DNA Damage-related Vascular Dysfunction

pathways and interventions

Paula K. Bautista-Niño

Colofon

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mechanismen en interventies

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Introduction

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ABSTRACT

Vascular aging plays a central role in health problems and mortality in older people. Apart from the impact of several classical cardiovascular risk factors on the vasculature, chronological aging remains the single most important determinant of cardiovascular problems. The causative mechanisms by which chronological aging mediates its impact, independently from classical risk factors, remain to be elucidated. In recent years evidence has accumulated that unrepaired DNA damage may play an important role. Observations in animal models and in humans indicate that under conditions during which DNA damage accumulates in an accelerated rate, functional decline of the vasculature takes place in a similar but more rapid or more exaggerated way than occurs in the absence of such conditions. Also epidemiological studies suggest a relationship between DNA maintenance and age-related cardiovascular disease. Accordingly, mouse models of defective DNA repair are means to study the mechanisms involved in biological aging of the vasculature. We here review the evidence of the role of DNA damage in vascular aging, and present mechanisms by which genomic instability interferes with regulation of the vascular tone. In addition, we present potential remedies against vascular aging induced by genomic instability. Central to this review is the role of diverse types of DNA damage (telomeric, non-telomeric and mitochondrial), of cellular changes (apoptosis, senescence, autophagy), cyclin-dependent kinase inhibitors, senescence-associated secretory phenotype (SASP)/senescence-messaging secretome (SMS), insulin and insulin-like growth factor 1 (IGF-1) signaling), the adenosine monophosphate-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR)-nuclear factor kappa B (NFκB) axis, reactive oxygen species (ROS) *vs.* endothelial nitric oxide synthase (eNOS)-cyclic guanosine monophosphate (cGMP) signaling, phosphodiesterase (PDE) 1 and 5 and diet restriction.

1. INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death worldwide, responsible for killing 17.3 million persons per year.[1] The onset of CVD is triggered by vascular endothelial alterations characterized by an impaired endothelium-dependent vasodilation, the overproduction of pro-inflammatory and prothrombotic molecules, and oxidative stress.[2] Age is the strongest independent predictor for CVD in risk scores in middle-aged persons, and an important determinant for cardiovascular health in the population aged 65 or older.[3, 4] Aging is characterized by the complex interaction of cellular and molecular mechanisms that leads to a collection of functional problems. Focusing on the vasculature, such problems are closely associated with each other, and include worsened vasodilation, increased arterial stiffness and overt remodeling of the extracellular matrix, diffuse intimal thickening and a dysfunctional endothelium.[4] The mechanisms through which age actually contributes to cardiovascular risk remain the subject of speculation. From a classical perspective, modifiable risk factors promote and modulate molecular mechanisms that, as time progresses, culminate in an imbalance in the production *vs.* scavenging of ROS (*i.e.*, superoxide anions, hydrogen peroxide and hydroxyl radicals), increasing ROS levels, and, as a consequence, reducing the bioavailability of nitric oxide (NO).[5, 6] NO is crucial in the maintenance of vascular homeostasis, including in the regulation of vascular dilation, the modulation of cell growth and the prevention of thrombosis.[7] In the absence of a healthy endothelium, these factors gradually increase the pathologic phenotype of the vasculature up to the point that cardiovascular events occur.

While this paradigm explains vascular aging in view of classical risk factors as causative mechanisms, a recently proposed alternative view on vascular aging has emerged that presents new mechanistic alternatives for understanding the process of vascular aging.[8] In this novel paradigm, causal mechanisms for the process of aging itself, most notably genomic instability, including telomere attrition, drive the detrimental changes occurring increasingly with (biological) aging (Figure 1). The involvement of these causal factors of aging in general have been discussed elsewhere.[9] In the present review we summarize the evidence that supports the role of genomic instability in vascular aging. In addition, we present mechanisms through which genomic instability generates the functional changes that are typical for the aging vasculature.

2. GENOMIC INSTABILITY AND AGING: A SHORT OUTLINE OF THE BASIC PRINCIPLES

2.1. DNA Repair Systems

The maintenance of genomic integrity is critical for the prevention of aging of organisms. To safeguard genomic integrity, cells are equipped with several genomic maintenance systems that sense and repair DNA damage.[10, 11] The sources of DNA damage are very diverse and range from intrinsic molecular reactions within DNA molecules such as hydrolysis, attacks by endogenous metabolic products, and ROS, to damage by exogenous physical and chemical entities such as chemotherapy and UVB light.[12] To account for the different types of DNA damage, cells are equipped with multiple DNA repair pathways. Each repair system is responsible for a specific subset of lesions, although partial overlap can occur depending on the type of DNA lesion that needs to be repaired. At least six DNA repair pathways can be listed in mammalian cells: (1) the direct reversal pathway, which executes the direct reversal of chemical modifications of nucleotides; (2) mismatch repair (MMR), which repairs base pair mismatches; (3) base excision repair (BER), repairing mainly oxidized and alkylation lesions in the nucleus and mitochondria, as well as single-strand breaks; (4) nucleotide excision repair (NER), to correct transcription-disturbing bulky adducts; (5) homologous recombination (HR); and (6) non-homologous end joining (NHEJ), which correct single- and double-strand breaks.[10, 13] Telomere maintenance requires further specialized proteins.[14] Hypothetically, the classical cardiovascular risk factors initiate ROS-induced DNA damage and thus contribute to genomic instability-related vascular aging (Figure 1). Although some factors that lead to (vascular) genomic instability have been identified, the road to identification of all relevant contributors is still long (Figure 1).[8, 15]

2.2. Aging: The Interplay between Genomic Damage, the Survival Response and Cellular Senescence

Unrepaired genomic damage enables the generation of harmful mutations that can be transferred to new cells during proliferation. This puts complex organisms at the potential risk of rapidly developing dysfunctional tissues or even tumors. As a protective measure, accumulating unrepaired DNA damage triggers a switch in biological pathways from a phenotype supporting growth to one favoring maintenance of the organism, a switch often referred to as the "survival response".[16] However, the switch is believed to contribute to the typical changes that occur during aging, as demonstrated in humans and animals with defective DNA maintenance.[16]

To avoid the harmful consequences of genomic instability, such as cancer, complex organisms have developed protective cellular mechanisms, namely apoptosis and cellular senescence. Whereas apoptosis embodies the loss of (dysfunctional) tissue due

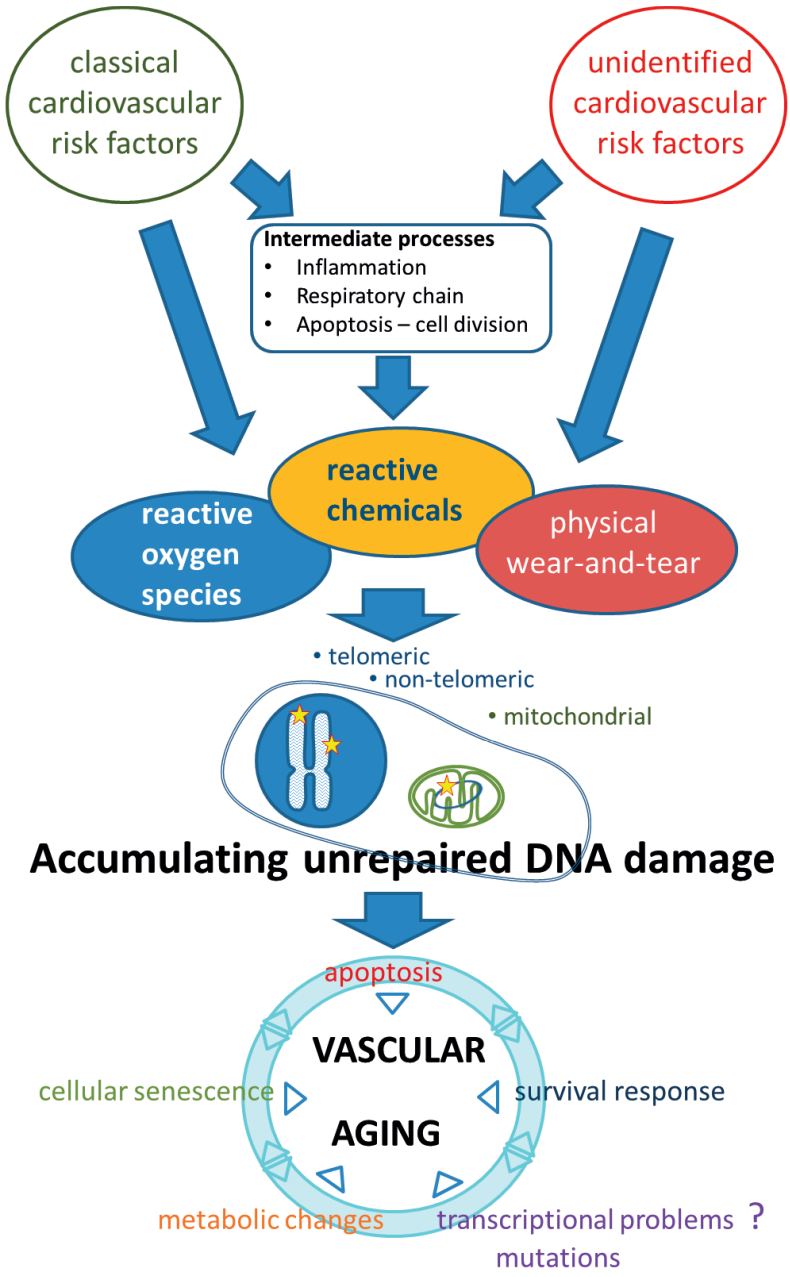

Figure 1. Etiology of vascular aging based on genomic instability as a causal factor. Classical and unidentified risk factors contribute to various types of DNA lesions. Unrepaired lesions accumulating during life lead to a growing set of pathophysiological changes that, either independently or in mutual interaction, lead to progressive vascular aging. The putative role of transcriptional problems or mutations herein needs to be established. The survival response may have beneficial (increased Nrf2-regulated antioxidants) as well as detrimental (decreased IGF-1 signaling, pro-inflammatory status) effects (see text and Ref. [8]).

to programmed cell death, which might account for loss of organ function, cellular senescence has a more intricate relationship with aging tissues. Senescent cells undergo cell cycle arrest and thus can no longer replicate, although they remain metabolically active and often acquire a SASP, an immunogenic phenotype consisting of interleukins, pro-inflammatory cytokines, and growth factors.[17] It is believed that this results in an increased susceptibility to age-associated disease, including cancer and cardiovascular disease.[17] As a consequence of cellular senescence, the organisms age and become susceptible to age-associated diseases. Paradoxically, the accumulation of senescent cells with age, which is believed to result from an inefficient clearance by the immune system, might also help delay tissue dysfunction through cell loss. Recently, however, it was shown that removal of senescent cells expressing the cyclin-dependent kinase inhibitor $p16^{INK4A}$ in genetically modified mice (INK-ATTAC mice) leads to a prolonged life and health span[18], supporting a fundamental role for cellular senescence in aging. The mechanisms through which removal of senescent cells leads to these effects remain to be elucidated.

3. GENOMIC INSTABILITY AS A CAUSAL FACTOR IN VASCULAR AGING: EVIDENCE IN HUMANS

There is ample evidence that genomic instability is involved in vascular aging in humans. The following section highlights the observations that have accumulated until the present.

3.1. Cardiovascular Disease in Progeria Syndromes

The role of DNA damage in aging is further highlighted in human progeria syndromes. Human syndromes of progeria arise from mutations in genes involved in genomic maintenance in at least 75% of the known cases.[19] Progeria syndromes provide a unique opportunity to study aging, but it should be noted that they are not a complete phenocopy, e.g., progeria patients show phenotypes that are rare during normal aging, such as clavicular agenesis in Hutchinson-Gilford progeria syndrome or the intensified risk of cancer in Werner syndrome.[20] The relation of progeria to normal aging remains debatable.

Despite this continuing debate, it is intriguing to observe that several progeria syndromes manifest severe, juvenile cardiovascular disease. Werner syndrome (WS) is characterized by the premature onset of clinical signs of aging, such as cancer, osteoporosis and cardiovascular disease (diabetes mellitus type II and atherosclerosis).[21] WS is caused by a WRN (Werner) gene mutation. WRN encodes a DNA helicase protein, *Escherichia coli* recQ-like helicase L2 (RECQL2), which is involved in DNA recombination, replication,

repair and transcription, and also in telomere maintenance.[22] WS patients develop a considerable burden of atherosclerotic plaques in the coronary arteries and the aorta; calcification of the aortic valve is also frequently observed. Consequently, most WS patients die during middle age (average life expectancy is 46 years) due to myocardial infarction and stroke.[21] A related disease called Bloom syndrome, a consequence of mutation of the RecQ helicase gene *BLM*, features telangiectasias (dilated blood vessels in the skin), but the function of blood vessels has not been extensively investigated, although the occurrence of diabetes in these patients might be an important confounder in such investigations.[23]

Hutchinson-Gilford progeria syndrome (HGPS), perhaps the best-known progeroid disorder, is characterized by hair loss, pain in the joints, wrinkled skin, and cardiovascular problems.[24] HGPS is caused, in most patients, by a point mutation in the lamin A gene (*LMNA*), which encodes the A-type nuclear lamins. The mutant lamin A, called progerin, remains fixed to the nuclear envelope causing various cellular changes, such as irregular nuclear shape and disorganization of heterochromatin, that lead to abnormal regulation of gene expression, therefore inducing premature aging. Death occurs around the age of 13 years mostly due to myocardial infarction or cerebrovascular events; however, in contrast to typical human aging or WS, atherosclerosis is very rare. Instead a major loss of vascular smooth muscle cells (VSMCs) in both big and small arteries is observed.[25] Interestingly, accumulation of prelamin A was observed in medial VSMCs and in atherosclerotic lesions from normally aged individuals. Moreover, prelamin A colocalized with β-galactosidase-positive VSMCs, *i.e.*, senescent VSMCs, and thus prelamin A was proposed as a marker of vascular aging in the general population.[26]

Excision repair cross-complementation group 1 (ERCC1)-xeroderma pigmentosum (XP) F is a structure-specific protein complex serving as an endonuclease that participates in the repair of several types of DNA lesions, mainly bulky, helix-distorting lesions that are repaired by the NER pathway, but also double-strand breaks and interstrand cross-links.[27-29] Progeroid syndromes arising from ERCC1-XPF mutations, often unique cases as each of the mutations found until now has been encountered in individual patients, have been repeatedly reported as being characterized by hypertension.[30] This is further accompanied by frailty, loss of subcutaneous fat, liver dysfunction, vision and hearing loss, renal insufficiency, bone marrow degeneration, and kyphosis.[31] Although the hypertension observed in this syndrome might point at accelerated vascular aging, this still needs to be confirmed, certainly if one takes into consideration the presence of renal insufficiency in the patients suffering from this type of syndrome.

For other progeroid syndromes related to mutations in genomic DNA repair enzymes, data concerning vascular function are not available. It is uncertain whether this is an indication for the absence of vascular aging. Rather, more prominent problems in other organ systems or a focus on increased susceptibility to cancer might mask the

presence of cardiovascular problems. In general, the patients are very frail, and cases are rare. Extensive cardiovascular characterization of such patients is, therefore, a very challenging task, and perhaps even not without risk for the patients themselves.

3.2. Indicators of a Role of Genomic Instability in the General Population

The role of genomic instability in disorders of the vasculature or the consequences thereof is a question that becomes increasingly important for the general population. If, indeed, this mechanism is central in age-related cardiovascular disease, there are major implications for prediction and detection and prevention. Research on the role of genomic instability in cardiovascular risk prediction opens a new window into expanding our understanding of the pathophysiology and causative risk factors in age-related diseases.[8] The use of emerging markers of DNA damage, identified in vascular and cardiac ischemic cells, has provided evidence for this role.[32] Part of the evidence comes from studies assessing the effect of ionizing radiation. An increased amount of circulating cell-free DNA and mitochondrial DNA (mtDNA) fragments has been observed in subjects exposed to low levels of ionizing radiation, suggesting the possible role of circulating DNA as a relevant biomarker of cellular damage.[33] In turn, it has been established that there is an association between radiation exposure and indicators of accelerated vascular aging, coronary artery disease and stroke in occupationally exposed groups. Andreassi *et al.* observed that long-term, low level radiation exposure is positively correlated to early atherosclerosis, as identified by increased subclinical cIMT (carotid intima media thickness), and to telomere shortening, an indicator for genomic instability.[34] This study also concluded that subjects with the Thr241Met polymorphism in the XRCC3 gene (gene coding for X-ray repair cross-complementing protein 3) have a greater susceptibility to radiation-induced vascular effects. Data of the Life Span study showed that people who had received an acute single dose of 1–2 Sv (sievert) had a significantly increased risk of mortality from myocardial infarction after 40 years of radiation exposure.[35] Other evidence is provided by observation of DNA damage markers in vascular tissue and circulating cells. Several groups observed elevated levels of oxidative DNA damage in human atherosclerotic plaques compared to non-atherosclerotic vessels or in circulating cells of persons with arterial disease [36, 37]. Likewise, several proteins involved in DNA repair including DNA-dependent protein kinase (DNA-PK), poly (ADP-ribose) polymerase 1 (PARP-1), p53, and Apurinic/apyrimidinic endonuclease 1/redox factor 1 (APE-1/Ref1), were up-regulated in plaques of carotid endarterectomy specimens compared with non-atherosclerotic arteries.[36] On the other hand, genetic association studies have shown a significant association of single nucleotide polymorphisms (SNPs) in NER-related genes with age-related vascular phenotypes. In the population of the AortaGen Consortium, comprising 20,634 participants from nine cohort studies, Durik *et al.* identified an association of the SNP rs2029298 (p-value: 1.04×10^{-4}) in the Damage-Specific DNA Binding

Protein 2 (DDB2) gene with carotid-femoral pulse wave velocity, a measure of vascular stiffness.[38] In addition, suggestive associations were found for eight SNPs located within or near ERCC5, ERCC6, general transcription factor IIH (GTF2H) subunit 1 and 3 (GTF2H3, GTF2H1), and ERCC2.[38] Verschuren *et al.* showed, in data from the GENDER (GENetic DEterminants of Restenosis) and PROSPER (Patient-centered Research Into Outcomes Stroke Patients Prefer and Effectiveness Research) studies comprising 6110 coronary artery disease (CAD) patients in total, that genetic variations in the NHEJ repair system are associated with risk for CAD.[39] In addition, several smaller studies have shown associations between polymorphisms in single DNA repair genes and risk of coronary artery disease, as reviewed elsewhere.[40] Interesting to note is also the finding that statins were found to improve DNA damage detection, which might be a mechanism leading to the improvement of atherosclerosis next to the reduction of lipids and oxidative stress.[41, 42]

3.3. Telomere Shortening

Human chromosomes are normally capped by telomeres that protect the end-segment of chromosomes between cell divisions. Since telomeres do not fully replicate during mitosis, they gradually become shorter as individuals age.[43] Defects in telomerase activity, abnormalities in DNA polymerase to synthesize terminal ends of the DNA, and the inhibition of the sheltering component telomeric repeat-binding factor 2 (TRF2) leads to an accelerated velocity of telomere shortening between cell divisions, which induces cellular senescence when the telomere reaches a critical length.[43] Telomere shortening promotes chromosome end fusion, chromosomal abnormalities and aneuploidy, suggesting that loss of chromosome end protection is correlated to genome instability.[44] Studies using knockout mouse models have established that the targeted deletion of 53BP1 and TRF2 genes is one of the main mechanisms involved in double-strand breaks and an increase of non-reciprocal translocations caused by telomere shortening.[45] In addition, RNA template of telomerase $(TERC)^{-/-}$ and high mobility group box 1 $(HMGB1)^{-/-}$ mice exhibit a reduced/null telomerase activity and telomere dysfunction, triggering aging-like cellular changes.[46, 47] Population-based studies suggest that telomere shortening plays a role in the onset of vascular aging-related phenotypes. Individuals with a shorter mean telomere length exhibit a two-fold risk of abdominal aortic aneurysm compared to those with a higher telomere length (odds ratio = 2.30, $p = 0.005$).[48, 49] Moreover, an association between telomere shortening and the following CVD risk factors has been found: atherosclerosis, arterial stiffness (as measured by carotid-femoral pulse wave velocity), smoking, insulin resistance, type 1 and type 2 diabetes, obesity, hypertension and up-regulation of the renin–angiotensin–aldosterone system.[50-57] Likewise, an increased level of telomere shortening, via increased oxidative DNA damage, has been identified in circulating endothelial progenitor cells (EPC) in CAD patients with metabolic syndrome.[58] These observations suggest that oxidative stress-

induced telomere shortening in EPC may accelerate vascular dysfunction, promoting the onset and progression of CAD due to a lack of vascular repair.[58] Despite the fact that the association of telomere shortening with aging and vascular-related disorders has been demonstrated, its potential use as a biomarker of age-related diseases remains unclear.[59-61]

3.4. Cyclin-Dependent Kinase Inhibitor 2 (CDNK2) A and B

Further exploring the role of genomic instability–induced cellular senescence in vascular aging, gene variations in senescence regulators have been associated with age-related vascular disease in humans. Several studies have provided insight about the risk association of the 9p21 locus with aging-related cardiovascular diseases such as atherosclerosis and aortic aneurysm.[62] This chromosomal region codes for two cyclin-dependent kinase inhibitor genes, *CDKN2A*, comprising codes for $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice), and *CDKN2B*, coding for $p15^{INK4B}$. These CDKs are key molecules involved in the regulation of cellular replication, among others in vascular cells.[63] Genetic polymorphisms in this chromosomal region have indicated that 9p21 variation has a significant influence in the genetic expression of *CDKN2A* and *CDKN2B* in VSMCs, which could increase the susceptibility to CAD.[64] In addition, differential expression of *CDNK2A* and *CDNK2B* has been observed in senescent cells *in vitro* and in aging tissues of rodents and humans.[65] Thus, the measurement of the expression of these genes has led to their use as a potential biomarker of biological age.[66] Most of the studies have determined the role of *CDNK2A* and *CDNK2B* in aging by focusing on human tumors, concluding that the deletion and silencing of the *CDKN2A–CDKN2B* locus are among the most frequent genetic events found in human cancer cells.[67] Thus, *CDNK2A* and *B* play a central role in diseases of aging.

4. GENOMIC INSTABILITY AS A CAUSAL FACTOR IN VASCULAR AGING: EVIDENCE FROM ANIMAL MODELS

4.1. Telomerase-Deficient ($TERC^{-/-}$ and $TERT^{-/-}$) Mice

Telomerase-deficient mouse models have been developed by knocking out TERC, $TERC^{-/-}$ mice, or the telomerase reverse transcriptase ($TERT^{-/-}$ mice). Homozygous $TERT^{-/-}$ and $TERC^{-/-}$ mice display short telomeres and a similar phenotype, but the $TERC^{-/-}$ mice have been studied more comprehensively. The telomeres of the $TERC^{-/-}$ mice shorten at a rate of ~5 kb in every subsequent generation (G). In conscious $TERC^{-/-}$ mice, higher systolic blood pressures were observed in mice from G1 compared with wild-type mice, whereas in G3 mice, both systolic and diastolic blood pressures were increased compared with wild-type and G1 mice.[68] The differences in blood pressure between $TERC^{-/-}$ and wild-

type mice appear to be caused by an increased production of endothelin-1 in the TERC$^{-/-}$.[68]

4.2. Mouse Models of Genomic Instability Associated with Human Progeria

Different mouse models of WS have been developed with either a complete knockout of the WRN protein, the transgenic expression of human WRN lacking helicase activity, or the in-frame deletion of the helicase domain. Depending on the model studied, the extent of the aging phenotype varies. The models lacking RecQ helicase activity show increased genomic instability and have increased visceral fat, high fasting triglycerides and cholesterol levels, insulin resistance and hyperglycemia.[69] Telomere shortening appears to be pivotal in the development of some of these metabolic changes[70], which are relevant analogues for human cardiovascular risk factors. In these models no vascular problems were reported, except, perhaps, decreased wound healing, which might implicate worsened angiogenesis.

A mouse model of HGPS expressing human progerin showed aberrations that were largely restricted to the vascular system. Recapitulating the vascular phenotype seen in patients with HGPS, these mice exhibited an increasing loss of VSMCs in the lamina media of large arteries.[71] Those changes were accompanied by a reduced vasodilator response to the NO donor sodium nitroprusside. Interestingly, the endothelium is initially undamaged, but with progression of the vessel damage, some loss in the endothelium is observed in 12-month-old mice.[71]

Mice lacking proper function of the versatile DNA repair enzyme ERCC1 show many features of accelerated aging. *Ercc1*$^{-/-}$ mice display a severe aging phenotype featuring frailty, osteoporosis, neurodegeneration, atrophic epidermis, sarcopenia, liver and kidney dysfunction and bone marrow degeneration.[72] *Ercc1*$^{-/-}$ mice only live four weeks, while mice with reduced ERCC1 function due to a combined exon 7 deletion allele and a null allele (*Ercc1*$^{d/-}$, *Ercc1*$^{-/\Delta 7}$ or *Ercc1*$^{\Delta/-}$) live longer (up to 30 weeks). The *Ercc1*$^{\Delta/-}$ mice are healthy up to an age of eight weeks, when they start developing a gradual aging phenotype. In our previous study we found that *Ercc1*$^{\Delta/-}$ mice had an increased systolic blood pressure compared to wild-type mice.[38] They also display a decreased vasodilator response in their microvasculature.[38] Microvascular function was assessed by hind leg reactive hyperemia using a laser Doppler technique, which measures superficial resistance vessels and possibly represents both endothelium-dependent and endothelium-independent relaxations.[73-75] Those microvascular changes resemble the ones seen in aged rodents as well as in humans, and, strikingly, these changes in humans are at least partly independent of classical cardiovascular risk factors.[73, 76] Another important characteristic of the human vascular aging phenotype that was recapitulated in the *Ercc1*$^{\Delta/-}$ mice includes greater stiffness, as measured by pressure-diameter relationships in the carotid arteries.[38] While aortic tissue from *Ercc1*$^{\Delta/-}$ contains increased amounts of

senescent cells, the contribution of cellular senescence to vascular dysfunction remains uncertain.[38]

Other genetic models add to the evidence, linking vascular senescence with vascular pathology and disease. Mice carrying the human XPD R722W mutation, so-called Xpd^{TTD} mice, show signs of accelerated vascular aging. In humans, the mutation in XPD causes trichothiodystrophy (TTD) which is characterized by postnatal growth failure, UV sensitivity, neurological degeneration, cachexia, osteoporosis and a shortened life span.[77] The Xpd^{TTD} mice show a similar phenotype, but the onset and severity of progeria is slower compared to *Ercc1*$^{\Delta/-}$ mice. We assessed vascular function in Xpd^{TTD} mice at 26 and 52 weeks and observed significantly reduced vasodilator responses to acetylcholine in aortic rings at 52 weeks compared to 26-week-old Xpd^{TTD} and wild-type mice.[38]

4.3. Mitochondrial DNA Maintenance Defects

ApoE-deficient mice lacking protein kinase ATM (ataxia telangiectasia mutated), a protein pivotal in DNA damage detection, showed accelerated development of atherosclerosis.[78] This was attributed to increased mtDNA damage leading to malignant metabolic changes. Further exploring the involvement of DNA (mtDNA), polymerase gamma (POLG) performs DNA synthesis inside the mitochondria, and thus mutations in POLG cause mitochondrial disorders. A mouse model with an mtDNA mutator phenotype, conferred by a homozygous mutation in POLG, has been used to study the role of mitochondrial function and aging. In early adulthood the POLG mutant mice develop many features of premature aging such as weight loss, reduced subcutaneous fat, kyphosis, osteoporosis, cardiomyopathy and a reduced life span.[79] Oxidative stress and respiratory chain dysfunction due to the accumulation of mtDNA point mutations in protein-coding genes of the respiratory chain complexes are considered to contribute to the premature aging phenotype of the POLG mutator mice. Using a double POLG/ApoE low-density lipoprotein (LDL) receptor knock-out, it was shown that instability of mtDNA might contribute to atherosclerosis. $POLG^{-/-}/ApoE^{-/-}$ mice had increased atherosclerosis in the brachiocephalic artery and descending aorta as compared to $POLG^{+/+}/ApoE^{-/-}$ controls. The $POLG^{-/-}/ApoE^{-/-}$ mice also exhibited reduced body weight, reduced fat mass, hyperlipidemia and apoptosis of VSMCs.[80] Apart from vascular effects, POLG mutant mice develop cardiac hypertrophy and dilatation, impairment of systolic and diastolic function, and increased cardiac fibrosis within 13 months of age.[81] Overexpression of catalase in the mitochondria of these mice attenuated the malignant cardiac phenotype, providing evidence for the role of oxidative stress in the development of cardiomyopathy due to mtDNA instability.

4.4. BubR1 Knockout

The spindle assembly checkpoint protein BubR1 has an important role in the maintenance of genomic stability by ensuring the correct microtubule-kinetochore attachment and segregation of chromatids during mitosis.[82]

Mice with reduced expression of BubR1 (10% of normal levels) develop progressive aneuploidy and exhibit a vascular aging phenotype characterized by reduced arterial elasticity, a reduced number of VSMCs, loss of endothelial-dependent relaxation, and increased production of superoxide anions. Apart from problems reminiscent of cardiovascular aging, BubR1 mice also show a variety of progeroid symptoms.[83, 84] Also, naturally aged wild-type mice have decreased BubR1 expression in different tissues, suggesting that BubR1 may be a regulator in normal aging.[18, 27, 83, 84] Clearance of $p16^{INK4A}$-positive senescent cells with the INK-ATTAC strategy in BubR1 progeroid mice ameliorates several of the progeroid hallmarks. However, the cardiovascular problems are not rescued, which corresponds with the observation that these features are $p16^{INK4A}$-independent in this model.[85] Importantly, vascular aging in ERCC1-defective mice appears to be associated with p53- and $p21^{Cip1}$ (or $p21^{Waf1}$)-related senescence,[8, 38] and this might further explain the ineffectiveness of removing p16-positive cells to improve cardiovascular function in BubR1 mice. Interestingly, it was recently shown that clearance of $p16^{INK4A}$-positive senescent cells in non-progeroid mice increases the life span and reduces cardiac stress sensitivity.[18, 85] This indicates that cellular senescence is indeed involved in deleterious cardiovascular phenotypes, involving both $p16^{INK4A}$ as well as p53- and p21-related senescence in a differential way.

4.5. Vascular Functional/Pharmacological Changes Due to Genomic Instability

4.5.1. NO-cGMP Signaling

NO is a key participant in many physiological pathways such as vasodilation, neurotransmission, and macrophage-mediated immunity. In the vascular endothelium, NO is produced from the substrate L-arginine by the enzyme eNOS (or nitric oxide synthase type III). The eNOS is activated by increased cytoplasmic Ca^{2+} levels, as induced, among others, by binding of vasodilatory (neuro)hormones to their G protein-coupled receptors.[86] Evidence provided up to date suggests that there is a reciprocal relationship between defective eNOS activity and genomic instability. During vascular aging, there is an increased production of ROS.[87] This ROS can be partly produced by eNOS, when the enzyme is in a so-called uncoupled state due to a reduced expression of the cofactor tetrahydrobiopterin (BH4), as has been shown in aging rats.[88] ROS coming from this and other sources, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or mitochondria, react with NO to form harmful free radicals, including peroxynitrite ($ONOO^-$) and N_2O_3. The overproduction of ROS not only leads to a gradual reduction

of NO bioavailability in the vasculature, but in addition can cause single-strand DNA breaks, 7,8-dihydro-8-oxoguanine and other oxidative lesions.[89]

The aberrant eNOS function is closely associated with dysfunction as observed in aged and diseased blood vessels. In $eNOS^{-/-}$ mice, systemic hypertension, altered vascular remodeling, dysfunctional angiogenesis and a prothrombotic phenotype have been observed.[90-94] In human atherosclerosis, eNOS mRNA expression was shown to be decreased in endothelial cells of advanced atherosclerotic plaques, which is accompanied by overt DNA damage.[36] In addition, eNOS uncoupling has been reported in patients with endothelial dysfunction as a consequence of diabetes, hypertension, hypercholesterolemia and smoking, linking the mechanism to classical risk factors.[95]

Apart from a potential role of eNOS dysfunction in the production of DNA lesions, genomic instability itself causes dysfunction of NO signaling. Organ bath studies and molecular analyses in *Ercc1*$^{\Delta/-}$ mechanistically explained the decreased vasodilator responses.[38] These experiments showed that NO-mediated responses, eNOS expression, and eNOS activation through phosphorylation serine 1177 were decreased. Increased generation of ROS, a central mechanism in age-related decreased NO-dependent vasodilation, was partly responsible for the diminished vasodilation in *Ercc1*$^{\Delta/-}$ mice since anti-oxidants such as *N*-acetylcysteïne and BH4 improved vasodilation.[38] Therefore, faulty eNOS activation and genomic instability appear to form a vicious circle leading to progressive endothelial dysfunction and accelerated vascular aging (Figure 3). In parallel to endothelial dysfunction, *Ercc1*$^{\Delta/-}$ mice show impaired VSMC function but whether the ERCC1 functional mutation has effects downstream of NO production (i.e. in cGMP responsiveness or metabolism in VSMCs), and whether this occurs at the level of cGMP generation by soluble guanylyl cyclase (sGC), protein kinase G Iα responsiveness to cGMP or cGMP metabolism by phosphodiesterases (PDEs) remains an important question in relation to therapeutic options (Figure 3).

5. PERSPECTIVES

5.1. Directions for Future Studies Establishing the Role of Genomic Instability

Although the evidence summarized above strongly indicates a major role of genomic instability in vascular aging, important questions remain to be solved. Firstly, there is the question as to whether the accelerated vascular aging features that are observed are due to local vascular processes, or whether they are the consequence of the general accelerated deterioration in mouse models of genomic instability. Tissue-specific inactivation of DNA repair enzymes is a means to explore this question. To this end, endothelial cell- and VSMC-specific Cre recombinase strains and mouse strains with Plox sites in DNA repair genes of interest are available, but still need to be combined.

Furthermore, the mechanisms that are the interface between genomic instability and derailment of vascular signaling systems need to be resolved. Cellular senescence has been mentioned as an option, but apoptosis is also a candidate mechanism. The role of cellular senescence *vs.* vascular cell apoptosis in vascular aging remains an important question. Although previously discussed mouse studies provide compelling evidence, the role of cellular senescence *in vivo* in human aging remains unclear, mostly due to the absence of specific biomarkers that can provide information about the state of cells in tissues.[96] Restricting this to vascular aging, no conclusive evidence for a causal role of cellular senescence in vascular aging, let alone that induced by genomic instability, has been published yet. The aforementioned results in INK-ATTAC models on a wild-type mouse background, and in ERCC1-defective mice, are, however, highly indicative [18, 38]. Models combining constructs to eliminate senescent cells in a background of vascular-specific genomic instability are putative tools to further establish this mechanism.

Another mechanism could be stem cell exhaustion, which requires comprehensive analysis of vascular cell progenitors in mice with increased genomic instability. In ERCC1-deficient mice, reduced hematopoietic progenitor cell reserves have been observed.[97] Since hematopoietic cells generate vascular progenitor cells[98], there is indeed a motive to explore this possibility.

Whether genomic instability in nuclear DNA outside of telomeres mediates vascular dysfunction through mutations or transcriptional dysfunction as caused by DNA lesions is a most important question that remains to be explored (Figure 1). Since DNA lesions have been repeatedly reported (see above), this is a very realistic option.

5.2. Towards New Interventions in Vascular Aging Caused by Genomic Instability

The awareness that genomic instability and cellular senescence arising thereof play a key role in general and in vascular aging opens new possibilities to prevent age-related cardiovascular disease. In particular, life-extending therapies that have been identified thus far are candidate interventions to decelerate vascular aging. In addition, interventions that prevent vascular genomic instability or readily improve NO-cGMP signaling are eligible for such purposes. We delineate the various options here.

5.2.1. Rapamycin, mTOR and Autophagy

During aging, increasing dysfunction related to a progressive failure of maintenance and repair pathways takes place as aberrant macromolecules, dysfunctional organelles and DNA damage may accumulate in cells and tissues.[99] Therefore, cellular maintenance mechanisms are crucial to preserve normal cellular functions. Autophagy, one of the main cellular preservation processes, is involved in the degradation of long-lived proteins and dysfunctional organelles as well as in the maintenance of the cell in case of

failure of macromolecule repair.[100] With age the rate of autophagy and protein degradation declines.[101, 102] Importantly, genetic ablation of Atg7, an important mediator of autophagy, causes an accelerated appearance of vascular aging hallmarks in mice.[103] Autophagy-modifying drugs, such as rapamycin, inhibit the mammalian target of rapamycin complex 1 (mTORC1) and control the activation of autophagy-related signaling pathways. Rapamycin (also known as sirolimus) increases longevity and delays pathological lesions in mice.[104] Furthermore, the therapeutic use of rapamycin or related drugs prevents age-related diseases such as cancer and cardiovascular diseases in animal models.[105, 106] In addition, the pleiotropic anti-atherosclerotic effects of rapamycin have allowed the implementation of rapamycin-based therapies to prevent or delay the pathogenesis of atherosclerosis.[107] Further, it has been reported that rapamycin improves endothelium-dependent vasodilation in old rodents.[108] A potential beneficial effect of mTOR inhibition on vascular aging independent from autophagy regulation was proposed.[109] This effect would be based on the regulation of a signaling network, consisting of mTOR, adenosine monophosphate-activated protein kinase (AMPK), and sirtuin (SIRT)-1. In this model, mTOR inhibition, in concert with SIRT-1 and AMPK activation, would counteract age-related vascular dysfunction thanks to modulation of the common transcription factors NFκB, FoxO and p53, that, when integrated, determine stress resistance, inflammation, ROS production, NO signaling, genomic instability and cellular senescence. Apart from this link to genomic instability and senescence, it has been found in a mouse model of Hutchinson-Gilford progeria that AMPK activation and mTOR inhibition occurs in conjunction with activation of autophagy.[110] Thus, models of genomic instability appear to implicate the proposed mTOR–AMPK signaling interaction, with a link to the regulation of autophagy (Figure 2). However, the effect on vascular aging as based on genomic instability remains to be explored.

