,463	5.923	0.195430
68	JOURNAL OF LIPID RESEARCH	28,376	5.922	0.019290
69	CELLULAR & MOLECULAR BIOLOGY LETTERS	2,216	5.787	0.002190
70	EXPERT REVIEWS IN MOLECULAR MEDICINE	2,042	5.600	0.000790
71	Reviews of Physiology Biochemistry and Pharmacology	865	5.545	0.000390
72	FEBS Journal	23,493	5.542	0.022540
73	Nucleic Acid Therapeutics	1,392	5.486	0.003070
74	JOURNAL OF MOLECULAR BIOLOGY	65,163	5.469	0.038400
75	BIOCHEMICAL SOCIETY TRANSACTIONS	14,862	5.407	0.014350
76	Food & Function	19,700	5.396	0.021550
77	BIOELECTROCHEMISTRY	6,107	5.373	0.004870
78	JOURNAL OF NEUROCHEMISTRY	40,281	5.372	0.019170
79	BIOORGANIC CHEMISTRY	10,576	5.275	0.010220
80	GENE THERAPY	8,112	5.250	0.004130
81	Frontiers in Molecular Biosciences	3,140	5.246	0.007660
82	CHROMOSOME RESEARCH	2,752	5.239	0.002250
83	CHEMICO-BIOLOGICAL INTERACTIONS	16,208	5.192	0.011950

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Age Impairs Soluble Guanylyl Cyclase Function in Mouse Mesenteric Arteries

Cheng Zhong [1], Minze Xu [1], Sengül Boral [2], Holger Summer [3], Falk-Bach Lichtenberger [1], Cem Erdoğan [1], Maik Gollasch [4,5], Stefan Golz [3], Pontus B. Persson [1], Johanna Schleifenbaum [1], Andreas Patzak [1,*] and Pratik H. Khedkar [1]

[1] Institute of Vegetative Physiology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Charitéplatz 1, 10117 Berlin, Germany; cheng.zhong@charite.de (C.Z.); minze.xu@charite.de (M.X.); falk.lichtenberger@charite.de (F.-B.L.); cem.erdogan@charite.de (C.E.); pontus.persson@charite.de (P.B.P.); johanna.schleifenbaum@charite.de (J.S.); pratik.khedkar@charite.de (P.H.K.)
[2] Institute of Pathology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Charitéplatz 1, 10117 Berlin, Germany; senguel.boral@charite.de
[3] Bayer AG, Research & Development, 42113 Wuppertal, Germany; holger.summer@bayer.com (H.S.); stefan.golz@bayer.com (S.G.)
[4] Experimental and Clinical Research Center (ECRC), Charité—Universitätsmedizin Berlin, 13125 Berlin, Germany; maik.gollasch@charite.de
[5] Department of Internal and Geriatric Medicine, University of Greifswald, Geriatric Medicine, 17475 Greifswald, Germany
* Correspondence: andreas.patzak@charite.de

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Abstract: Endothelial dysfunction (ED) comes with age, even without overt vessel damage such as that which occurs in atherosclerosis and diabetic vasculopathy. We hypothesized that aging would affect the downstream signalling of the endothelial nitric oxide (NO) system in the vascular smooth muscle (VSM). With this in mind, resistance mesenteric arteries were isolated from 13-week (juvenile) and 40-week-old (aged) mice and tested under isometric conditions using wire myography. Acetylcholine (ACh)-induced relaxation was reduced in aged as compared to juvenile vessels. Pretreatment with L-NAME, which inhibits nitrix oxide synthases (NOS), decreased ACh-mediated vasorelaxation, whereby differences in vasorelaxation between groups disappeared. Endothelium-independent vasorelaxation by the NO donor sodium nitroprusside (SNP) was similar in both groups; however, SNP bolus application (10^{-6} mol L^{-1}) as well as soluble guanylyl cyclase (sGC) activation by runcaciguat (10^{-6} mol L^{-1}) caused faster responses in juvenile vessels. This was accompanied by higher cGMP concentrations and a stronger response to the PDE5 inhibitor sildenafil in juvenile vessels. Mesenteric arteries and aortas did not reveal apparent histological differences between groups (van Gieson staining). The mRNA expression of the α1 and α2 subunits of sGC was lower in aged animals, as was PDE5 mRNA expression. In conclusion, vasorelaxation is compromised at an early age in mice even in the absence of histopathological alterations. Vascular smooth muscle sGC is a key element in aged vessel dysfunction.

Keywords: aging; mesenteric artery; nitric oxide; soluble guanylyl cyclase; soluble guanylyl cyclase activator

1. Introduction

Aged arteries contribute to cardiovascular disease [1,2]. Age-related decline in arterial function includes reduced vasorelaxation and increased vasoconstriction, which compromise organ perfusion and function. The decrease in endothelium-dependent vasodilatation that comes with age is not necessarily accompanied by structural changes in the arterial wall [3]. Disturbances in the generation or breakdown of endothelium-derived vasoactive

autacoids play an important role. What is typical is the attenuated response to vasodilators or to the blockade of the nitric oxide (NO) system, as shown by numerous studies in humans, rats, and mice [3–7]. The NO system, prostacyclin-derived metabolites, and the endothelium-derived hyperpolarizing factor (EDHF) are major components of the endothelial dilatory function.

Although all of these systems contribute to endothelial dysfunction with age, NO is the main dilatory factor in many vascular beds such as those of the kidney, the mesentery, and the retina [8,9]. NO, produced by endothelial NO synthase, diffuses into smooth muscle cells to activate its receptor, soluble guanylyl cyclase (sGC), which catalyses cyclic guanosine monophosphate (cGMP) production. cGMP activates phosphoglycerate kinase (PGK), which in turn modulates the function of various channels and enzymes resulting in reduced cytosolic calcium levels and muscular relaxation [10]. Potential mechanisms behind age-related decline in NO system functioning comprise reduced endothelial NO synthase (eNOS) expression and function on top of increased NO scavenging by reactive oxygen species (ROS) [11–13]. Both mechanisms diminish endothelial NO bioavailability. Furthermore, the remodelling and functional decline of vascular smooth muscle (VSM) components, eventually leading to increased vessel stiffness, affect endothelial dilatation [14].

In contrast to the widely investigated role of the endothelial compartment, there is very limited knowledge regarding NO-induced signalling in aged VSM cells. Studies do report decreased cGMP levels and reduced sGC expression in old animals as compared to young ones [15,16], which suggest the role of sGC in the age-related dysfunction of the NO system. To test the hypothesis that sGC function is reduced with age, we investigated the mesenteric arteries of juvenile (≈13-week-old) and aged mice (≈40-week-old). Aged animals, not old, were included in the study to exclude atherosclerosis and vascular remodelling, which commonly occur in old mice and markedly influence vascular function. Resistance mesenteric arteries were used, as this is a preferred model for the study of arterial function [17].

2. Results

2.1. Vessel Activity and Pre-Contraction

The activity of juvenile and adult vessels was tested using a high-potassium physiological solution (K-PSS, 123.7 mmol L^{-1} KCl, Carl Roth, Karlsruhe, Germany). Aged vessels showed a 14% reduced response to K-PSS as compared to the juvenile vessels (3.16 ± 0.12 ($n = 48$) vs. 3.66 ± 0.13 mN ($n = 52$), Mann–Whitney test, $p < 0.01$). Phenylephrine (PE, 10^{-5} mol L^{-1}) was used to pre-contract the vessels for the vasorelaxation experiments. The responses to PE, which were normalized to the respective KCl-induced contraction, were similar in both groups (120.61 ± 5.64 (aged, $n = 31$) vs. 118.56 ± 7.92% (juvenile, $n = 38$), Mann–Whitney test, $p > 0.05$).

2.2. Aging Impairs Endothelium-Dependent Relaxation

Vascular aging is accompanied by endothelial dysfunction [3]. To test endothelium-dependent vasorelaxation, resistance mesenteric arteries from mice were pre-contracted with PE (10^{-5} mol L^{-1}) and treated with cumulatively increasing concentrations of ACh (10^{-9} to 10^{-5} mol L^{-1}) (Figure 1A). Both juvenile ($n = 10$, $EC_{50} = (8.03 \pm 2.05) \times 10^{-8}$ mol L^{-1}) and aged ($n = 11$, $EC_{50} = (5.60 \pm 0.35) \times 10^{-8}$ mol L^{-1}) vessels showed concentration-dependent relaxation in response to Ach, with comparable sensitivity (EC_{50}: Mann–Whitney U test, $p > 0.05$). However, aged vessels showed a significantly smaller maximum response to ACh than juvenile vessels (Figure 1B). To test the contribution of the endothelial NO system in this context, vessels were pre-treated with a non-selective inhibitor of NO synthases, *N*ω-nitro-L-arginine methylester hydrochloride (L-NAME, 10^{-4} mol L^{-1}), for 30 min [8]. The responses to ACh decreased more strongly in the juvenile group after L-NAME treatment as compared to the aged group, which resulted in similar concentration–

response curves. This observation suggests that NO bioavailability is reduced in aged vessels (Figure 1C,D).

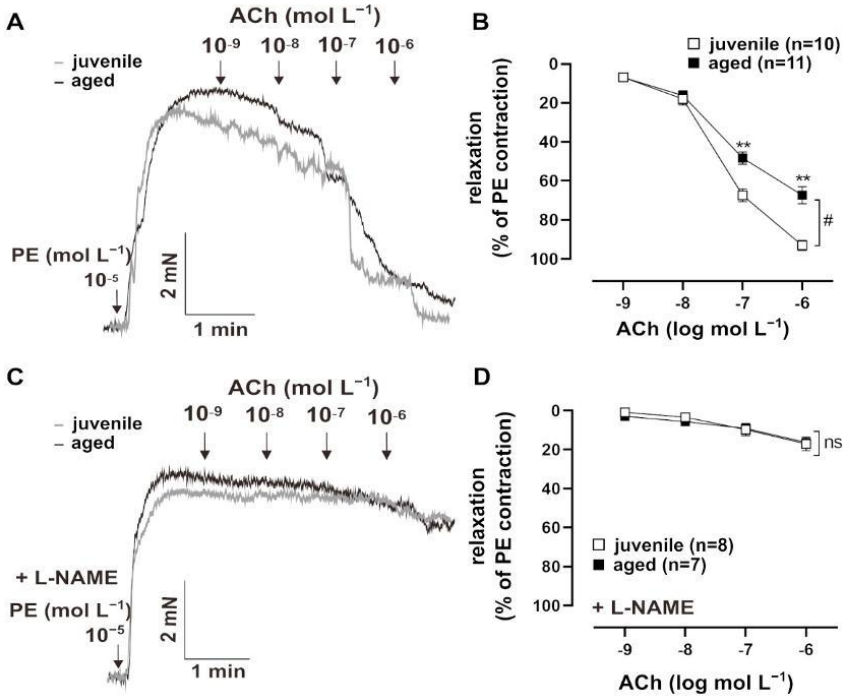

Figure 1. Age-related differences in relaxation to acetylcholine (ACh). (**A**) Representative traces and (**B**) concentration–response curves showing the relaxation induced by 10^{-9}–10^{-6} mol L^{-1} ACh in juvenile and aged mesenteric arteries. Vessels were pre-contracted using 10^{-5} mol L^{-1} phenylephrine (PE). Juvenile vessels showed a higher maximum response to ACh compared to aged vessels ($^{\#}$ $p < 0.05$ Brunner test, ** $p < 0.01$ Mann–Whitney test). Both aged and juvenile vessels had a similar sensitivity to ACh (EC_{50}: $(5.60 \pm 0.35) \times 10^{-8}$ mol L^{-1} (aged), $(8.03 \pm 2.05) \times 10^{-8}$ mol L^{-1} (juvenile)). (**C**) Representative traces and (**D**) concentration–response curves showing the relaxation induced by 10^{-9}–10^{-6} mol L^{-1} ACh in juvenile and aged vessels. Vessels were pre-treated for 30 min with L-NAME (10^{-4} mol L^{-1}, indicated as "+L-NAME"), a non-selective NOS inhibitor, and pre-contracted with PE. L-NAME largely reduced ACh-mediated relaxation in both aged and juvenile vessels. There were no differences between the aged and juvenile groups.

2.3. Endothelium-Independent Relaxation

To assess whether NO signalling in VSM contributes to the reduced relaxation in aged vessels, their response to the endothelium-independent vasodilator, sodium nitroprusside (SNP, 10^{-11} to 10^{-5} mol L^{-1}), was measured. Vessels were pre-treated with L-NAME for 30 min, followed by a pre-contraction with 60 mmol L^{-1} KCl (Figure 2A). The sensitivity of both juvenile (n = 12, EC_{50} = $(1.73 \pm 0.46) \times 10^{-7}$ mol L^{-1}) and aged (n = 9, EC_{50} = $(7.85 \pm 4.71) \times 10^{-7}$ mol L^{-1}) vessels to SNP were similar (Mann–Whitney U test, $p > 0.05$). SNP also induced similar maximum responses in juvenile and aged vessels (Figure 2B). Remarkably, bolus application of SNP for 10 min revealed a faster relaxation in juvenile vessels as compared to aged vessels, which was most prominent during the first 2 min after NO-application (Figure 2C,D).

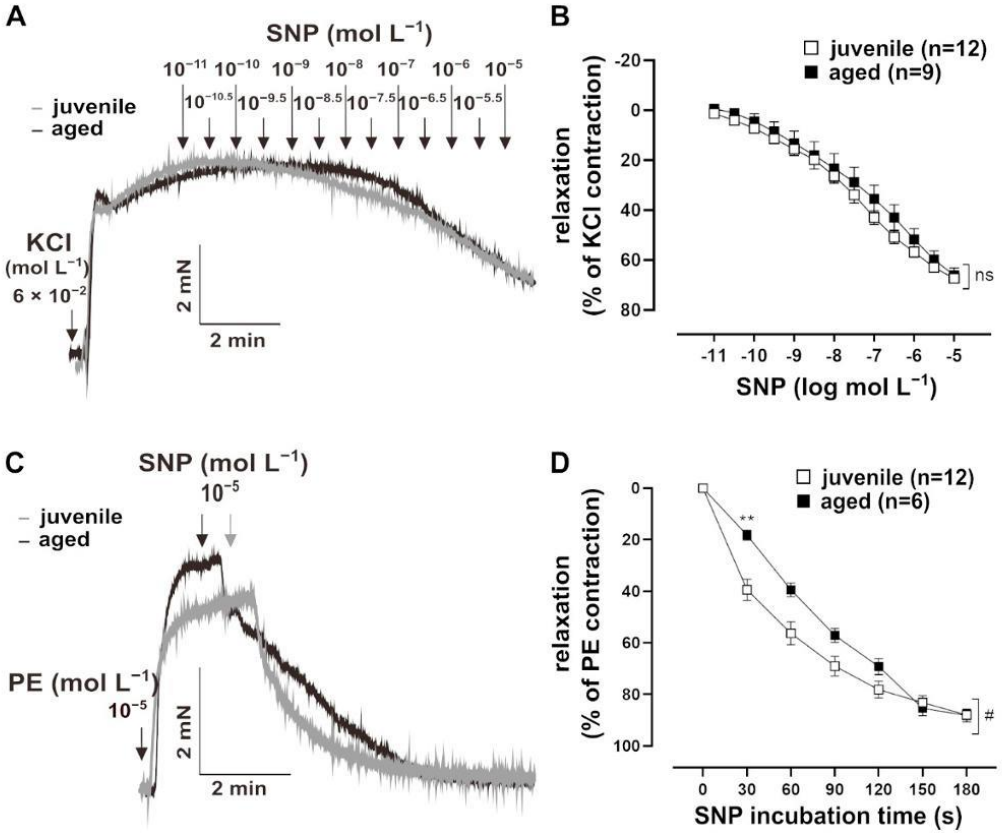

Figure 2. Effect of the NO donor, sodium nitroprusside (SNP), on mouse mesenteric arteries. (**A**) Representative traces and (**B**) concentration–response curves showing the relaxation induced by 10^{-11}–10^{-5} mol L^{-1} SNP in juvenile and aged mesenteric arteries. Vessels were pre-treated with L-NAME (10^{-4} mol L^{-1}) and pre-contracted using potassium chloride (KCl, 60 mmol L^{-1}). Juvenile and aged mesenteric arteries showed similar sensitivity (EC_{50}: (3.52 ± 3.34) × 10^{-6} mol L^{-1} (juvenile), (7.85 ± 4.71) × 10^{-6} mol L^{-1} (aged)) and maximum response (Brunner test, p >0.05, Mann–Whitney test, $p > 0.05$) to SNP. (**C**) Representative traces and (**D**) time–response curves showing relaxation induced by SNP (10^{-5} mol L^{-1}) in juvenile and aged vessels over time. Vessels were pre-treated with L-NAME and pre-contracted with phenylephrine (PE). SNP caused faster relaxation in juvenile as compared to aged mesenteric arteries in the physiologically important range of up to 3 min ($^{\#}$ $p < 0.05$ Brunner test, ** $p < 0.01$ Mann–Whitney test).