Discouraging the use of mTOR inhibition is the fact that rapamycin significantly attenuates both endothelial function and the expression of eNOS in human endothelial cell lines *in vitro*, although it does not cause endothelial cell death.[111] Studies with mTOR-inhibiting drugs, among others applied on coronary stents in patients with advanced arterial aging, have reported deleterious effects of such drugs on various variables of endothelial (dys)function, although conflicting results are abundant.[112-116] It is unclear whether the conflicting results are dependent on the concentration of the mTOR inhibitor to which the endothelial cells are exposed, which presumably is very high in the case of drug-eluting stents. Although in cultured endothelial cells the increasing anti-inflammatory effect of increasing concentrations of mTOR inhibitors parallels the increasing cytostatic effect,[117] this issue needs further inspection. We have also shown that rapamycin actually induces PAI-1 expression in cultured endothelial cells and *in vivo* in mice,[118] so the net benefit of this drug in preventing senescence may be mixed at best.

In summary, mTOR inhibition, on the one hand, seems to be an attractive hypothetical option to reduce vascular aging in relation to genomic instability, but the idea should be approached cautiously.

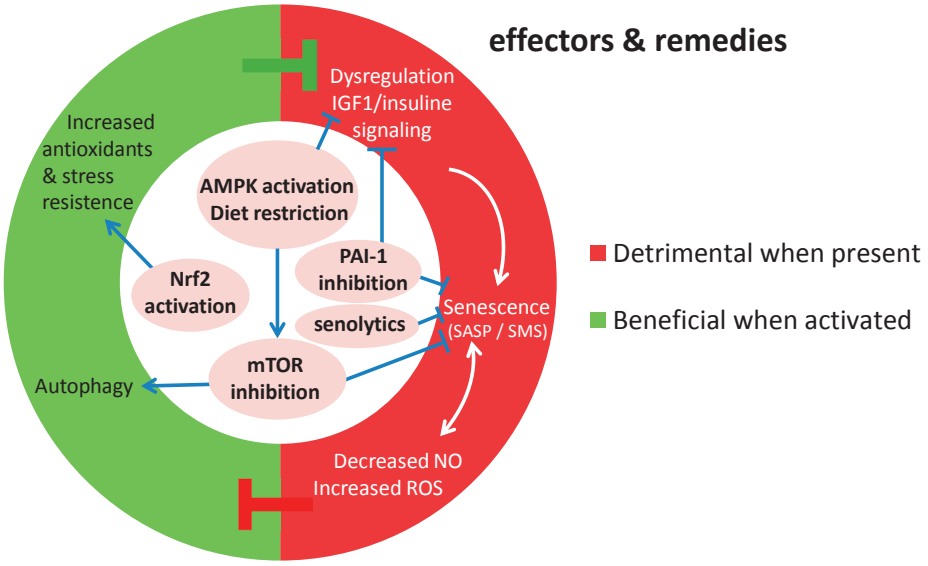

Figure 2. Molecular effectors of genomic instability that contribute to vascular aging, and the potential remedies (center of the chart) against that currently under development. Senescence, imbalanced NO *vs.* ROS production, inflammation and changes in insulin signaling are detrimental when present while autophagy, apoptosis and stress resistance have a beneficial contribution to vascular aging. IGF-1 putatively has a detrimental effect, although this needs further scrutiny (Ref. [8]). Pointed arrows indicate stimulatory processes, while blunted arrows indicate inhibitory processes.

5.2.2. Senolytics and Inhibitors of Senescent Cell Signaling

Cellular senescence and the overproduction of SASP-associated proteins, also referred to as the senescence-messaging secretome (SMS), contributes to local and systemic dysfunction and disease. Therefore, the implementation of "senolytic" therapies has been approached as an intervention to specifically target senescent cells (Figure 2), eliminate them, and thus diminish the contribution of SASP and SMS.[119] The use of senolytic drugs including dasatinib and quercetin has been effective in eliminating senescent primary mouse embryonic fibroblasts and senescent human fat cell progenitors. *In vivo,* the combination of these drugs reduced senescent cells in normal aged, radiation-exposed mice, and in *Ercc1*$^{\Delta/-}$ mice.[119] In addition, this study showed that periodic drug administration extended the health span in *Ercc1*$^{\Delta/-}$ mice and delayed age-related symptoms and pathology, osteoporosis, and loss of intervertebral disc proteoglycans. Despite the evidence suggesting that interventions that reduce the number of senescent cells could mitigate age-related tissue dysfunction, the burden of cell senescence biomarkers and

SASP needs to be further studied and validated in humans. Therefore, the implementation of new therapies to reduce senescent cell number and SASP must be characterized.

Pioneer results from our group showed for the first time that modulation of the SMS can actually prevent the development of senescence in kl/kl mice, a mouse model of accelerated aging.[120] In addition to contributing to the molecular fingerprint of senescence, plasminogen activator inhibitor-1 (PAI-1) is essential and even sufficient for the induction of replicative senescence *in vitro* and is a critical downstream target of the tumor-suppressor p53.[121, 122] The contribution of PAI-1 to cellular senescence is broadly relevant in the organism as a whole, and age-dependent increases in plasma PAI-1 levels have been identified in wild-type mice as they age, in murine models of accelerated aging (*Klotho* and *BubR1*$^{H/H}$), and in humans.[18, 123, 124] We observed that forced decrease of PAI-1 attenuated levels of the SMS factors insulin-like growth factor-binding protein 3 (IGFBP3) and interleukin-6 in plasma of kl/kl mice to levels seen in wild-type (WT) mice. In addition, telomere integrity was partially protected in numerous tissues. Furthermore, the nuclear accumulation of the senescence marker p16^{INK4A} was prevented. Similar observations were made in another aging-related model.[125] It is important to note that IGFBP3 is also strongly affected in DNA repair-defective progeroid models, as are other components of the IGF-1 growth factor signaling pathway, placing this pathway in the center of genomic instability-related (vascular) aging.[8, 126] Moreover, this link raises the exciting possibility that PAI-1 might be involved in genomic instability-related vascular aging (Figure 2). As a still remote possibility, PAI-1 might act as part of the SMS from cells that become senescent due to unrepaired DNA damage, thus transmitting a harmful signal to cells in which the genomic integrity is still warranted (Figure 3). Application of genetic or pharmacological inhibition of PAI-1 in models of genomic instability is therefore an attractive approach to test this hypothesis.

5.3. Dietary Restriction

In search of treatment perspectives, it is of course important to consider more general anti-aging and longevity-increasing interventions. Apart from the previously discussed possibility to employ rapamycin against vascular aging, dietary restriction (DR) is perhaps the most important and well-known option. DR is a reduction of intake of food to the level that it results in low-normal levels of energy intake while avoiding malnutrition.[127] Claims of an effect of diet restriction on longevity date back as far as 3000 years. Studies that have taken place over many decades over the last century indeed confirm such an effect in various species, including yeast, worms, flies, spiders, rotifers, fish and rodents, demonstrating that DR is the most effective intervention to slow down aging and extend life expectancy.[128-134]

DR is known also to protect against age-related cardiovascular disease. Two main mechanisms can be involved: (1) reduction of the intake of harmful food, such as carbo-

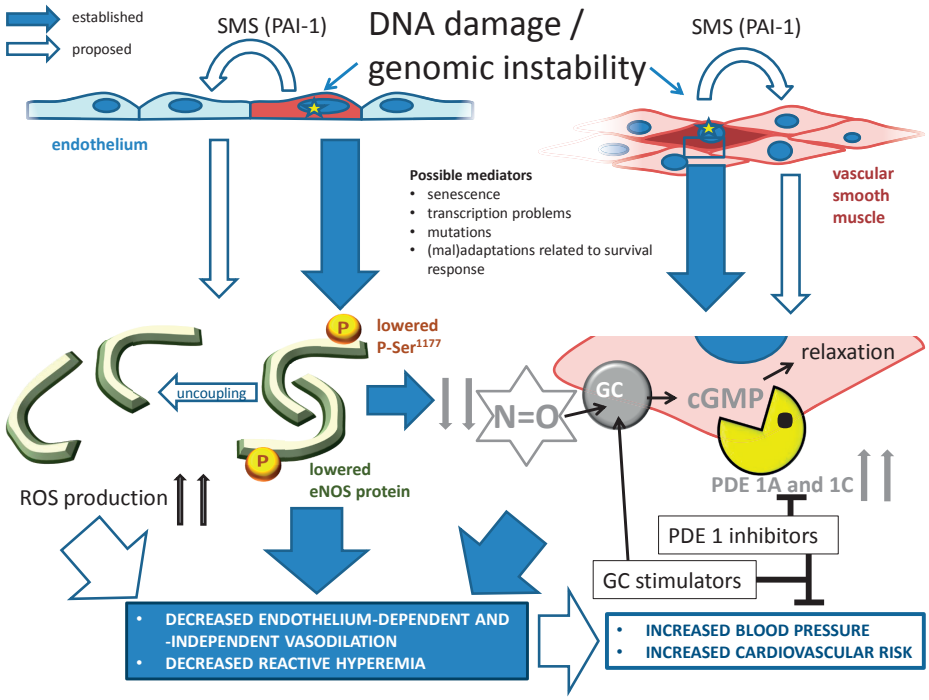

Figure 3. The role of genomic instability (indicated by yellow stars) on NO-cGMP signaling, and its consequences for age-related cardiovascular disease. Large, blue closed arrows indicate established relationships: genomic instability primarily leads to endothelial eNOS dysfunction in endothelial cells (Section 4.5.1). Large, blue open arrows refer to proposed mechanisms that were not fully explored: cellular senescence caused by unrepaired DNA could affect healthy cells through SASP/SMS (Section 5.2.2.). The affected cells in turn might worsen vascular function through changes in eNOS-cGMP signaling. PDE1 subtype inhibitors and guanylyl cyclase (GC) stimulators are promising drugs to at least acutely improve vascular function. Their value for prevention of genomic instability and vascular aging needs to be assessed. PDE1A and 1C have a putative role in atherosclerosis, arteriosclerosis, reduced blood flow and hypertension (see Section 5.4). The role of both PDE1 subtypes in aging-related vasomotor dysfunction remains to be studied. Small, thick arrows pointing up or down indicate up- and down-regulation respectively. Blunt arrows indicate inhibition, pointed thine arrow indicate stimulation.

hydrates and polysaturated fats;[135, 136] or (2) slowing down of the aging process itself. It has been shown that chronic DR improves the aging-related rise of blood pressure and vascular wall remodeling, as shown in rodents.[137, 138] This effect can be attributed to the improvement of vascular relaxation, a consequence of decreased ROS and increased NO bioavailability. In addition, DR has been reported to attenuate cardiovascular disease in nonhuman primates.[139, 140]

It is not clear what the main mechanisms of the anti-aging effect of DR are. However, the reduction of genomic instability is a possibility. In a previous review we discussed that effects on oxidative stress-induced DNA and macromolecular damage are a putative mechanism.[8] Reports have shown a possible effect of specific nutrient restriction

and of caloric restriction on markers of DNA damage and DNA repair capacity, and a plethora of publications regarding the association between food consumption and telomere length is available.[141-144] This observation pleads for evaluation of the effects of DR on the general and vascular aspects of aging in models of genomic instability. Alternatively, effects on IGF-1/ growth hormone (GH) signaling, SIRT-1 and nutrient-sensing pathways might be at play (Figure 2).[8] Since IGF-1/GH signaling is suppressed both after DR and in mouse models of genomic instability, this pathway apparently shares a common function in DR and the survival response in progeroid mice. Mouse models in which GH signaling is intentionally knocked out display increased longevity, and share features of the genetic program with genomic instability models.[126] Therefore, IGF-1/GH suppression is a point of convergence between DR, genomic instability and longevity. Whether this convergence takes place after genomic instability to improve survival, contributes to improved genomic integrity, as proposed above, or both remains to be elucidated. The effect of dietary restriction therefore needs to be explored in models of genomic instability, importantly those involving evaluation of vascular aging. The role of altered GH *vs.* IGF-1/insulin therein on vascular function needs special attention as these pathways appear to have opposite effects, as previously explained.[8]

We here propose that the aforementioned relationship with mTOR and AMPK might also be important in DR effects (Figure 2). There is evidence that DR deactivates the mTOR-dependent signaling pathways, slowing aging and delaying aging-related diseases.[145] This suggests that DR and rapamycin can act together but have different effects on several pathways related to an increased longevity in young mice; therefore, the combination of both therapies could cause and exponential rise of lifespan in mice.[146] It would be interesting to investigate if such an interaction also exists for the attenuation of vascular aging.

5.4. PDE Inhibition

An attractive aspect of PDE1 and 5 as drug targets is that there are several experimental and clinically approved drug candidates that might overcome the increased PDE activity. One is the selective PDE1 inhibitor IC86340, but unfortunately this drug appears not to be available anymore.[147, 148] Other PDE1 inhibitors are under development.[149] Further, there is the possibility to inhibit PDE5, or both PDE1 and 5. Sildenafil is a PDE5 inhibitor which also blocks PDE1 at high doses.[150] Sildenafil was found to reduce both diastolic and systolic blood pressure in untreated hypertensive patients. However, due to Sildenafil's short duration of action, research is focusing on new inhibitors such as tadalafil.[151] Vinpocetine is a PDE inhibitor with a preferential affinity for PDE1 over PDE5. Vinpocetine is an Food and Drug Adminstration (FDA)-approved nutriceutical and a registered drug in Eastern Europe, used to enhance cerebral blood flow and improve memory.[152] PDE1 inhibitors were also developed for the treatment of cognitive impairment associated

with schizophrenia.[149] Such treatment inhibits injury-induced hypertrophy in human and rodent vessels, and decreases atherosclerosis in ApoE knockout mice.[153, 154] Therefore PDE1 inhibition is an attractive option for treating age-associated cardiovascular diseases. Until now, vinpocetine never found widespread application, for reasons that are unclear. Nevertheless, PDE1 inhibition has been identified as a potential drug target in atherosclerosis, and also in heart failure. In addition, this possibility should also be explored for DNA damage-induced decrease of VSMC relaxation, as explained earlier.

5.5. Reconsideration of Antioxidant Therapies

As explained above, ROS have been identified as a source of DNA damage, and therefore ROS scavenging is a potential treatment modality. Clinical studies applying ROS scavengers (antioxidants) have, however, not resulted in benefits for patients suffering from cardiovascular diseases.[155] Although this might be due to the fact that such interventions might require the onset of intervention early in life, there is also a shortcoming in that the drugs might not reach the right place at the right time or even hamper healthy cellular signaling that is performed by ROS.[156] A better targeted interaction of antioxidant enzymes and ROS might overcome the latter shortcomings of exogenously applied ROS scavengers.

Nrf2 has been proposed as a "master regulator" of cytoprotective mechanisms and it could be associated with increased longevity and attenuating age-related diseases in mice.[157] Therefore, Nrf2 gene regulation and the enhancement of the endogenous antioxidant capacity (Figure 2) could be an important therapeutic target to diminish the production of ROS, reducing DNA damage and their effects on vascular aging. Certainly, several drugs have been developed and tested to stimulate the bioavailability of NO through the regulation of the Nrf2/antioxidant response element (Nrf2/ARE). The combined action of NO and Nrf2/ARE signaling could improve vascular function and confer protection against vascular diseases.[158] On the other hand, several alternatives to increase Nrf2 have been currently explored, including calorie restriction, ozone therapy, hyperbaric oxygen and physical exercise.[159]

5.6. Other vascular pathways potentially relevant in vascular aging

5.6.1 The Renin-Angiotensin system

A potential mediator of blood pressure increase and decreased endothelium-dependent relaxation caused by DNA damage is activation of the renin-angiotensin system (RAS). The most prominent role of the RAS is considered to be blood pressure and fluid balance control; indeed, Angiotensin (Ang) II, the main bioactive hormone of this system, is strongly involved in hypertension, arteriosclerosis, vascular DNA damage and cell senescence, inflammation, oxidative stress, longevity and health span.[160] Also, Ang II inhibits eNOS - NO - cGMP signaling.[161]

A schematic overview of the RAS and clinically available pharmacological interventions targeting this system is presented in Figure 4. Briefly, production of Ang II starts with angiotensinogen that is produced predominantly by the liver. Angiotensinogen is cleaved by renin to produce Ang I. Ang I is converted by angiotensin-converting enzyme (ACE) into Ang II, which can bind to either the Ang II type 1 (AT_1) receptors or the Ang II type 2 (AT_2) receptors. Signaling through the AT_1 receptors has vasoconstrictive, pro-inflammatory and hypertrophic effects.[162] For this reason the Ang II / AT_1 receptor signaling is associated with hypertension, heart failure and renal failure. Interestingly, the Ang II / AT_1 axis seems to be involved in aging of vascular smooth muscle cells and of endothelial progenitor cells.[163] This evidence supports a role for Ang II / AT_1 signaling in DNA damage related vascular dysfunction.

On the other hand, the effects of AT_2 receptor stimulation in cardiovascular processes seem to be beneficial and include vasodilation and natriuresis. Besides AT_2 effects, there is an endogenous pathway within the RAS called the ACE2/Ang-(1–7)/Mas receptor axis that counterregulates Ang II/AT_1 effects. The ACE2/Ang-(1–7)/Mas receptor axis is antifibrotic, antiproliferative, natriuretic, vasodilatory, and endothelium-protective.[164] The RAS is affected by aging, but findings are contradictory. In general, it is believed that RAS activation plays a role in aging, and in age-related vascular dysfunction.[165] Ang II, through ROS production, has also been reported to damage DNA.[166] Nevertheless, the effect of DNA damage on RAS remains to be studied.

5.6.2 Endothelium-derived Hyperpolarization

In addition to the eNOS-NO-cGMP pathway the endothelium-derived hyperpolarization (EDH) pathway has a major contribution in the regulation of vasodilation. It is considered that while the eNOS-NO-cGMP pathway is more important in conduit vessels, EDH has a more prominent role in resistance vessels. EDH does not refer to one single molecule. Instead, several candidates have been proposed to act as EDH factors including the C-type natriuretic peptide, lipoxygenases, arachidonic acid metabolites, potassium ions and H_2O_2. In general, EDH factors induce relaxation by activating small and intermediate calcium-dependent potassium channels.[167]

The importance of EDH is increased in certain conditions, such as in heart failure, essential hypertension and hypercholesterolemia, where a suppression of the eNOS-NO-cGMP axis is observed. In these cases, preserved or upregulated EDH responses attenuate the consequences of impaired NO signaling. Moreover, part of the beneficial effects on the cardiovascular system achieved with interventions like ACE inhibition or PDE3 inhibition are due to enhanced EDH signaling.[167] Hitherto it is not known how EDH is affected by DNA damage; although it has been reported that in murine saphenous arteries, the contribution of EDH to endothelium-dependent relaxations diminishes as the mice age.[168]

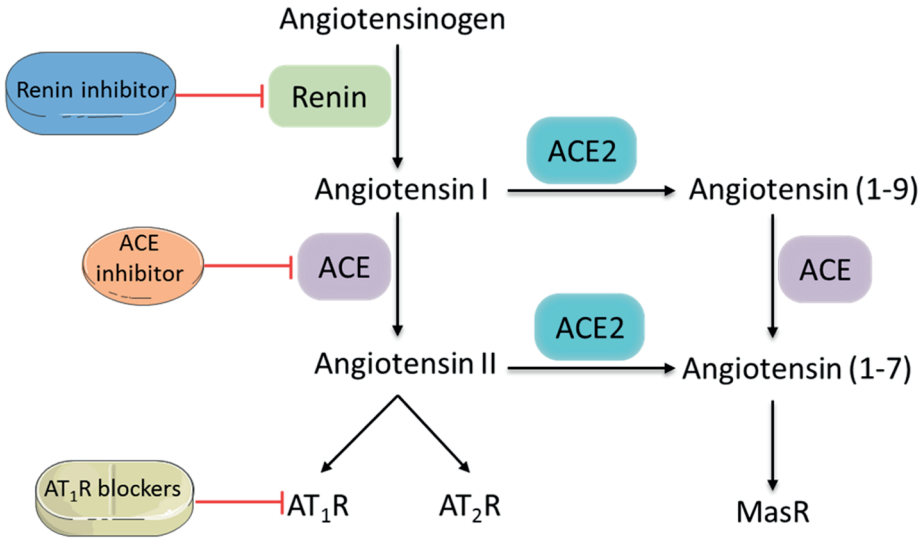

Figure 4. Schematic overview of the Renin Angiotensin System and the clinically available interventions targeting the RAS. Renin cleaves Angiotensinogen to produce Angiotensin I which in turn is converted to Angiotensin II by the the Angiotensin-converting enzyme (ACE). Ang II can bind to AT_1 or AT_2 receptors. ACE2 can convert Ang I to Ang (1-9) and Ang II to Ang (1-7). Ang (1-7) then binds to the Mas receptor.

6. SUMMARY

There is ample evidence that genomic instability is involved in vascular aging. Nuclear DNA lesions, among which is telomere erosion, and mitochondrial DNA damage are strongly associated with several main features of vascular aging, such as diminished vasodilator capacity and increased vasoconstriction, increased blood pressure, increased vascular stiffness and atherosclerosis. Pivotal cellular biological changes involved in these pathological features comprise cellular senescence, apoptosis, autophagy, stem cell exhaustion and altered proliferative capacity of vascular cells. The role of gene mutation and of compromised transcription remains unknown (Figure 1). Potential mediating signaling pathways involved include components of the survival response (Figure 1), notably antioxidants under regulation of Nrf2 (beneficial), increased inflammatory status (detrimental) and decreased IGF-1/GH signaling (detrimental), as well as the interplay between mTOR, AMPK and NFκB, SIRT-1, and PAI-1, p53- and p21- and p16-related signaling. Proposed remedies against genomic instability–related vascular aging include PAI-1 inhibition, mTOR inhibition, DR, senolytics, PDE1 and 5 inhibitors, drugs stimulating EDH and blockers of the RAS.

7. AIMS OF THIS THESIS

In this thesis the effect of DNA damage on the development of vascular damage is investigated. Also some potential interventions to treat the DNA damage-related vascular dysfunction are evaluated. In the present **Chapter (1)** we have summarized the evidence that supports the role of genomic instability in vascular aging and present mechanisms through which genomic instability generates the functional changes that are typical for the aging vasculature.

Previously, we identified in a mouse model of genomic instability due to defective DNA repair (*Ercc1*$^{\Delta/-}$ mice), that accelerated age-related vascular dysfunction takes place and that this was characterized by a decreased NO/cGMP responsiveness and increased cellular senescence in the vascular smooth muscle layer. Therefore in **Chapter 2** we investigate which component of the eNOS-NO-cGMP pathway is dysregulated in vascular smooth muscle cells, whether cellular senescence plays a role in this dysregulation, and if genetic variants of involved components of this pathway are involved in the increased risk of age-related vascular disease in humans.

Despite the evidence presented in Chapter 2 and previous work, one important question that remains is whether the observed accelerated decline of vasodilation in *Ercc1*$^{\Delta/-}$ *mice* arises from defective local vascular DNA repair or if it is a consequence of the systemic deterioration these mice endure. To address if local DNA repair defects leads to changes as observed in *Ercc1*$^{\Delta/-}$ mice, in **Chapters 3** and **4** we respectively investigate cardiovascular function in mouse models with specific loss of genome maintenance in vascular endothelial and smooth muscle cells. In Chapter 3 *Ercc1* was specifically knocked-out out in endothelial cells while in Chapter 4 DNA maintenance was crippled by specific deletion of the FACE1/*Zmpste24* gene. This model was chosen because of the observation by Shanahan *et al.* that faulty prelamin A to lamin A conversion characterizes vascular aging in healthy humans and Hutchinson Gilford progeria patients.[26] When lamin A is not properly processed it remains fixed to the nuclear envelope, causing various cellular changes such as irregular nuclear shape, disorganization of heterochromatin and defective recruitment of DNA repair factors that lead to abnormal regulation of gene expression, thereby inducing premature aging.

In **Chapter 5** we examine the effects of two well-known vasoprotective interventions: lifestyle intervention, with dietary restriction (DR), vs. pharmacotherapy, with chronic blockade of the Angiotensin II (Ang II) AT_1 receptor. DR is known to be an effective anti-aging intervention. It was found that DR ameliorates the vasodilator dysfunction observed in *Ercc1*$^{\Delta/-}$ mice, however; it is not known which vasodilatory signaling pathway is improved. Ang II has an important role in hypertension, arteriosclerosis, vascular DNA damage, inflammation and oxidative stress. Moreover, Ang II inhibits eNOS-NO-cGMP signaling. However, it is not known how genomic instability influences RAS

activity. Hence, in Chapter 5 we set out to identify which vasodilatory signaling pathway is improved by DR in *Ercc1*$^{\Delta/-}$ mice and if DR affects the vasoconstrictor responses to Ang II. Also, we evaluated the effect of chronic AT_1 receptor blockade on endothelial function and blood pressure in AL-fed *Ercc1*$^{\Delta/-}$ mice.

Aging changes the palette of major contributors to disturbed tone regulation. It is known that in humans decreased eNOS-NO-cGMP signaling contributes to the endothelial dysfunction observed in aging and cardiovascular diseases. In some conditions such as essential hypertension and hypercholesterolemia, impaired eNOS-NO-cGMP signaling is compensated by endothelium-derived hyperpolarization (EDH), and in fact EDH is unaffected by genomic instability. In **Chapter 6** we discuss findings from a recent study presenting proof of principle for a new class of antihypertensive drugs. The candidate lead compound targets EDH based on the oxidative activation of PKGIα, independently of cGMP. Drugs that improve EDH signaling could have great therapeutic relevance beyond hypertension, and might also be useful in the context of vascular aging due to genomic instability.

Complementary to chapter 6, in **Chapter 7** we investigate the involvement of PKGIα in EDH-induced vasodilation of porcine coronary arteries exposed to bradykinin, light, H_2O_2 and L-*S*-nitrosocysteine. Finally, **Chapter 8** provides a summary and future perspectives.

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AMPK	adenosine monophosphate-activated protein kinase
Ang	Angiotensin
APE-1/Ref1	Apurinic/apyrimidinic endonuclease 1/redox factor 1
ApoE	ApoE, Apolipoprotein E
ARE	antioxidant response element
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
ATM	ataxia telangiectasia mutated
BER	base excision repair
CAD	coronary artery disease
CDNK2	cyclin-dependent kinase inhibitor 2
cGMP	cyclic guanosine monophosphate
cIMT	carotid intima media thickness
CVD	cardiovascular diseases
DDB2	Damage-Specific DNA Binding Protein 2
DNA-PK	DNA-dependent protein kinase
DR	dietary restriction
EDH	endothelium-derived hyperpolarization
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
Ercc1	excision repair cross-complementation group 1
GH	growth hormone
GTF2H	general transcription factor IIH
HGPS	Hutchinson-Gilford progeria syndrome
HMBG-1	high mobility group box 1
hMSC	human mesenchymal stem cells
HR	homologous recombination
IGF1	insulin-like growth factor 1
IGFBP3	insulin-like growth factor-binding protein 3
INK-ATTAC mice	genetically modified mice in which cells expressing the cyclin-dependent kinase inhibitor p16INK4A are being removed by apoptosis due to caspase 8 activation
LMNA	lamin A gene
MMR	mismatch repair
MtDNA	mitochondrial DNA
mTOR(C1)	mammalian target of rapamycin (complex 1)
NADPH	nicotinamide adenine dinucleotide phosphate
NER	nucleotide excision repair
NHEJ	non-homologous end joining

NfkB	nuclear factor kappa B
NO	nitric oxide
Nrf2	transcription factor NF-E2-related factor-2
PAI-1	plasminogen activator inhibitor-1
PARP-1	poly [ADP-ribose] polymerase 1
PDE	phosphodiesterase
POLG	polymerase gamma
RAS	Renin angiotensin system
RecQ	*Escherichia coli* recQ-like helicase
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
(s)GC	(soluble) guanylyl cyclase
SIRT-1	sirtuin-1
SMS	senescence-messaging secretome
SNP	single nucleotide polymorphism
T2DM	type 2 diabetes mellitus
TERC	RNA template of telomerase
TERT	telomerase reverse transcriptase
TRF2	telomeric repeat-binding factor 2
TTD	trichothiodystrophy
VSMC	vascular smooth muscle cell
WRN	Werner gene
WS	Werner Syndrome
XP	xeroderma pigmentosum
XRCC3	gene coding for x-ray repair cross-complementing protein 3

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Chapter 2

Phosphodiesterase 1 regulation is a key mechanism in vascular aging

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ABSTRACT

Reduced nitric oxide – cyclic guanosine monophosphate (cGMP) signaling is observed in age-related vascular disease. We hypothesize that this disturbed signaling involves effects of genomic instability, a primary causal factor in aging, on vascular smooth muscle cells, and that the underlying mechanism plays a role in human age-related vascular disease. To test our hypothesis we combined experiments in mice with genomic instability (*Ercc1*$^{\Delta/-}$ mice), human VSMC cultures and population GWAs studies. Aortic rings of *Ercc1*$^{\Delta/-}$ showed 43% reduced responses to the soluble guanylyl cyclase (sGC) stimulator sodium nitroprusside (SNP). Inhibition of phosphodiesterase (PDE) 1 and 5 normalized SNP relaxing effects in *Ercc1*$^{\Delta/-}$ to wild-type (WT) levels. PDE1C levels were increased in lung and aorta. cGMP hydrolysis by PDE in lungs was higher in *Ercc1*$^{\Delta/-}$. No differences in activity or levels of cGMP-dependent protein kinase 1 or sGC were observed in *Ercc1*$^{\Delta/-}$ mice vs. WT. Senescent human VSMC showed elevated *PDE1A* and *PDE1C* and *PDE5* mRNA levels (by 11.6-, 9- and 2.3-fold respectively), which associated with markers of cellular senescence. Conversely, PDE1 inhibition lowered expression of these markers. Human genetic studies revealed significant associations of *PDE1A* single nucleotide polymorphisms with diastolic blood pressure (β= 0.28, p= 2.47x10^{-5}) and carotid intima media thickness (β= -0.006, p= 2.89x10^{-5}). In summary, these results show that genomic instability and cellular senescence in VSMC increase PDE1 expression. This might play a role in to aging-related loss of vasodilator function, VSMC senescence, increased blood pressure and vascular hypertrophy.

INTRODUCTION

Since the identification of endothelial nitric oxide (NO) as an important vasodilator signal in blood vessels acting through second messenger cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells (VSMC), many studies have shown that this vascular signaling pathway is disturbed in cardiovascular diseases.[1, 2] It is now widely assumed that disturbed NO-cGMP signaling is importantly involved in the pathogenesis of age-related vascular dysfunction and cardiovascular disease resulting thereof.[3, 4] However, actual evidence in humans is not abundant. A recent combined human genetic study with mouse experiments shows that mutations in the human cGMP-producing machinery, consisting of soluble guanylyl cyclase and chaperonin containing t-complex polypeptide subunit η (CCTη), a protein that stabilizes soluble guanylyl cyclase, increases the risk of myocardial infarction due to increased thrombogenesis.[5] Since this is a rare variant leading to increased familial risk for myocardial infarction, it is unclear whether this potential pathogenic mechanism acts in the general population. Even more recently it was found that genetic variants in phosphodiesterase (PDE) type 1A, an enzyme that degrades cGMP, might contribute to dysregulation of blood pressure.[6] Notably, dysregulation of PDE1 sub-types has been suggested to play a role in atherosclerosis and a potential target for treatment of VSMC and cardiomyocyte dysfunction.[7-12] However, it is unclear which function of cGMP would be involved. Also it is unclear what circumstances related to vascular aging would lead to decreased levels of this cyclic nucleotide.

Previously, we identified that genomic instability, a principle causal factor of the biological process of organismal aging, contributes to vascular aging.[13] A pivotal finding in this previous work was that in mice with genomic instability due to genetically induced defective DNA repair accelerated age-related vascular dysfunction takes place, and that this was hallmarked by a decreased NO-cGMP responsiveness and increased cellular senescence in the VSMC layer. We therefore hypothesize that genomic instability causes dysregulation of the cGMP production and metabolism machinery in VSMC, that cellular senescence plays a role in this dysregulation, and that genetic variants of involved components of this machinery are involved in increased risk of age-related vascular disease in humans.

To test our hypothesis we first explored the effect of genomic instability in mice on cGMP-induced vasodilator function and key enzymes of this signaling pathway. Subsequently, we explored the role of cellular senescence on the cGMP pathway in primary cultures of human arterial VSMC. Finally, we used genetic association studies in human cohorts to explore if there is a role of genetic variants in PDE genes on age-related human vascular disease.

MATERIALS AND METHODS

To accomplish our aims we followed an integrative approach combining experiments in animal models, human VSMC cultures and population GWAs studies.

Animals

A thorough description of the generation and of the overall phenotype of *Ercc1*$^{\Delta/-}$ mice can be found in previous publications.[14, 15] *Ercc1*$^{\Delta/-}$ mice of 16 weeks in an F1 hybrid Fvb/C57Bl/6, background and their wild-type littermates (WT) of the same age were bred at the Erasmus MC animal facility. The animals were housed in individually ventilated cages with access to normal chow and water *ad libitum*. As required by Dutch law, all animal studies were approved by an independent Animal Ethical Committee.

Experiments in human VSMC

To assess the effect of cellular senescence PDE sub-types, human VSMCs were obtained from normal medial aortic explants from 5 donors (20-54 years old) and cultured in M199 with 20% FBS and antibiotic supplements and passaged 1:2 as described previously.[16] Serial passaging until senescence was performed, and RNA was isolated at different passages. cDNA was prepared from 5 µg of total RNA to quantify expression of the *PDE1A*, *PDE1C* and *PDE5A* genes and senescence markers *p16* and *p21*.

Reciprocally, the effect of PDE1 and PDE5 inhibition on p16 and p21 was tested in n=6 samples from 3 independent duplicate experiments. Coronary artery smooth muscle cells Passage 6 (CC-2583, Lonza) cultured in DMEM + 10% FCS were treated with vinpocetine 10 µmol/L or sildenafil 100 nmol/L and incubated for 24 hours after which RNA isolation was performed and. cDNA was prepared from 500 ng of total RNA.

Organ bath experiments

Ercc1$^{\Delta/-}$ and WT mice were asphyxiated in a CO_2 chamber. The thoracic aortas were isolated and stored overnight in cold, oxygenated Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) solution. The following day, vessel segments were mounted in 6-mL organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer at 37°C and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure. Maximum contractile responses were determined using 100 mmol/L KCl. After washout of KCl, preconstriction was elicited with 30 nmol/L U46619 resulting in 50-100% of the previously obtained 100 mmol/L KCl precontraction. During preconstriction, relaxation concentration-response curves (CRCs) were constructed to sodium nitroprusside (SNP) and diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA NONOate). To detect a possible

NO/sGC/cGMP independent vasorelaxant activity of SNP, we performed SNP CRCs after inactivation of sGC with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µmol/L). To investigate the involvement of increased activity of PDE enzyme in VSMC dysfunction of *Ercc1*$^{\Delta/-}$ mice, segments were preincubated for 20 minutes with 100 nmol/L sildenafil or 10 µmol/L vinpocetine. Sildenafil and vinpocetine were used together with 100 µmol/L L-NAME, to allow the measurement of the response to exogenous NO donor SNP in the absence of endogenous NO. To test for differences in cGMP-dependent protein kinase 1α (PKGI) sensitivity, CRCs to 8- Bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclicmonophosphate (8-Br-PET-cGMP) were constructed during NOS inhibition with L-NAME (100 µmol/L).

Next, we investigated which part of the downstream signaling of sGC is altered, focusing on cGMP production, PKG activation and PDE activity.

Cyclic GMP measurement

To measure SNP-induced cGMP production, vessel segments (5–10 mg) were exposed to SNP (100 µmol/L) in 6 mL oxygenated Krebs bicarbonate solution for 5 min at 37ºC in the presence of the PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) (100 mmol/L). Tissues were subsequently frozen in liquid nitrogen, and stored at -80ºC. To determine cGMP, frozen tissues were homogenized in 0.5 mL 0.1 mol/L HCl using a stainless-steel ultraturrax (Polytron). Homogenates were centrifuged at 3300 *g*, and cGMP was measured in 300 µL supernatant by ELISA following acetylation (R&D Systems, Minneapolis, U.S.A.). Experiments were performed in quadruplicate, and results are expressed as pmol/mg protein. The lower limit of detection was 0.1 pmol/mg protein. The protein concentration was determined using bicinchoninic acid (BCA) protein assay.

Measurement of PDE5 activity

To measure the cGMP hydrolyzing activity of PDE5, lung tissue of *Ercc1*$^{\Delta/-}$ and WT mice was isolated, frozen in liquid nitrogen and stored at -80ºC. Frozen tissues were crushed in liquid nitrogen and then homogenized at 0ºC in a buffer solution containing 50 mmol/L Tris HCl pH 7.5, 1.5 mmol/L EDTA, 1 mmol/L DTT, Roche complete protease inhibitor cocktail, phosphatase Inhibitor Cocktail 1, and 1 mmol/L sodium orthovanadate. In order to assure appropriate linear kinetics of cGMP hydrolysis, protein concentration of all samples was determined and subsequently they were diluted to yield 15%-30% of total hydrolysis. Diluted sample, containing 0.9 mg/mL protein was added to an assay buffer (40 mmol/L Tris HCl, pH 7.5, 10 mmol/L $MgCl_2$, 0.2 mg/mL BSA, 1 mmol/L EGTA, 1 µmol/L cGMP) containing 10^5 counts per minute [^{3}H]-cGMP per sample. Homogenization buffer without protein was used as a negative control and undiluted protein solution (8 mg/mL) was used to determine full hydrolysis. Assay mixture was incubated at 30ºC for 30 minutes. After that the reaction was stopped by heating the mixture to 100ºC. Subse-

quently the mixture was cooled by placing it on ice and [^{3}H]-5′-GMP was further hydrolyzed by incubation for 30 min at 37°C with 0.25 mg/mL *Ophiophagus hannah* snake venom to ^{3}H-guanosine. Following incubation, 1 mL of Dowex ion exchange slurry was added (1:0.9:0.1 mixture of Dowex AG-1X8/water:methanol) and shaken for 2 minutes on an Eppendorf shaker. The mixture was centrifuged for 4 minutes at 18000 *g* and 700 µL of supernatant was added to a scintillation cocktail and the number of counts per minute was determined.