In addition to NO, which is a natural agonist of sGC, the artificial activator runcaciguat (BAY60-2770, Bayer AG, Wuppertal, Germany) was tested. Runcaciguat activates sGC in its haem-free configuration of NO (Figure 3A) [18]. This pharmacological activation of sGC provided additional information about its function. Runcaciguat was applied as a bolus at a maximal concentration of 10^{-6} mol L^{-1} for 10 min. It induced a faster response in juvenile vessels as compared to aged vessels, most prominently at 2 min post application (Figure 3B). At this time point, cGMP levels were lower in aged vessels (Figure 3C). Furthermore, the pharmacological inhibition of PDE5, which degrades cGMP, induced stronger relaxation in PE-pre-contracted juvenile vessels than in aged vessels (Figure 4A,B).

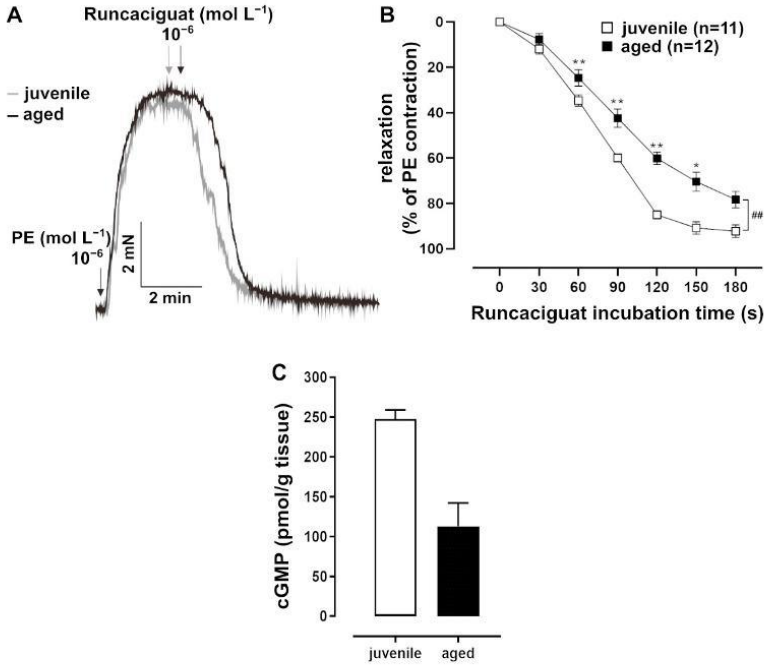

Figure 3. Effects of the soluble guanylate cyclase (sGC) activator runcaciguat. (**A**) Representative traces and (**B**) time–response curves showing relaxation induced by runcaciguat (10^{-6} mol L^{-1}) in juvenile and aged mesenteric arteries. Vessels were pre-treated with L-NAME (10^{-4} mol L^{-1}) and pre-contracted with PE. Juvenile vessels showed a significantly faster relaxation in response to runcaciguat (10^{-6} mol L^{-1}) as compared to aged vessels in the physiologically important range of up to 3 min ($^{\#\#}$ $p < 0.01$ Brunner test, * $p < 0.05$, ** $p < 0.01$ Mann–Whitney test). (**C**) A direct cGMP ELISA was performed on tissue lysates obtained from L-NAME-treated and PE-pre-contracted arteries 2 min after bolus application of runcaciguat (10^{-6} mol L^{-1}). Juvenile vessels had higher cGMP levels than aged vessels (juvenile: $n = 2$ pooled samples, each with mesenteric arterial tissue from at least 5 mice; adult: $n = 2$ pooled samples, each with tissue from 3 mice).

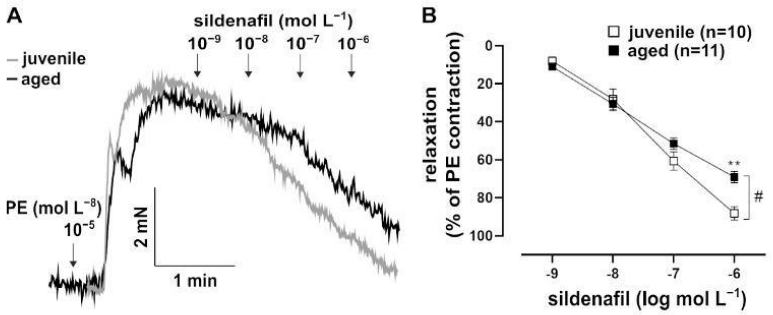

Figure 4. Effects of the PDE5 inhibitor, sildenafil. (**A**) Representative traces and (**B**) concentration–response curve showing the relaxation induced by 10^{-9}–10^{-6} mol L^{-1} sildenafil in juvenile and aged vessels. Vessels were pre-contracted with PE. Juvenile vessels showed a higher maximum response to sildenafil as compared to aged vessels ($^{\#}$ $p < 0.05$ Brunner test, ** $p < 0.01$ Mann–Whitney test). The sensitivity of both groups to sildenafil was not significantly different (EC_{50}: $(5.99 \pm 1.64) \times 10^{-8}$ mol L^{-1} (juvenile), $(4.93 \pm 1.14) \times 10^{-8}$ mol L^{-1} (aged)).

2.4. Vessel Histology Is Similar between Juvenile and Aged Mice

In old mice (>12 months), vessel dysfunction is accompanied by irreversible histological changes characterized by atherosclerotic plaques and vascular wall remodelling [19]. In the present study, a relatively early advanced life span was investigated. Interestingly, ACh-induced vasorelaxation was already reduced in aged mice (40 weeks) as compared to juvenile mice (13 weeks). Histological investigations of mesenteric arteries and aortae showed no visible changes in the vessel structure (Figure 5A–D). Furthermore, the media-to-lumen ratios of mesenteric arteries as well as aortae were not different between the aged and juvenile groups (Figure 5E).

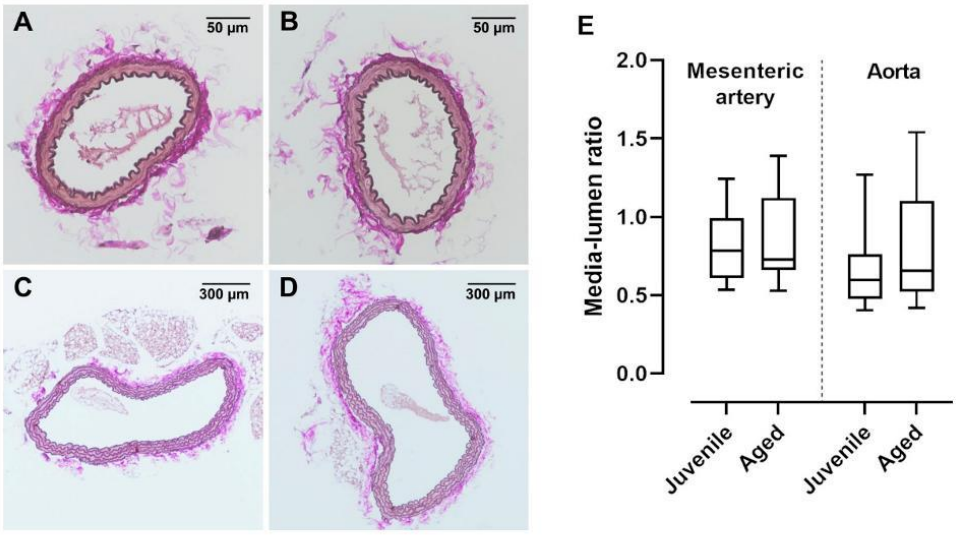

Figure 5. van Gieson-stained cross sections of mouse mesenteric artery and aorta. Vascular walls of both (**A**) juvenile and (**B**) aged mesenteric arteries showed no structural differences and had a similar number of elastic fibres. Neither (**C**) juvenile (n = 36) nor (**D**) aged (n = 19) aortae showed atherosclerotic alterations. (**E**) The media lumen ratios of juvenile mesenteric arteries (n = 26) and aortae (n = 20) were not significantly different from aged mesenteric arteries (n = 31) and aortae (n = 19), respectively ($p > 0.05$, Mann–Whitney test).

2.5. Differential Expression of mRNA of sGC Subunits and PDE5

To examine the effect of aging on the NO–sGC pathway on a molecular level, we quantified the mRNA expression of the enzymes involved in the mesenteric arteries of juvenile and aged mice. Relative expression levels were determined using the ΔCt method and expressed as a ratio of the target gene's expression level to that of the housekeeping gene (Figure 6). While there were no differences in the expression levels of nNOS and eNOS between the two groups, nNOS was expressed at extremely low levels in both groups. Expression of iNOS was below the detection level in both groups (data not shown). The α1 (GUCY1A1) and α2 (GUCY1A2) subunits of sGC were expressed at significantly lower levels in aged vessels as compared to juvenile vessels. The β1 subunit (GUCY1B1), however, was expressed at comparable levels in both groups. PDE5 expression levels in aged mice were also lower than in juvenile mice (Figure 6). PDE3A expression levels were lower and PDE3B levels were higher in aged as compared to juvenile vessels (Figure 6).

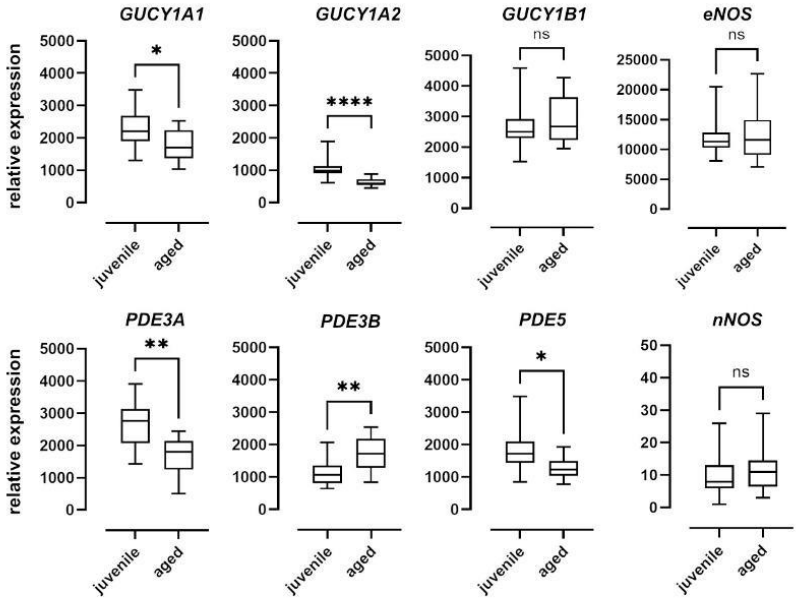

Figure 6. mRNA expression of sGC subunits and isoforms of PDE and NOS. Expression levels of the alpha subunits GUCY1A1 and GUCY1A2 of soluble guanylyl cyclase (sGC) were significantly higher in juvenile mesenteric arteries ($n = 16$) compared to aged vessels ($n = 10$). The expression of the beta subunit GUCY1B1, however, was not significantly different. Juvenile mesenteric arteries ($n = 16$) showed a significantly higher expression of PDE3A and PDE5 compared to aged vessels ($n = 10$); however, the expression of PDE3B was significantly lower. Expression levels of both eNOS and iNOS were not significantly different between the two groups (juvenile: $n = 23$, aged: $n = 17$). Data are represented as box and whisker plots of mRNA expression levels normalized to the housekeeping gene RPL32 (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, Mann–Whitney test).

3. Discussion

In the present study, we demonstrated that the age-dependent decline in the relaxing ability of mesenteric resistance arteries of male mice is associated with an impaired sGC function. Aged vessels relaxed slower than juvenile arteries in response to the native dilatator NO and to the NO-independent sGC activator, runcaciguat. In addition, the mRNA-expression of two sGC alpha subunits was significantly lower in aged compared to juvenile vessels. The data suggest the important contribution of NO-related pathways in VSM cells to the impaired dilatory vessel function during aging.

A decrease in endothelium-dependent vasodilatation is a main and well-known feature of vessel aging, and it has been demonstrated in numerous studies in animals as well as in humans [3–7]. Defects in the NO pathway, deficits in EDHF-mediated responses, and changes in the function of the prostaglandin system contribute to endothelial dysfunction in the process of aging [3,20]. Endothelial dysfunction is characterized by reduced flow-mediated dilatation [21]. Furthermore, agonist-induced dilatation is impaired [22]. In the present study, ACh-induced vasorelaxation was reduced in aged animals as compared to young ones, in agreement with previous observations in rats and humans [4,15,23]. However, the non-selective inhibition of NOS using L-NAME did not only decrease the overall response of both juvenile and aged vessels to ACh, but also abolished the difference between the group responses. This strongly suggests the critical role of the NO system in the dilatation of small mesenteric arteries of C57BL6 mice and in the age-related reduction in

the dilatory capacity of these vessels, which is in line with previous studies demonstrating impaired NO-mediated relaxation in large arteries, resistance vessels, and arterioles [24–27].

Endothelium-independent vasorelaxation was tested by the cumulative application of the NO donor, SNP. The concentration-dependent relaxations did not differ between juvenile and aged vessels, suggesting that NO signalling pathways were not impaired in VSM cells. In the literature, results regarding endothelium-independent relaxation are inconsistent. For example, endothelium-independent NO-mediated relaxation in three to four-month-old rats was similar as compared to 20-month-old rats [15], while it was reduced in 45-week-old rats as compared to 12-week-old rats [16]. These contrary observations may reflect different aging models. Furthermore, it cannot be excluded that differences in the nutrition, motor activity, and environmental conditions of the animals contribute to the inconsistent observations. For a more detailed investigation of NO signalling in VSM cells, endothelium-independent relaxation in response to the bolus application of NO was followed up for 10 min. This experimental design enabled a closer view of the dynamics of NO signalling in VSM cells. The experiment revealed prompt relaxation in juvenile vessels in contrast to sluggish relaxation in older vessels. More notably, this difference could be seen in the physiologically important range of up to 2 min. Several mechanisms of vascular adaptation to metabolic demands and vessel-based autoregulation of organ perfusion include NO signalling via sGC. Most of these dynamic adaptations of local and systemic circulation work within a time period that lasts from seconds to minutes; one example is the myogenic response [28]. The NO-system modulates this important mechanism for the control of organ perfusion. It has also been shown that NO release varies with the frequency of the fluctuations of blood pressure and blood flow, respectively, and dampens blood pressure variations in a low-frequency range. The latter may be an antihypertensive effect [29,30]. Thus, studying these dynamics is important for understanding age-related differences in cardiovascular control.