Quantitative Real-Time PCR analyses

Quantitative real-time PCR was performed in a StepOne thermal cycler (UK, Applied Biosystems) using the SYBR Green PCR Master Mix (UK, Applied Biosystems) following manufacturer's recommendations. *β*-actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT-1*) and TATA-box-binding protein (*TBP*) DNA quantitation was performed in parallel on all samples and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency.

The sense and antisense primers for human PDE were: *PDE1A*: 5′-CCT-ATG-TGG-CAA-GCA-GCT-CA-3′; 5′-CCC-ATC-ACT-CAT-GGA-GCC-TT-3′; *PDE1C*: 5′-GCA-GCC-AGA-AGC-CAT-TGA-AA-3′; 5′-GGA-GTG-ACA-TTG-TCC-AGC-GA-3′; *PDE5A*: 5′-GAT-TGC-TGC-ACT-AAG-CCA-CG-3′; 5′-AGT-GGA-TGT-TCA-CTT-CGC-TGT-3′.

The sense and antisense primers for human p16 and p21 were: *p16:* 5′-TCG-CGA-TGT-CGC-ACG-GTA-3′, 5′-ATC-GGG-GAT-GTC-TGA-GGG-AC -3′; *p21:* 5′-CCA-GCA-TGA-CAG-ATT-TCT-ACC-AC-3′, 5′-CTT-CCT-GTG-GGC-GGA-TTA-GG-3′. Expression values of PDE subspecies and p16 and p21 were corrected for expression levels of at least two of the following house-keeping genes: *TBP, HPRT-1*, *ACTB*, or tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*). All PCR reactions were performed in duplicates. Generally, results did not show qualitative difference after correction for the different house-keeping genes. The house-keeping gene that gave the most conservative result with respect to differences between groups was chosen for data presentation and statistical analyses. Data were considered to be unreliable if less than 2 of the house-keeping genes could be measured, if C_T was on average higher than 34 or if duplicate measurements showed strong dissimilarities. Data of senescent human cells from each of the 5 cultures are expressed relative to the expression at the lowest passage of cells from each explant, which was set at 1. The lowest passage is the passage number needed to obtain sufficient amounts of mRNA and protein for PCR and Western blot analyses, which in this study occurred at passage number 6.

Western blot analyses

From a separate group of mice, 4 aortic ring segments per mouse were mounted in organ baths to evoke responses to KCl, U46619, acetylcholine and finally SNP in order to recreate the organ bath experimental conditions of our previous studies.[13] Immediately after the final SNP responses, the rings of each individual mouse were pooled and snap frozen in liquid nitrogen. Aortic tissue was crushed at -80°C and resuspended in ice cold radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate and 1 mmol/L EDTA) containing protease and phosphatase-inhibitors (1mmol/L PMSF, 1 mmol/L $NaVO_4$, 1 mmol/L NaF, 1 μg/mL aprotinin, 1 μg/mL pepstatin and 1 μg/mL leupeptin) to extract total protein. The lysates were cleared by centrifugation.

Protein content of the murine aortic and human VSMCs lysates was determined with the BCA method (Thermo Scientific). Lysates were subsequently run on a criterion bis/tris gradient gel, and blotted to a nitrocellulose membrane.

For sGCα1, sGCβ1 and PKGIα membranes were blocked with 1:1 odyssey blocking buffer:PBS and were subsequently incubated with antibodies diluted in 1:1 odyssey blocking buffer:PBS against sGCα1 and sGCβ1 (provided by HHHW Schmidt[17]; 1:2000 and 1:1000 respectively) and PKGIα (Santa Cruz Biotechnology, diluted 1:750). Primary antibodies were detected with secondary antibodies labeled with the near infrared dye IRdye800 and visualized with an Odyssey detection system. Protein expression levels were normalized to the house-keeping protein glyceraldehyde-3-phosphate dehydrogenase(GAPDH) in each run.

For PDE1A, membranes were blocked with 5% BSA Tris Buffered Saline with Tween (TBS-T), whereas for PDE1C and PDE5, 5% dried skimmed milk powder in TBS-T was used. Subsequently incubation with the primary antibodies was as follows. In mice lung tissue: PDE1A (Santa Cruz, SC-67734 1:500 in 5% BSA TBS-T), PDE1C (Santa Cruz, SC-67323 1:1000 in 5% dried skimmed milk powder in TBS-T), PDE5 (Alexis Biochemicals, ALX-210-099 1:1000 in 5% dried skimmed milk powder in TBS-T) and p-PDE5 (FabGennix, PPD5-140AP 1:1000 in 5% dried skimmed milk powder in TBS-T).

In human VSMCs: PDE1A (Santa Cruz, SC-67737 1:500 dilution in 5% BSA in TBS-T) and (Santa Cruz, SC-374602 1:100 in 5% dried skimmed milk powder in TBS-T), PDE1C (Santa Cruz, SC-376474 1:100 in 3% dried skimmed milk powder in TBS-T), PDE5 (Alexis Biochemicals, ALX-210-099 1:1000 dilution in 5% % dried skimmed milk powder in TBS-T) and p21 (Cell Signalling, Cat: 2947 1:250 in 2% BSA TBS-T).

Primary antibodies were detected with a HRP- conjugated antibody (Bio-Rad 1:2000 in 1% dried skimmed milk powder in TBS-T). For visualization, we used an enhanced chemiluminiscent substrate for detection of HRP (Pierce ECL Western Blotting Substrate, Thermo Scientific). Protein expression levels were normalized to the house-keeping protein Actin in each run.

Genetic association studies

We investigated the association of single nucleotide polymorphisms in the *PDE1A*, *PDE1C* and *PDE5A* genes with blood pressure (BP), pulse pressure (PP), carotid-femoral pulse wave velocity (CFPWV), common carotid intima media thickness (cIMT) and coronary artery disease (CAD). To provide sufficient statistical power, we used data from different consortia as follows: The look-up for the association between the *PDE1A*, *PDE1C* and *PDE5A* single nucleotide polymorphisms and BP and PP was done in the International Consortium of Blood Pressure Genome-Wide Association Studies (ICBP-GWAS).[18] ICBP-GWAS includes ~ 200,000 participants of European ancestry from 35 studies. Analyses were adjusted for sex, age, age squared, body mass index and ancestry principal components. Individuals who had received treatment for hypertension were imputed to have 15 mmHg higher systolic blood pressure (SBP) and 10 mmHg higher diastolic blood pressure (DBP) than the observed measurements.

For CFPWV we used data from The AortaGen Consortium which comprises 20,634 individuals of European ancestry from 9 cohort studies.[19] As CFPWV increases nonlinearly and displays striking variance inflation with advancing age, its distribution is strongly right skewed. CFPWV also varies depending on the method used to measure transit distance. Therefore, genetic association analyses were performed using a sex-specific standardized residual that was based on the inverse of CFPWV, which normalizes the distribution, and that was further adjusted for age, age squared, height, and weight.

The look-up for the association between the PDE single nucleotide polymorphisms and cIMT was done using data from the Cohorts for heart and aging research in genomic epidemiology (CHARGE) GWAS meta-analysis, which includes 31,210 participants of European ancestry. cIMT was determined by ultrasonography and analyses are age and sex adjusted.[20]

The look-up for CAD was done using the CARDIoGRAM GWAS meta-analysis data.[21] This meta-analysis comprises 22 GWAS studies of European ancestry involving 22,233 cases and 64,762 controls. Analyses were adjusted for age (onset of the first event for cases or time of recruitment for controls) and gender.

Statistical methods

Data are presented as mean ± SEM. Statistical analysis between the groups of single values was performed by two-sided Student's *t* test. Differences in dose-response curves were tested by ANOVA for repeated measures (general linear model – repeated measures (GLM-RM); under the assumption of sphericity of the data). Differences were considered significant at $p<0.05$. Log_{10}-transformed SNP concentrations at which the half-maximal response occurred (pEC_{50}) were estimated with sigmoid curve fitting software (GraphPad Prism5).

For the genetic association studies 868 single nucleotide polymorphisms were selected: 400 inside the *PDE1A* gene, 358 in the *PDE1C* and 110 in the *PDE5A* gene .To ob-

tain the number of independent tests single nucleotide polymorphisms in high linkage disequilibrium, as defined by a pairwise $r^2 \geq 0.7$, were excluded using the *prune* option in Plink.[22] This resulted in 174 independent single nucleotide polymorphisms. A significance threshold of $p < 2.87 \times 10^{-4}$ after applying Bonferroni correction ($0.05/174 = 2.87 \times 10^{-4}$) was considered significant.

RESULTS

Studies in mice

Vascular smooth muscle function in Ercc1$^{\Delta/-}$ mice

The responses of *Ercc1$^{\Delta/-}$* mouse aortae to the highest concentrations of SNP were reduced by 43% (P=0.0002, Table 1) and by 20% in response to the highest dose of DEA-NONOate (P=0.0014). Due to the higher variability of the responses to DEA NONOate all further studies were performed with SNP.

The SNP response was entirely dependent on sGC activation, since ODQ fully eliminated the responsiveness to SNP in WT and *Ercc1$^{\Delta/-}$* (Figure 1).

Table 1. Pharmacodynamic parameters of SNP concentration-response curves

	pEC_{50}			Emax		
	control	**sildenafil**	**vinpocetine**	**control**	**sildenafil**	**vinpocetine**
WT	-7.8±0.2	-8.8±0.1 #	-8.4±0.1	-85.5±4.3	-93.4±3.1	-96.4±1.2
Ercc1$^{\Delta/-}$	-7.7±0.4	-8.4±0.1 #	-8.2±0.1 #	-48.6± 7.6	-74.9±5.2 #	-87.4±3.1 #

pEC_{50}: Log_{10}-transformed SNP concentrations at which the half-maximal response occurred. Emax: maximal effect of SNP (% of U46619 precontraction). # p<0.05, t-test compared with control

Key enzymes in cGMP signaling

Soluble guanylate cyclase: We did not observe a significant reduction of cGMP production in *Ercc1$^{\Delta/-}$* aortae during PDE inhibition with IBMX (Figure 2A). Moreover, the protein levels of α- and β- subunits of sGC measured by Western blotting in aortic tissue were not different in *Ercc1$^{\Delta/-}$* as compared to WT animals (Figures 2B, 2C).

Phosphodiesterase activity: Both the PDE5 inhibitor sildenafil (100 nmol/L) and the PDE1 inhibitor vinpocetine (10 μmol/L) significantly increased responses to SNP as compared over the entire concentration-effect range in *Ercc1$^{\Delta/-}$* and WT mice (p<0.05, GLM-RM test). In *Ercc1$^{\Delta/-}$* the increase of SNP responses induced by vinpocetine was significantly higher than by sildenafil (p<0.05 GLM-RM on entire concentration effect range for SNP, sildenafil vs. vinpocetine). Looking further into qualitative aspects of the SNP effects, potency of SNP increased significantly by ~10-fold in *Ercc1$^{\Delta/-}$* for both PDE inhibitors, and also for sildenafil in WT mice, whereas in WT mice potentiation by vinpocetine did not reach significance (Table 1). In addition, the 2 drugs increased Emax in

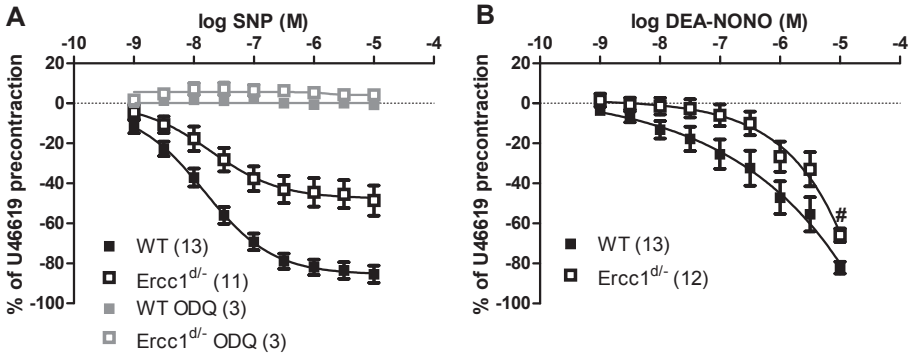

Figure 1. VSMC relaxing responses in progeroid *Ercc1*$^{\Delta/-}$ mice. Relaxation of thoracic aortas from 16 week old *Ercc1*$^{\Delta/-}$ and WT mice in response to the endothelium-independent vasodilator SNP (A) and to DEA-NONOate (B) were measured in organ bath setups during precontraction with U46619. Due to the higher variability of the responses to DEA NONOate all further studies were performed with SNP. The sGC inhibitor ODQ was added 20 min before SNP. *P<0.05 (GLM-RM, control compared with PDE inhibition). Numbers of observations are given in parentheses.

Ercc1$^{\Delta/-}$ mice by 53% (p=0.03) and 80% (p=0.0023), respectively, whereas no statistically significant increase was observed in WT animals (Figures 3A-3D).

Using an enzyme-kinetic assay to measure PDE activity, we observed a 35% increase (P=0.0119) of cGMP-hydrolyzing activity in the lungs of *Ercc1*$^{\Delta/-}$ mice as compared to WT mice. Sildenafil 100 nmol/L completely abolished hydrolysis in both *Ercc1*$^{\Delta/-}$ and WT animals (Figure 3E). The results from qPCR experiments point out that *PDE5* mRNA is downregulated in both aorta and lung of *Ercc1*$^{\Delta/-}$ (Figure 4A). When checking for protein expression, which was only possible in lung samples, PDE5 protein followed the same pattern as PDE5 mRNA, i.e. levels were decreased (Figure 4B). In addition, phosphorylation of PDE5 protein at Ser^{92} was decreased (Figure 4B). Subsequently, PDE1 sub-types were measured. The aortic PDE1A and PDE1C mRNA expression were highly variable, and although the average in aortic PDE1C was higher in *Ercc1*$^{\Delta/-}$ no significant difference with WT animals were found (Figure 4C). Aortic PDE1A mRNA levels were very low (C_T > 34) and could only be measured in 4 out of 6 samples (Figure 4C). Aortic PDE protein levels were too low to be measured. In an attempt to obtain an impression of PDE1 protein in the vasculature we performed Western blots in the highly vascularized lung. As in agreement with aortic mRNA levels, lung PDE1A protein levels were not different, whereas PDE1C protein was significantly increased in *Ercc1*$^{\Delta/-}$ mice (Figure 4D).

cGMP-dependent protein kinase type-1: The responses to the cell-permeable PKGI agonist PT-8Br-cGMP were identical in *Ercc1*$^{\Delta/-}$ and WT aortas (Figure 5A). In agreement with this observation, the protein levels of PKGI in aortic tissue, measured by Western blotting were not different in *Ercc1*$^{\Delta/-}$ as compared to WT animals (Figure 5B).

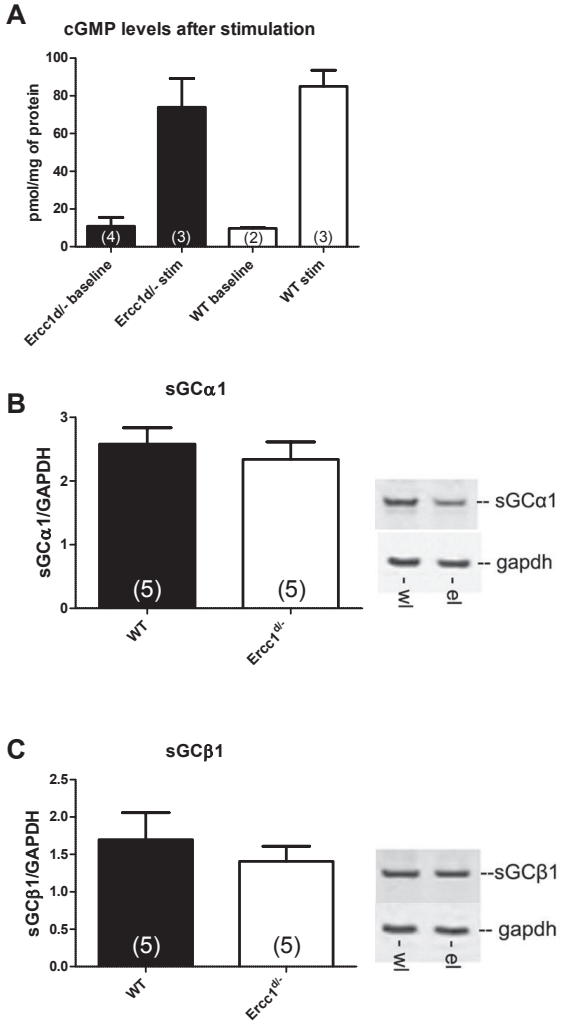

Figure 2. cGMP production and sGC subunit protein levels in thoracic aortas of *Ercc1*$^{\Delta/-}$ mice. Production of cGMP in aortic tissue of *Ercc1*$^{\Delta/-}$ and WT mice with and without stimulation with SNP 10^{-5} mol/L for 5 minutes (A). sGC α1 and β1 sub-types (B,C) protein levels were measured with Western blot and corrected for GAPDH levels. Numbers of observations are given in parentheses

Studies in human VSMC

To study the relation between PDE1 expression and cellular senescence, passage-dependent expression of PDE1 and p16 and p21, a cyclin-dependent kinase and an accepted senescence marker[13, 23, 24] was studied in 5 VSMC cultures, typical results from one of the cultures are presented in figures 6A- 6D. There was a passage-dependent increase of *PDE1A* and *PDE1C* mRNA levels, and this corresponded with an increase in *p16* and *p21*. Summary results are presented in figure 6E, senescent human aortic VSMC showed a significant 11.6- and 9-fold increase of *PDE1A* and *PDE1C* mRNA levels respectively (Figure 6A and 6B), whereas the *PDE5* level was only modestly increased (Figure 6E, p=0.06). The pattern on the PDE1C protein expression (Figure 7A) corresponded with

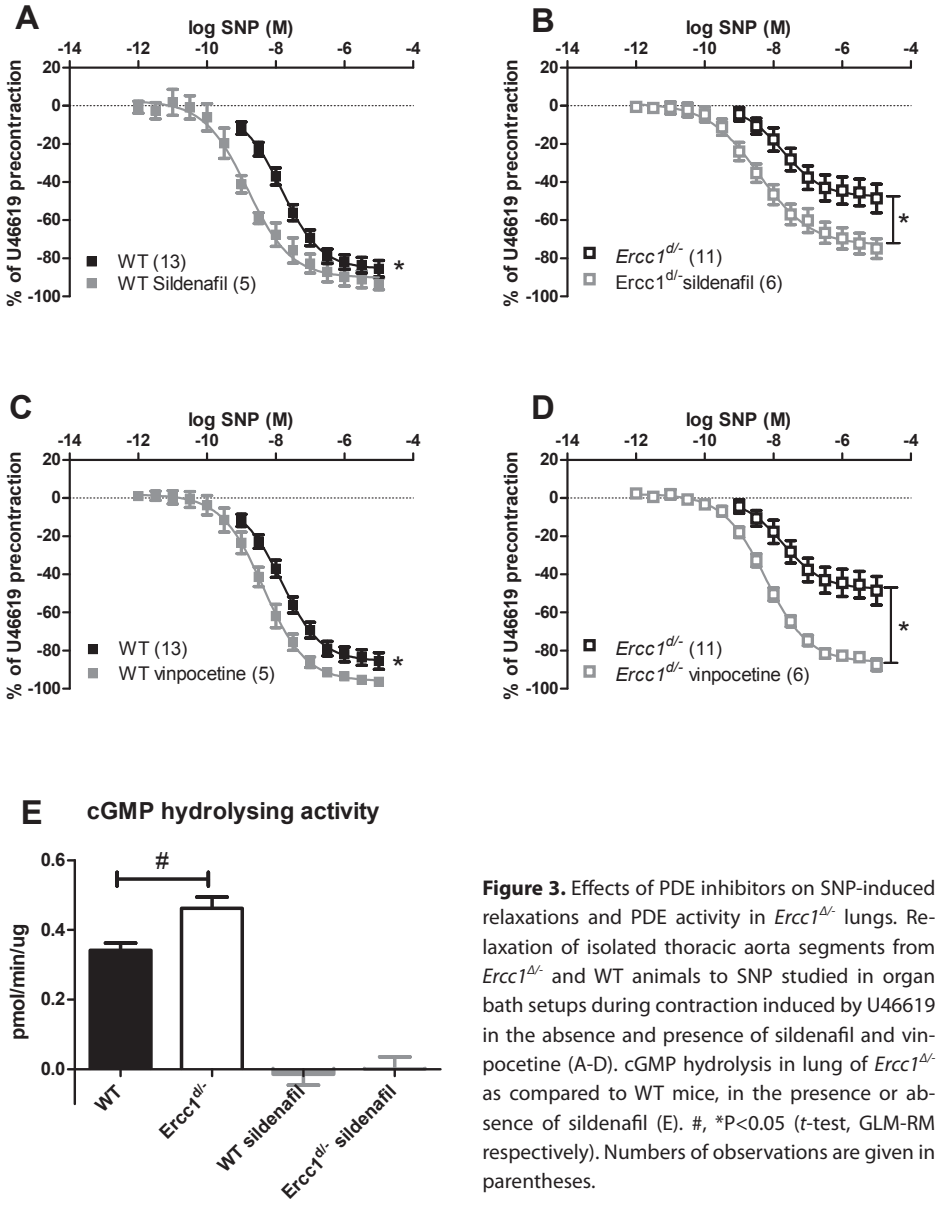

Figure 3. Effects of PDE inhibitors on SNP-induced relaxations and PDE activity in *Ercc1*$^{\Delta/-}$ lungs. Relaxation of isolated thoracic aorta segments from *Ercc1*$^{\Delta/-}$ and WT animals to SNP studied in organ bath setups during contraction induced by U46619 in the absence and presence of sildenafil and vinpocetine (A-D). cGMP hydrolysis in lung of *Ercc1*$^{\Delta/-}$ as compared to WT mice, in the presence or absence of sildenafil (E). #, *P<0.05 (*t*-test, GLM-RM respectively). Numbers of observations are given in parentheses.

qPCR data and p21 protein expression (Figure 7B) but we could not specifically detect PDE1A nor PDE5 protein in human samples despite trying several different antibodies. When we treated VSMCs with the PDE1 and 5 inhibitors vinpocetine and sildenafil to accomplish loss-of-function of the PDEs we found that vinpocetine (predominant PDE1 inhibition) lowered both p16 and p21 expression, whereas sildenafil (predominant PDE5 inhibition) only appeared to affect p21 (Figure 7C and 7D).

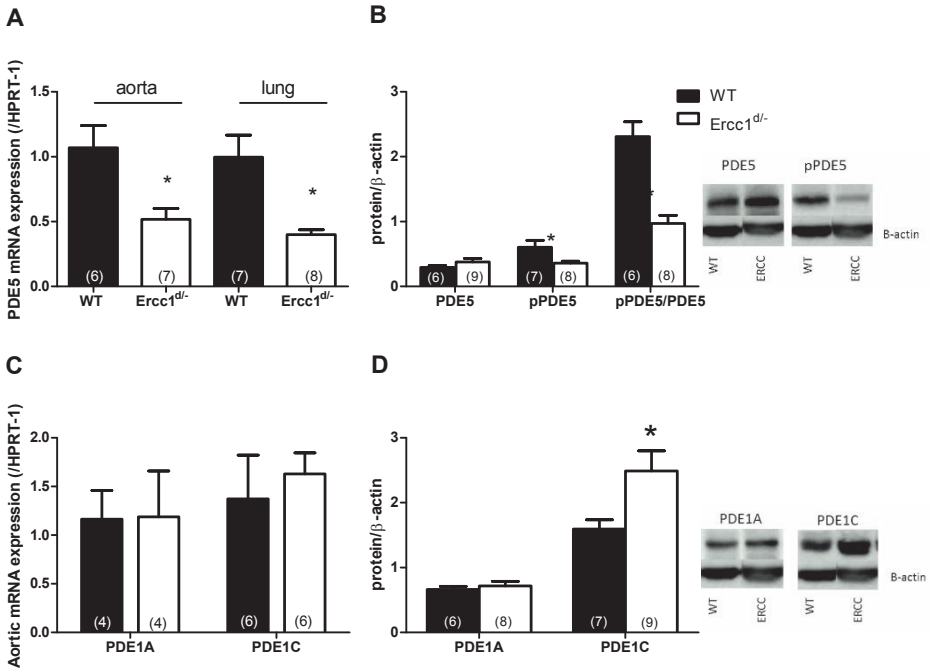

Figure 4. Expression of PDE1 and 5 sub-types in mouse tissues. Aortic and lung PDE5 mRNA (A) and lung protein expression (B), PDE1A and 1C aortic mRNA expression (C) and lung protein expression (D) were measured with qPCR and WB, correspondingly. *P<0.05 (*t*-test). Numbers of observations are given in parentheses.

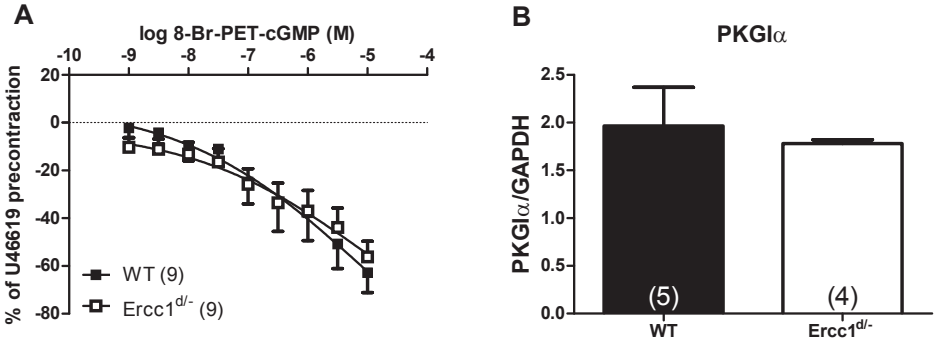

Figure 5. Responses to PKGI activator and PKGIα protein levels in aortic tissue of *Ercc1*$^{\Delta/-}$ mice. Relaxation of thoracic aortas from 16 week old *Ercc1*$^{\Delta/-}$ and WT mice to cell membrane-permeable PKGI activator PT-8Br-cGMP during U46619-induced contraction, as measured in organ bath setups (A). PKGIα protein levels were measured with Western blot and corrected for GAPDH levels (B). Numbers of observations are given in parentheses.

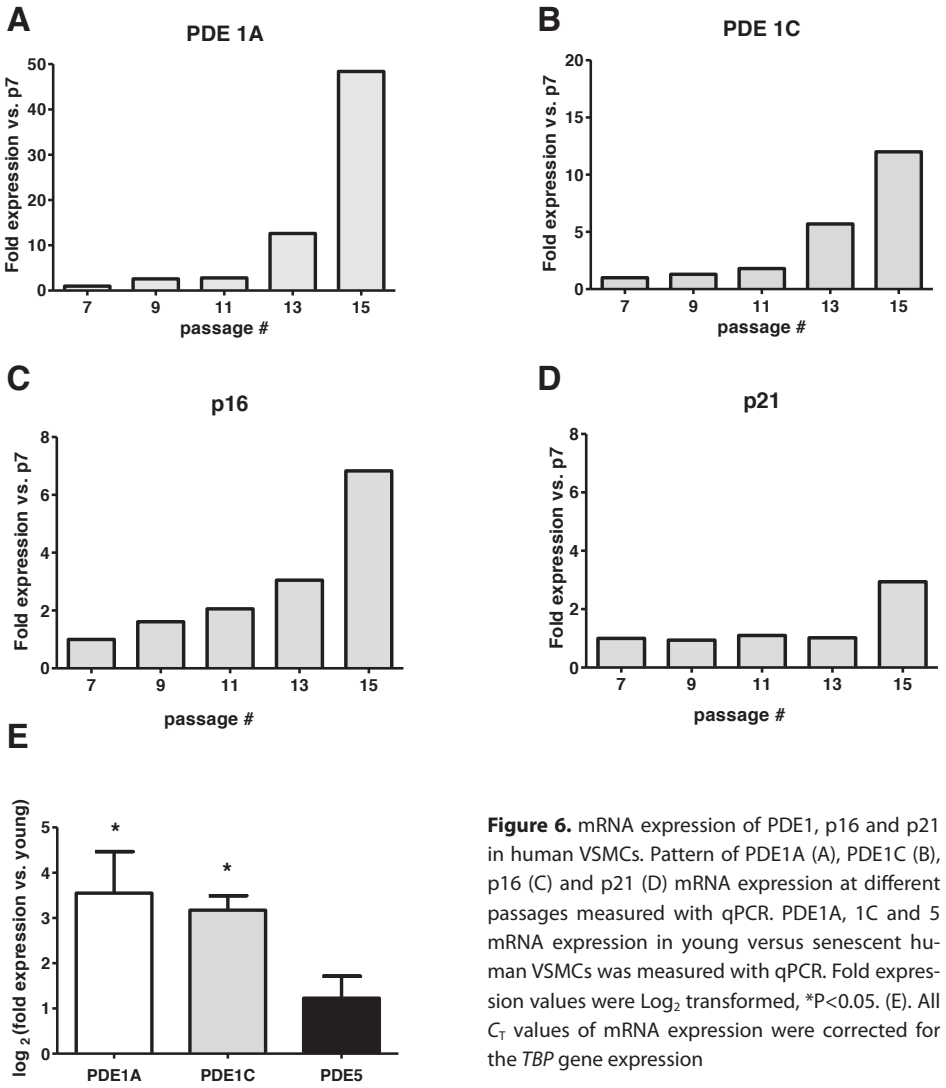

Figure 6. mRNA expression of PDE1, p16 and p21 in human VSMCs. Pattern of PDE1A (A), PDE1C (B), p16 (C) and p21 (D) mRNA expression at different passages measured with qPCR. PDE1A, 1C and 5 mRNA expression in young versus senescent human VSMCs was measured with qPCR. Fold expression values were Log_2 transformed, *P<0.05. (E). All C_T values of mRNA expression were corrected for the *TBP* gene expression

Genetic association studies

Since in vitro cellular senescence is not synonymous for vascular aging, we tested the involvement of PDE1 and PDE5 by means of genetic association studies. After Bonferroni correction for multiple testing, 14 SNPs in the *PDE1A* gene remained significantly associated with DBP at the threshold of $p \leq 2.87x\ 10^{-4}$ (Table 2, Figure 8A). The biggest effect size was found for the SNP rs1430158 ($\beta= 0.281$, $p= 2.47x10^{-5}$). We found 6 SNPs in the *PDE1A* gene significantly associated with cIMT (Table 2, Figure 8B). Among all rs2887202 showed the lowest p value ($\beta= -0.0061$, $p= 2.89x10^{-5}$)

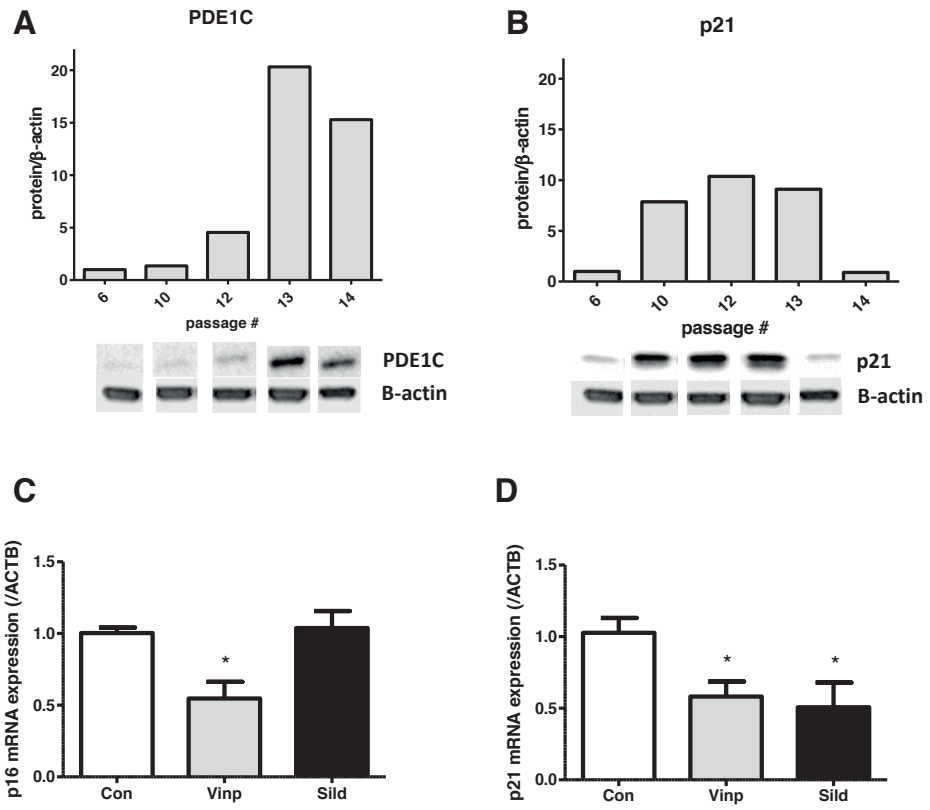

Figure 7. Protein expression of PDE1C and p21 in human VSMCs and mRNA expression of p16 and p21 in human VSMCs treated with PDE1 and 5 inhibitors. Pattern of PDE1C (A) and p21 (B) protein expression at different passages measured with WB. Protein levels were corrected for β-actin levels. p16 (C) and p21 (D) mRNA expression in control, vinpocetine- and sildenafil-treated VSMCs. *P<0.05. All C_T values of mRNA expression were corrected for the *ACTB* gene expression.

The results for the association of the SNPs in the *PDE1A*, *PDE1C* and *PDE5* genes with SBP, DBP, PP, CFPWV, cIMT and CAD at $2.87 \times 10^{-4} < p < 0.05$ are presented in supplementary table 1. There are some suggestive associations of *PDE5A* with SBP (rs6844263, β= 0.312, p= 1.60×10^{-3}) and DBP (rs1155577, β= 0.214, p= 5.16×10^{-4}), and of *PDE1A* with PP (rs17343395, β= -0.354, p= 5.05×10^{-3}) and cIMT (rs12989198, β= -0.0053, p= 3.28×10^{-4}). One SNP in the *PDE1C* gene showed a suggestive association with CAD (rs10226190, β= 0.080, p= 5.75×10^{-3}). CFPWV did not show any association that might be relevant.

Table 2. Significant associations at the threshold of $p \leq 2.87x\ 10^{-4}$ of the single-nucleotide polymorphisms (SNP) in the PDE1A gene with DBP and cIMT

Chromosome			Allele					
SNP	**n**	**Position**	**Coded**	**Frequency**	**Beta**	**se**	**p**	**Outcome**
rs1430158	2	182970373	T	0.77	0.282	0.067	2.47×10^{-5}	DBP
rs833166	2	182917392	T	0.78	0.278	0.068	4.85×10^{-5}	DBP
rs10497597	2	182971998	T	0.23	0.269	0.068	6.84×10^{-5}	DBP
rs2623431	2	182922232	T	0.78	0.266	0.068	8.61×10^{-5}	DBP
rs833168	2	182919236	T	0.78	0.264	0.068	9.56×10^{-5}	DBP
rs16823124	2	182932372	G	0.78	0.254	0.066	1.35×10^{-4}	DBP
rs16823150	2	182952598	T	0.81	0.254	0.067	1.65×10^{-4}	DBP
rs1438065	2	182927238	G	0.78	0.249	0.067	1.79×10^{-4}	DBP
rs11682598	2	182927925	G	0.78	0.248	0.067	1.90×10^{-4}	DBP
rs864417	2	182918692	T	0.75	0.250	0.067	1.94×10^{-4}	DBP
rs12693302	2	182919688	G	0.26	0.248	0.067	1.96×10^{-4}	DBP
rs12996836	2	182920273	T	0.25	0.248	0.067	1.97×10^{-4}	DBP
rs7558737	2	182948186	T	0.22	0.243	0.067	2.84×10^{-4}	DBP
rs10931009	2	182949102	T	0.22	0.243	0.067	2.87×10^{-4}	DBP
rs2887202	2	183029113	A	0.58	-0.006	0.002	2.89×10^{-5}	cIMT
rs934264	2	183033988	T	0.58	-0.006	0.002	4.14×10^{-5}	cIMT
rs1897104	2	183058624	A	0.42	0.006	0.002	8.91×10^{-5}	cIMT
rs10931012	2	182998273	A	0.40	0.006	0.002	1.70×10^{-4}	cIMT
rs11684406	2	183001113	T	0.60	-0.006	0.002	1.70×10^{-4}	cIMT
rs17343416	2	182994934	T	0.59	-0.005	0.002	2.38×10^{-4}	cIMT

DISCUSSION

In our studies in mice with accelerated vascular aging due to genomic instability we found decreased vasodilator function and increased cGMP metabolism in lung tissue that can both be restored by PDE1 and 5 inhibition, whereas the highly vascularized lung shows increased PDE1C expression. In humans, VSMC senescence, which can be caused by genomic instability, also leads to increased PDE1, and possibly also PDE5 activity, while in the general population SNPs in the *PDE1A* gene are associated with increased DBP and cIMT, two hallmarks of human age-related vascular dysfunction. Therefore, in general our data suggest that increase of PDE1A and C in VSMC might play a pivotal role ageing-related vasodilator dysfunction and vascular hypertrophy, and that this increase is linked to cellular senescence, a consequence of genomic instability. A similar observation, but with more reservations, can be made for PDE5.