To support the assumption that the age difference in relaxation dynamics is related to a change in sGC activity, cGMP concentrations in the vessels were measured. For this purpose, the NO- and haem-independent sGC activator, runcaciguat, was applied as a bolus. Again, the relaxation was slower in aged vessels as compared to juvenile vessels. More importantly, the slower relaxation was accompanied by lower cGMP levels 2 min after runcaciguat was applied. Thus, both the natural as well as the pharmacological stimulation of sGC point to a decline in its activity with increasing age. The smaller effect of PDE5 inhibition in aged vs. juvenile mice is in agreement with this conclusion. However, the lower PDE5 mRNA expression in aged animals also has to be considered in this context.

sGC is a heterodimeric haemoprotein, consisting of an alpha and a beta subunit, each of which has two isoforms in vertebrates (α1, α2, and β1, β2) [31]. The α1/β1 heterodimer is probably most widely expressed in mammals. However, the α2/β1 variant has also been found in several tissues [32]. Thus, sGCα1/β1 and sGCα2/β1 isoforms seem to be the physiologically active heterodimers [33]. Mice lacking the α1 subunit of sGC show decreased ACh-induced vasodilation and are prone to hypertension, while the deletion of the β1 subunit in smooth muscle induces a complete loss of sGC function [34–37]. Experiments with mice deficient in either α1- or α2-sGC underscore the functional importance of both sGCα1/β1 and sGCα2/β1 heterodimers in aortic relaxation [38]. In mice, sGCα1/β1 is mainly responsible for the modulation of renal blood flow and systemic blood pressure. Interestingly, sGCα2/β1 can replace sGCα1/β1 function [37]. In the present study, mRNA for α1 (GUCY1A1), α2 (GUCY1A2), and β1 (GUCY1B1) was detected in resistance mesenteric vessels, with the lowest expression for GUCY1A2, which agrees with observations in the rat aorta [38]. GUCY1A1 and GUCY1A2 were expressed at lower levels in aged as compared to juvenile vessels, which might explain the decline in sGC function. The cGMP-degrading enzyme, PDE5, was also differentially expressed when juvenile and aged vessels were compared. We assumed a compensatory decrease in PDE5 due to reduced cGMP levels. In contrast to the findings of the present study, an upregulation of PDE5 has been observed in the iliac arteries of middle-aged rats and in senescent human VSM

cells [39,40]. PDE5 expression seems not to be influenced by sGC, because sGCα1/β1-deficient mice show comparable PDE5 expression to that in wild-type mice [41]. We also observed a significant reduction in the mRNA expression of the functionally less significant PDE3A isoform in aged mice compared to juvenile mice. The activation of sGC in rat pulmonary artery smooth muscle cells upregulates the protein expression and activity of PDE3A, an effect reversed by sGC inhibitors [42]. A similar effect is seen in sGC knock-out mice, which have a 50% reduction in the expression and activity of PDE3A compared to that found in wild-type mice [43]. Taken together, GUCY1A1, GUCY1A2, PDE3A, and maybe PDE5 mRNA expression patterns correspond with the decline in the dynamics of sGC activity shown here and support the notion of altered NO signalling and cGMP metabolism, respectively, in old VSM cells. Protein expression data are not shown because the specificity of antibodies for the sGC subtypes and PDEs is lower than that for qPCR probes. Therefore, mRNA data may reflect gene expression better than protein expression data in this case. In order to exclude a possible significant contribution of eNOS to an age-related decrease in relaxation, its expression was analysed. Results revealed no difference in eNOS mRNA levels between groups. The nNOS expression was also investigated. It was expectedly very low and may not reach biological significance. The expression of iNOS was below the detection level, which suggests an absence of inflammatory processes in aged vessels and is in agreement with the histological finding that there is no atherosclerotic remodelling of aged vessels. The observation corresponds with the literature and reflects the comparatively lower contribution of NO from nNOS to vasorelaxation [44,45]. Studies on the effect of age on eNOS expression do not provide a uniform picture and report either unaltered or decreased eNOS mRNA and protein expressions [46–50]. Other studies have also found increased eNOS mRNA expression [50,51]. Although the majority of the results may point to the contribution of eNOS to a decreased endothelial relaxing function in old vs. young animals, further studies on the contribution of eNOS to vasorelaxation in general as well as with respect to age are warranted.

In conclusion, altered NO signalling in VSM impairs the age-related vasorelaxation of resistance arteries in male mice. Blunted sGC activity in the VSM compartment hallmarks vascular aging, which occurs early in life, in the absence of overt histopathological changes.

4. Materials and Methods

4.1. Experimental Animals

Male mice (C57BL/6) were maintained at the animal facility of the Charité—Universitätsmedizin Berlin under a 12 h light/dark cycle in enriched cages. Juvenile (age: 91–97 days ~13 weeks, n = 65) and aged (age: 280–288 days ~40 weeks, n = 45) were fed with standard pellet food sniff (ssniff-Spezialdiäten GmbH, Soest, Germany, sodium content: 0.24%) and allowed access to tap water ad libitum.

4.2. Chemicals and Drugs

The drugs used in this experiment—acetylcholine (ACh), phenylephrine (PE), L-NAME ($N\omega$-nitro-L-arginine methylester hydrochloride)—were purchased from Sigma, St. Louis, MO, USA; SNP was purchased from Carl Roth, Karlsruhe, Germany; sildenafil citrate from BioVision, Milpitas, CA, USA. Runcaciguat was provided by Bayer AG, Research & Development, Pharmaceuticals (Wuppertal, Germany). All salts, glucose and HEPES used to prepare buffer solutions were purchased from Carl Roth, Karlsruhe, Germany. PE and ACh were dissolved in distilled water and stored at −20 °C. The SNP solution was prepared in distilled water immediately before use and stored in an amber tube. L-NAME was dissolved using ultrasound in distilled water. Runcaciguat was initially prepared in dimethylsulphoxide (DMSO, Thermo Scientific, Bellefonte, PA, USA) and stored at −20 °C.

4.3. Vessel Function

Mesenteric arteries with the size of resistance arteries (diameter of non-pressurized arteries: 59–67 µm) were used in the present study. To isolate the vessels, the mice were killed

by cervical dislocation under isoflurane. The intestines were removed and placed in ice-cold and oxygenated buffer (146 mmol L^{-1} NaCl, 4.5 mmol L^{-1} KCl, 1.2 mmol L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 1 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 5.5 mmol L^{-1} glucose, 0.025 mmol L^{-1} Na (EDTA), 5 mmol L^{-1} HEPES, and 1.6 mmol L^{-1} $CaCl_2 \cdot 2H_2O$; pH 7.4). The mesenteric arterial network was isolated from the tissue and 2 mm long segments in the size of resistance arteries were cut. The segments were then mounted onto two stainless steel wires (diameter: 40 µm) in a small vessel myograph (model 410A, Danish Myo Technology A/S, Hinnerup, Denmark), under isometric conditions. The vessel segments were allowed to equilibrate in carbogenated (95% O_2, 5% CO_2) experimental solution (119 mmol L^{-1} NaCl, 4.7 mmol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 1.2 mmol L^{-1} $MgSO_4 \cdot 7H2O$, 6.1 mmol L^{-1} glucose, 25 mmol L^{-1} $NaHCO_3$, and 2.5 mmol L^{-1} $CaCl_2 \cdot 2H_2O$; pH 7.4) at 37 °C. The resting tension of the arteries was set according to Mulvany's normalization procedure [52]. The diameter of the vessel was set to 90% of that calculated for a transmural tension of 100 mm Hg, as described by Mulvany and Halpern [53].

To test the activity of the mounted and equilibrated vessels, the chamber solution was changed to K-PSS (123.7 mmol L^{-1} KCl, 1.2mmol L^{-1} KH_2PO4, 1.2 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 6.1 mmol L^{-1} glucose, 25 mmol L^{-1} $NaHCO_3$, and 2.5 mmol L^{-1} $CaCl_2 \cdot 2H_2O$; pH 7.4). After maximum contraction was reached, the K-PSS was washed out and replaced with an experimental solution. To measure the relaxing ability of arteries, they were precontracted with phenylephrine (PE, 10^{-5} mol L^{-1}) or 60mM KCl (63.7 mmol L^{-1} NaCl, 60 mmol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 25 mmol L^{-1} $NaHCO_3$, 1.2 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 11.1 mmol L^{-1} glucose, 0.026 mmol L^{-1} Na (EDTA), and 1.6 mmol L^{-1} $CaCl_2 \cdot 2H_2O$). Endothelium-dependent relaxation was measured by applying ACh in cumulative concentrations (10^{-9}~10^{-6} mol L^{-1}, each concentration for 50 s), and endothelium-independent relaxation was measured by the application of sodium nitroprusside (SNP, 10^{-11}~10^{-5} mol L^{-1}, each concentration for 30 s). Vessels were pre-incubated with L-NAME (10^{-4} mol L^{-1}) in the chamber for 30 min to estimate the contribution of the NO system. The PDE5 inhibitor sildenafil (10^{-9}~10^{-6} mol L^{-1}, each concentration for 30 s) was used to characterize the NO–sGC–cGMP axis. The NO- and haem-independent sGC function was tested using the sGC activator, runcaciguat (10^{-6} mol L^{-1}).

4.4. Direct cGMP ELISA

Mesenteric arteries were isolated. After normalization and KCl-testing, arteries were incubated with L-NAME (10^{-4} mol L^{-1}) for 30 min and pre-contracted with PE (10^{-5} mol L^{-1}). Then, runcaciguat (10^{-6} mol L^{-1}) was applied as bolus. After 2 min, the arteries were shock-frozen and stored at −80 °C until cGMP concentration was measured using a direct cGMP ELISA kit (Enzo life Science, Lausen, Switzerland) according to the manufacturer's instructions.

4.5. Gene Expression Analysis

Isolated mesenteric arteries were frozen and stored at −80 °C. RNA was isolated with RNA-Bee-reagent (Biozol, Eching, Germany) and reverse transcribed with the High-Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. qPCR was performed using a Lightcycler LC480 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol using the hydrolysis probe fluorescent detection of the DNA generated during PCR. The expression levels of mRNA were normalized to the housekeeping gene RPL32 (primer sequences: see Table 1) and relative expression levels were calculated by ΔCt method.

4.6. Morphometric Analysis

Aortae (thoracic and abdominal) and small branches of mesenteric arteries were dissected and placed in 4% buffered formalin (SAV Liquid Production GmbH, Flintsbach, Germany) for fixation. The fixed vessels were embedded in paraffin, sliced (4 µm for aorta and 6 µm for mesenteric arteries), and stained using van Gieson's solution (1% acid fuchsin

and saturated aqueous solution of picric acid mixed 1:10) for elastic fibres. Image J 1.48 was used to measure arterial dimensions and to calculate the media lumen ratio (digital images: Color Camera Nikon DS-Ri2 Numerical Aperture) [54].

Table 1. Sequences of primers and probes used for gene expression analysis.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	TaqMan Probe (5′–3′)
nNOS	CCCGACAGGCCAAAGAAATA	ACGTCCCCGCAGACATAAAT	GCTGGCCGAGTCTGTGTACCGCGCCC
iNOS	AGCGGCTCCATGACTCCCA	GGCACCCAAACACCAAGCT	ATGCGGCCTCCTTTGAGCCCTTT
eNOS	GACCCTCACCGCTACAACAT	ATGAGGTTGTCCTGGTGTCC	AGGATGTGGCTGTGTGCATGGATCT
GUCY1a1	ATTTCATGCTGGACCGAGAC	TTCCCTTGGAAGTCCCTCTT	TAACGGCATCAGAAGGCTGGTGAAC
GUCY1a2	CTGGACTCACTAGGCGAAAG	GTCATGTGTATCGTCTGAGGC	CAGCCTCCTGACGGCGCCCTT
GUCY1b1	AGCCCTTACACCTTCTGCAA	CATTGCCACACTGAGTGACC	CCTTTTCACATCATATTTGACCGGAACC
GUCY1b2	CAGGTGTTGTGGGAGACAAG	TCCTAGAGGCCGTGTTTACG	CCCGGTACTGCCTGTTTGGTGACAC
PDE5	CCGACTTCAGCTTCAGTGACTT	GGTCAGTGAACATCCGAATTG	TGTCTGATCTGGAAACAGCGCTGTG
RPL32	TTCATCAGGCACCAGTCAGA	TTGTCAATGCCTCTGGGTTT	TGTGAAAATTAAGCGAAACTGGCGG

4.7. Statistics

Mean, standard error of the mean (SEM), and EC50 were calculated using GraphPad Prism 9.0.1 (GraphPad software, San Diego, CA, USA). Data for time and concentration-dependent vessel responses are presented as mean ± SEM. Data were tested for normal distribution using the Shapiro–Wilk test. However, most data sets were not normally distributed. Therefore, non-parametric tests were used for all of the comparisons. Time and concentration-dependent differences between groups were tested by the Brunner test, a non-parametric counterpart of the two-way ANOVA, which tests the hypotheses of a global difference between the two groups and a global effect of change [55]. The Mann–Whitney U test (GraphPad Prism 9.0.1) was used post hoc to analyse the differences between the vessel responses of the two groups at individual time points and concentrations, as well as the EC50. Morphometry and mRNA expression data are presented as median, box, and whiskers (min., max.), and differences were tested using the Mann–Whitney U test.

Author Contributions: Conceptualization and methodology, C.Z., P.H.K., A.P. and M.G.; formal analysis, C.Z., M.X. and P.H.K.; investigation, C.Z., P.H.K., M.X., S.B., H.S., S.G., J.S., C.E. and F.-B.L.; resources, A.P., P.B.P. and S.G.; data curation, C.Z. and P.H.K.; writing—original draft preparation, C.Z.; writing—review and editing, P.H.K., A.P., P.B.P. and M.G.; visualization, C.Z. and P.H.K.; supervision, A.P. and M.G.; project administration, A.P.; funding acquisition, A.P., P.B.P., J.S. and M.G. All authors have read and agreed to the published version of the manuscript.