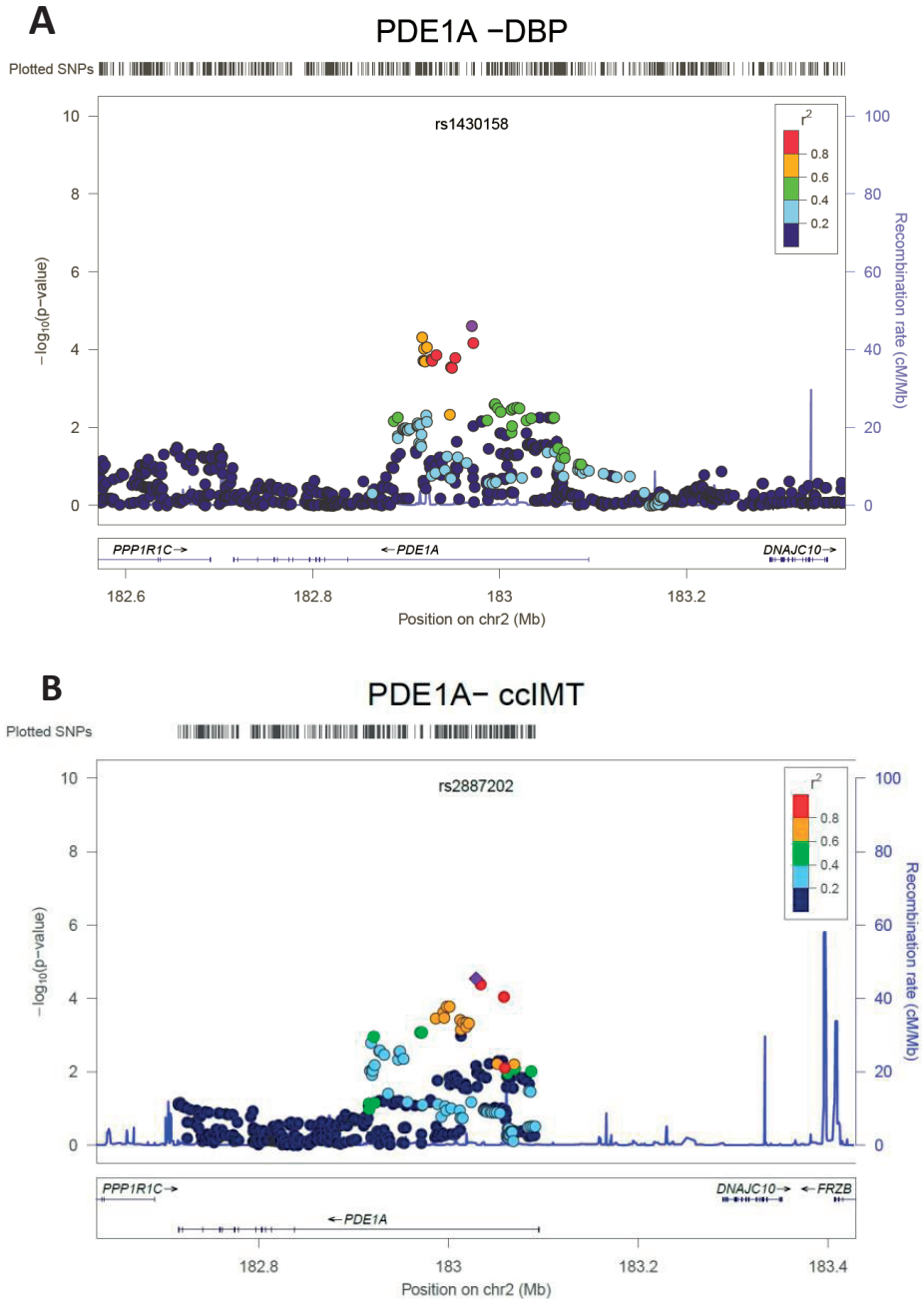

Figure 8. Regional plots for the association between PDE1A Single Nucleotide Polymorphisms and Diastolic Blood Pressure and Intima Media Thickness. Regional association plot showing $-\log_{10}$ (p-values) for all PDE1A SNPs ordered by their chromosomal position and DBP (A) and IMT (B).
Each SNP is colored according to its correlation with the SNP showing the lowest p-value (index SNP) within the region as specified in the color scheme.

Several pieces of evidence demonstrate that reduced cGMP responsiveness is important in the context of vascular aging. Our currently reported data, and those previously reported, showed reduced responses to SNP in *Ercc1*$^{\Delta/-}$ mice, which can be restored by the preferential PDE1 inhibitor vinpocetin. Also, others have shown in aged rats that arterial relaxing responses to SNP progressively reduced with increasing age.[25, 26] In comparison to our previous study[13], the present study shows that apart from reduced eNOS signaling, genomic instability causes reduced responsiveness to cGMP due to an increased metabolism of this nucleoside and apparently not due to changes in cGMP production or PKG activity. This is suggested by our observation that PDE inhibition fully restored SNP responses in *Ercc1*$^{\Delta/-}$ to the level of WT, and that sGC and PKG do not appear to be changing in activity or in expression levels. The complete restoration of SNP responses by vinpocetine and sildenafil also suggest that it is unlikely that other sub-types than PDE 1 and 5 are involved. In contrast to our previous study[13], we here show the comparison with both vinpocetine and sildenafil on complete SNP dose response curves to explore the relative involvement of PDE1 and PDE5. Our comparisons show that in WT sildenafil has the more pronounced effect in WT, whereas in *Ercc1*$^{\Delta/-}$ the effect of vinpocetine appears to dominate. Although sildenafil and vinpocetine are not strictly specific inhibitors, and have affinity for both PDE sub-types, these data suggest that in *Ercc1*$^{\Delta/-}$ the contribution of PDE1 relative to PDE5 with regard to decreased cGMP signaling is increased. This is also supported by the western blot and PCR data obtained in aortic and in lung tissue. Unfortunately, we were not able to quantify PDE protein levels in the mouse aorta to provide more evidence for which PDE sub-type is involved in the vasculature. Yet, the highly vascularized lung tissue did show increased PDE1C levels, and similar observations were made in human senescent VSMCs, suggesting that particularly this sub-type is of interest for further investigation.

Compared to other studies on the effect of aging on SNP responses, a reduction of maximal SNP response, as observed in our mice, only appears in normal male Wistar rats at an age of 90 weeks or higher, and at 130 weeks and older even a decline in the response to 8-Br-cGMP has been observed.[26] In humans, a reduction of VSMC responsiveness to NO donors in relation to age is not observed in brachial arteries as readily as the decline of endothelial function,[27, 28] even with concomitant essential hypertension,[29, 30] although in some studies this reduction did occur.[31] VSMC dilator dysfunction also occurs in the later stages of chronic heart failure (CHF),[32-37] while in mild-to-moderate heart failure, such VSMC dysfunction was found to be predictive of worsening CHF.[38] When taking the data of normally aging animals and humans together, it seems that VSMC dysfunction develops in a progressive stage of vascular aging, concomitant with vessel disease. In line with this observation, in our model, where aging of both endothelial cells and VSMC is accelerated by ablation of DNA repair, both cell types are affected, and VSMC dysfunction can be demonstrated at a young age.

Relevant to advanced vascular aging is the occurrence of senescence vascular cells. When we measured the gene expression of three PDEs sub-types in human VSMCs, we found that predominantly *PDE1A* and *PDE1C* mRNA levels increased with increasing passages and *p16* and *p21* expression. The association between VSMC senescence and PDE1 sub-types was confirmed when we treated VSMCs with vinpocetin, which caused a reduced *p16* and *p21* mRNA expression. PDE5 inhibition only reduced *p21* mRNA expression, again pointing to a stronger association of senescence with PDE1. Overall, our results show that PDE1 and cell senescence are reciprocally associated and suggest that PDE1 might even be involved in regulation of VSMC senescence. Other roles for PDE1 in the aging vasculature have been proposed previously. As VSMCs age, their phenotype changes from a contractile to a synthetic state in which they exhibit increased proliferation, migration and production of extracellular matrix proteins thus contributing to the neointima formation in aged vessels. It has been shown that PDE1A localization is different in contractile compared with synthetic VSMCs; in contractile VSMCs in the media PDE1A is predominantly cytoplasmic, whereas in synthetic VMSCs in the neointima, it is nuclear and associated with VSMC proliferation.[39] The impact of cytoplasmic PDE1 subtypes on decreased vasodilator capacity is most probably reflected in our organ bath studies in mice and the genetic association with DBP in humans. The role of *PDE1A* gene variants in cIMT may be related to nuclear localization of the enzyme.

In our candidate gene look-up using GWAS data we found several single nucleotide polymorphisms in the *PDE1A* gene that confirms a role in DBP and cIMT. A role for DBP regulation by *PDE1A* was also suggested recently in another genetic association study[6]; however, the mechanism underlying this association was not described. There is also a suggestive role for *PDE5A* in DBP. Although none of the associations with SBP, PP, CFPW and CAD reached our significance threshold, there are several suggestive associations.

The variability in associations with respect to the clinical variable that is explored suggests that PDE1 and 5 would be involved in very specific functions in the vascular system. For instance, DBP might be relatively more dependent on basal tone of vascular smooth muscle cells and medial thickness and less dependent on cardiac contractility and vascular compliance, as determined by changes in extracellular matrix, than SBP and PP. CAD is an even more complex phenomenon, involving also inflammation, dyslipidemia and thrombogenesis. In other words, our genetic association study might hint at the most relevant biological role of PDE1A, and perhaps also of PDE5, which would be in regulation of vasomotor function and vascular remodeling by increasing VSMC proliferation and migration[39]; remodeling processes that alter the vessel tone and therefore blood pressure.[40]

It might seem remarkable that *PDE1C* SNPs did not show associations, with the exception of perhaps a weak relationship with PP and CAD. However, this observation does not necessarily mean that PDE1C is not important for human vascular disease. It

might simply be explained by the absence of a relevant genetic variant. Our mouse data and previous studies in human VSMC and atherosclerotic tissue are very suggestive for a relevant role of PDE1C.[10, 41] In addition, the fact that the mRNA and protein levels of PDE1A and PDE1C are increasing more than those of PDE5 does not mean that both type 1 sub-types are more important. Our data show relative increases of gene product, and this is not indicative for the absolute contribution of each PDE sub-type. In fact, previous studies show that PDE1 sub-type expression might be low in cultured VSMC, hence leading to high increases of gene expression relative to baseline even in the case that absolute increases are small.[42]

Drugs affecting cGMP levels are potential pharmacological tools for the treatment of age-related cardiovascular disease. NO donors for the treatment of angina pectoris and PDE5 inhibitors for the treatment of erectile dysfunction and reduction of blood pressure in pulmonary hypertension are already being used in patients.[43-45] Vinpocetine is a PDE inhibitor with a preferential affinity for PDE1 over PDE5, which it also inhibits. Vinpocetine is a Food and Drug Administration (FDA)-approved nutraceutical and a registered drug in Eastern Europe, used to enhance cerebral blood-flow and improve memory.[46, 47] It inhibits injury-induced hypertrophy in human and rodent vessels, and decreases atherosclerosis in ApoE knockout mice.[7, 8] From our results we anticipate that PDE inhibition, particularly PDE1-specific inhibition, might also be interesting as a potential pathway subject to modification to chronically improve genomic stability-related vascular dysfunction by increasing signaling through the NO/sGC/cGMP axis.

In summary, we have identified PDE1 as a genetic risk factor and a potential treatment target in age-related vascular disease, where it concerns disturbed NO-cGMP signaling in VSMCs. This is specifically important for blood pressure regulation and vascular hypertrophy and possibly also in regulation of VSMC senescence. Our study warrants further exploration of PDE1 inhibitors as drugs to improve healthy cardiovascular aging in the general population.

Clinical Perspectives

After the failure of several clinical trials to show effectiveness of PDE5 inhibitors in chronic cardiovascular disease, our result underpin the previously suggested importance to further evaluate the potential of PDE1 as a treatment target. Our current study suggests that vascular aging related to genomic instability might be a major focus in this endeavor. Inhibition of specific PDE sub-types is an attractive option because their strict localization-function relationship, which was explained above for PDE1A, theoretically allows very specific pharmacological interventions. Unfortunately, the PDE1-specific inhibitor IC86340 has been discontinued, and a search for new inhibitors is required. Stimulators or activators of sGC might be an alternative drug group, although it might not provide the same specificity.

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Data on coronary artery disease / myocardial infarction have been contributed by CARDIoGRAMplusC4D investigators and have been downloaded from www.CARDIOGRAMPLUSC4D.ORG.

Data on pulse wave velocity was provided by the Aorta Gen Consortium.We acknowledge the Subclinical working group of the CHARGE Consortium for the cIMT data.

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Chapter 3

Local endothelial DNA repair defect causes aging-resembling endothelial-specific dysfunction

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ABSTRACT

We have identified genomic instability as a causative factor in vascular aging, a major contributor to cardiovascular disease and death. Loss of endothelium-dependent and -independent vasorelaxation, a major hallmark of age-related vascular disease, is accelerated in mice with a DNA repair defect in all cells of the organism. Also blood pressure and vascular stiffness is increased. It is not known if these changes are due to local or systemic DNA damage. We here investigated cardiovascular function in a mouse model with specific loss of *Ercc1* in vascular endothelial cells (EC-KO) at the age of 3 and 5 months, and identified the endothelium-derived factor that play a role. *In vivo* measurements of vascular function revealed decreased microvascular vasodilation in the skin, decreased lung perfusion, decreased aortic distensibility, and decreased cardiac stroke volume and output at the age of 5 months in EC-KO vs. wildtype controls. This was absent at 3 months. At the age of 5.5 – 6 months EC-KO suddenly died, which was associated with renal papillary and tubular necrosis. This was preceded by extravasation of red blood cells in medular microvessels surrounding the tubuli at 5 months. *Ex vivo* vasodilatory function in aorta and iliac artery were normal at 3 months but decreased at 5 months after birth. Coronary artery dilator function was decreased at 3 months and to a higher degree at 5 months of age. Vasodilation to endothelium-derived nitric oxide was abolished in aorta and coronary artery, whereas endothelium-derived hyperpolarization and responses to nitric oxide donor sodium nitroprusside were completely intact. Expression of eNOS mRNA and protein was decreased in EC-KO. Carotid arteries showed no difference in stiffness. Blood pressure was increased at 3 months, but normal at 5 months. Scanning electron microscope showed no endothelial denudation and aortic von Willebrand factor expression levels were normal in EC-KO. Expression levels of senescence markers were unchanged. In summary, DNA damage in the endothelium leads to very specific, non-congenital disturbance of nitric oxide signaling in the endothelium, leaving endothelium-derived hyperpolarization and smooth muscle cell responses to nitric oxide intact. The phenotypic changes resemble those reported for eNOS knockout mice, including mild, temporarily increased blood pressure and gradual decrease of cardiac output. Thus, the effects of local endothelial DNA damage might be largely attributed to loss of nitric oxide signaling.

INTRODUCTION

Despite the currently available prevention and treatment options, cardiovascular diseases (CVD) continue to be a main cause of morbidity and mortality worldwide. Even when traditional risk factors, such as smoking, high cholesterol, diabetes or a sedentary lifestyle, are absent or controlled, cardiovascular problems remain a major health issue due to the independent risk factor age.[1] Aging, which is not synonymous to age, is a natural but very complex process leading to the decline and ensuing loss of organ function. The accumulation of damaged DNA is considered as one of the primary causes driving the process of aging, and this involves various processes.[2] Firstly, cells with unrepaired damaged DNA enter into apoptosis or senescence; apoptosis can lead to organ function decline due to the loss of cells or tissue, and senescent cells acquire and secrete a senescence-associated secretory phenotype (SASP) that affects surrounding cells and ultimately renders the organism more susceptible to age-associated diseases.[3] This principle is thought to be applicable for both cancer as well as cardiovascular disease. Further, DNA damage might simply lead to mutated gene products. Last, but perhaps most importantly, accumulating DNA damage leads to a so-called 'survival response' that switches the organism's physiological status from one that promotes growth to one that suppresses growth and focuses on maintenance.[4] The contribution of these moduli to age-related disease putatively depends on classical disease risk factors and yet uncharted DNA damage sources. In all likelihood, in human populations this is subjected to individual genetic and environmental variations, explaining individual differences in the rate of aging. Also, this process potentially leads to variation in the rate of aging between organs. This so-called segmental aging is clearly observed in mouse models of accelerated aging.[4]

Several mouse models of accelerated aging due to the accumulation of damaged DNA repair have been generated.[4] One of the models that shows many features of human-like aging is the *Ercc1*$^{\Delta/-}$ mouse. In these heterozygous mice the function of the Excision Repair Cross Complementation group 1 (*ERCC1*) protein is impaired because of one null-allele and one N-terminal protein truncation due to a deletion mutation in exon 7 of the *Ercc1* gene.[5] The ERCC1 protein is important for several DNA repair mechanisms including global genome-and transcription-coupled nucleotide excision repair and interstrand crosslink repair; therefore, in *Ercc1*$^{\Delta/-}$ mice there is an overt accumulation of DNA damage.[6] *Ercc1*$^{\Delta/-}$ mice live an average of 24-28 weeks and within 12 weeks of age develop many human-like features of accelerated aging like neurodegeneration and osteoporosis, and also display increased blood pressure, increased vascular stiffness and loss of macro- and microvascular dilator function.[7] Like in humans and aging wildtype rodents, the vasodilator dysfunction in *Ercc1*$^{\Delta/-}$ mice is explained by reduced NO-cGMP signaling, which was due to decreased endothelial nitric oxide synthase (eNOS) expres-

sion and activation and increased metabolism of cGMP by phosphodiesterase (PDE) 1 and PDE5.[7, 8]

Ercc1 $^{\Delta/-}$ mice display segmental progeria, leaving some processes, like the occurrence of brain or spinal cord vacuolization, and tumor development, unaffected.[5] Conversely, this implies that affected organs might suffer from the impact of local rather than systemic DNA damage. Thus, the observed accelerated decline of endothelium-dependent vasodilation in *Ercc1*$^{\Delta/-}$ mice might arise from defective endothelial DNA repair. To address if local endothelial DNA repair leads to specific changes as observed in *Ercc1*$^{\Delta/-}$ mice we investigated cardiovascular function in a mouse model with specific loss of *Ercc1* in vascular endothelial cells.

METHODS

Animals

We evaluated the effect of endothelial genomic instability on cardiovascular function in a mouse model of endothelium-specific DNA repair defectiveness (*Tie2Cre*$^{+}$ *Ercc1*$^{fl/-}$ mouse model).

The Cre-loxP system was used to generate a conditional mouse model expressing Cre-recombinase under the control of the vascular endothelial cell receptor tyrosine kinase (*Tie2*) promoter (Tie2Cre). *Tie2Cre*$^{+/-}$ female mice were crossed with *Ercc1*$^{+/-}$ male mice to generate *Tie2Cre*$^{+/-}$ *Ercc1*$^{+/-}$ mice in a C57BL/6J background. The females were then crossed with *Ercc1*$^{fl/fl}$ male mice in a FVB/N background to produce *Tie2Cre*$^{+}$ *Ercc1*$^{fl/-}$ mice in a C57BL6/FVB F1 hybrid background [9]. These *Tie2Cre*$^{+}$ *Ercc1*$^{fl/-}$ mice were homozygous for *Ercc1*, after deletion of the floxed allele in endothelial cells expressing Cre-recombinase. These mice are referred throughout this manuscript as endothelial cell-knock out mice (EC-KO). Littermates (genotypes: *Tie2Cre*$^{+}$ *Ercc1*$^{fl/+}$, *Tie2Cre*$^{-}$ *Ercc1*$^{fl/+}$, *Tie2Cre*$^{-}$ *Ercc1*$^{fl/-}$) were used as controls. These mice are referred to as control mice in the manuscript.

Mice were kept in individually ventilated cages, in a 12 h light/dark cycle and fed normal chow and water *ad libitum*. All animal studies were approved by the Animal Care Committee of Erasmus University Medical Center Rotterdam (protocol number 118-13-03).

EC-KO mice were dying suddenly when they were around 5.5-6 months-old, because of this measurements were performed at 3 and 5 months.

Pathological examination, tissue collection and blood analysis

One of the EC-KO mice unexpectedly died prematurely during daytime, and was submitted to whole body fixation in formalin for 48 hours and pathological examination.

Tissues were processed by paraffin embedding technique, sectioned by the microtome, and then stained with Hematoxiline Eosin. Slides were examined by a board-certified veterinary pathologist.

Before sacrifice, blood was collected from the vena porta and analyzed for cell counts. Immediately after sacrifice we collected vascular tissue, lungs, kidneys and heart.

Cardiac function

Cardiac geometry and function were measured by performing 2-D guided short axis M-mode transthoracic echocardiography (Vevo770 High-Resolution Imaging System, VisualSonics) equipped with a 35-MHz probe. Left ventricular (LV) external and lumen diameters were traced, and heart rate, LV mass and fractional shortening were subsequently calculated using the VisualSonics Cardiac Measurements Package. Mice were under anesthesia with 2.5% isoflurane, intubated and connected to a pressure-controlled ventilator, and body temperature was kept at 37°C.

Blood pressure measurement

Blood pressure (BP) was measured non-invasively in conscious mice using the tail cuff technique (CODA High-Throughput device, Kent Scientific). BP was measured on 5 consecutive days and each session consisted of 30 measurement cycles for each mouse. The first 4 days were taken as acclimatization sessions to minimize stress-induced changes in the measurements. A measurement was considered invalid if the minimum blood volume required for a blood pressure reading to be made was not reached. BP values reported here correspond to the average of all valid measurements recorded at day 5.

Aortic stiffness

Using the data on systolic and diastolic aortic diameters acquired by transthoracic echocardiography we calculated aortic dilatation by subtracting the diastolic aortic diameter from the systolic aortic diameter.

Microvascular vasodilator function and lung perfusion *in vivo*

Three to seven days after blood pressure measurement we assessed *in vivo* vasodilator function using Laser Doppler perfusion imaging. Reactive hyperemia, defined as the increase of the hindleg perfusion after temporary occlusion of the blood flow, was calculated. Blood flow was measured in the left hindleg one day after removing the leg's hair using a hair removal cream. The hindleg was kept still with help of a fixation device. After recording baseline perfusion for 5 minutes, blood flow was occluded for 2 minutes with a tourniquet. To record hyperemia and the return of the blood flow to the post-occlusion baseline, blood flow was monitored for 10 minutes after releasing the tourniquet. During all measurements mice were under 2.8% isoflurane anesthesia, and

temperature was constantly monitored and maintained between 36.4-37.0 °C. For each mouse we calculated the maximum response to occlusion and the area under the curve relative to the post-occlusion baseline. Only the area above the baseline was considered. Values below the baseline were set at 0.

In a separate set of 5 months-old control vs. EC-KO mice (n= 3 vs 3) lung perfusion was measured by microCT imaging. Mice were anesthesized with 2.5% isoflurane in O_2 and received IV injection with eXIA160 (Binitio Biomedical Inc., Canada) contrast agent. The mice were placed in a imaging cassette and restrained to prevent movement. Mice were scanned using intrinsic cardiac respiratory gating to reduce artifacts caused by irregular breathing. Blood flow was assessed during diastole.

Ex vivo vascular assessment

Immediately after sacrifice thoracic aorta, iliac and left anterior descending coronary arteries were carefully dissected from mice and kept in cold Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,$CaCl_2$ 2.5, $MgSO_4$ 1.2 , KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3 in distilled water; pH 7.4). Vessel rings of 1.5-2 mm length were mounted in small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing 6 mL of Krebs-Henseleit buffer oxygenated with 95% O_2 and 5% CO_2. After warming, the tension was normalized by stretching the vessels in steps until 90% of the estimated diameter at which the effective transmural pressure of 100 mmHg is reached. Thereafter the viability of the vessels was tested by inducing contractions with 30 and 100 mmol/L KCl. After the maximum response to KCl had been reached vessels were washed.

To evaluate vasodilatory responses, aortic and iliac segments were first pre-constricted with 30 nmol/L of the thromboxane A_2 analogue U46619, resulting in a preconstriction corresponding with 50-100% of the response to 100 mmol/L KCl. After this, concentration-response curves (CRCs) to the endothelium-dependent vasodilator acetylcholine (ACh) were constructed by adding cumulative doses (10^{-10}-10^{-5} mol/L). When the CRC to ACh was completed, the vessel segments were exposed to the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). Complete CRCs to SNP (10^{-10}-10^{-4} mol/L) were performed in parallel rings preconstricted with 30 nmol/L U46619.

The contribution of nitric oxide (NO) and prostaglandins in the aortic ACh responses was explored by performing the experiments in the presence of the endothelial nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester salt (L-NAME, 10^{-4} mol/L) and the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10^{-5} mol/L). Inhibitors were added to the organ bath 20 minutes prior to U46619.

In coronary arteries we investigated endothelium-dependent vasodilation by performing CRCs to ACh and to the nucleotides adenosine diphosphate (ADP) and uridine triphosphate (UTP). The intracellular signalling activation caused by ADP and UTP, has

not been studied in detail in the mouse heart. However, studies on blood pressure and cerebral arterioles in the eNOS-/- mice have shown that the two nucleotides do not cause vasodilation through the same mechanisms. It is thought that UTP-induced vasodilation exclusively involves endothelium-dependent hyperpolarization (EDH) [10], while ADP acts through both NO and EDH on a 50%/50% basis.[11] CRCs to ADP and UTP were performed in coronary arteries precontracted with U46619 and then in coronary segments pre-contracted with 30 mM KCl. The latter was done to elucidate the contribution of EDH in ADP- and UTP-induced vasodilation because when arteries are precontracted with 30 mmol/L KCl, EDH cannot occur because the artery is too strongly depolarized.[12-14] VSMC dilatory function was tested by constructing CRCs to the NO donor SNP.

In iliac rings, after washing out KCl 100 mmol/L, we investigated contractile responses to angiotensin II (AngII, 10^{-10}-10^{-7} mol/L), endothelin-1 (ET-1 10^{-10}-10^{-6} mol/L) and phenylephrine (PE, 10^{-9}-10^{-5} mol/L). The involvement of Ang II type 2 (AT_2) receptors in AngII responses was tested by adding PD123319 (10^{-7} mol/L) 30 minutes prior to starting the CRCs.

Mechanical properties of the vascular wall

Carotid arteries explanted from 5 months old mice were mounted in the pressure myograph (Danish Myograph Technology, Aarhus, Denmark) in calcium free buffer (in mmol/L: NaCl 120, KCl 5.9, EGTA 2, MgCl2 3.6, NaH2PO4 1.2, glucose 11.4, NaHCO3 26.3; pH 7.4) to investigate the passive properties of the vessel. The intraluminal pressure of the carotid artery was increased stepwise by 10 mm Hg starting at 0 mm Hg and reaching 120 mm Hg. Following each 10 mm Hg step the vessel was allowed to equilibrate for 3 minutes and then, lumen and vessel diameter were measured and used to calculate mediastrain and mediastress. Mediastrain, calculated as pressure-induced increases in lumen diameter, relates to the elasticity of the vessel. Mediastress indicates distending force on the vessel wall or wall tension, and was calculated as described by Resch et al.[15]

eNOS activation

Freshly isolated lungs were cut in portions of about 0.5 x 0.5 mm, which were carefully incised with cuts made with microsurgical scissors to allow rapid, free entry of buffer and its contents into the lung vasculature. The portions were allowed to equilibrate for 1 hour in Krebs-Henseleit buffer oxygenated with 95% O2 and 5% CO2 at 37°C, freely hanging in the center of 10 ml-organ baths. After this, half of the lung portions were stimulated with 10 µmol/L acetylcholine for 10 minutes, and the other half remained unexposed. The lung portions were then removed and snap-frozen in liquid nitrogen to be stored at -80°C until use for determination of eNOS phosphorylation levels applying Western blotting.

Quantitative real-time PCR

Total RNA was isolated from aortic tissue and cDNA was prepared, which was amplified by Real-time PCR on a StepOne thermocycler (Applied Biosystems). Each reaction was performed in duplicate with SYBR Green PCR Master Mix (Applied Biosystems). β-actin and HPRT-1 were used for normalization. Results from unreliable duplicates or melting-curves were discarded (values were excluded of Ct ≥ 31). The relative amount of genomic DNA in DNA samples was determined as follows: $RQ = 2^{(-\Delta\Delta Ct)}$. Sequences of the primers used are provided in Table 1.

Table 1. Sequences of the primers used for qPCR analyses.

Gene name	Forward primer	Reverse primer
p16	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA
p19	GCCGCACCGGAATCCT	TTGAGCAGAAGAGCTGCTACGT
p21	GTCCAATCCTGGTGATGTCC	GTTTTCGGCCCTGAGATGT
TNFα	AGGGTCTGGGCCATAGAACT	CAGCCTCTTCTCATTCCT
IL-1α	TCAACCAAACTATATATATCAGGATGTGG	CGAGTAGGCATACATGTCAAATTTTAC
IL-6	GACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
MMP13	ACTTCTACCCATTTGATGGACCTT	AAGCTCATGGGCAGCAACA
GAPDH	TGCACCACCAACTGCTTA	TGGATGCAGGGATGATGTTC

Western blots

Frozen tissues were homogenized in ice cold RIPA buffer (50 mmol/L HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate and 1 mmol/L EDTA) containing protease and phosphatase inhibitors (1 mmol/L PMSF, 1 mmol/L $NaVO_4$, 1 mmol/L NaF, 1 µg/mL aprotinin, 1 µg/mL pepstatin and 1 µg/mL leupeptin) using a stainless-steel ultraturrax (Polytron). Homogenized tissues were centrifuged and protein concentration was measured in the supernatants using the BCA method (Thermo Scientific, USA). The samples were run on a criterion bis/tris gradient gel, and blotted to nitrocellulose membranes.

For eNOS, membranes were blocked with 5% milk TBS-T; for pSer1177- eNOS membranes were blocked with 5% BSA TBS-T. After blocking, membranes were incubated overnight with the primary antibodies as follows: eNOS (Santa Cruz, SC-654 1:500 in 5% milk TBS-T) and pSer1177- eNOS (Santa Cruz, SC-21871-R 1:500 in 5% BSA TBS-T).

We used an HRP- conjugated antibody (Bio-Rad 1:2000 in 1% milk-TBS-T) to detect the primary antibodies. For visualization we used an enhanced chemiluminiscent substrate for detection of HRP (Pierce ECL Western Blotting Substrate, Thermo Scientific). All protein expression levels were normalized to actin.

Statistical methods

Data are presented as mean and SD. Statistical analysis between the groups of single values was performed by two-sided t-test. Differences in dose-response curves were

tested by ANOVA for repeated measures (sphericity assumed). Differences were considered significant at $p < 0.05$.

RESULTS

General health features

Tie2Cre Ercc1$^{f/-}$ mice had a reduced lifespan, with a median of 24.6 weeks (Figure 1A). Up until several hours before death occurred EC-KO mice did not show any signs of discomfort or morbidity and body weights were normal (Figure 1B). However, shortly before death, immobility and rapid breathing was observed in 2 of the EC-KO mice. These mice died quickly during daytime. All other mice died during night-time when they were not observed. Blood cell analyses showed no significant changes (Table 2). One of the mice that died at the age of 22.6 weeks during daytime was collected timely enough to be submitted to whole body fixation in formalin for 48 hours and pathological examination. Organs included were brain, heart, skeletal muscle, aorta, sciatic nerve, liver, spleen, lung and kidney. The spleen showed moderate lymphopenia and lymphocyte apoptosis (Figure 1C), however this was not an obvious potential death cause. The kidney was showing severe necrosis spreading from the papilla to the medulla (Figure 1D-E). Since this a potential cause of death, e.g. by rapidly causing severe electrolyte disturbances, we investigated if 5 months old EC-KO mice showed aberrations in the kidney. Kidney microsections were stained with Mason's trichrome for microscopic examination. 5 Months-old EC-KO mice displayed extravasation of red blood cells in the proximity of the tubules (Figures 1F-G). The data suggest that endothelial DNA damage strongly affects the renal medullary microvasculature, and that this is a potential mechanism of mortality.

Table 2. Blood cell counts at 3 and 5 months

Parameter	3 months			5 months		
	KO (n=10)	**Control** (n=17)	**p-value**	**KO** (n=8)	**Control** (n=17-19)	**p-value**
Leucocytes	7 (3.0)	6 (2.9)	0.70	7 (2.8)	9 (5.2)	0.22
% Lymphocytes	63 (7.6)	60 (8.0)	0.43	63 (6.5)	56 (16.6)	0.30
% Monocytes	4 (0.5)	4 (0.8)	0.10	4 (1.1)	4 (1.1)	0.24
% Granulocytes	33 (7.5)	36 (7.5)	0.33	34 (5.7)	35 (12.0)	0.80
Erythrocytes	10 (0.8)	10 (0.7)	0.35	10 (0.6)	10 (0.5)	0.71
Hemoglobin	9 (0.8)	9 (0.6)	0.87	10 (0.6)	9 (0.6)	0.15
Hematocrit	0.5 (0.04)	0.5 (0.03)	0.77	0.5 (0.03)	0.5 (0.03)	0.20
Platelets	383 (217)	343 (250)	0.67	451 (325)	497 (313)	0.74

Values are Mean (SD).

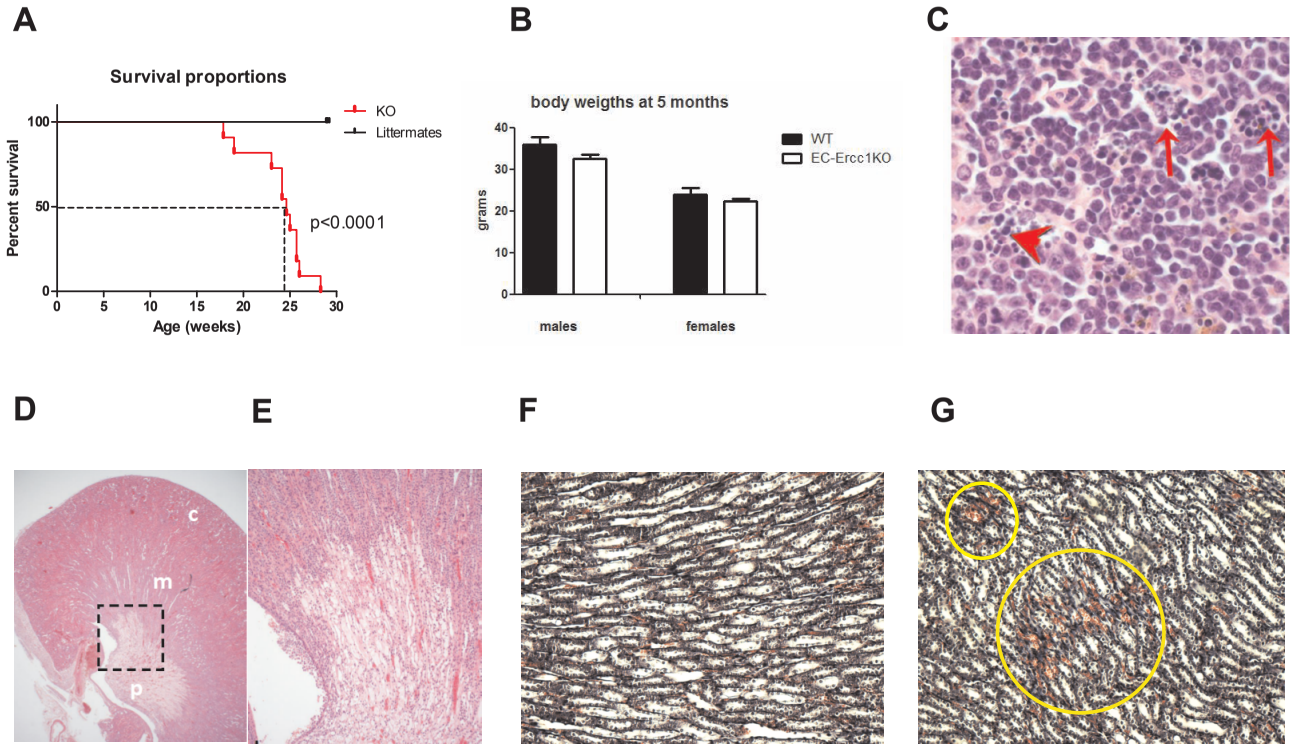

Figure 1. General health and pathology findings. Kaplan-Meier survival analyses of KO vs control mice (A). Body weights at 5 months (B). Spleen sections of a KO mice stained with H&E x40, showing scattered tangible body macrophages (arrow) which contain intracytoplasmic fragments of apoptotic lymphocytes (arrowhead) (C). x2, section showing cortex (c), medulla (m) and renal papilla (p), note the sharply-delineated area of papillary necrosis characterized at this magnification by the loss of staining affinity (D). x10, higher magnification of the inset in figure D showing the junction between normal and necrotic medulla (E). Mason's trichrome staining of kidney sections from a control mouse (F) and a KO mouse (G). The area encircled shows extravasation of red blood cells in the proximity of the tubules.