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References

1. Veerasamy, M.; Ford, G.A.; Neely, D.; Bagnall, A.; MacGowan, G.; Das, R.; Kunadian, V. Association of aging, arterial stiffness, and cardiovascular disease: A review. *Cardiol. Rev.* **2014**, *22*, 223–232. [CrossRef]
2. Koopman, J.J.E.; Kuipers, R.S. From arterial ageing to cardiovascular disease. *Lancet* **2017**, *389*, 1676–1678. [CrossRef]
3. Brandes, R.P.; Fleming, I.; Busse, R. Endothelial aging. *Cardiovasc. Res.* **2005**, *66*, 286–294. [CrossRef] [PubMed]
4. Egashira, K.; Inou, T.; Hirooka, Y.; Kai, H.; Sugimachi, M.; Suzuki, S.; Kuga, T.; Urabe, Y.; Takeshita, A. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation* **1993**, *88*, 77–81. [CrossRef] [PubMed]
5. Hongo, K.; Nakagomi, T.; Kassell, N.F.; Sasaki, T.; Lehman, M.; Vollmer, D.G.; Tsukahara, T.; Ogawa, H.; Torner, J. Effects of aging and hypertension on endothelium-dependent vascular relaxation in rat carotid artery. *Stroke* **1988**, *19*, 892–897. [CrossRef] [PubMed]
6. Welter, J.; Pfitzer, G.; Grisk, O.; Hescheler, J.; Lubomirov, L.T. Epac-mediated relaxation in murine basilar arteries depends on membrane permeability of cyclic nucleotide analogues and endothelial aging. *Gen. Physiol. Biophys.* **2020**, *39*, 157–168. [CrossRef] [PubMed]
7. Singh, N.; Prasad, S.; Singer, D.R.; MacAllister, R.J. Ageing is associated with impairment of nitric oxide and prostanoid dilator pathways in the human forearm. *Clin. Sci.* **2002**, *102*, 595–600. [CrossRef]
8. Wennysia, I.C.; Zhao, L.; Schomber, T.; Braun, D.; Golz, S.; Summer, H.; Benardeau, A.; Lai, E.Y.; Lichtenberger, F.B.; Schubert, R.; et al. Role of soluble guanylyl cyclase in renal afferent and efferent arterioles. *Am. J. Physiol. Renal. Physiol.* **2021**, *320*, F193–F202. [CrossRef]
9. Gericke, A.; Goloborodko, E.; Sniatecki, J.J.; Steege, A.; Wojnowski, L.; Pfeiffer, N. Contribution of nitric oxide synthase isoforms to cholinergic vasodilation in murine retinal arterioles. *Exp. Eye Res.* **2013**, *109*, 60–66. [CrossRef]
10. McDonald, L.J.; Murad, F. Nitric oxide and cyclic GMP signaling. *Proc. Soc. Exp. Biol. Med.* **1996**, *211*, 1–6. [CrossRef]
11. Yang, C.; DeMars, K.M.; Candelario-Jalil, E. Age-Dependent Decrease in Adropin is Associated with Reduced Levels of Endothelial Nitric Oxide Synthase and Increased Oxidative Stress in the Rat Brain. *Aging Dis.* **2018**, *9*, 322–330. [CrossRef]
12. Eskurza, I.; Monahan, K.D.; Robinson, J.A.; Seals, D.R. Effect of acute and chronic ascorbic acid on flow-mediated dilatation with sedentary and physically active human ageing. *J. Physiol.* **2004**, *556 Pt 1*, 315–324. [CrossRef] [PubMed]
13. Hamilton, C.A.; Brosnan, M.J.; McIntyre, M.; Graham, D.; Dominiczak, A.F. Superoxide excess in hypertension and aging: A common cause of endothelial dysfunction. *Hypertension* **2001**, *37 Pt 2*, 529–534. [CrossRef] [PubMed]
14. Lacolley, P.; Regnault, V.; Laurent, S. Mechanisms of Arterial Stiffening: From Mechanotransduction to Epigenetics. *Arterioscler. Thromb. Vasc. Biol.* **2020**, *40*, 1055–1062. [CrossRef] [PubMed]
15. Ma, L.; Wang, K.; Shang, J.; Cao, C.; Zhen, P.; Liu, X.; Wang, W.; Zhang, H.; Du, Y.; Liu, H. Anti-peroxynitrite treatment ameliorated vasorelaxation of resistance arteries in aging rats: Involvement with NO-sGC-cGKs pathway. *PLoS ONE* **2014**, *9*, e104788.
16. Guo, Y.; Xu, C.; Man, A.W.C.; Bai, B.; Luo, C.; Huang, Y.; Xu, A.; Vanhoutte, P.M.; Wang, Y. Endothelial SIRT1 prevents age-induced impairment of vasodilator responses by enhancing the expression and activity of soluble guanylyl cyclase in smooth muscle cells. *Cardiovasc. Res.* **2019**, *115*, 678–690. [CrossRef] [PubMed]
17. Outzen, E.M.; Zaki, M.; Abdolalizadeh, B.; Sams, A.; Boonen, H.C.; Sheykhzade, M. Translational value of mechanical and vasomotor properties of mouse isolated mesenteric resistance-sized arteries. *Pharmacol. Res. Perspect.* **2015**, *3*, e00200. [CrossRef]
18. Hahn, M.G.; Lampe, T.; El Sheikh, S.; Griebenow, N.; Woltering, E.; Schlemmer, K.H.; Dietz, L.; Gerisch, M.; Wunder, F.; Becker-Pelster, E.M.; et al. Discovery of the Soluble Guanylate Cyclase Activator Runcaciguat (BAY 1101042). *J. Med. Chem.* **2021**, *64*, 5323–5344. [CrossRef]
19. Ting, K.K.; Coleman, P.; Zhao, Y.; Vadas, M.A.; Gamble, J.R. The aging endothelium. *Vasc. Biol.* **2021**, *3*, R35–R47. [CrossRef]
20. Giles, T.D.; Sander, G.E.; Nossaman, B.D.; Kadowitz, P.J. Impaired vasodilation in the pathogenesis of hypertension: Focus on nitric oxide, endothelial-derived hyperpolarizing factors, and prostaglandins. *J. Clin. Hypertens.* **2012**, *14*, 198–205. [CrossRef]
21. Laurent, S. Defining vascular aging and cardiovascular risk. *J. Hypertens.* **2012**, *30*, S3–S8. [CrossRef] [PubMed]
22. Matz, R.L.; Schott, C.; Stoclet, J.C.; Andriantsitohaina, R. Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products. *Physiol. Res.* **2000**, *49*, 11–18. [PubMed]
23. Chang, F.; Flavahan, S.; Flavahan, N.A. Superoxide inhibition restores endothelium-dependent dilatation in aging arteries by enhancing impaired adherens junctions. *Am. J. Physiol. Heart Circ. Physiol.* **2018**, *314*, H805–H811. [CrossRef] [PubMed]
24. Luscher, T.F.; Barton, M. Biology of the endothelium. *Clin. Cardiol.* **1997**, *20* (Suppl. 2), II-3-10. [CrossRef]
25. Hirai, D.M.; Copp, S.W.; Hageman, K.S.; Poole, D.C.; Musch, T.I. Aging alters the contribution of nitric oxide to regional muscle hemodynamic control at rest and during exercise in rats. *J. Appl. Physiol.* **2011**, *111*, 989–998. [CrossRef] [PubMed]
26. Soucy, K.G.; Ryoo, S.; Benjo, A.; Lim, H.K.; Gupta, G.; Sohi, J.S.; Elser, J.; Aon, M.A.; Nyhan, D.; Shoukas, A.A.; et al. Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness. *J. Appl. Physiol.* **2006**, *101*, 1751–1759. [CrossRef] [PubMed]
27. Laughlin, M.H.; Turk, J.R.; Schrage, W.G.; Woodman, C.R.; Price, E.M. Influence of coronary artery diameter on eNOS protein content. *Am. J. Physiol. Heart Circ. Physiol.* **2003**, *284*, H1307–H1312. [CrossRef] [PubMed]
28. Schiffrin, E.L. How Structure, Mechanics, and Function of the Vasculature Contribute to Blood Pressure Elevation in Hypertension. *Can. J. Cardiol.* **2020**, *36*, 648–658. [CrossRef]

29. Nafz, B.; Wagner, C.D.; Persson, P.B. Endogenous nitric oxide buffers blood pressure variability between 0.2 and 0.6 Hz in the conscious rat. *Am. J. Physiol.* **1997**, *272 Pt 2*, H632–H637. [CrossRef]
30. Just, A.; Wittmann, U.; Nafz, B.; Wagner, C.D.; Ehmke, H.; Kirchheim, H.R.; Persson, P.B. The blood pressure buffering capacity of nitric oxide by comparison to the baroreceptor reflex. *Am. J. Physiol.* **1994**, *267 Pt 2*, H521–H527. [CrossRef]
31. Derbyshire, E.R.; Marletta, M.A. Structure and regulation of soluble guanylate cyclase. *Annu. Rev. Biochem.* **2012**, *81*, 533–559. [CrossRef]
32. Montfort, W.R.; Wales, J.A.; Weichsel, A. Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor. *Antioxid. Redox Signal.* **2017**, *26*, 107–121. [CrossRef] [PubMed]
33. Russwurm, M.; Koesling, D. Isoforms of NO-sensitive guanylyl cyclase. *Mol. Cell Biochem.* **2002**, *230*, 159–164. [CrossRef] [PubMed]
34. Buys, E.S.; Raher, M.J.; Kirby, A.; Shahid, M.; Baron, D.M.; Hayton, S.R.; Tainsh, L.T.; Sips, P.Y.; Rauwerdink, K.M.; Yan, Q.; et al. Genetic modifiers of hypertension in soluble guanylate cyclase alpha1-deficient mice. *J. Clin. Investig.* **2012**, *122*, 2316–2325. [CrossRef] [PubMed]
35. Friebe, A.; Mergia, E.; Dangel, O.; Lange, A.; Koesling, D. Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7699–7704. [CrossRef]
36. Groneberg, D.; Konig, P.; Wirth, A.; Offermanns, S.; Koesling, D.; Friebe, A. Smooth muscle-specific deletion of nitric oxide-sensitive guanylyl cyclase is sufficient to induce hypertension in mice. *Circulation* **2010**, *121*, 401–409. [CrossRef]
37. Mergia, E.; Thieme, M.; Hoch, H.; Daniil, G.; Hering, L.; Yakoub, M.; Scherbaum, C.R.; Rump, L.C.; Koesling, D.; Stegbauer, J. Impact of the NO-Sensitive Guanylyl Cyclase 1 and 2 on Renal Blood Flow and Systemic Blood Pressure in Mice. *Int. J. Mol. Sci.* **2018**, *19*, 967. [CrossRef]
38. Mergia, E.; Friebe, A.; Dangel, O.; Russwurm, M.; Koesling, D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *J. Clin. Investig.* **2006**, *116*, 1731–1737. [CrossRef]
39. Justo, A.F.O.; de Oliveira, M.G.; Calmasini, F.B.; Alexandre, E.C.; Bertollotto, G.M.; Jacintho, F.F.; Antunes, E.; Monica, F.Z. Preserved activity of soluble guanylate cyclase (sGC) in iliac artery from middle-aged rats: Role of sGC modulators. *Nitric Oxide* **2021**, *106*, 9–16. [CrossRef]
40. Bautista Nino, P.K.; Durik, M.; Danser, A.H.; de Vries, R.; Musterd-Bhaggoe, U.M.; Meima, M.E.; Kavousi, M.; Ghanbari, M.; Hoeijmakers, J.H.; O'Donnell, C.J.; et al. Phosphodiesterase 1 regulation is a key mechanism in vascular aging. *Clin. Sci.* **2015**, *129*, 1061–1075. [CrossRef]
41. Stegbauer, J.; Friedrich, S.; Potthoff, S.A.; Broekmans, K.; Cortese-Krott, M.M.; Quack, I.; Rump, L.C.; Koesling, D.; Mergia, E. Phosphodiesterase 5 attenuates the vasodilatory response in renovascular hypertension. *PLoS ONE* **2013**, *8*, e80674. [CrossRef]
42. Busch, C.J.; Graveline, A.R.; Jiramongkolchai, K.; Liu, H.; Sanchez, L.S.; Bloch, K.D. Phosphodiesterase 3A expression is modulated by nitric oxide in rat pulmonary artery smooth muscle cells. *J. Physiol. Pharmacol.* **2010**, *61*, 663–669.
43. Dunnes, S.; Voussen, B.; Aue, A.; Groneberg, K.; Nikolaev, V.; Groneberg, D.; Friebe, A. Phosphodiesterase 3A expression and activity in the murine vasculature is influenced by NO-sensitive guanylyl cyclase. *Pflugers Arch.* **2018**, *470*, 693–702. [CrossRef]
44. Arce, C.; Vicente, D.; Segura, V.; Flacco, N.; Monto, F.; Almenar, L.; Aguero, J.; Rueda, J.; Jimenez-Altayo, F.; Vila, E.; et al. Activation of alpha1A -adrenoceptors desensitizes the rat aorta response to phenylephrine through a neuronal NOS pathway, a mechanism lost with ageing. *Br. J. Pharmacol.* **2017**, *174*, 2015–2030. [CrossRef] [PubMed]
45. Cacanyiova, S.; Berenyiova, A.; Malekova, M.; Kristek, F.; Dovinova, I.; Krenek, P.; Pivackova, L.; Pifkova, I. Different vasoactive effects of chronic endothelial and neuronal NO-synthase inhibition in young Wistar rats. *J. Physiol. Biochem.* **2014**, *70*, 749–760. [CrossRef] [PubMed]
46. Luttrell, M.; Kim, H.; Shin, S.Y.; Holly, D.; Massett, M.P.; Woodman, C.R. Heterogeneous effect of aging on vasorelaxation responses in large and small arteries. *Physiol. Rep.* **2020**, *8*, e14341. [CrossRef] [PubMed]
47. Novella, S.; Dantas, A.P.; Segarra, G.; Vidal-Gomez, X.; Mompeon, A.; Garabito, M.; Hermenegildo, C.; Medina, P. Aging-related endothelial dysfunction in the aorta from female senescence-accelerated mice is associated with decreased nitric oxide synthase expression. *Exp. Gerontol.* **2013**, *48*, 1329–1337. [CrossRef] [PubMed]
48. Woodman, C.R.; Price, E.M.; Laughlin, M.H. Shear stress induces eNOS mRNA expression and improves endothelium-dependent dilation in senescent soleus muscle feed arteries. *J. Appl. Physiol.* **2005**, *98*, 940–946. [CrossRef]
49. Tanabe, T.; Maeda, S.; Miyauchi, T.; Iemitsu, M.; Takanashi, M.; Irukayama-Tomobe, Y.; Yokota, T.; Ohmori, H.; Matsuda, M. Exercise training improves ageing-induced decrease in eNOS expression of the aorta. *Acta Physiol. Scand.* **2003**, *178*, 3–10. [CrossRef] [PubMed]
50. Chou, T.C.; Yen, M.H.; Li, C.Y.; Ding, Y.A. Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension* **1998**, *31*, 643–648. [CrossRef]
51. Cernadas, M.R.; Sanchez de Miguel, L.; Garcia-Duran, M.; Gonzalez-Fernandez, F.; Millas, I.; Monton, M.; Rodrigo, J.; Rico, L.; Fernandez, P.; de Frutos, T.; et al. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ. Res.* **1998**, *83*, 279–286. [CrossRef] [PubMed]
52. Mulvany, M.J.; Halpern, W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* **1977**, *41*, 19–26. [CrossRef] [PubMed]
53. Mulvany, M.J.; Halpern, W. Mechanical properties of vascular smooth muscle cells in situ. *Nature* **1976**, *260*, 617–619. [CrossRef] [PubMed]

54. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675. [CrossRef] [PubMed]
55. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2014. Available online: http://www.r-project.org/ (accessed on 15 May 2021).

11. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12. Complete list of publications

Original publications

C Zhong, M Xu, S Boral, H Summer, F-B Lichtenberger, C Erdoğan, M Gollasch, S Golz, PB Persson, J Schleifenbaum, A Patzak, PH Khedkar. Age Impairs Soluble Guanylyl Cyclase Function in Mouse Mesenteric Arteries. *Int J Mol Sci***.** 2021 Oct 22;22(21):11412. *Impact Factor (2020): 5.923*

EG. Avery, H Bartolomaeus, A Rauch, C-Y Chen, GN'Diaye, U Löber, TU.P. Bartolomaeus, R Fritsche-Guenther, AF. Rodrigues, A Yarritu, **C Zhong**, LY Fei, D Tsvetkov, M Todiras, J-K Park, L Markó, A Maifeld, A Patzak, M Bader, S Kempa, JA. Kirwan, SK. Forslund, DN. Müller, N Wilck. *Quantifying the impact of gut microbiota on inflammation and hypertensive organ damage*. *Cardiovasc Res*. 2022 Jul 29;cvac121.doi: 10.1093/cvr/cvac121. *Impact Factor (2021*): *13.081*

G Fan, M Kaßmann, Y Cui, C Matthaeus, S Kunz, **C Zhong**, S Zhu, Y Xie, D Tsvetkov, O Daumke, Y Huang, M Gollasch. Age attenuates the T-type Ca_V 3.2-RyR axis in vascular smooth muscle. *Aging Cell*. 2020 Apr;19(4):e13134. *Impact Factor (2020): 9.304*

C Matthaeus, X Lian, S Kunz, M Lehmann, **C Zhong**, C Bernert, I Lahmann, DN Müller, M Gollasch, O Daumke. eNOS-NO-induced small blood vessel relaxation requires EHD2-dependent caveolae stabilization. *PLoS One*. 2019 Oct 10;14(10):e0223620. *Impact Factor (2019): 2.74*

Review

C Zhong, J Schleifenbaum. Genetically Encoded Calcium Indicators: A New Tool in Renal Hypertension Research. *Front Med (Lausanne**).*** 2019 Jun 13;6:128.

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