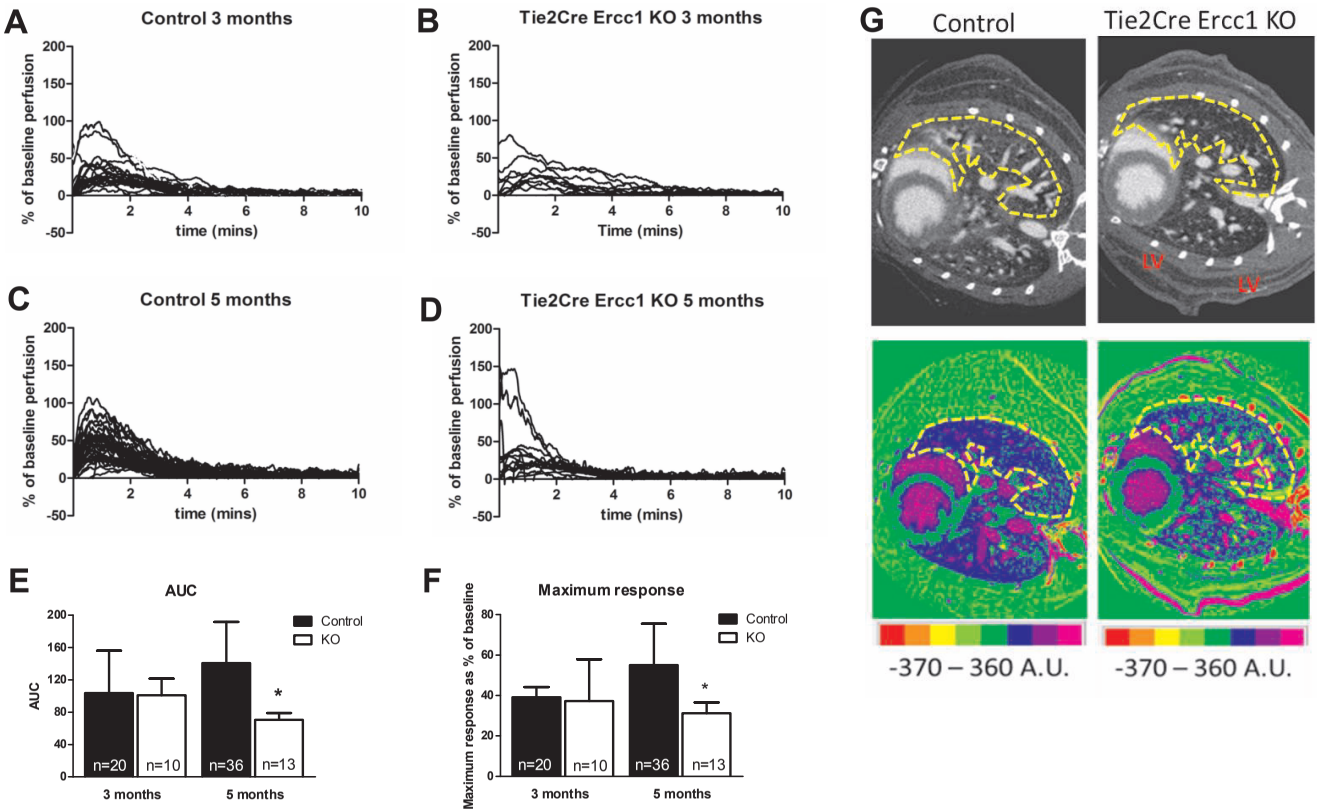

Figure 2. In vivo vasodilator function was assessed using Laser Doppler perfusion imaging and µCT-based contrast-aided perfusion. Functional differences between skin reperfusion after 2 minutes of occlusion between control (A) and KO mice (B) at 3 months; and between control (C) and KO mice (D) at 5 months. Calculated area under the curve (E), and average maximum response (F) for the observed differences in skin reperfusion. µCT-based contrast-aided perfusion measurement, showing lower lung perfusion (encircled) in KO vs. control (71 vs. 111 A.U.) during diastole. LV: left ventricle (G). *P<0.05 (*t*-test).

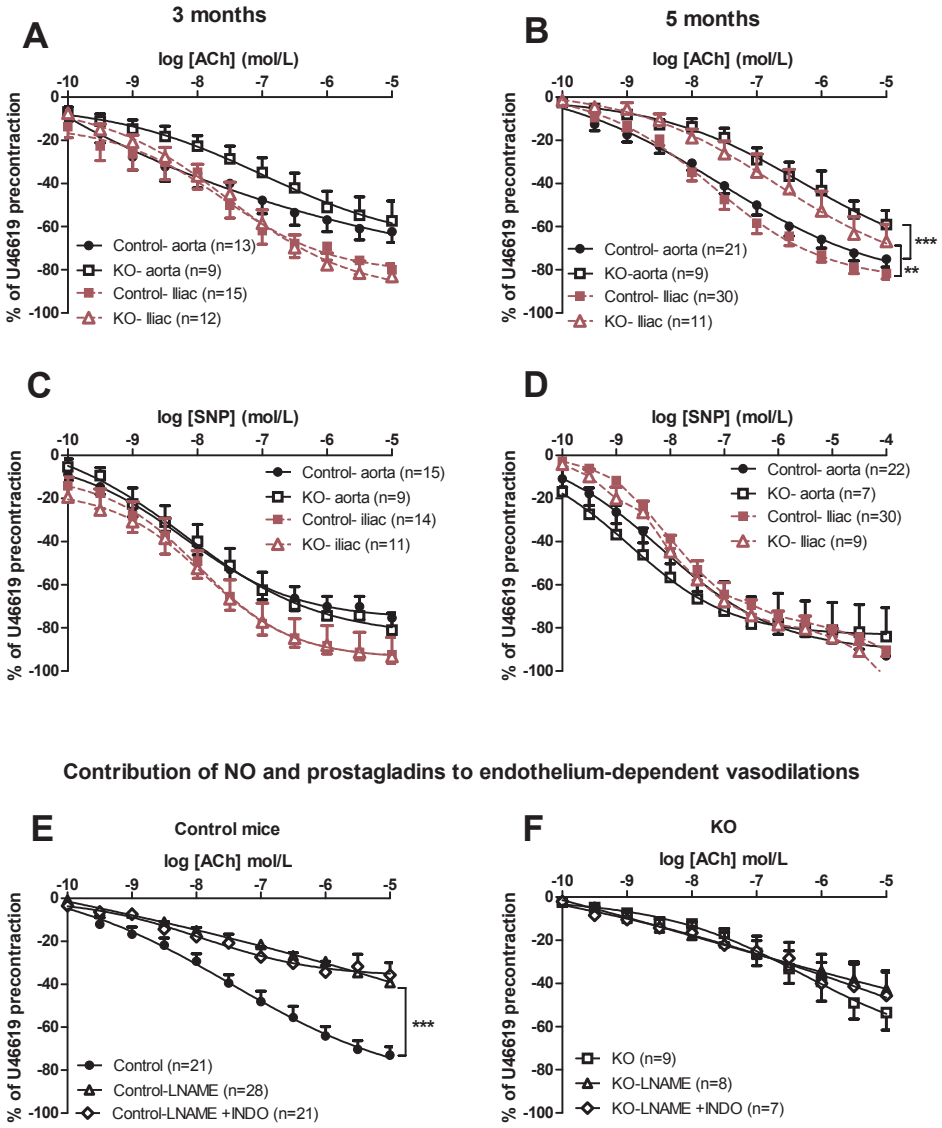

Figure 3. Endothelium-dependent and independent relaxations in isolated aortic and iliac rings measured *ex vivo* in small wire organ bath set-ups. ACh-induced vasodilation of KO and control mice in aorta and iliac artery at 3 months (A), and at 5 months (B). Endothelial-independent relaxations induced by SNP in aortic and iliac rings at 3 months (C) and at 5 months (D). Contribution of NO and prostaglandins to ACh-induced vasodilation in control (E) and KO mice (F). **P<0.001; ***P<0.0001 (GLM-RM).

In vivo microvascular function

At 3 months we did not observe significant differences between EC-KO and control mice in reactive hyperemia after 2-min occlusion of blood flow (Figures 2A, B, E, F). However, when measured at 5 months, EC-KO mice showed decreased reactive hyperemia in

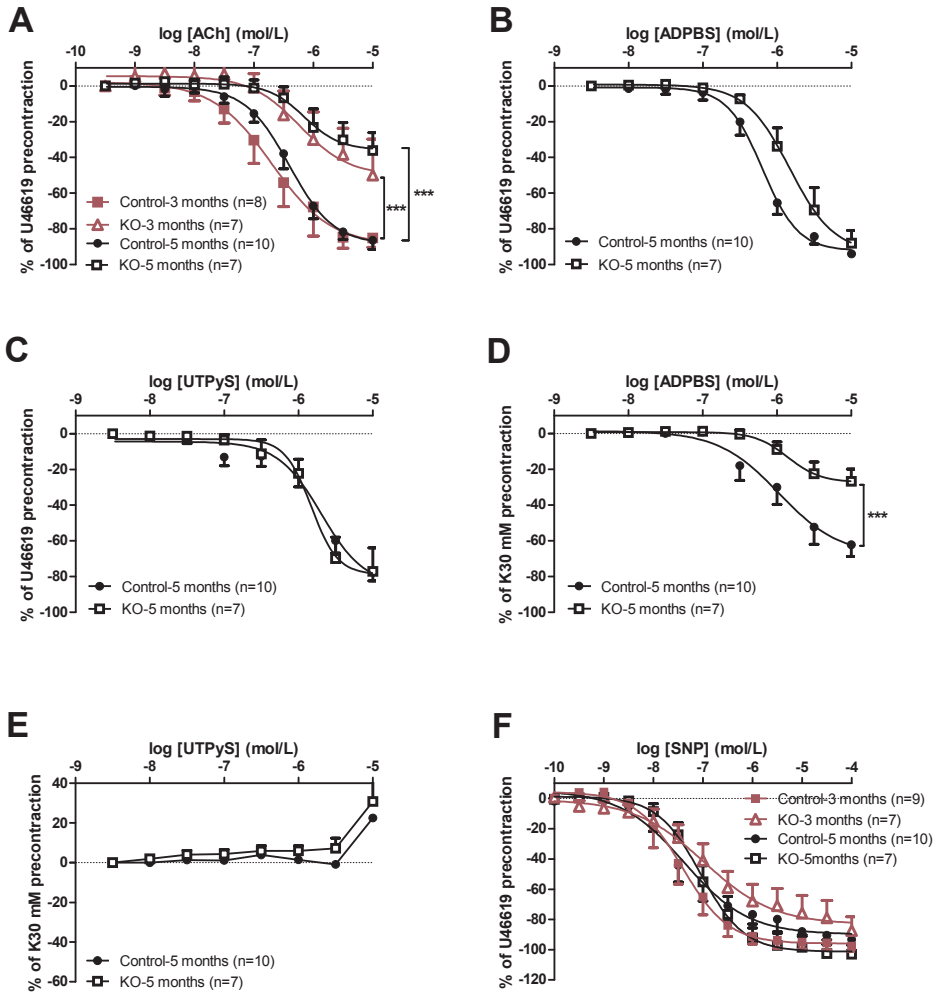

Figure 4. Vasodilation in coronary arteries measured *ex vivo* in small wire organ bath set-ups. Relaxations to ACh (A), ADP (B) and UTP (C) in coronary rings precontracted with U46619. Relaxations to ADP (D) and UTP (E) in coronary rings precontracted with KCl 30mM. SNP induced vasodilation (F). ***P<0.0001 (GLM-RM).

hindlimbs (Figures 2C-F). Of note, when passing from the age of 3 to 5 months WT mice tended to show increased reactive hyperemia, whereas this tended to decrease in EC-KO mice. Lung perfusion was successfully measured in only 1 pair of control vs. EC-KO mice and results suggest that blood supply to the lung is decreased in EC-KO mice (Figure 2G); however, this experiment will have to be repeated in a representative sample of mice to draw definitive conclusions.

Ex vivo vasodilator function

Loss of vasodilator capacity is an important feature in the pathogenesis of aging-related cardiovascular disease. Therefore, vasodilator responses were measured. Arterial tissue from diverse embryonic origin, i.e. descending aorta vs. iliac artery vs. coronary artery, were included, since it is known that this origin creates differential pathogenic changes.[16]

Vasodilator responses in aorta and iliac arteries

Ex vivo vascular function tests revealed an age-dependent decrease in endothelium-dependent relaxations to acetylcholine in EC-KO mice in aortic and iliac artery, being absent at 3 months (Figure 3A) but present at 5 months (Figures 3B). Vascular smooth muscle dilatory function to the NO donor SNP was intact in both aorta and iliac arteries at 3 and 5 months (Figures 3C-D).

To investigate which vasodilator pathways were involved in the decreased endothelium-dependent relaxations to ACh in aorta at 5 months we performed the CRCs in the presence of the eNOS inhibitor L-NAME and the COX inhibitor INDO. In control mice, approximately half of the ACh responses were mediated by NO (responses to ACh were reduced by ~50% in the presence of the eNOS inhibitor L-NAME) (Figure 3E). No apparent contribution of prostaglandins was observed since adding the COX inhibitor did not cause further reductions in the vasodilator responses (Figure 3E). Compared to control mice, in EC-KO mice there was no contribution of the NO or prostaglandins pathways to the ACh vasodilatory responses (Figure 3F). In both WT and KO mice a residual ACh response was observed, suggesting that the contribution of EDH is intact.

Vasodilator responses in coronary arteries

In coronary arteries endothelial dysfunction was already present at 3 months (Figure 4). Endothelium-dependent relaxation to ACh was significantly decreased in EC-KO mice at 3 months and more pronounced at 5 months (Figure 4A).

At 5 months we evaluated responses to the nucleotides ADP and UTP. Relaxations to ADP were found to be partly reduced in EC-KO mice (Figure 4B) while responses to UTP were similar in EC-KO and control mice (Figure 4C). Figures 4D and 4E show the responses to ADP and UTP in arteries precontracted with 30 mmol/L KCl instead of U46619. In such arteries, EDH cannot occur because the artery is too strongly depolarized; [12-14] therefore the relaxation responses seen in Figure 4D are mediated by NO, confirming that in EC-KO mice NO signaling is affected and that ADP causes relaxation via mixed NO-EDH signaling. In Figure 4E no relaxation responses are observed (either in EC-KO or control mice), indicating that the relaxations responses induced by UTP (shown in Figure 4C) are entirely dependent on EDH. Therefore, gathering the results from Figures 4A-4E and as expected from the results in aorta we conclude that in EC-KO mice NO signaling is nearly abolished while EDH signaling is preserved. In agreement with the results in aorta and

iliac arteries, vascular smooth muscle dilatory function to NO in coronary arteries was intact at 3 and 5 months (Figure 4F).

Levels of eNOS, expression of endothelial vs. VSMC components, and scanning electron microscopy

Western blot analysis showed a tendency towards reduced baseline eNOS protein level, corrected for β-actin, in EC-KO vs. WT (Figure 5A). The ratio of eNOS-activating phosphorylation of the serine residue at position 1177 (pSer1177-eNOS) to total eNOS protein was not different at baseline. (Figure 5A). Expression of eNOS mRNA also appeared to be suppressed in EC-KO, further explaining the loss of eNOS protein and signaling (Figure 5B). To elucidate vascular cell-specific effects of the parallel presence of Tie2-driven Cre expression with Plox sites in the mouse genome, we measured the expression of *Ercc1* mRNA, endothelium-specific (von Willebrand factor, vWF) and VSMC-specific (SM22α actin) markers in aortic tissue of EC-KO vs. control mice (Figure 5B). We selected 3 samples representing EC-KO mice and 5 samples representing control mice; however, one of the EC-KO samples did not contain sufficient RNA to successfully perform PCR.

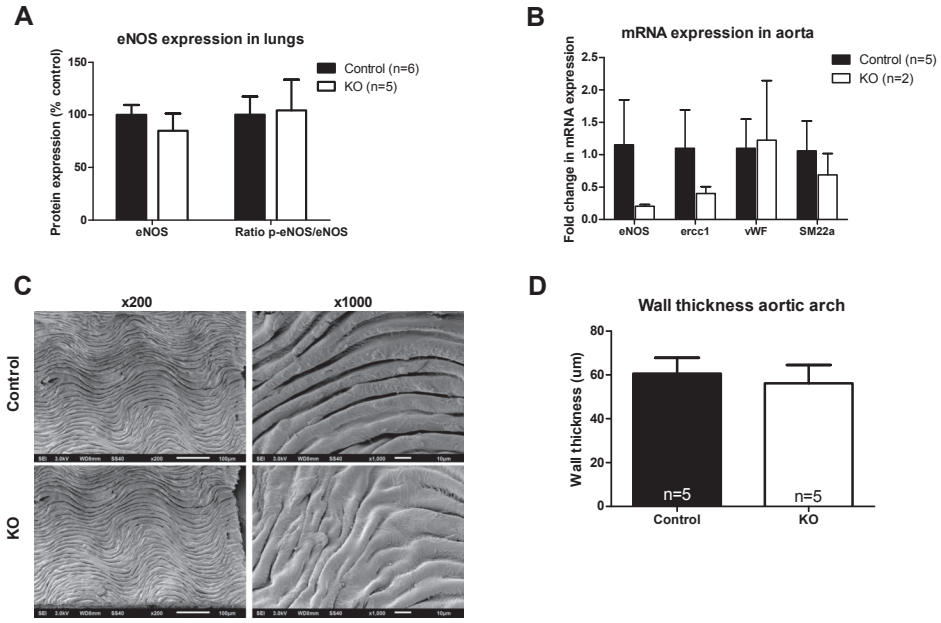

Figure 5. Levels of eNOS, expression of endothelial vs. VSMC components, and scanning electron microscopy. Protein expression levels in lung of eNOS and p-eNOS (A), mRNA expression of eNOS, ercc1, von Willebrand factor and SM22α actin (B). Representative scanning electron microscopy images of the endothelium (C). Wall thickness measured in HE-stained aortic microsections (D).

Although statistical analyses could not be performed, in EC-KO mice there was a striking decrease in Ercc1, whereas vWF and SM22α actin expression remained similar to expression in control mice. The data suggest that Ercc1 was suppressed but did not lead to loss of ECs or VSMCs. To examine the integrity of the endothelial cell layer distal parts of the abdominal aorta from 5 months-old animals (5 control vs. 5 EC-KO mice) were fixated in glutaraldehyde 2% and used for scanning electron microscopy after platinum sputtering. Scanning Electron Microscopy of the luminal surface also did not reveal a loss of endothelial cells (Figure 5C). Also, wall thickness measured microscopically in HE-stained aortic microsections was similar between EC-KO and control mice (Figure 5D).

Blood pressure

Since endothelium-dependent vasodilations were altered we explored if this had consequences for blood pressure. Systolic blood pressure was higher in EC-KO mice at 3 months (138 mm Hg in EC-KO vs 125 mm Hg in control mice) but no differences were observed at 5 months. No differences were observed in diastolic blood pressure at 3 or 5 months (Table 3).

Table 3. Cardiac function, blood pressure and aorta distensibility at 3 and 5 months.

Parameter	3 months			5 months		
	KO (n=10-19)	**Control** (n=11-21)	**p-value**	**KO** (n=11-17)	**Control** (n=12-31)	**p-value**
SV (uL)*	30 (4.5)	31 (4.1)	0.56	25 (5.2)	29 (3.5)	**0.03**
FS (%)	34 (5.1)	34 (5.7)	0.71	33 (6.5)	33 (6.0)	0.94
HR (bpm)	502 (37.4)	492 (37.0)	0.38	494 (40.1)	498 (49.2)	0.78
CO (ml/min)*	15 (2.3)	15 (2.6)	0.92	13 (2.4)	14 (1.7)	0.06
SBP (mmHg)	138 (14.6)	125 (11.9)	**0.04**	128 (22.8)	125 (20.6)	0.72
DBP (mmHg)	94 (15.2)	89 (15.6)	0.46	88 (19.3)	86 (20.7)	0.86
Aorta distensibility (mm)	0.3 (0.1)	0.3 (0.04)	0.69	0.2 (0.1)	0.3 (0.1)	0.05

Values are Mean (SD). SV, stroke volume; FS, fractional shortening; HR, heart rate; bpm, beats per minute; CO, cardiac output; SBP, systolic blood pressure; DBP, diastolic blood pressure. * Values are corrected for heart weight, expressed as per 100 mg of heart weight.

Cardiac function

The change in blood pressure followed an unexpected course, as it is slightly elevated at an age that vasodilation only started to develop (3 months), and becomes normal again when endothelium-dependent vasodilation is strongly decreased (5 months). Cardiac function was explored as a possible explanation. Since we included female and male mice, we corrected the values where applicable for heart weight No differences between EC-KO and control mice were observed in heart rate, stroke volume (SV), cardiac output (CO) and fractional shortening at 3 months. However, stroke volume was significantly decreased in 5 month-old EC-KO compared to control mice (Table 3). Also, SV and CO

significantly decreased in the EC-KO mice at 5 months compared with measurements performed when they were 3 months old.

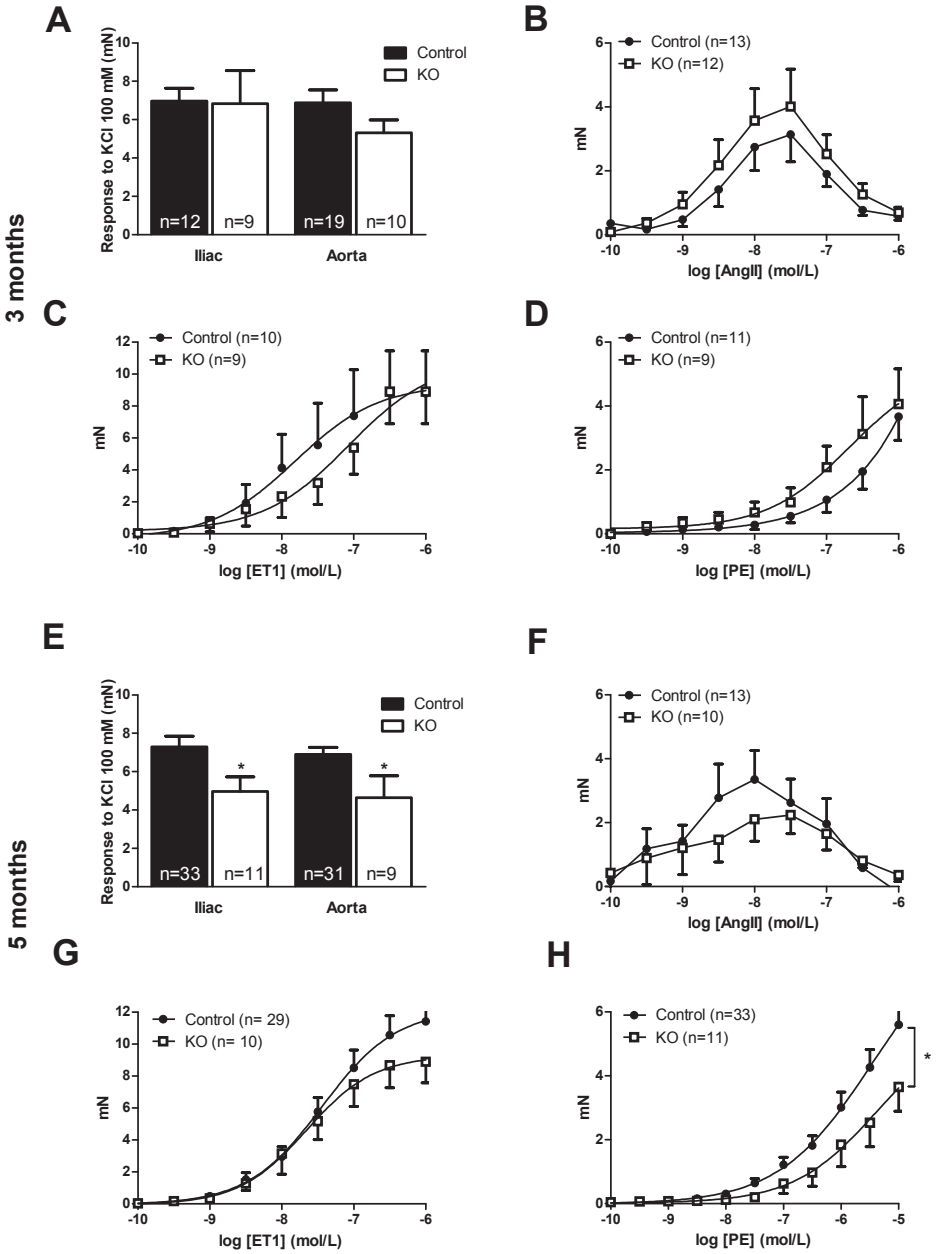

Figure 6. Constrictor responses at 3 and 5 months in aorta and iliac arteries. Contractile responses (in milli Newtons) to KCl 100 mM in aorta and iliac arteries (A, E), and to angiotensin II (B, F), endothelin-1 (C, G) and phenylephrine (D, H) in iliac arteries at 3 and 5 months. *P<0.05 (GLM-RM).

Ex vivo vasoconstrictor responses

Another possibility to explain the course of blood pressure change is adaptation of contractile responses. Hence, we tested receptor-independent constriction due to hyperpolarization with KCl, and receptor-mediated constriction to the central nerve system-regulated neurotransmitter PE, the both locally and systemically regulated hormone Ang II, and the locally regulated endothelium-derived constricting peptide ET-1. At 3 months, no differences were observed between EC-KO and control mice in the responses to KCl, Ang II, ET-1 or PE (Figures 6A-D). At 5 months, EC-KO mice exhibited significantly weaker contractions to KCl in aorta and iliac arteries (Figure 6E). In iliac artery, constrictor responses to Ang II and ET-1 (Figures 6F-G) remained normal, but those to PE were significantly decreased (p-value = 0.016) (Figure 6H).

Mechanical properties of the vascular wall

Increase of vascular stiffness is another factor contributing to increased blood pressure. Since we previously found that arterial stiffness was increased in *Ercc1*$^{\Delta/-}$ mice,[7] we measured aortic wall movement during cardiothoracic echography, and calculated distensibility of the aorta by subtracting the diastolic aortic diameter from the systolic aortic diameter.

At 3 months no differences were found between EC-KO and control mice but at 5 months distensibility was decreased in EC-KO (Table 3).

Since the decreased distensibility might be an indicator of increased wall stiffness we evaluated the mechanical properties of carotid arteries (Figure 7). No significant differences in lumen diameter or wall thickness under similar perfusion pressure increments were observed between EC-KO and control mice (Figures 7A-B). No differences in mediastrain (indicating elasticity) or mediastress (which indicates distending force on the vessel wall) were observed (Figures 7C-D).

mRNA expression of SASP components

Since we previously found an increase of p53, p21 and senescence-associated β-galactosidase staining in *Ercc1*$^{\Delta/-}$ mice, a role of cellular senescence in the observed endothelial function differences was suggested [7]. Although our previous report could detect only few senescent cells in the medial layer, and we only expect EC senescence in our EC-KO, which should be difficult to detect, we decided to perform qPCR for several SASP components. We found no significant differences in aortic mRNA levels of p16, p19, p21, TNFα, IL-1α, IL-6 and MMP13 (Figure 8).

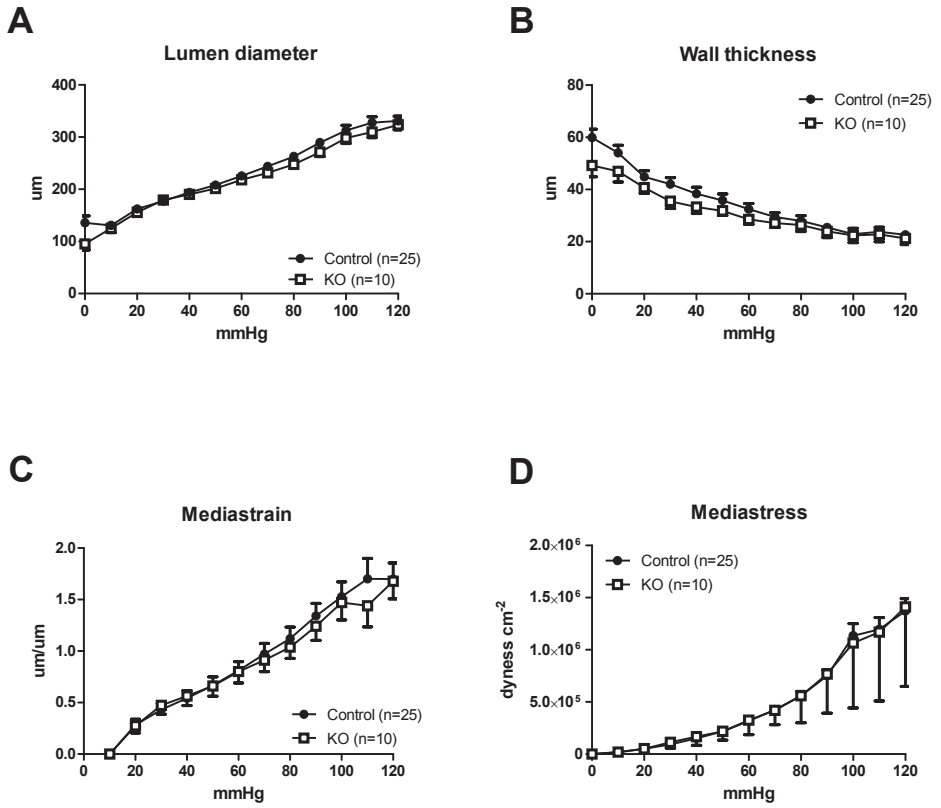

Figure 7. Mechanical properties of the carotid artery measured in a vascular perfusion set-up at 5 months. Lumen diameter (A), wall thickness (B), mediastrain (C) and mediastress (D) measured at increasing intraluminal pressures.

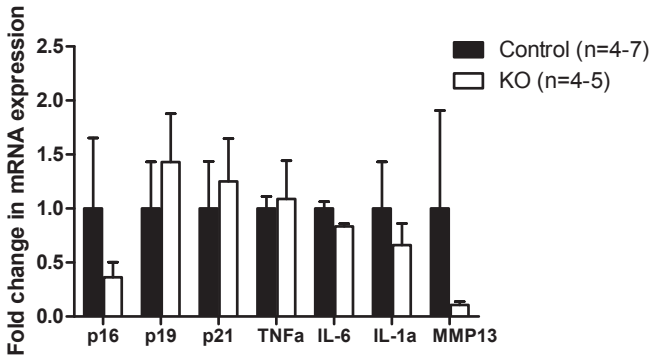

Figure 8. mRNA expression of several SASP components measured in aorta. aortic mRNA levels of p16, p19, p21, TNFα, IL-1α, IL-6 and MMP13 in 5 months-old Control vs KO mice.

DISCUSSION

We investigated the role of local endothelial genomic instability on cardiovascular function in a mouse model with specific loss of *Ercc1* in vascular endothelial cells. We found that local endothelial genomic instability causes macrovascular and microvascular vasodilator dysfunction due to specific loss of endothelium-dependent NO signaling, i.e., not EDH or prostaglandins. Likely, there is a reduced NO availability, taken the decreased eNOS expression, and the normal responses of VSMC to exogenously administered NO. The preserved endothelial cell layer, as confirmed by SEM, intact vWF levels and normal EDH excludes the loss of NO through reduced EC numbers. Notably, the rate of development of vasodilator function is vessel-specific, at 3 months of age being observed in coronary arteries only but not in iliac artery, aorta, or the skin microvasculature. This suggests a non-congenital origin of the vasomotor dysfunction and a non-homogenous regulation of eNOS over the vascular bed under otherwise healthy conditions. The reduction of vasodilator response was only associated with a temporary blood pressure increase. Apart from vasodilator dysfunction, EC-KO mice showed a severely compromised microvascular barrier function in the kidney, potentially leading to premature death. Furthermore, cardiac output was affected at 5 months of age. In addition, aortic distensibility measured by echography was reduced, suggesting increased vascular stiffness, but this was not observed by stress and strain measurements in carotid arteries. In summary, local endothelial DNA damage leads to very specific loss of NO signaling, affecting organ function and potentially also blood pressure under otherwise healthy conditions. The impact on vascular stiffness remains to be further explored.

Our present study shows that the effect of DNA damage on NO signaling is very specifically evoked within the endothelium, possibly even cell-autonomously. This answers an important question remaining from our previous study in the *Ercc1*$^{\Delta/-}$ mouse model, showing that NO signaling was disturbed both in EC and VSMC.[7,8] Hence, the endothelial effect of DNA damage is not dependent on genomic lesions elsewhere. Nevertheless, the endothelium being important in all organs, *Ercc1* KO mice share some features, including a worsened renal morphology and a shortened lifespan.[5] While *Ercc1*$^{\Delta/-}$ live an average of 24-28 weeks, endothelial-specific KO live until an average age of 25 weeks. However, in endothelial-specific KO mice death is sudden, without preceding weight loss or obvious behavioral changes, whereas *Ercc1*$^{\Delta/-}$ mice start deteriorating at an early age, showing consistent weight loss and problems of neuronal origin.[5] Renal tubular malfunction leading to sudden death caused by severe electrolyte disturbances was proposed as one of the possible causes of death in the endothelial KO mice, whereas in *Ercc1*$^{\Delta/-}$ mice the neurodegeneration and general frailty are very prominent and considered as possible cause of death. Nevertheless, one cannot entirely exclude that renal dysfunction contributes in both models. Indeed, cellular senescence and DNA

damage response markers are associated with reduced viability of the kidney when transplanted.[17, 18] More attention is needed for further exploration of the role of DNA damage in renal aging and chronic kidney disease, where knowledge is lacking.

On the basis of the present study we cannot entirely rule out that sudden death arose from effects on the circulation in other organs. Indeed, the coronary circulation was affected relatively early. Lowered cardiac output was observed at the age of 5 months. Several hours before death at least a part of the animals were showing respirational problems, which might relate to cardiac events. Alternatively, lung perfusion might be severely undermined, as was suggested by the observation employing thoracic uCT flowmetry. A close monitoring of cardiac function, both left and right ventricle as wells as electrocardiograms, pO_2, and thrombotic status, which is also regulated by EC and NO, would be needed to further elucidate the cause of death. Nevertheless, the current findings point out that local endothelial DNA damage has very specific and clinically relevant consequences independently from accelerated aging in other organs. This underlines the potential of the present model as a model to explore the role of endothelial-specific aging on health.

A striking finding was the temporary presence of increased blood pressure, being increased at 3 months, which is in line with increased blood pressure in 8 weeks old *Ercc1*$^{\Delta/-}$ mice found previously,[7] but normal again at 5 months. The normalization of blood pressure is associated with a lower left ventricular stroke volume and cardiac output. Also, vasoconstrictive responses to KCl and phenylephrine were decreased. Thus the blood pressure rise might be lost in EC-KO at the age of 5 months because of reduced vasoconstrictor responses. Whether this is an adaption response or a pathological consequence of DNA damage is unclear. On the one hand, the specific reduction of PE vs. ET-1 and Ang II responses might point at an adaptation, since the adrenergic receptors acted upon by PE are part of the acute, vasomotoric blood pressure regulation by the sympathetic nervous system, whereas Ang II and ET-1 are more associated to chronic vascular remodeling. On the other hand, the altered cardiac function might be a consequence of reduced coronary vasodilator capacity. Whether it be the one, the other or a combination of both processes, at least the almost total loss of NO signaling does not immediately lead to dramatic consequences for blood pressure or cardiac function at the age studied. This is in striking resemblance with earlier findings in eNOS knockout mice, where it was shown that blood pressure increase was also biphasic, and that cardiac hypertrophy only develops much later, at 21 months of age.[19] Of course, the presence of compensatory mechanism in an otherwise healthy animal, which holds for both our EC-KO as well as eNOS KO mice, will enable the animals to maintain blood pressure homeostasis. Although prematurely dying, aging eNOS KO mice live on average 4 times longer than EC-KO, and therefore the sudden death observed here cannot be explained by lack of eNOS function alone.

Increased vascular stiffness is an important hallmark of vascular aging. We showed previously that DNA damage accelerates the increase of vascular stiffness. The origin of this increase, either in humans or in our model of accelerated aging, is not completely understood. Here we show that EC-KO mice display reduced aortic distensibility at 5 months of age. However since increased stiffness could not be confirmed in carotid arteries, the change observed in aorta might be due to the reduced stroke volume of the heart. On the other hand, compliance is normally calculated correcting vascular distension for blood pressure, and the latter is unchanged in 5 month-old EC-KO. Regretfully, blood pressure was not measured in parallel with compliance. Again in agreement, eNOS KO mice of similar age as our EC-KO show decreased aortic compliance. However, their carotid compliance is higher, albeit marginally.[20] Since eNOS decrease is only partial in our model, a slower development of increased vascular stiffness might be expected, and the reduced aortic distensibility might be an early sign of such aberration. Further scrutiny with more exact measurements, combining distensibility, pulse wave velocity and blood pressure is warranted.

Our results could not be correlated to cellular senescence. This could be because cellular senescence might not be required for induction of vasodilator dysfunction. Alternatively, our analysis might not allow detection of EC-specific changes, ECs being in strong minority compared to VSMC in aortic tissue causing signal dilution. In a previous study we showed that in *Ercc1*$^{\Delta/-}$ mice senescent cells were VSMC, whereas senescent EC were not observed. Senescent cells were only very few, increasing the chance for senescent EC to remain undetected. Nevertheless, one has to question how senescence in very few cells could lead to the presently observed, strong effects on vasodilation. Hypothetically, the SASP signal might be conveyed through cell-cell communication within the endothelium, but until now only the reverse, i.e. the rescue from senescence of endothelial cells by endothelial progenitor cells, has been reported.[21] Alternatively, a strongly localized paracrine pro-inflammatory process could take place. Indeed, this would not be detected with our present analysis. In general, the knowledge regarding this question is rather minimal, which can be attributed to the complicated detection of cell senescence and local inflammation in the endothelium, and the lack of studies regarding cell-cell communication in senescent EC. Nevertheless, it has been suggested previously that SASP in few EC might cause a more generalized dysfunction of circulatory function, much in a way that SASP of tumor fibroblasts might promote cancer progression.[3]

In summary, we found that local endothelial genomic instability causes sudden death, clear microvascular complications in the skin, kidney and possibly also in the lung, coronary vasodilator dysfunction associated with lower cardiac stroke volume, and macrovascular dilation problems. Potentially, early sign of increased vascular stiffness are present. Measurement of NO signaling and the striking resemblance with eNOS

knockout mice indicate that the loss of this signaling pathway might play a major role in the observed phenotype. Interventions that normalize this signaling pathways, such as pharmacotherapy with guanylyl cyclase stimulators or activators and specific phosphodiesterase inhibitors are indicated. Further, diet restriction is a potential treatment option. This has already been shown to be effective in *Ercc1*$^{\Delta/-}$ mice [6, 8, 22], in accordance with its generally known anti-vascular aging effect in humans.

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Chapter 4

Local VSMC DNA damage causes dilation of the proximal aorta and aging-resembling macrovascular motor dysfunction

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ABSTRACT

DNA damage is a leading causal factor in aging, and has been implicated in aging-related cardiovascular disease. Amongst others it contributes to decreased vasodilator responses, increased vascular stiffness and increased blood pressure. Recently, we showed that genetic ablation of the DNA repair in endothelial cells in mice specifically undermines endothelium-derived nitric oxide-induced vasodilation. Since this does not represent the full array of functional changes when compared to whole body DNA repair mutant mice we hypothesized that local DNA damage in vascular smooth muscle cells (VSMC) is also contributing to vascular aging, and that this would cause cell autonomous, i.e. VSMC-specific changes. To this end we removed the *Zmpste24* gene, which is pivotal in the maturation of the genome maintenance protein lamin A, specifically in smooth muscle. The smooth muscle-specific DNA mutant mice had a shortened longevity, living on average 26 weeks, morbidity and subsequent death developing rapidly. Half of the mice developed dilated proximal aortic arches, which was accompanied by aortic regurgitation. Despite a modest decrease of ejection fraction in both mice with and without aortic regurgitation (~18% relative to wildtype mice) there were no signs of heart failure. Systolic blood pressure was increased by ~10 mmHg, but was normal in the mice with aortic regurgitation. Smooth muscle-specific vasodilation to nitric oxide was decreased, whereas endothelium-dependent dilations were normal by virtue of replacement of nitric oxide responses by increased prostaglandin and endothelium-dependent hyperpolarization. Carotid arteries showed less compliance to increasing intraluminal pressure. The lamina media showed less cellularity, and contracted less to Ca^{2+}. There was a trend towards loss of AT_2 receptor-mediated vasoconstrictions, a hallmark of aging-related vascular disease and hypertension. Markers of DNA damage and senescence were increased in the knockout mice. Most of the changes were already present in animals of 3 months of age, except for increased SBP and aortic dilatation / regurgitation. In summary, a defective DNA repair specifically in smooth muscle cells leads to a rapid development of a broad array of aging-related vascular problems, but all related to smooth muscle-specific defects. Thus, the effect of DNA damage appears to be cell-autonomous. This mouse represents a possible model for type A aortic dissection, a life threatening condition.

INTRODUCTION

Age is the most important risk factor for cardiovascular diseases, as age-induced changes in vascular structure and function lead to impaired arterial compliance, allowing disease to progress.[1, 2] Hallmarks of vascular ageing in the clinic include decreases in vasodilatory capacity, organ perfusion and angiogenesis, and increases in wall thickness, blood pressure (BP) and atherogenesis. Underlying pathogenic processes can be retraced to dysfunction of the endothelium, and involve reduced levels of endothelium-derived relaxing factors, a pro-inflammatory ('activated') state of the endothelial cells (EC), leakiness of the cell-cell contacts, and a disbalance in cell fate (apoptosis vs. senescence vs. proliferation).[3-6] In Chapter 3 we showed that DNA damage, a causative factor in general ageing, forces EC to assume a state of dysfunction that promotes the above-mentioned pathogenic processes. Comparison of the vascular phenotype of mice with EC-specific knockout of the endonuclease ERCC1 (Tie2Cre:Ercc1f/- mice, or EC-KO) with whole body Ercc1 gene mutants (*Ercc1*$^{\Delta/-}$) revealed that both mice display reduced endothelium-dependent vasodilation, whereas in *Ercc1*$^{\Delta/-}$ the vascular smooth muscle cells (VSMC) responsiveness to nitric oxide (NO) is additionally disturbed. Furthermore, in *Ercc1*$^{\Delta/-}$ mice there is a quick development of carotid artery stiffness, which is absent in EC-KO, and BP increase appears to be more pronounced in Ercc1$^{\Delta/-}$ mice. Senescence markers were observed only in the aorta of *Ercc1*$^{\Delta/-}$ mice. This suggests that DNA damage effects are cell(-type)-autonomous, and that damage in >1 cell type is required to induce the full array of changes observed in the aging (human) vasculature. Indeed, multiple studies support the hypothesis that DNA damage is a major driver of (vascular) ageing by inducing cellular apoptosis and senescence.[7-9] This not only impairs tissue regeneration, but also results in the secretion of various cytokines and growth factors, leading to pathological remodeling of the extracellular matrix (ECM), and ultimately causing changes in arterial structure and function.[6] Structural remodeling in aged arteries includes an increase in lumen diameter, diffuse medial thickening, and obvious ECM remodeling.[4, 6, 10] This contributes to clinical symptoms, in particular systolic hypertension, increased arterial stiffness and increased pulse wave velocity (PWV).[11, 12] Our observations in *Ercc1*$^{\Delta/-}$ and EC-KO point at a role of DNA damage in these processes, at least in EC. The contribution of DNA damage in VSMC is still unknown.

One of the leading observations that demonstrates the putative importance of DNA damage in humans is the association between lamin A dysfunction and vascular aging. Lamin A has an important structural role in nuclear scaffolding and is involved in the regulation of nuclear pore formation and chromatin organization.[13, 14] Defective maturation of lamin A results in the accumulation of prelamin A. Prelamin A (like progerin) induces DNA damage, apoptosis and senescence, e.g. in cells from patients with Hutchinson-Gilford Progeria Syndrome (HGPS), in cells from aged humans, and in cells from transgenic

animals that display premature ageing.[14-19] This is due to the fact that prelamin A impairs the recruitment of DNA damage repair proteins and checkpoint activation, thus resulting in defective DNA damage repair.[15, 17, 19-22] HGPS patients and mice with defective lamin A maturation show an array of aging features at young age, such as (premature) death, weight loss, alopecia, growth retardation, lipodystrophy, and, most importantly, cardiovascular disease.[16, 23-27] The latter is featured by myocardial infarction or cerebrovascular events, and a major loss of VSMCs in both big and small arteries.[28] Thus, prelamin A accumulation is expected to produce a large array of vascular aging features observed in the general population in humans. VSMC DNA damage due to prelamin A accumulation, like such damage in Ercc1-defective mice (Chapter 2), may disturb VSMC relaxation.

The aim of this study was to determine how prelamin A accumulation in VSMCs affects the physiology and function of the cardiovascular system. To this end, the SMC-specific Zmpste24 KO mouse was used. This is a novel model where the accumulation of prelamin A is restricted to SMC in the vasculature and/or other SM22α-expressing tissues, i.e. SMC outside the vasculature.

METHODS

Animals

The SMC-specific *Zmpste24* KO mouse model that was used in this study is a novel model that has not been used before to study the effects of prelamin A accumulation in vivo. This model, unlike the other *Zmpste24* KO mouse models, restricts the accumulation of prelamin A to the vasculature and/or tissues with cell types that express the SMC marker SM22α.The mice used in this study were *Zmpste24*$^{fl/fl}$ *Cre*$^{-/-}$ and *Zmpste24*$^{fl/fl}$ *Cre*$^{+/-}$. Ear DNA was amplified by PCR and resolved by agarose gel to determine mouse genotypes. In this study, *Zmpste24*$^{fl/fl}$ *Cre*$^{-/-}$ and *Zmpste24*$^{fl/fl}$ *Cre*$^{+/-}$ mice will be referred to as WT and *Zmpste24* SMC-KO mice respectively.

Mice were kept in individually ventilated cages, in a 12 h light/dark cycle and fed normal chow and water *ad libitum*. All animal studies were approved by the Animal Care Committee of the King's College of London and Erasmus MC.

Cardiac function

Left ventricular functions were assessed using M-mode, or motion mode, from the parasternal long axis, which gives a view of the left ventricle and atrium and the aorta (Vevo2100 High-Resolution Imaging System, VisualSonics). M-mode imaging modality was used to determine the ejection fraction and left ventricle dimensions. Mice were under anesthesia with 2.5% isoflurane, intubated and connected to a pressure-controlled ventilator, and body temperature was kept at 37°C.

Blood pressure measurement

We measured BP non-invasively in conscious mice using the tail cuff technique (MK-2000ST NP-NIBP monitor, Muromachi, Japan). Mice were trained for 4 days to minimize stress-induced changes in the measurements, and measurements were then taken on the 5^{th} day.

Ex vivo vascular function

Thoracic aorta, iliac and left anterior descending coronary arteries were collected from mice within 5 minutes after sacrifice and kept in cold Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,$CaCl_2$ 2.5, $MgSO_4$ 1.2 , KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3 in distilled water; pH 7.4). Vessel segments of maximum 2 mm length were mounted in small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at which the effective transmural pressure is 100 mmHg. We tested the viability of the vessel segments by inducing contractions with 30 and 100 mmol/L KCl.

Vasodilator responses

After washout of KCl, coronary and aortic segments were pre-constricted with 30 nmol/L U46619, a thromboxane A_2 analogue, resulting in 50-100% of the previously obtained contraction to 100 mmol/L KCl. Following pre-constriction, concentration-response curves (CRCs) to the endothelium-dependent vasodilator acetylcholine (ACh) were constructed by adding cumulative doses (10^{-10}-10^{-5} mol/L). After the CRC to ACh was finished, the vessel segments were exposed to the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). Complete CRCs to SNP (10^{-10}-10^{-4} mol/L) were done in parallel rings preconstricted with 30 nmol/L U46619.

The involvement of nitric oxide (NO), prostaglandins and the endothelium-dependent hyperpolarization (EDH) in ACh responses was investigated in aortic segments by performing the experiments in the presence of the endothelial nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester salt (L-NAME, 10^{-4} mol/L), the cyclo-oxygenase (COX) inhibitor indomethacin (10^{-5} mol/L) and the intermediate and small conductance Ca^{2+}-dependent K^+ channel (IK_{Ca}/SK_{Ca}) blockers TRAM34 (10^{-5} mol/L) and apamin (10^{-7} mol/L). The involvement of phosphodiesterase (PDE) in SNP vasodilation was investigated by performing the experiments in the presence of the PDE inhibitor sildenafil (10^{-7} mol/L). At this concentration the inhibitor is expected to predominantly block PDE5 and to a lesser degree possibly also PDE1. Inhibitors were added to the organ bath 20 minutes prior to U46619.

Constrictor responses

In aorta arteries, we assessed contractile responses to phenylephine (PE, 10^{-9}-10^{-5} mol/L) after washing out KCl 100 mmol/L. In coronary arteries, we evaluated contractile responses to KCl, calcium and endothelin-1 (ET-1). In iliac arteries, we assessed contractile responses to angiotensin II (Ang II, 10^{-10}-$10^{-7.5}$ mol/L), ET-1 (10^{-10}-10^{-6} mol/L) and the calcium ionophore ionomycin (10^{-10}-10^{-5} mol/L). Irbesartan (10^{-7} mol/L) was used to test the involvement of Ang II type 1 (AT_1) receptors and PD123319 (10^{-7} mol/L) to test the involvement of Ang II type 2 (AT_2) receptors. CRCs to ET-1 were also performed in the presence of the ET_A receptor antagonist BQ123 (10^{-6} mol/L) and the ET_B receptor antagonist BQ788 (10^{-6} mol/L). The CRC to ionomycin was also done under RhoA-kinase inhibition (Y27632, 10^{-5} mol/L). Inhibitors were added 30 minutes prior starting the CRCs.

Mechanical properties of the vascular wall

Passive properties of the vessel were measured in the carotid artery mounted in pressure myograph (Danish Myograph Technology, Aarhus, Denmark) in calcium free buffer (in mmol/L: NaCl 120, KCl 5.9, EGTA 2, $MgCl_2$ 3.6, NaH_2PO_4 1.2, glucose 11.4, $NaHCO_3$ 26.3; pH 7.4). The intraluminal pressure of the carotid artery was increased stepwise by 10 mm Hg starting at 0 mm Hg and reaching 120 mm Hg. After each 10 mm Hg increment the vessel was allowed to equilibrate for 3 minutes and then, lumen and vessel diameter were measured. With these data, mediastress and mediastrain were calculated.

Genetic association studies

We investigated the association of single-nucleotide polymorphisms (SNPs) in the *LMNA* and *ZMPSTE24* genes with systolic and diastolic BP (SBP, DBP) and carotid-femoral pulse wave velocity (CFPWV) using data from different consortia with large population samples. The look-up for the association between the *LMNA* and *ZMPSTE24* SNPs and BP was done in the International Consortium of Blood Pressure Genome-Wide Association Studies (ICBP-GWAS).[29] ICBP-GWAS includes data from approximately 200,000 participants of European ancestry from 35 studies. Linear regression analyses were adjusted for sex, age, age squared, body mass index and ancestry principal components. Individuals who had received treatment for hypertension were imputed to have 15 mm Hg higher SBP and 10 mm Hg higher DBP than the observed measurements. Associations with CFPWV were tested using data from the AortaGen Consortium which comprises around 20,000 individuals of European ancestry from 9 cohort studies.[30] Since CFPWV has a right skewed distribution due to nonlinear increases and high variance with advancing age, together with differences due to the method used by the different cohorts, association analyses were performed using a sex-specific standardized residual based on the inverse of CFPWV, this normalizes the distribution. The standardized residual was adjusted for age, age squared, height, and weight.

Statistical analysis

Data are presented as mean and SEM. Statistical analysis between the groups of single values was performed by two-sided t-test, 1-way or 2-way ANOVA as appropriate. Differences in dose-response curves were tested by ANOVA for repeated measures (sphericity assumed). Differences were considered significant at $P<0.05$. For the genetic association studies 35 SNPs were selected: 17 inside the *LMNA* gene and 18 in the *ZMPSTE24* gene. The significance threshold was calculated using Bonferroni correction for multiple testing. P-values $<1.43x10^{-3}$ (=0.05/35) were considered significant.

RESULTS

Lifespan and body weight

Figure 1A shows the Kaplan-Meier analysis comparing the survival rates between WT and SMC-KO mice. SMC-KO mice had a median survival of 26 weeks, whereas WT mice lived beyond 52 weeks. WT and SMC-KO mice body weights were measured from a period of 1 to 6 months before early death for both males and females. Figure 1B shows that *Zmpste24* SMC-KO mice gained weight normally from birth as their WT counterparts until about 5 months when the weight started to decrease. SMC-KO mice showed significant weight loss at 6 months when they weighed 27.61±1.06 g compared with WT mice 31.62±1.47 g (2-way ANOVA $p<0.05$). At this age the SMC-KO mice developed dilated bowels, which might relate to SMC DNA repair knockout and the observed weight loss. The gastrointestinal tract remains to be further explored in relation to SMC apoptosis.

DNA damage and senescence markers in SMC-KO mice at 6 months

Deletion of Zmpste24 in SMC is expected to increase DNA damage and cause cellular senescence in the medial layer of arterial tissue. Ascending aorta sections from 6 months-old WT and SMC-KO mice were stained with a γH2AX antibody. As shown in Figure 1C-J, SMC-KO mice displayed cell loss in the media layer and extensive DNA damage in the medial layer compared with WT mice. In agreement with this finding, mRNA expression of the senescence markers p16 and p21 was significantly increased in aorta from 6 months-old SMC-KO mice (Figure 1K-L).

Cardiac function and blood pressure

Echocardiography was used to assess the cardiac function in WT and SMC-KO mice at 3 and 6 months. Using color Doppler imaging to visualize blood flow from the left ventricle to the aorta, it was discovered that SMC-KO mice exhibit aortic insufficiency, also known as aortic regurgitation. There was normal flow of blood from the left ventricle to the aorta in WT mice; however, in SMC-KO mice, this was very quickly followed by a

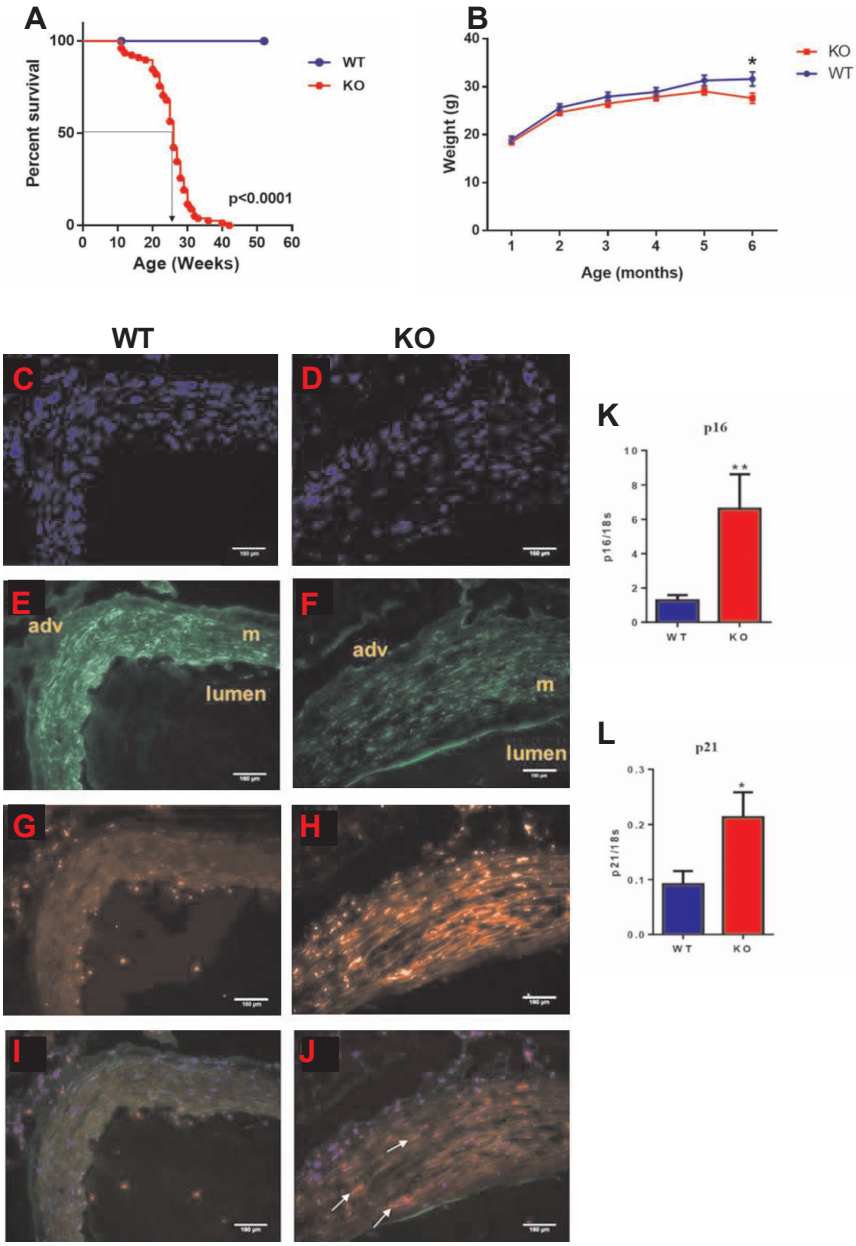

Figure 1. Lifespan, body weights, DNA damage and senescence markers. Kaplan-Meier survival analysis of *Zmpste24* WT and SMC-KO mice (A) and body weights from 1 to 6 months (B); n= 9-25 mice per group, *P<0.05 (2-way ANOVA). DAPI staining of 6 months-old WT and KO mice ascending aorta showing cell loss in the media of KO mice (C-D). Positive smooth muscle actin staining in WT and SMC-KO aortic sections (E-F). Positive DNA damage (γH2A) stain in KO mice ascending aorta sections (G-H). Merged DAPI, SMA and γH2A stains (I-J). n=4-5. mRNA expression of p16 (K) and p21 (L) in aorta; n= 8-9 mice per group, *P<0.05; **P<0.01 (*t*-test).

jet of flow back into the left ventricle from the aorta. Of the 23 SMC-KO mice scanned at 3 and 6 months over half (52%) had aortic regurgitation (AR). AR is often associated to proximal aortic dilation. Echography indeed revealed dilation of the aortic root and ascending aorta in SMC-KO mice with AR at 6 months (Figure 2A and B). In SMC-KO mice without AR aortic root, diameter was wider than in WT mice and no differences in the ascending aorta diameter were observed.

From the age of 3 months fractional shortening (FS) and, thus, ejection fraction (EF), started to decrease in SMC-KO compared to WT, which became significant at the age of 6 months (Figures 2C and 2D, 2-way ANOVA, $P<0.05$). There was no difference in EF and FS between SMC-KO mice with AR vs without AR at both time points (not shown). The difference in FS and EF remained small, and accordingly left ventricular diastolic and systolic diameter were not different between WT and SMC-KO mice at both ages (Figures 2E and 2F). To explore if there were any further hemodynamic consequences, BP and heart rate measurements were performed at the age of 4, 5 and 6 months. SBP was increased in SMC-KO mice vs. WT at 6 months of age (Figure 3A), whereas DBP (Figure 3B), pulse pressure (not shown) and heart rate (Figure 3C) were unchanged. When separating SMC-KO without AR from those with AR, only mice without AR displayed increased SBP, and also increased DBP (Figure 3D and 3E). No differences were observed at the age of 4 and 5 months in BP or heart rate (not shown). Post-mortem histological analyses revealed no differences in heart weight corrected for tibia length or in left ventricular posterior wall thickness at the age of 6 months (not shown). Thus, despite the small change in FS, and as a consequence EF, there were no signs of heart failure yet, nor of the typical BP changes one can expect in AR (decreased DBP and increased PP, normal or increased SBP). Moreover, increased SBP was only observed in animals without AR.

Ex vivo vascular function

To find an explanation for the (AR-independent) cardiac dysfunction and high SBP observed in SMC-KO mice, we investigated vasodilator and vasoconstrictor responses.

Vasodilator responses

The aortic responses to the endothelium-dependent vasodilator ACh at the age of 5 months were similar in SMC-KO and WT mice (Figure 4A). Yet, the response to the NO donor SNP was disturbed in SMC-KO mice (Figure 4B). Findings at an earlier age (3 months) were identical (Figures 4A and 4B). These findings are suggestive for an upregulation of non-NO endothelium-derived relaxing factors employed by ACh. Therefore, in 5 months-old mice we evaluated the contribution of NO, prostaglandins and EDH to the ACh relaxation responses. In WT mice, approximately half of the ACh response was mediated by NO (responses to ACh were reduced by ~50% in the presence of the NOS inhibitor L-NAME). Of the remaining half, a small fraction was due to prostaglandins

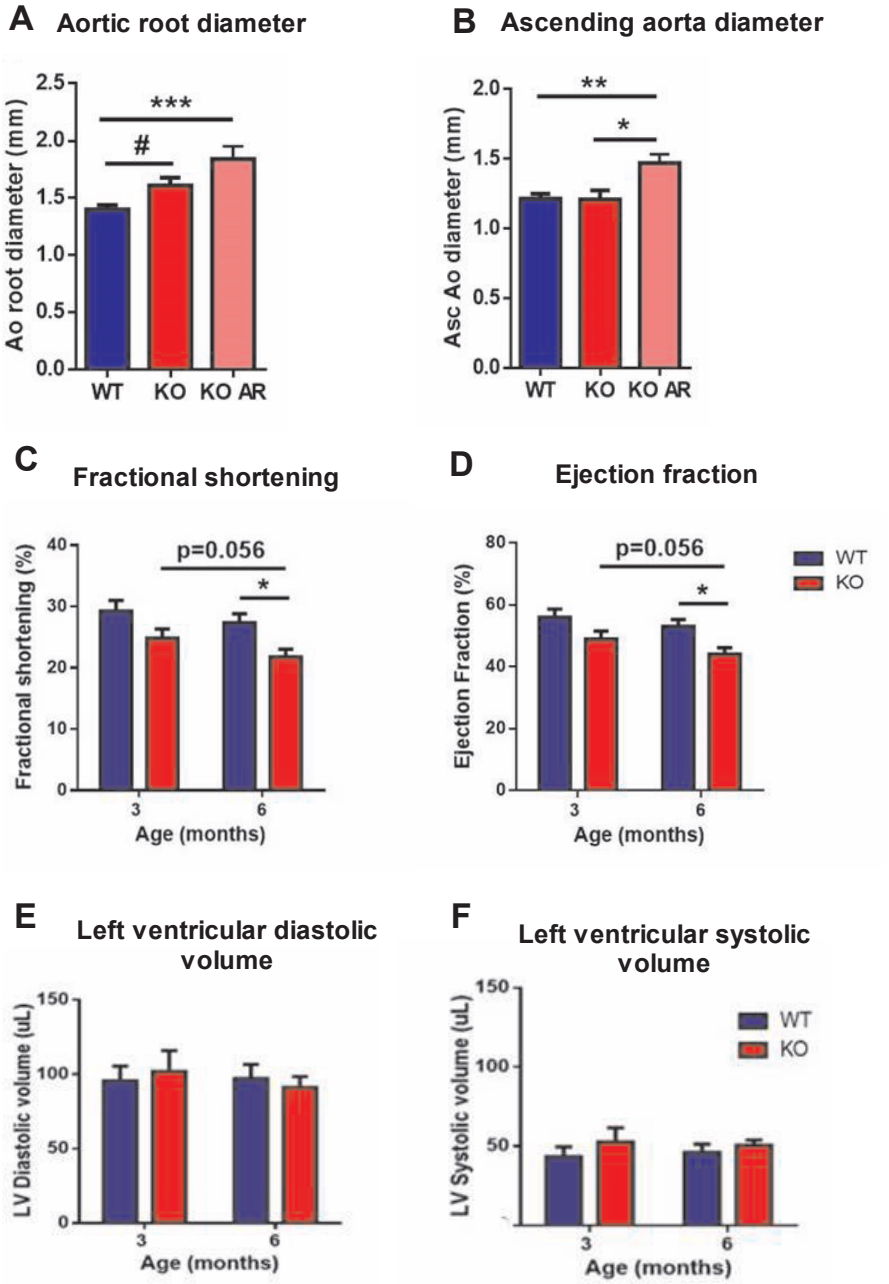

Figure 2. Aortic diameters and changes in cardiac function following a 3-month period. Diameters of the aortic root (A) and ascending aorta (B) measured using images obtained from echocardiography scans at 6 months in WT and *Zmpste24* SMC-KO mice with or without aortic regurgitation (AR). n= 8-13 mice per group. *P<0.05; **P<0.01; ***P≤0.0001 (1-way ANOVA); #P<0.05 (*t*-test). Fractional shortening (C), ejection fraction (D), left ventricular diastolic (E) and systolic volume (F). n=8-9 mice per group. * P<0.05 (2-way ANOVA).

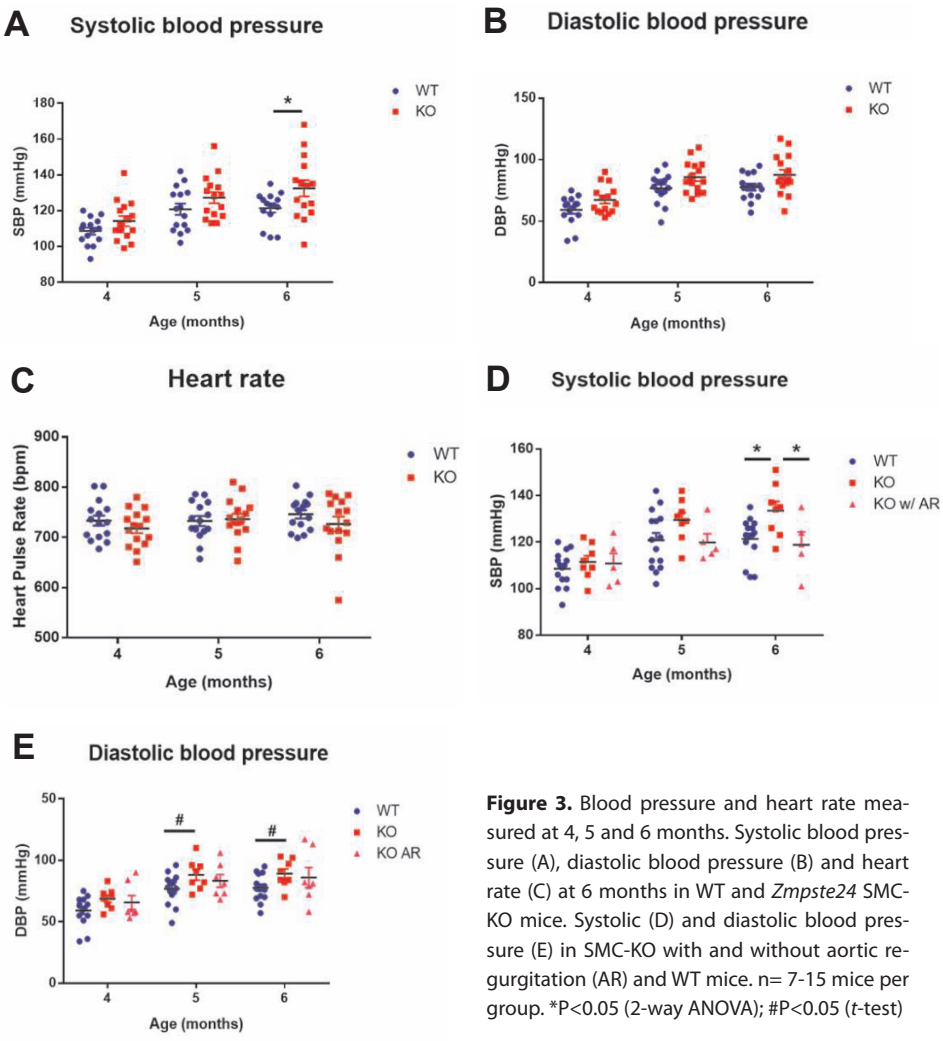

Figure 3. Blood pressure and heart rate measured at 4, 5 and 6 months. Systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) at 6 months in WT and *Zmpste24* SMC-KO mice. Systolic (D) and diastolic blood pressure (E) in SMC-KO with and without aortic regurgitation (AR) and WT mice. n= 7-15 mice per group. *P<0.05 (2-way ANOVA); #P<0.05 (*t*-test)

(i.e., blocked by indomethacin), while there appeared to be no role for EDH (Figure 4C). Compared to WT, the NO contribution to the ACh response in SMC-KO was diminished, while the contributions of prostaglandins and EDH were upregulated (Figure 4D). Thus, non-NO-mediated responses compensate for the diminished role of NO in the ACh-mediated relaxation of the aorta of SMC-KO.

To investigate whether increased PDE1 or 5 activity underlies the diminished response to SNP, the SNP effect was studied in the presence of the PDE inhibitor sildenafil and L-NAME. This combination abolished the difference between WT and SMC-KO (Figure 4E), confirming that enhanced NO degradation by PDE is indeed responsible for the decreased SNP response in SMC-KO.

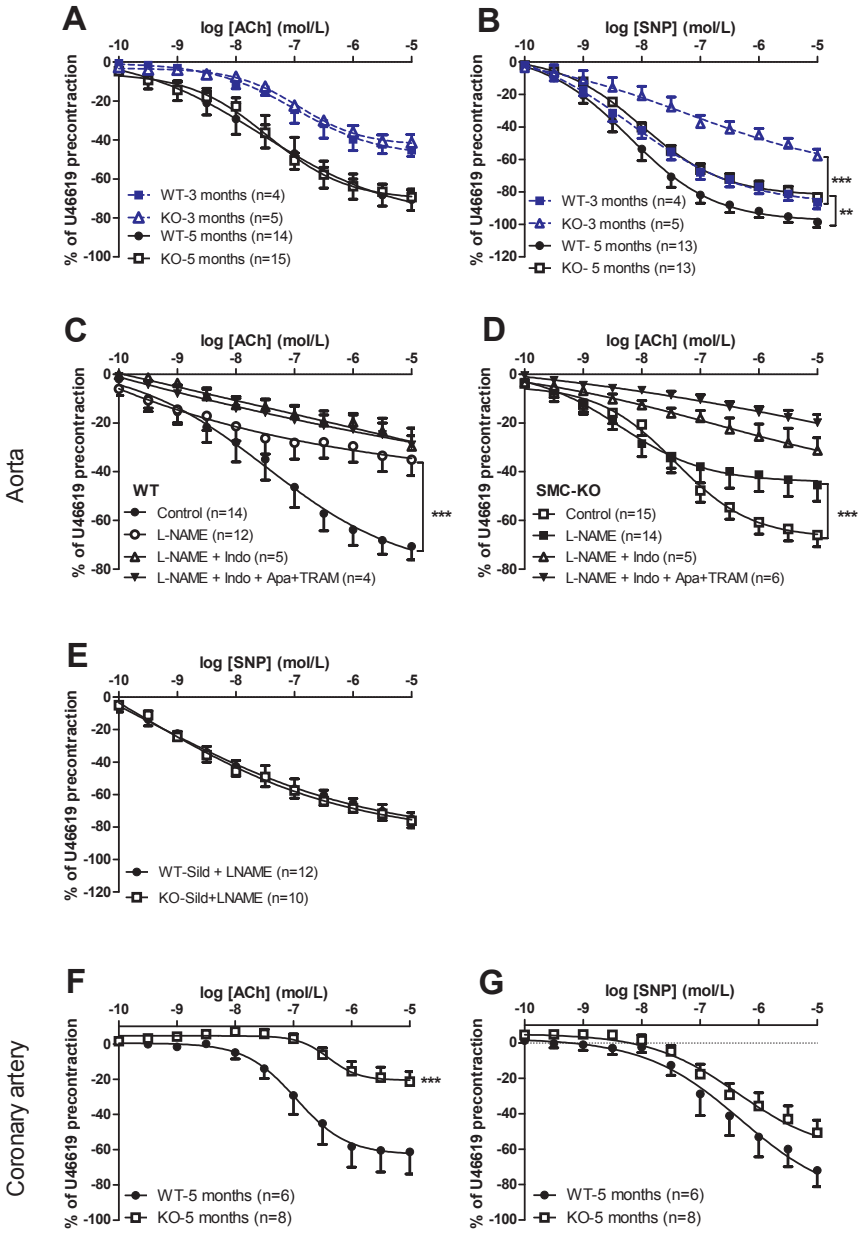

Figure 4. Vasodilation in aorta and coronary arteries. Endothelium-dependent relaxations to acetylcholine (ACh) in aortic rings at 3 and 5 months (A) in WT and *Zmpste24* SMC-KO mice. Endothelium-independent relaxations to sodium nitroprusside (SNP) in aorta at 3 and 5 months (B). Contribution of NO, prostaglandins and endothelium-dependent hyperpolarization to ACh-induced vasodilation in WT (C) and SMC-KO mice (D) at 5 months. SNP-induced vasodilation in the presence of L-NAME and sildenafil in WT and SMC-KO mice at 5 months (E). Relaxations to ACh (F) and SNP (G) in coronary artery at 5 months. **P<0.001; ***P<0.0001 (GLM-RM).

Given the compromised cardiac function in SMC-KO mice, we also evaluated coronary vasodilatory function in 5 months-old mice. In this preparation, both the endothelium-dependent relaxation to ACh ($P<0.0001$) and the response to SNP ($P=0.06$) were diminished in SMC-KO mice vs. WT mice (Figures 4F and 4G).

Morphological and mechanical properties of the vascular wall

Increased vascular stiffness can contribute to increased BP. Hence, at 5 months we evaluated the mechanical properties of carotid arteries (Figure 5). SMC-KO mice exhibited less increases in lumen diameter with stepwise increments in intraluminal pressure compared to WT mice (Figure 5A), while no differences were observed in wall thickness (Figure 5B). Strain (calculated as pressure-induced increases in lumen diameter and indicating compliance) tended to be lower, although not statistically significant, in SMC-KO mice (Figure 5C). Stress, which indicates distending force on the vessel wall, was significantly reduced in SMC-KO mice (Figure 5D).

Changes in stiffness of aorta and arteries in aging mice and humans are associated with increased wall thickness and decreased cellularity [31]. Wall thickness did not appear to change in carotids. Histological examination revealed increased wall thickness in the aorta of SMC-KO mice as well (Figure 5E), combined with decreased cellularity based on nuclei counting (Figure 5F).

Constrictor responses

Decreased cellularity and increased wall stiffness are associated with decreased KCl-induced vasoconstriction in aged mice and humans.[31,32] Such changes might affect BP regulation, and we therefore measured responses to various vasoconstrictors in iliac and coronary arteries. The former are known to display an increased response to Ang II in *Ercc1*$^{\Delta/-}$ mice, most likely due to the loss of loss of AT_2 receptor-mediated counterregulation of AT_1 receptor signaling.[33]

KCl was given to induce vasoconstriction independently of receptors. At 5 months, but not at 3 months of age, SMC-KO mice displayed lower iliac responses to KCl than WT mice (Figure 6A). To explore if calcium responsiveness could explain the differences in KCl response, CRCs to the calcium ionophore ionomycin were constructed in iliac arteries of 3 and 5 months- old mice (Figure 6B). Indeed, iliac arteries of 3 months-old WT and SMC-KO mice showed no difference in their response to ionomycin, whereas at 5 months ionomycin induced smaller constrictions in SMC-KO iliac arteries. RhoA kinase is known to be activated in the aging artery and contributes to vasoconstriction.[34] Blockade of the effect of ionomycin by the RhoA kinase inhibitor Y27632 was significant in iliac arteries of SMC-KO mice only (Figures 6C and 6D). Coronary constrictor responses to both KCl and calcium were also diminished in 5 months-old SMC-KO mice (Figures 6E and 6F), and the same was true for the KCl-induced constriction of the aorta (data not shown).

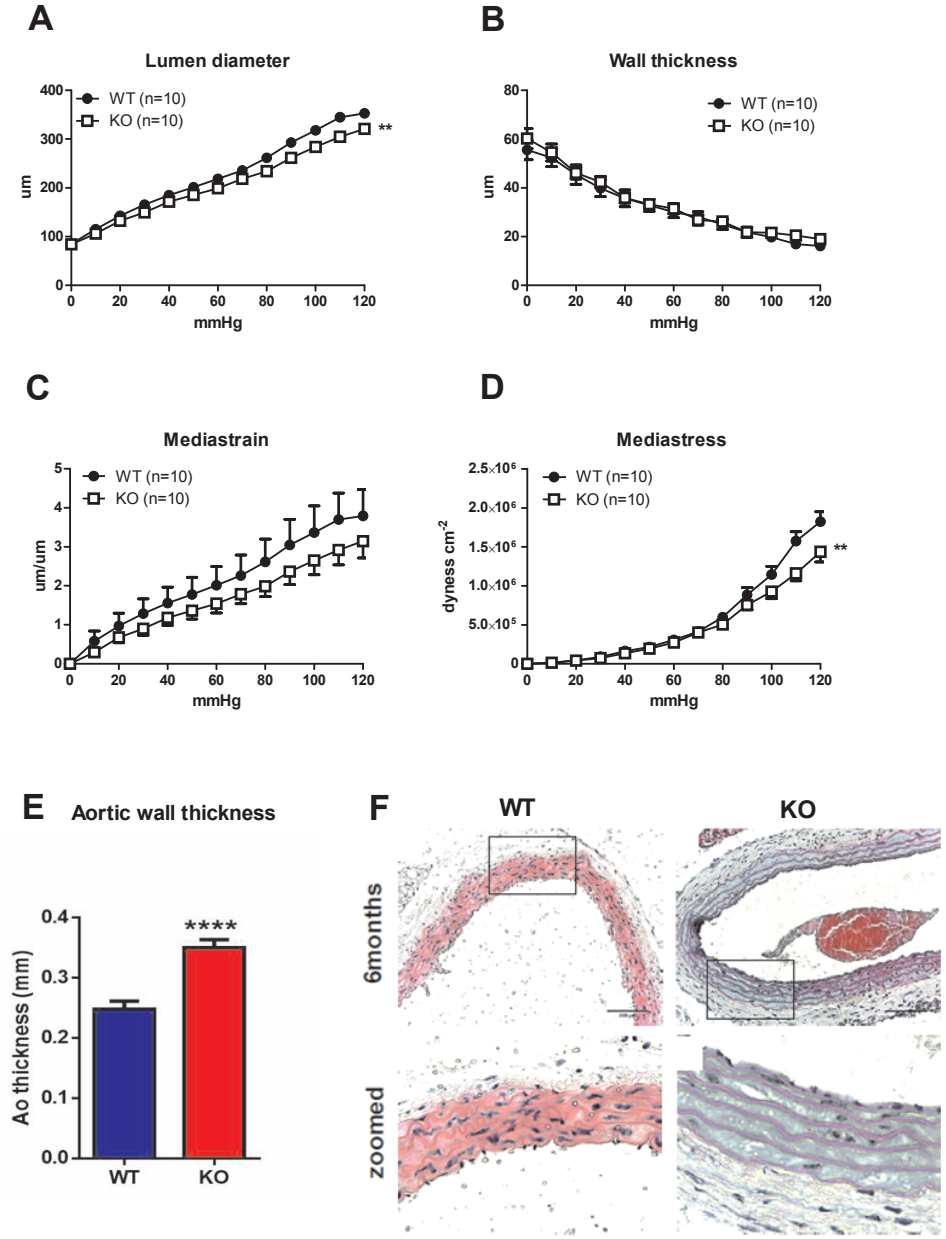

Figure 5. Mechanical properties of the carotid artery measured in a vascular perfusion set-up. Lumen diameter (A), wall thickness (B), strain (C) and stress (D) at increasing intraluminal pressures in WT and *Zmpste24* SMC-KO mice. **P<0.001 (GLM-RM). Aortic wall thickness (E) and representative images showing ascending aorta sections of WT and SMC-KO mice at 6 months stained with HE (F). ***P≤0.0001 (*t*-test).

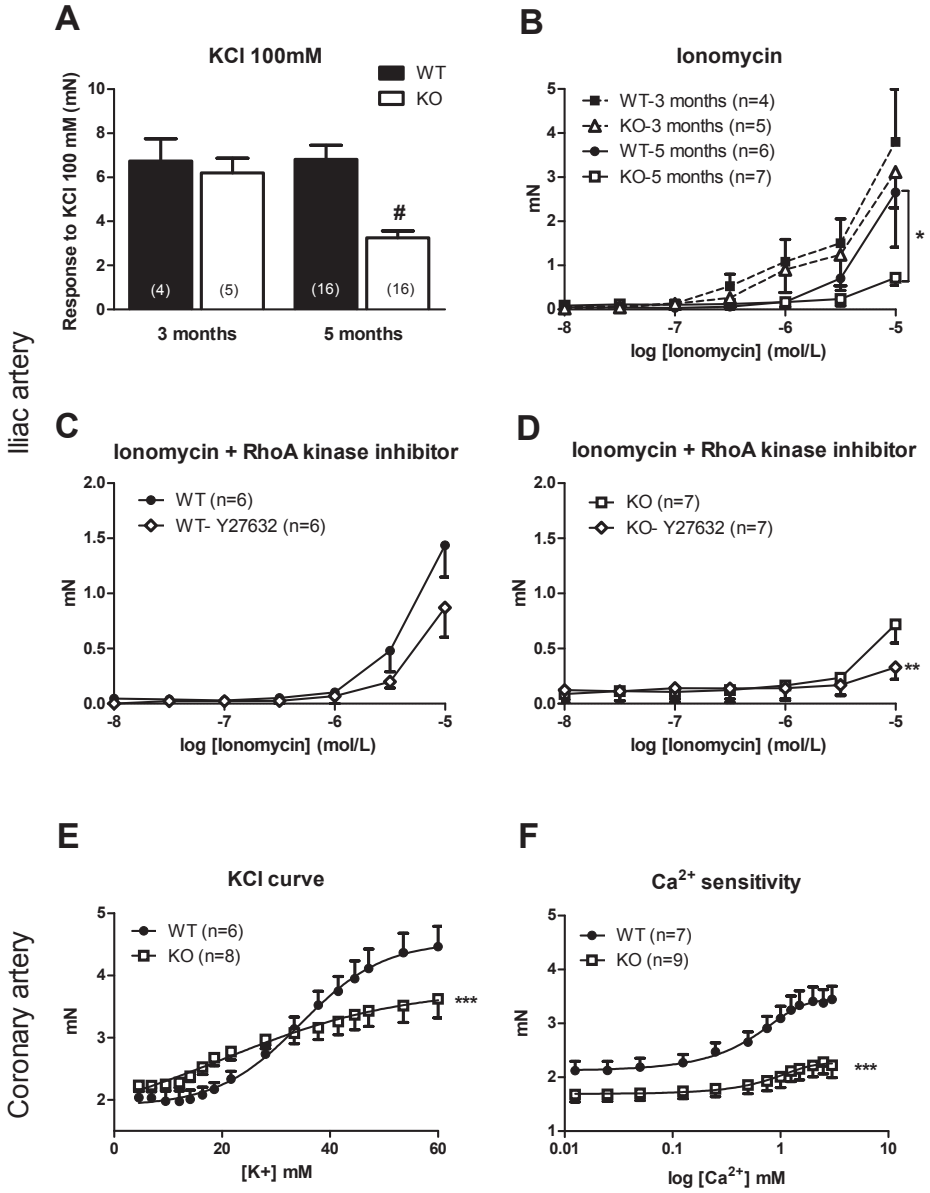

Figure 6. Receptor-independent constrictor responses in iliac and coronary arteries. Contractile responses to 100 mM KCl (A) and to the calcium ionophore ionomycin in iliac arteries of WT and *Zmpste24* SMC-KO mice at 3 and 5 months (B). Contribution of the RhoA kinase to the ionomycin response at 5 months in WT (C) and SMC-KO mice (D). Contractile responses to increasing concentrations of KCl (E) and to increasing concentrations of calcium in coronary arteries at 5 months (F). #P<0.05 (*t* test); *P<0.05, **P<0.001; ***P<0.0001 (GLM-RM).

Ang II responses were studied in iliac arteries in the presence or absence of the receptor antagonists PD123319 (AT_2 receptor) and irbesartan (AT_1 receptor). Given the general decrease of contractility in SMC-KO mice, the Ang II-induced responses of iliac arteries of WT and SMC-KO mice at the age of 5 months (E_{max} 1.2±0.40 mN vs. 0.35±0.09 mN, P<0.05) were corrected for the difference in response to 100 mM KCl. After this correction, Ang II responses were identical in both strains. Irbesartan almost totally blocked these responses (Figure 7A), while PD123319 tended to increase the Ang II response in WT mice, but not in SMC-KO mice (Figure 7B). To explore if this change in receptor function was specific for Ang II, we also investigated responses to ET-1. ET_A and ET_B receptors, similarly to AT_1 and AT_2 receptors, show opposite functions, and this difference is lost in hypertensive conditions.[35] At 5 months of age, the ET-1 responses were stronger in WT than in SMC-KO mice (E_{max} 7.3±1.3 mN vs. 2.6±0.7 mN, P<0.05), but correction for KCl responses abolished this difference (Figure 7C). Antagonism of the ET_A and ET_B receptors with BQ123 and BQ788, respectively, led to similar responses in SMC-KO and WT mice. ET_A receptor antagonism decreased the constrictor responses to ET-1 whereas ET_B receptor antagonism did not affect the responses to ET-1 (Figures 7D and 7E).

Genetic association studies

We found 6 SNPs in the *LMNA* gene to be significantly associated with CFPWV (Table 1). Four SNPs were associated with increased CFPWV and 2 with decreased CFPWV. SNP rs2485668 showed the lowest P value (β= 0.074, $P=7.1x10^{-4}$). No significant associations were found with SBP or DBP.

Table 1. Single nucleotide polymorphism in the LMNA gene associated with pulse wave velocity

SNP ID	chromosome		Allele		Beta	Standard error	p-value
	nr	Position	Effect allele	Frequency			
rs2485668	1	154368173	C	0.080015	0.075	0.022	0.000707
rs582690	1	154354880	C	0.919953	-0.075	0.022	0.000722
rs509551	1	154366673	C	0.920109	-0.077	0.022	0.000798
rs584025	1	154355150	C	0.921043	-0.074	0.022	0.000825
rs9427236	1	154360030	C	0.920747	-0.072	0.022	0.001034
rs2485676	1	154361496	G	0.079207	0.071	0.022	0.001401

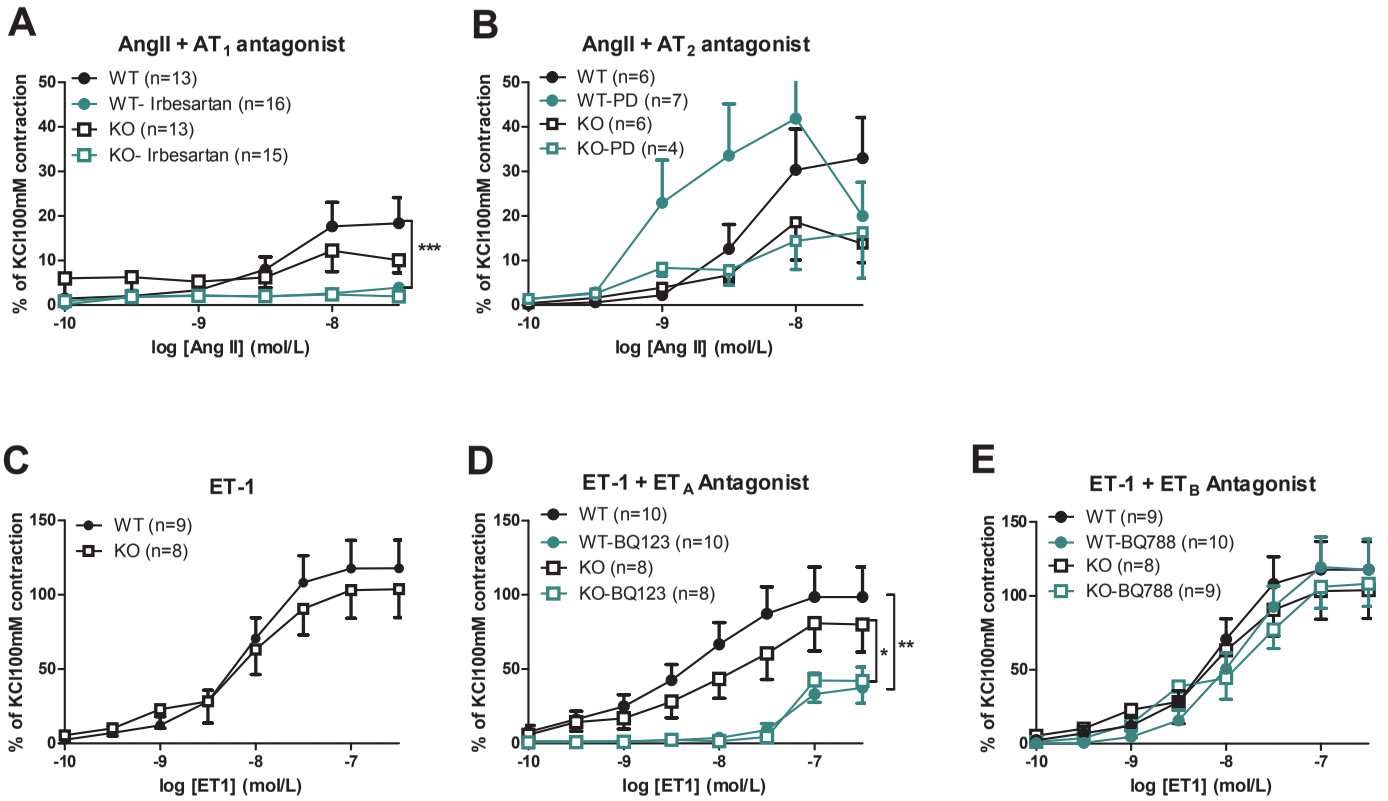

Figure 7. Receptor-dependent constrictor responses in iliac artery. Responses to Ang II in WT and *Zmpste24* SMC-KO mice at 5 months in the presence and absence of the AT_1 receptor antagonist irbesartan (A) or the AT_2 receptor antagonist PD123319 (B). Responses to ET-1 at 5 months (C). Responses to ET-1 at 5 months in the presence and absence of the ET_A receptor antagonist BQ123 (D) and in the presence and absence of the ET_B receptor antagonist BQ788 (E). *P<0.05, **P<0.001; ***P<0.0001 (GLM-RM).

DISCUSSION

In this study we explored whether genomic instability, caused by the presence of prelamin A, an accepted marker of vascular aging, would cause vasomotor disturbances and possible cardiac problems. Indeed, cardiac function was modestly altered, as characterized by a ≈16% decrease of FS and EF in SMC-KO mice vs. WT mice. SBP additionally increased by 11 mm Hg. A decreased NO-mediated VSMC relaxation and increased stiffness could underlie the increased SBP. The dilation of the ascending aorta, and the concomitant AR, might also affect BP. In fact, type A aortic dissection, for which the dilation in mice might model, can be accompanied by hypotension. Normally this is an ominous event possibly pointing at tamponade of the cardiac muscle or imminent rupture of the ascending aorta. The reduced EF and the normalized BP in AR vs. non-AR mice might be indicative for such a situation, but this could not be established in the current study. At least, there were no signs of dilated cardiomyopathy that could explain the cardiac function changes, or indicate that chronic hypertension has caused aortic dilatation and AR. Therefore the remodeling of the aortic root most likely arises from local changes induced by DNA damage. DNA damage was indeed observed in the aorta of SMC-KO. We cannot exclude that the SMC-KO mice are either compensating for the loss of NO-mediated vasodilation by adaptation of cardiac function, or that changes in the digestive tract contribute to BP changes. Nevertheless, it is clear that local inhibition of DNA maintenance in SMC cause VSMC-specific decrease of NO-mediated vasodilation, which can be compensated by the endothelium by means of recruitment of prostaglandin and EDH. The endothelial compensation of NO loss is a well-known phenomenon, for example in models of heart failure, ischemia-reperfusion and hypercholesterolemia.[36] Furthermore, constriction responses to KCl and Ca^{2+} were diminished, as occurs also in humans.[32] This might be the result of increased stiffness and the loss of VSMC, an observation also made in aged WT mice.[31] Thus, DNA damage is causing vasomotor and aortic morphological changes disturbance that are also observed in aging-related situations with clinical relevance.

The findings in SMC-KO mice contrast with those in mice with EC-KO of Chapter 3. EC-KO mice show a specific decrease of endothelium-dependent NO-mediated relaxation, whereas SMC-KO mice display specific decrease of endothelium-independent NO-mediated relaxation. EC-KO mice do not compensate for loss of ACh-induced dilations, whereas SMC-KO do, namely by increasing prostaglandin-induced dilatations and recruiting EDH, similar to observations in eNOS knockout mice.[37-39] Apparently, local DNA damage in ECs undermines the compensatory function of the endothelium, while reduced NO responsiveness caused by local DNA damage in VSMCs can be compensated by the endothelium. This novel finding, with potential relevance for vascular disease, agrees with the observation in Chapter 5 that diet restriction allows *Ercc1*$^{\Delta/-}$ mice to recruit dilator prostaglandins, and that dietary restriction conserves genomic integrity in various tissues.[40]

Another striking finding is that EC-KO mice do not show increased carotid stiffness whilst SMC-KO mice do. VSMC dysfunction may more rapidly result in increased stiffness than endothelial dysfunction, because VSMC produce matrix and are expected to directly contribute to loss of compliance, as evidenced in isolated arteries under calcium-free conditions in both studies. In EC-KO mice, in-vivo aorta distensibility was decreased, perhaps heralding the initiation of vascular stiffness increase. However, since EC can actively contribute to changes in compliance through the release of relaxing and contracting factors, a more complete assessment of vascular stiffness is needed. This should include examination of matrix changes, elastic properties, *in vivo* autonomic regulation, and local regulation of hemodynamic function. This is particularly important because of the observation that stress is lower in SMC-KO mice compared to WT. There is a chance that the decrease in compliance is due to the relatively lower forces per area surface applied in the SMC-KO carotid arteries. The difference in stress is surprising, and might be explained by differential changes in radial vs. longitudinal elastic properties. To answer this question dedicated equipment for measurement of bidirectional elasticity is needed. Regardless of the present limitations the observations in SMC-KO mice are in agreement with observations in *Ercc1*$^{\Delta/-}$ mice and suggest that DNA damage in VSMC contributes to the increase of vascular stiffness that is seen in aging. This is further supported by the findings from the genetic association look-up where polymorphisms in the *LMNA* gene were associated with PWV in a large population sample.

Apart from changes in vasodilation, also vasoconstriction was hampered in SMC-KO mice. This is due to lowered Ca^{2+}-induced constriction, occurring independently from voltage-gated calcium channels. This might simply be a consequence of the increased vascular stiffness and loss of medial cellularity, as proposed recently.[31] Alternatively, Ca^{2+} sensitivity might be decreased. This possibility is supported by the observation that both in EC-KO and SMC-KO mice KCl responses are decreased, while medial acellularity and increased stiffness cannot have played a role in EC-KO mice. The loss of KCl responses was also observed in aging human coronary arteries,[32] illustrating yet another common feature of human and mouse vascular aging.[31, 41] In addition, RhoA kinase contributed exclusively to Ca^{2+}-induced vasoconstriction in SMC-KO mice; an increase of RhoA kinase in aging VSMC has been reported previously,[34] supporting the concept that DNA damage closely resembles aging.[42] However, it should be noted that responses in WT mice were more variable, therefore our current results are only suggestive and the upregulation of RhoA kinase in mouse models of accelerated vascular aging should be further studied.

Typically, receptor-mediated constrictions, when corrected for this loss of Ca^{2+} responsiveness, were not found to be altered in SMC-KO, both in the case of Ang II and ET-1. Dilator AT_2 receptor responsiveness appeared to be lost, as has been seen before in disease conditions.[33, 43] Yet, this did not lead to enhanced AT_1 receptor-induced constric-

tion, possibly because the AT_1 receptor-induced effects were modest anyway due to the upregulated non-NO endothelial compensatory pathways in this model. Markers of DNA damage might help to determine which aged patients, if any, benefit from renin-angiotensin system blockade.

A remaining issue in DNA repair defective mice is the lack of hypertrophy of the arterial and aortic lamina media. This is often observed in human aging. The short longevity in combination with the medial cell loss might prevent hypertrophy from occurring in the mouse models. To explore this possibility the application of hypertrophic stimuli, such as dyslipidemia, strongly increased arterial pressure, endothelial removal at young age, luminal wire injury, peri-arterial cuff placement or chronic Ang II infusion in EC- and SMC-KO mice might provide better insight.

In summary, SMC-KO mice recapitulate aspects of aging-related human vascular problems such as increased SBP, decreased vasodilation to NO, dilation of the ascending aorta with concomitant aortic regurgitation, a potential increase in arterial stiffness, and, although with reservation, a loss of AT_2 receptor function. These characteristics are complementary to the earlier observed aging-like changes in EC-KO mice. The sum of changes in EC-KO and SMC-KO mice well resembles the vascular phenotype in *Ercc1*$^{\Delta/-}$ mice, albeit that SMC-KO mice uniquely shows aortic dilation. Thus both DNA damage in EC and VSMC, in a cell-autonomous way, lead to specific changes that are relevant for aging-related vascular disease. The present results refer to several clinical phenomena that are encountered in aging humans, such as type A aortic dissection, loss of coronary artery function and ejection fraction, hypertension and altered responsiveness to Ang II blocking drugs, Ca^{2+} channel blockers and associated medicines. Thus, the SMC-KO model, in complement to EC-KO and *Ercc1*$^{\Delta/-}$ mice, can help to pinpoint the etiology of specific aging-related cardiovascular problems. The application of stress factors, e.g. those mimicking classical risk factors that contribute to DNA damage, may provide further clinical relevant results. With the creation of cell-type specific DNA repair defective mice this has now become technically feasible.

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Chapter 5

Dietary restriction but not angiotensin II type 1 receptor blockade improves DNA damage-related vasodilator dysfunction in rapidly aging *Ercc1*$^{\Delta/-}$ mice

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ABSTRACT

DNA damage is an important contributor to endothelial dysfunction and age-related vascular disease. Recently, we demonstrated in a DNA repair-deficient, prematurely aging mouse model (*Ercc1*$^{\Delta/-}$ mice) that dietary restriction (DR) strongly increases life- and health span, including ameliorating endothelial dysfunction, by preserving genomic integrity. In this mouse mutant displaying prominent accelerated, age-dependent endothelial dysfunction we investigated the signaling pathways involved in improved endothelium-mediated vasodilation by DR, and explore the potential role of the renin-angiotensin system. *Ercc1*$^{\Delta/-}$ mice showed increased blood pressure and decreased aortic relaxations to acetylcholine in organ bath experiments. Nitric oxide (NO) signaling and phospho-Ser^{1177}-eNOS were compromised in *Ercc1*$^{\Delta/-}$ DR improved relaxations by increasing prostaglandin-mediated responses. Increase of cyclo-oxygenase 2 and decrease of phosphodiesterase 4B were identified as potential mechanisms. DR also prevented loss of NO signaling in vascular smooth muscle cells and normalized angiotensin II vasoconstrictions, which were increased in *Ercc1*$^{\Delta/-}$ mice. *Ercc1*$^{\Delta/-}$ mutants showed a loss of Angiotensin II type 2 receptor-mediated counterregulation of Angiotensin II type 1 receptor-induced vasoconstrictions. Chronic losartan treatment effectively decreased blood pressure, but did not improve endothelium-dependent relaxations. This result might relate to the aging-associated loss of treatment efficacy of renin-angiotensin system blockade with respect to endothelial function improvement. In summary, dietary restriction effectively prevents endothelium-dependent vasodilator dysfunction by augmenting prostaglandin-mediated responses, whereas chronic Angiotensin type 1 receptor blockade is ineffective.

INTRODUCTION

Age is a major risk factor for the development of cardiovascular diseases (CVD), independently from traditional risk factors.[1] An important factor that contributes to organismal aging, including vascular aging, is genomic instability.[2, 3] We recently demonstrated that mutation of the DNA repair endonuclease excision repair cross complementing 1 in mice (*Ercc1*$^{\Delta/-}$ mice) accelerates important characteristics of vascular aging-related vasomotor dysfunction.[4, 5] In general, *Ercc1*$^{\Delta/-}$ mice rapidly and faithfully mimic natural human aging compared to aged wild-type (WT) mice.[6] Accordingly, mouse models of accelerated vascular aging due to genomic instability can be used as tools complementary to models representing the impact of classical risk factors, such as hypertension and dyslipidemia.

We demonstrated that dietary restriction (DR, 30% reduced food intake without malnutrition), a universal intervention extending lifespan in numerous species, tripled remaining lifespan and strongly improved health span in *Ercc1*$^{\Delta/-}$ animals, by far exceeding the relative lifespan extension in WT mice. We found that this dramatic anti-accelerated aging effect in the mutant was at least in part due to preserving genomic integrity by reducing DNA damage accumulation.[2] The improvement of health span included prevention of endothelial dysfunction, which in humans is one of the major contributors to morbidity and mortality due to a decline in vascular function.[1] In humans, DR has a beneficial effect on cardiovascular risk, which is attributed to the reduction in diet-related risk factors such as dyslipidemia, high blood pressure (salt intake), and hyperglycemia.[7] This in turn reduces oxidative stress and augments the nitric oxide (NO) – cGMP pathway, an important endothelial signaling axis involved in blood flow, blood pressure and cardiovascular growth regulation.[7] Our results in *Ercc1*$^{\Delta/-}$ mice have added a novel paradigm, namely that DR preserves genomic integrity and thus in this manner protects against vascular aging.

In this new paradigm it is not known which vasodilatory signaling pathway is improved. In our previous studies we have shown that, comparable to human aging, *Ercc1*$^{\Delta/-}$ mice display a reduction of NO– cGMP signaling and increased oxidative stress.[4, 5] Therefore, we here set out to identify which vasodilatory signaling pathway is improved by DR in *Ercc1*$^{\Delta/-}$ mice. In addition, the impact of DR on endothelium-independent relaxation was investigated.

A potential mediator of blood pressure increase and decreased endothelium-dependent relaxation caused by DNA damage is activation of the renin-angiotensin system (RAS). Angiotensin (Ang) II, the main bioactive hormone of this system, is strongly involved in hypertension, arteriosclerosis, vascular DNA damage and cell senescence, inflammation, oxidative stress, longevity and health span.[8] Also, Ang II inhibits eNOS - NO - cGMP signaling.[9] Given that the RAS is sensitive to salt and LDL cholesterol, it may also respond to DR.[10, 11] However, it is not known how genomic instability influences

RAS activity, let alone whether RAS activation would mediate its detrimental effects on the vascular wall. Therefore, we additionally studied the vasoconstrictor responses of the *Ercc1*$^{\Delta/-}$ mouse vasculature to Ang II under *ad libitum* (AL) feeding and DR. Also, we evaluated the effect of chronic AT_1 receptor blockade on endothelial function and blood pressure in AL-fed *Ercc1*$^{\Delta/-}$ mice.

METHODS

Animals and interventions

Animal experiments were performed at RIVM and Erasmus MC in accordance with the Principles of Laboratory Animal Care and with the guidelines approved by the Dutch Ethical Committee in full accordance with European legislation.

Dietary restriction studies

Ercc1$^{\Delta/-}$ mice and their wild-type littermates (WT) (Bl6/FVB F1 hybrids) underwent DR intervention from resp. 7 and 11 weeks after birth until sacrifice as described extensively in our previous publication, and in the Methods supplement.[2] At the age of 16 weeks, AL-fed *Ercc1*$^{\Delta/-}$ weigh on average ~13 grams, whereas DR-fed *Ercc1*$^{\Delta/-}$ animals weigh ~10 grams.[2]

Losartan intervention study

From 5 weeks of age, *Ercc1*$^{\Delta/-}$ and WT mice (Bl6/FVB F1 hybrids) were divided into two groups per strain, which were either treated with losartan (100 mg/kg/day) in drinking water, or drinking water only until the age of 12 weeks when the animals were sacrificed. Blood pressure was measured by tail cuff at the age of 11 weeks. At the studied age, *Ercc1*$^{\Delta/-}$ mice weigh ~15 grams on average.[2] The study rationale and animal numbers are described in the Methods supplement.

Organ bath experiments

Tissue harvesting and preparation procedures, and detailed description of the organ bath experiments can be found in the Methods supplement.

In short, thoracic aorta and iliac arteries were collected and tested in small wire organ bath setups. Vasodilations to cumulative concentrations of acetylcholine (ACh) and sodium nitroprusside (SNP) were measured in vessels preconstricted with U46619 to construct concentration-response curves (CRCs). When sufficient aortic tissue was available, the involvement of nitric oxide (NO) and prostaglandins in ACh responses was investigated by performing the experiments in the presence of the endothelial nitric oxide synthase (eNOS) inhibitor N^G-Methyl-L-Arginine acetate salt (L-NMMA, 10^{-5} mol/L),

the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10^{-5} mol/L) or both inhibitors. In iliac arteries Ang II (10^{-10}-10^{-7} mol/L) CRCs were constructed. PD123319 (10^{-7} mol/L) was used to test the involvement of Ang II type 2 (AT_2) receptors, and the guanylyl cyclase inhibitor 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10^{-5} mol/L) to test the role of NO-cGMP signaling. Inhibitors were added 15 minutes prior to U46619 or Ang II.

Quantitative real-time PCR

Total RNA was isolated and cDNA was prepared, which was amplified by real-time PCR to perform $\Delta\Delta C_T$ quantification, either with the use of SYBR green or Taqman analysis. Further details are found in the Supplementary information.

Western blots

Protein was isolated from lung tissue, run on bis/tris gradient gels, blotted to nitrocellulose membranes and then incubated with primary antibodies. Details are provided in the Supplementary information.

Plasma renin concentration

Blood was collected from 12-wk-old WT and *Ercc1*$^{\Delta/-}$ mice by cardiac puncture and transferred to EDTA coagulation vials. Blood samples were centrifuged at 4600 rpm for 10 minutes to collect plasma. Plasma renin concentration was determined by an enzyme-kinetic assay as described previously.[12]

Statistical methods

Data are presented as mean±SEM. SNP-corrected ACh responses were calculated as follows: (response to ACh as % of U46619 preconstriction / response to 10^{-4} mol/L SNP as % of U46619) x 100 (to indicate as a percentage) x -1 (to indicate that it is a relaxation). Statistical testing for differences between single values expressed in bar graphs was performed by t-test or 1-way ANOVA followed by appropriate post-hoc tests. Differences in CRC were tested by general linear model for repeated measures (GLM-RM, sphericity assumed). Differences were considered significant at $p<0.05$.

RESULTS

The effect of DR on acetylcholine responses in WT and *Ercc1*$^{\Delta/-}$ mice

We first investigated the effect of genomic instability and DR on the diminished ACh response at different ages in *Ercc1*$^{\Delta/-}$ and WT mice. As previously reported, AL-fed *Ercc1*$^{\Delta/-}$ mutants showed a lifespan of 19 weeks (median age), which was extended by DR to a median age of 44 weeks 2. ACh responses in the *Ercc1*$^{\Delta/-}$ aorta of AL-fed animals age-

dependently decreased between the age of 7 to 16 weeks (Figure 1A), and at the latter age were significantly decreased compared to 20-wk old WT. WT aortas did not show any change in ACh response between 11 to 20 weeks (data not shown). To explore if DR would protect against endothelial dysfunction until an age at which AL-fed *Ercc1*$^{\Delta/-}$ mice have already succumbed (predominantly occurring from neurodegeneration), we proceeded to an age of 30 weeks in DR-fed animals. In our initial publication on the effect of DR on general health 2 we demonstrated that DR improved the response to ACh in 16-wk-old *Ercc1*$^{\Delta/-}$ mice. Here we show that the improvement of ACh responses persisted in 30-wk-old DR-fed *Ercc1*$^{\Delta/-}$ mutants (Figure 1B), well after the AL mice had died. In WT animals DR had no effect on ACh-induced relaxation (Figure 1B). Thus, *Ercc1*$^{\Delta/-}$ mice showed decreased aortic relaxations to ACh with increasing age, which could partly be prevented by DR.

Endothelial vs. non-endothelial responses

Ercc1$^{\Delta/-}$ aortas displays a pronounced decrease of endothelium-independent responses to NO.[4, 5] We therefore investigated the effect of DR on responses to SNP, which entirely rely on direct release of NO and subsequent cGMP production in vascular smooth muscle cells (VSMC), as evidenced by the complete blockade of this response by the guanylyl cyclase inhibitor ODQ (data not shown). Dilatory responses to 10^{-4} mol/L SNP, which was given on top of ACh, progressively decreased in AL-fed *Ercc1*$^{\Delta/-}$ mice, reaching statistical significance in 16-wk-old mice as compared to 7-wk-old mice (Figure 1C, $p<0.05$ one-way ANOVA on 7-, 11- and 16-wk AL-fed mice with Dunnett post-hoc test). DR significantly prevented the age-dependent decline in dilator responses in 11- and 16-wk-old mice (Figure 1C, $p<0.05$, t-test). Even 30-wk-old DR-fed *Ercc1*$^{\Delta/-}$ mutants still displayed a better SNP response as compared to 16-wk AL-fed *Ercc1*$^{\Delta/-}$ mice ($p<0.05$, t-test). In WT animals no age- or diet-related changes were observed (Figure 1D, 11-wk animals not shown), and SNP responses were similar to those in 7-wk AL-fed and DR-fed *Ercc1*$^{\Delta/-}$ mice, which is expected as WT mice at 20 weeks of age do not (yet) display an aging-phenotype

To exclude any influence of ACh on SNP responses and to explore dose-related effects of SNP, we generated SNP CRCs in 16-wk-old *Ercc1*$^{\Delta/-}$ and in 20-wk-old WT animals (Figure 1E). The data confirmed that in AL-fed *Ercc1*$^{\Delta/-}$ mice SNP responses were strongly reduced, and that they were fully restored to the level of WT animals by DR. In WT animals no significant changes occurred.

The response to ACh depends on the amount of relaxing factors that is released from the endothelium as well as the responsiveness of the VSMC to these factors. The present observation that responses of VSMC to NO are fully restored by DR (Figure 1C, E) while the responses to ACh are not (Figure 1B), suggests that the release of endothelial-derived relaxing factors is compromised. Therefore, we studied the contribution of these factors to vasodilation.

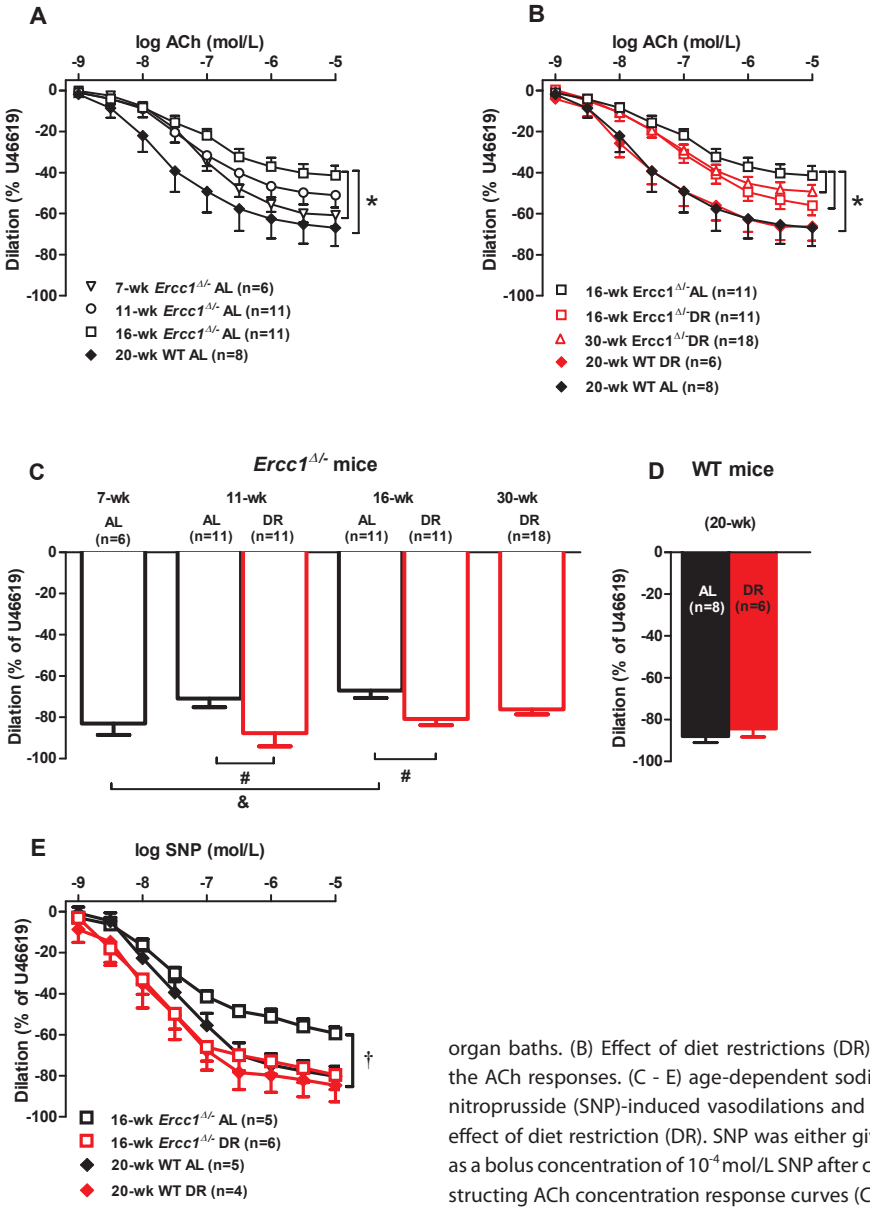


Figure 1. The effect of dietary restriction on endothelial and non-endothelial vasodilations in Ercc1$^{\Delta/-}$ and WT mice at different ages (A) Age-dependent acetylcholine (ACh)-induced vasodilation in aortic segments from *ad libitum* (AL) fed wildtype (WT) and *Ercc1*$^{\Delta/-}$ mice as measured *ex vivo* in small wire organ baths. (B) Effect of diet restrictions (DR) on the ACh responses. (C - E) age-dependent sodium nitroprusside (SNP)-induced vasodilations and the effect of diet restriction (DR). SNP was either given as a bolus concentration of 10^{-4} mol/L SNP after constructing ACh concentration response curves (C, D) or administered in cumulative concentrations immediately after preconstriction (E). Responses are expressed as % relaxation of the U46619. Error bars: S.E.M. *P<0.05, GLM-RM. &P<0.05 16-week (16-wk) vs. 7-week (7-wk) *Ercc1*$^{\Delta/-}$AL, one-way ANOVA, Dunnett post-hoc test, #P<0.05 *t*-test, †P<0.05, 16-wk *Ercc1*$^{\Delta/-}$AL compared to all other groups, GLM-RM.

The role of endothelial signaling compounds in genotype- and diet-related effects

Dilations in AL-fed vs. DR-fed WT mice did not differ and results were therefore pooled. ACh responses were almost completely dependent on NO in WT animals since adding the eNOS inhibitor L-NMMA blocked the response to ACh (Figure 2A). As expected from our previous study,[4] NO also mediated a large part of the vasodilation to ACh in AL-fed *Ercc1*$^{\Delta/-}$ mice (Figure 2B). The residual response suggests the emergence of an endothelium-derived hyperpolarizing factor (EDHF), which did not appear to be COX-dependent, since it was not affected by indomethacin (Figure 2B). This result, together with the observation that inhibition of vasodilation by L-NMMA was much more pronounced in WT confirms the specific loss of NO signaling in *Ercc1*$^{\Delta/-}$ aorta's. Remarkably, the DR-induced facilitation of the ACh response in *Ercc1*$^{\Delta/-}$ mice appeared to be due to an upregulation of a vasodilator prostaglandin pathway, since now indomethacin did further reduce the response of ACh on top of L-NMMA, while the effect of L-NMMA alone was unaltered (Figure 2C). We studied total eNOS and pSer1177-eNOS (eNOS-activating phosphorylation of the serine residue 1177) protein expression in lung tissue (Figure 3A). No significant differences in eNOS levels were found between groups, although as observed in a previous publication,[4] total eNOS protein tended to be lower in AL-fed *Ercc1*$^{\Delta/-}$ mice as compared to AL-fed WT. In contrast, DR significantly increased the pSer1177-eNOS/eNOS ratio in WT mice but not in *Ercc1*$^{\Delta/-}$ (Figure 3A).

Prostaglandins are produced by COX-1 or 2, and exert their vasodilator effects through the IP receptor using adenylyl cyclase (AC) 5/6 – cAMP signaling as a second messenger system. cAMP is prone to degradation by phosphodiesterase type 4B/D (PDE4).[13-15] To investigate which of these components could be responsible for the upregulated prostaglandin response we first quantified mRNA expression in blood vessel-rich lung tissue. Ct values for the IP receptor, PDE4D and AC6 mRNA levels were on average >34, and therefore we considered these levels too low for reliable detection. COX-1 expression was significantly increased, and COX-2 showed a tendency to increase in DR-fed *Ercc1*$^{\Delta/-}$ (supplementary Figure 1). PDE4B mRNA was decreased in both *Ercc1*$^{\Delta/-}$ mouse groups compared with AL-fed WT mice (supplementary Figure 1). Thereupon, we decided to look further into protein levels of COX-1, COX-2 and PDE4B (Figure 3B). AL-fed *Ercc1*$^{\Delta/-}$ mice had significantly higher protein levels of COX-1 compared to the other 3 groups. DR had a significant effect on *Ercc1*$^{\Delta/-}$ COX-2 levels vs. AL-fed *Ercc1*$^{\Delta/-}$, but no effect was observed in the WT groups. Sirtuins are one of the mechanisms that could upregulate COX-2 expression and activity;[16] however we did not observe any significant differences in sirtuin 1 (SIRT-1) protein levels in any of the groups (Figure 3B). PDE4B was decreased again in *Ercc1*$^{\Delta/-}$ regardless of the diet. Surprisingly, PDE4B was also decreased in DR-fed WT. The results indicate that both an increase of prostaglandin production by COX-2 in combination with a decreased metabolism by PDE4B might support the

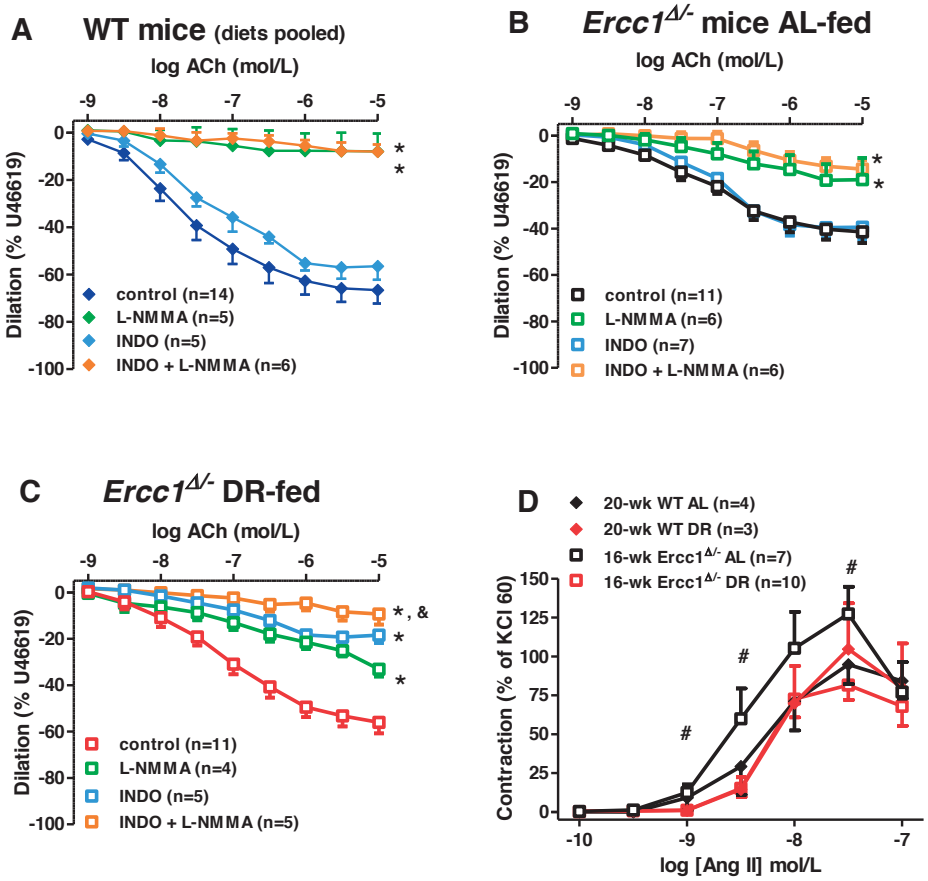


Figure 2. The effect of dietary restriction on endothelial signaling pathways and vasoconstriction responses to Angiotensin II. Contribution of nitric oxide (NO) and prostaglandins to ACh-induced vasodilation of aortic segments from 20-wk-old AL- and DR-fed wild-type (WT) (A), and 16-wk-old AL-fed (B) and DR-fed (C) *Ercc1*$^{\Delta/-}$ mice measured in organ baths. L-NMMA (10^{-5} mol/L) and INDO (10^{-5} mol/L) resp. inhibit NO and prostaglandin synthesis. Responses are expressed as % relaxation of the U46619 preconstriction. (D) Vasoconstriction to Ang II expressed as % of contraction to 100 mM KCl. Error bars: S.E.M. *P<0.05 vs. non-pretreated segments. &P<0.05 vs. L-NMMA-treated segments. #P<0.05, t-test on individual concentrations of Ang II of AL-fed *Ercc1*$^{\Delta/-}$ mice vs. WT littermates.

emergence of prostaglandin – cAMP signaling, and improved vasodilation after DR in *Ercc1*$^{\Delta/-}$ mice.

Effects of genomic instability and DR on Ang II responses

To explore a possible role of the renin-angiotensin system we first investigated vasoconstriction to Ang II in a subset of the diet intervention mice. Ang II responses were in general highly variable within each strain, and tended to be higher in AL-fed *Ercc1*$^{\Delta/-}$ vs. AL-fed WT mice (Figure 2D), although this did not reach significance over the entire CRC

(GLM-RM). DR-fed *Ercc1*$^{\Delta/-}$ animals showed a trend for a decreased response to Ang II as compared to AL-fed *Ercc1*$^{\Delta/-}$ mutants (GLM-RM, p=0.059). The results suggest a genomic instability-induced upregulation of the Ang II response, which is normalized by DR.

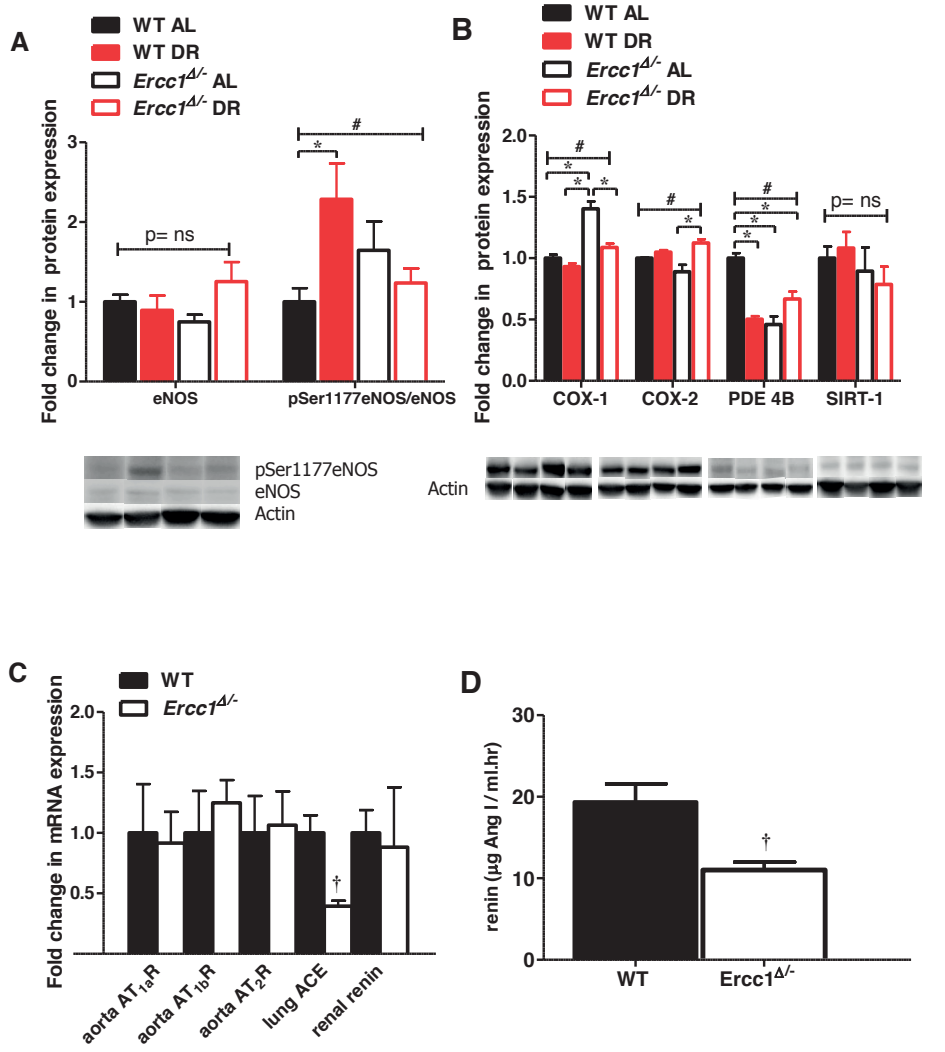


Figure 3. Expression of relevant proteins in the dietary restriction study and mRNA expression of RAS components and plasma renin levels in the Losartan intervention study. Relative protein expression levels in lung tissue of eNOS and pSer1177-eNOS/eNOS ratio (A), and of COX-1, COX-2, PDE4B and SIRT-1 (B) in 16-wk-old *Ercc1*$^{\Delta/-}$ mice and 20-wk-old wild-type (WT) littermates from the diet intervention study. All protein values are corrected for β-actin and normalized to WT-AL expression levels. Relative mRNA expression levels of AT_{1a}-, AT_{1b}- and AT_2-receptors in abdominal aortic tissue, of ACE in lung tissue and of renin in renal tissue of 12-wk-old *Ercc1*$^{\Delta/-}$ mice and WT littermates from the losartan treatment study (C). Plasma renin concentration in *Ercc1*$^{\Delta/-}$ mice and WT littermates from the losartan treatment study (D). Error bars: S.E.M. #P<0.05, one way- ANOVA; *P<0.05, Bonferroni's post-hoc tests; †P<0.05 vs. WT, *t*-test.

The losartan intervention study

In a separate cohort of *Ercc1*$^{\Delta/-}$ and WT mice we evaluated the effect of chronic AT_1 receptor blockade on blood pressure and vascular function. In agreement with our previous study[4] blood pressure tended to be slightly higher in *Ercc1*$^{\Delta/-}$ mice, mainly reflected by systolic blood pressure ((SBP), 140.6 mmHg in *Ercc1*$^{\Delta/-}$, 128.2 in WT; p-value = 0.076), and to a lesser extent by diastolic blood pressure (DBP), 104.2 mmHg in *Ercc1*$^{\Delta/-}$ mice, 97.3 mmHg in WT; p= 0.163) (Figure 4). Chronic AT_1 receptor blockade by losartan significantly lowered SBP and DBP in both mouse strains (p< 0.01 in all cases) .

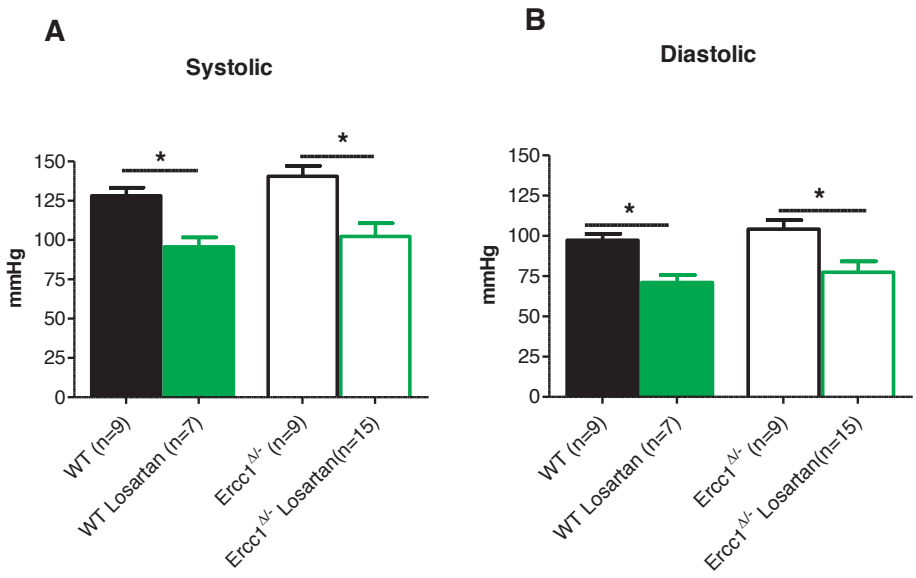


Figure 4. Blood pressure measurements in the losartan intervention study. (A) Systolic and (B) diastolic blood pressure in conscious *Ercc1*$^{\Delta/-}$ and wild-type (WT) mice of the losartan intervention study as measured by the tail cuff method. *P<0.05, *t*-test

Vasomotor responses to Ang II in the losartan intervention study

In the losartan intervention cohort, *Ercc1*$^{\Delta/-}$ mice displayed an exaggerated response to Ang II as compared to WT mice (Figure 5A). Vasoconstrictions are mediated by AT_1 receptors and we therefore explored other indicators of increased AT_1 receptor activity such as negative feedback on renin activity and ACE expression. Plasma renin activity was reduced (Figure 3D). This was not due to a change in mRNA level in the kidney (Figure 3C). ACE mRNA in the lung was reduced (Figure 3C). Both findings are in agreement with increased AT_1 receptor activity.

To further explore mechanisms leading to increased Ang II vasoconstrictions we studied vascular AT_1 and AT_2 receptor expression and function. We and others previously

reported that AT_2 receptor stimulation counteracts AT_1 receptor-mediated vasoconstriction[17]. To explore the effect of genomic instability on AT_2 receptor activity, Ang II responses in *Ercc1*$^{\Delta/-}$ and WT animals in the presence of AT_2 receptor antagonist PD123319 were compared to those in the absence of this antagonist. PD123319 did not change the Ang II response in *Ercc1*$^{\Delta/-}$ mice, but tended (p=NS) to increase this response in WT (Figure 5A). Chronic treatment with losartan, starting from week 5 after birth until the end of week 12, normalized this exaggerated response in the *Ercc1*$^{\Delta/-}$ mice (Figure 5B), but had no effect on the Ang II response in WT mice (data not shown). Therefore, genomic instability leads to loss of counterregulation of AT_1 receptor-mediated vasoconstriction by AT_2 receptor, and not due changes in receptor expression.

To explore the possible involvement of counterregulation of Ang II-induced constriction by NO-cGMP signaling, which can be the result of endothelial AT_2 recep-

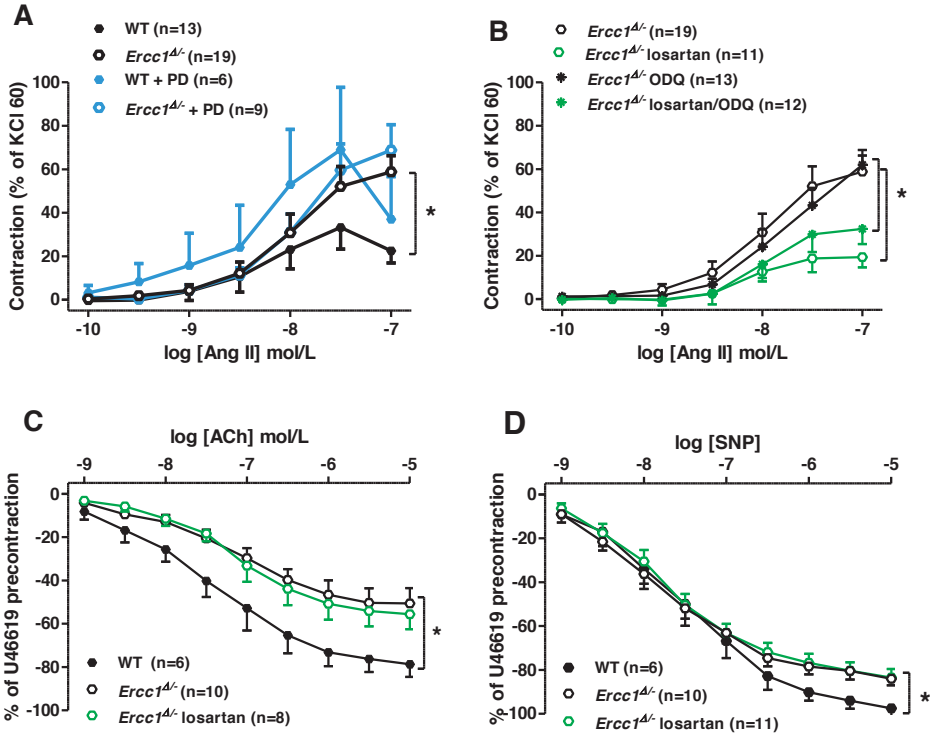


Figure 5. Ex-vivo vascular function in *Ercc1*$^{\Delta/-}$ and WT mice from the losartan intervention study. (A) Vasoconstriction to Ang II of isolated iliac arteries in the presence and absence of PD123319. (B) Ang II responses in isolated iliac arteries from untreated and losartan-treated *Ercc1*$^{\Delta/-}$ mice in the presence and absence of ODQ. (C and D) Vasodilation in isolated aortic tissue of the mice from the losartan intervention study to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator, NO-donor SNP. All responses were measured ex vivo in small wire organ baths. Aortic tissue used to measure vasodilator responses was preconstricted with thromboxane analog U46619. Error bars: S.E.M. *P<0.05, GLM–RM.

tor stimulation, Ang II responses were studied in the presence of the guanylyl cyclase inhibitor ODQ. This approach, rather than adding an eNOS inhibitor, was chosen because *Ercc1*$^{\Delta/-}$ mice show both changes in endothelial NO production as well as in cGMP responses of VSMC. Although the presence of ODQ tended to increase Ang II responses, the increase was very modest and did not reach significance in *Ercc1*$^{\Delta/-}$ animals (Figure 5B), nor in WT (not shown). Apparently, the loss of NO-cGMP signaling cannot entirely explain the increase Ang II vasoconstrictions.

Since increased AT1 receptor signaling is believed to be involved in vascular disease related to endothelial dysfunction, a role that might both provoke as wells as be mediated by increased blood pressure, we tested the effect of chronic losartan treatment on vasodilator function in *Ercc1*$^{\Delta/-}$ mice.

Effect of chronic losartan treatment on accelerated age-related vasodilator dysfunction

In the 12-wk-old *Ercc1*$^{\Delta/-}$ mutants vasodilator responses to ACh were significantly decreased as compared to WT animals (Figure 5C). The dilation response to a SNP concentration-response curve was also decreased in *Ercc1*$^{\Delta/-}$ mice (Figure 5D). Chronic AT_1 receptor blockade with losartan *in vivo* did not significantly change any of the responses. Our findings indicate that the observed vasodilator dysfunction (persistent after losartan) was not blood pressure-dependent and that the detrimental effect of genomic instability cannot be opposed by chronic AT_1 receptor blockade with Losartan.

DISCUSSION

In the present study we explored potential mechanisms that lead to DR-mediated improvement of vasodilator dysfunction caused by DNA damage in rapidly aging *Ercc1*$^{\Delta/-}$ mice. These mice are known to display a stochastic increase of transcription stalling, indicative for increased DNA damage.[2] In the liver, this could be partly reversed by DR.[2] Such measurements were not possible in aorta due to its small size, and therefore direct evidence for decreased DNA damage in vascular tissue cannot be provided at this stage. Instead we focused on the identification of the involved vasomotor signaling pathways. Loss of vasodilation turned out to be entirely due to loss of NO-cGMP signaling, both as a result of decreased endothelial NO release and decreased VSMC responsiveness to NO. In previous publications we already observed that the decreased NO function in *Ercc1*$^{\Delta/-}$ mice was due to decreased eNOS expression and activation, increased PDE1 and possibly also increased PDE5 activity, and for a small part due to increased ROS production.[4, 5] The present results now indicate that DR improves vascular dilation up to an age of at least 30 weeks. This is because DR enables aortic tissue to recruit endothelium-derived

vasodilatory prostaglandins, which are normally absent. In addition, the responsiveness of VSMC to NO is improved. A possible explanation for the emerging prostaglandin response is the increase in COX-2, combined with a decrease in PDE4B, which together should lead to improved vasodilator cAMP signaling.

We additionally explored the possible involvement of Ang II. Although defective DNA repair increased vasoconstrictive responses to Ang II, chronic blockade of AT_1 receptors with losartan did not rescue vasodilator responses. Also, blood pressure itself is not a driving mechanism in the observed vasodilator dysfunction, since blood pressure-lowering did not affect this dysfunction. Ang II-induced constriction most likely increased due to the loss of counterregulatory effects of AT_2 receptors when *Ercc1*$^{\Delta/-}$ mutants age. We found that chronic losartan treatment reversed the enhanced Ang II-induced vasoconstriction in *Ercc1*$^{\Delta/-}$ mice. This reduction could not be explained by physiological antagonism through effects on endothelial function, since the latter was unaffected by losartan. Furthermore, blood pressure lowering per se, increased renin release (resulting in AT_1 receptor downregulation by upregulating Ang II), and the sustained presence of losartan (or its active metabolite) after transferring arterial tissue to organ baths, are also unlikely causes of this reduction, since the conditions were identical in the losartan-treated WT mice, where no reduction in Ang II response occurred. Thus, chronic AT_1 receptor antagonism was capable of restoring the WT situation in a blood pressure-, circulating RAS- and endothelium-independent manner.

Preservation of endothelium-dependent responses by (lifelong) DR has been previously reported in aging WT rodents, involving nuclear factor erythroid-2-related factor-2 (Nrf2)-mediated upregulation of antioxidants.[18] Vermeij et al. showed that in various tissues of AL-fed *Ercc1*$^{\Delta/-}$ mice Nrf2-related antioxidants are already increased as a protective mechanism ([2] and unpublished observations), and that after DR, Nrf2 is further activated.[2] Previously, we showed that decreased SNP responses in *Ercc1*$^{\Delta/-}$ involved ROS.[4] In the present study SNP responses were normalized after DR (Figure 1), in agreement with a potential role of Nrf2. In addition to upregulation of anti-oxidant defenses, DR is known to increase eNOS expression and activation, the activation being mediated by eNOS deacetylation of the calmodulin-binding region through SIRT-1.[19, 20] These observations, made in WT rodents, are reminiscent of our result that DR increased baseline p-eNOS levels in WT mice. In a previous study we demonstrated that eNOS phosphorylation at Ser^{1177} was prohibited in (AL-fed) *Ercc1*$^{\Delta/-}$.[4] We now show that DNA damage prevents the DR effect on baseline p-eNOS. Apparently, in conditions of deteriorated integrity, regulation of (p-)eNOS is swiftly lost. Strikingly, SIRT-1 was not increased by DR in *Ercc1*$^{\Delta/-}$ nor in WT mice. Therefore, an effect through deacetylation of eNOS by SIRT-1 seems unlikely. It should be noted that we performed measurements of eNOS in lung tissue, based on the assumption that measurements in this highly vascularized organ are representative for vascular tissue in general.[4, 5]

In the light of failing (up-)regulation of NO signaling it is not surprising that DR in *Ercc1*$^{\Delta/-}$ mice recruits another system to enhance endothelial function, namely vasodilatory prostaglandin signaling, acting through cAMP as a second messenger. To achieve this, COX-2 expression increased in DR-fed *Ercc1*$^{\Delta/-}$ mice, on top of already decreased PDE4B expression in those mice. It needs to be noted, however, that the changes were very modest. The molecular mechanism underlying the DR-induced COX2 and PDE4B upregulation could not be addressed in the present study. One possibility is that this involved a DR-induced effect on blood pressure. However, since 16 week-old AL fed *Ercc1*$^{\Delta/-}$ (unlike the 12 week-old mice used in the losartan study) are too frail for blood pressure measurement, this could not be investigated. Altogether, the fate of COX products in the aging vasculature is certainly not uniform in diverse studies in rodents and humans.[21] Nevertheless, our results confirm the observation that DR prevents the decline of plasma and renal prostacyclin levels in aging rats.[22, 23] Kim et al. show rather diverse changes, claiming lower levels of both vasoconstrictive and vasodilatory COX products after DR in the aging rat aorta.[24] DR was reported to increase SIRT-1, which in turn might increase COX-2 expression and activity.[16, 20] However, our results exclude the possibility that this happens in *Ercc1*$^{\Delta/-}$. Clearly, multiple studies indicate the participation of COX products in DR-induced changes, and future studies should now investigate the underlying mechanism(s).

Ang II responses increased in AL-fed *Ercc1*$^{\Delta/-}$ mice compared to WT, whereas plasma renin activity decreased. The latter result is in agreement with the observation that renin levels decrease with age.[25, 26] Although renin levels start decreasing already when approaching middle age, our present findings indicate that the aging process might contribute to this decrease. With respect to the effect of aging on AT_1 receptor-mediated vasoconstriction many contrasting findings have been described, depending on the species and the vessels that have been used.[27-31] Nevertheless, our observation is in agreement with findings showing that in older persons blood pressure and blood flow responses to Ang II are elevated, especially in the presence of diabetes and/or in the absence of counterregulation by AT_2 receptors.[29, 32-34] Counterbalancing of AT_1-mediated pathogenesis is the basis for development of AT_2 receptor agonists as clinical drugs against cardiovascular diseases.[35, 36] Conversely, increased Ang II activity via AT_1 receptors due to a loss of counterregulation by AT_2 receptor is a mechanism observed in various disease models and in aging wild-type rats.[35, 37] The counterbalancing effect by AT_2 receptors is often ascribed to endothelial NO release, or might relate to a change in dimerization of the two Ang II receptor subtypes.[38] Loss of NO signaling clearly does not play a role in *Ercc1*$^{\Delta/-}$ mutants or their WT littermates given the absence of an effect of ODQ, leaving receptor interaction as the alternative mechanism. The vascular dysfunction observed in the present study was clearly not related to Ang II and increased blood pressure. In patients, the effect of chronic AT_1 receptor blockade on preservation

of vasodilator function is variable, having either a protective effect or not.[39] It was assumed that this might depend on the underlying disease or the vessel type that is investigated. However, our study suggests that the aging process might explain such variation. Unfortunately, most of the human studies exploring the effect of chronic AT_1 receptor blockade focus on patients around 60 and younger, and not on the oldest old. However, there are clues that aging affects the effectiveness of AT_1 receptor blockade. It has been shown that losartan becomes gradually less effective in aging rats, especially in the presence of hypertension and after loss of endothelium-independent NO function.[40] Clinical observations show that losartan/antihypertensive treatment can lead to adverse cognitive effects in elderly, which is ascribed to perfusion problems.[41] However, the same study suggests that this perfusion problem is a result of both blood pressure lowering and a persisting vascular dysfunction, at least in the brain. This implicates that vasodilator function is not improved. More dedicated studies in the oldest patients are necessary to resolve this paradox. There are mechanistic explanations available for this paradigm. In patients or in animal models of heart failure, hypertension, and/or diabetes, Ang II receptor blockade largely improves endothelial function due to an acute reduction of ROS formation by NADPH oxidase, increasing NO bioavailability.[9] DNA damage largely lowers NO independently from ROS,[4] and apparently this undermines the treatment efficacy of AT_1 receptor antagonists in our mouse model. The findings in our study might therefore be limited to *Ercc1*$^{\Delta/-}$ mice. Although this model, like comparable DNA repair models, is considered as a more humanized aging model than WT mice,[6] its acceptance as such awaits further confirmation. Ideally, tools become available to investigate whether the limited effectiveness of RAS treatment in humans is also due to DNA damage. A multitude of DNA lesion types exist, and current tools are not sufficient to reliably engage such an enterprise. However, novel techniques such as single-cell RNA and DNA sequencing performed with nanopore sequencing techniques, as to avoid PCR-related aberrations, might be used to detect stochastic stalling of transcription of long RNAs or to count single cell DNA mutations; two surrogate markers for DNA damage. The cells used for this purpose have to be long-lived to detect accumulated lesions. Yet it should be possible to obtain them in a non-invasive manner. A potential nucleotide source could be circulating (adult) endothelial cells. It might be worthwhile to explore such possibilities: suboptimal effectiveness of and treatment response variability to RAS inhibition are well-known phenomena, but remain largely unexplained, and therefore drive studies that attempt to find prediction markers for therapy effectiveness.[42, 43]

Clinical perspectives

- DR is a very efficient intervention to prevent vasodilator dysfunction caused by genomic instability. In this study we set out to identify potential mechanisms that lead to DR-mediated improvement of vasodilator dysfunction caused by DNA damage.

- Improvement of prostaglandin-mediated endothelium-dependent signaling and of VSMC responses to NO were identified as mechanisms. Vascular dysfunction induced by genomic instability is not reversible with chronic losartan treatment.
- Mouse models of genomic instability appear to represent the RAS blockade-resistant part of aging-related vascular disease, and might be tools to further explore this clinically relevant issue. Further study on the effect of genomic instability might offer a novel source of mechanistic explanations and markers with potential for clinical translation.

SUPPLEMENTARY INFORMATION

Diet restriction studies

Ercc1$^{\Delta/-}$ mice lacking adequate ERCC1 endonuclease function due to partial removal of the *Ercc1* gene have been described in detail elsewhere (Dollé MET, *Pathobiol. Aging Age-rel. Dis.* 2011). *Ercc1*$^{\Delta/-}$ mice and their wild type littermates (WT) were bred at the animal facility of the Centre for Health Protection, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The diet restriction intervention has been described extensively in our previous publication.[2] In short, the animals used in this study were 7-, 11-, and 16-week-old (-wk) *Ercc1*$^{\Delta/-}$ mice, and 11-, 20-wk wildtype (WT) mice that were either exposed to AL feeding or to DR. In *Ercc1*$^{\Delta/-}$ DR was started at 7 weeks of age, when they reach their maximum weight, with a 10% restriction, intensifying DR to 20% and 30% in weeks 8 and 9 respectively. Thereafter, 30% DR was maintained until the day of sacrifice, upon which vascular tissue was harvested for organ bath experiments and molecular analyses. WT animals were put on DR from the age of 11 weeks, when they were in a comparable phase of their growth curve respective to *Ercc1*$^{\Delta/-}$,[2] and then followed a similar procedure to *Ercc1*$^{\Delta/-}$ mice. Consequently, 16-wk old *Ercc1*$^{\Delta/-}$ mice were as long on DR as 20-wk old WT. As the DR group allowed longer survival of the *Ercc1*$^{\Delta/-}$ mice (~25 vs. ~55 weeks, AL vs. DR, resp.),[2] an additional group of 30-wk old mice was explored to assess if any protective effect on the vasculature would last at least as long as the maximal life expectancy of AL-fed *Ercc1*$^{\Delta/-}$.

Losartan intervention study

Male and female *Ercc1*$^{\Delta/-}$ (n=28) and wild type mice (n=17) for this study were bred at the Erasmus MC animal facility. Changes in blood pressure and vascular function were visible from an age as young as 8 weeks.[4] At the age of 7 weeks a non-significant trend towards endothelial dysfunction was already observed, which becomes significant between the age of 11 to 16 weeks.[2,4] Blood pressure in conscious *Ercc1*$^{\Delta/-}$ mice can be measured reliably from the age of 8 weeks at which point it is elevated. At the age of 16 weeks and beyond the *Ercc1*$^{\Delta/-}$ mice become very frail and reliable blood pressure measurement was not possible anymore. Ang II vasoconstrictions in iliac artery were measured in *Ercc1*$^{\Delta/-}$ mice from the age of 12 weeks to 18 weeks and found to be higher than in WT (see Results section). Therefore, we anticipated that a treatment with AT_1 receptor antagonist losartan started at the age of 5 weeks, the earliest possible time point, and sustained until the age of 12 weeks would be an adequate approach to test its expected protective and blood pressure effect. From 5 weeks of age, *Ercc1*$^{\Delta/-}$ and WT mice were divided into two groups per strain, which were either treated with losartan (100 mg/kg/day) in drinking water, or drinking water only. Thus, the following groups were created: *Ercc1*$^{\Delta/-}$ losartan (n=15); *Ercc1*$^{\Delta/-}$ water (n=19); WT losartan (n=7); WT water

(n=13). The distribution of performed measurements amongst the animals within this study are depicted in Table 1.

Table 1. Numbers of animals included in the diverse evaluations

Variable	WT water total: (n=13)	*Ercc1*$^{\Delta/-}$ water (n=19)	WT losartan (n=7)	*Ercc1*$^{\Delta/-}$ losartan (n=15)
Vasodilator function	6	10	4	8
Ang II constriction	9	13	5	12
BP	9	9	7	15
Real-time PCR	10	12	7	12

Tail cuff experiments

The animals were first trained for 4 consecutive days to acclimatize to measurement conditions. On the subsequent 5th day representative blood pressures were measured. Thereafter, the animals were sacrificed and vascular tissue was taken out to measure vasomotor function in organ bath set-ups, and to perform molecular analyses.

Tissue harvesting and preparation

Thoracic aorta and iliac arteries were collected from mice within 5 minutes after sacrifice by asphyxiation, and stored overnight in cold, oxygenated (5% CO_2; 95% O_2) Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,$CaCl_2$ 2.5, $MgSO_4$ 1.2 , KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3 in distilled water; pH 7.4) solution. The following day, vessel segments were mounted in 6-mL small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer at 37°C and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at which the effective transmural pressure is 100 mmHg. Viability of the tissue was tested through induction of contractions by exposure to respectively 30, 60, and 100 mmol/L KCl.

Testing of vasodilator function

In aortic segments, after washout of KCl, a pre-constriction was elicited with 30 nmol/L U46619, a thromboxane A_2 mimetic, resulting in 50-100% of the previously obtained contraction to 100 mmol/L KCl. Following pre-constriction, relaxation for the endothelium-dependent vasodilator acetylcholine (ACh) were constructed by giving cumulative doses (10^{-9}-10^{-5} mol/L), followed by exposure to the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). To further explore the endothelium-independent dilator we cumulatively added SNP (10^{-9}-10^{-5} mol/L) in parallel rings preconstricted with 30 nmol/L U46619. When sufficient aortic tissue was available, the involvement of nitric oxide (NO) and prostaglandins in ACh responses was investigated by performing the experiments in the presence of the endothelial nitric oxide synthase inhibitor

N^G-Methyl-L-Arginine acetate salt (L-NMMA, 10^{-5} mol/L), the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10^{-5} mol/L) or both inhibitors. Inhibitors were added to the organ bath 10 minutes prior to U46619.

Quantitative real-time PCR

Total RNA was isolated with the Nucleospin RNA II kit (Machery-Nagel). RNA was reverse-transcribed by use of Quantitect Rev. Transcription Kit (Qiagen). Four nanograms of cDNA was amplified by Real-time PCR on a Bio-Rad CFX96 thermocycler using iQ™ SYBR Green Supermix (Bio-Rad Life Sciences). β-actin was used for normalization, and comparison with B2M and HPRT-1 as a household gene was performed to further verify reliability. Since this yielded the same result, these data are not shown. Each reaction was run in duplicate and generation of specific PCR products was confirmed by melting-curve analysis and gel electrophoresis. Results from unreliable duplicates or melting-curves were discarded (values were excluded of Ct ≥ 31). Each reaction was performed in duplicate with SYBR Green PCR Master Mix (Applied Biosystems). The relative amount of genomic DNA in DNA samples was determined as follows: $RQ = 2^{(-\Delta\Delta Ct)}$. COX-1 and -2, adenylate cyclase (AC)-5 and 6, phosphodiesterase (PDE)-4B and 4D, and the prostaglandin-I_2 (IP) receptor. Details of primers are provided in Table 2.

Table 2. Primer sequences for SYBR green qPCR

Marker	Forward primer	Reverse primer
COX-1	TACTCACAGTGCGGTCCAAC	GTACAGAGGGCAGAATGCGA
COX-2	GGGCCATGGAGTGGACTTAAA	TCCATCCTTGAAAAGGCGCA
AC5	CCAGTGTACTGCCCAAGAACT	GTAAACAGTGATTCTCCGCAGC
AC6	CTGCGGTGAGGGAGAATCACT	AGCCCTGACACGCAGTAGT
PDE4B	ACTGATGCACAGCTCAAGCC	CCAGCTCCTTGGCTAGATGA
PDE4D	GCAGACTTGCGAAGCGAATC	CCATTGTCCACATCGAAACCAC
IP receptor	ATGTACCGCCAACAGAGACG	CCTCGGATCATGAGAGGCAG
AT_{1a} receptor	CCCACGTGTCCCTGTTACTAC	TTTGGGGACAGTACAGGTTTC
AT_{1b} receptor	CTGTGAAATTGCGGACGTAGT	AAGCCATAAAACAGAGGGTTCAG
AT_2 receptor	TACCCGTGACCAAGTCCTGA	TACCCATCCAGGTCAGAGCA
β-Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG

ACE (lung) and renin (kidney) expression was studied using TaqMan® assays using probe sets from ThermoFisher Scientific with the following identities: β-Actin, Mm00607939_s1; ACE, Mm00802048_m1; renin, Mm02342889_g1.

Western Blots

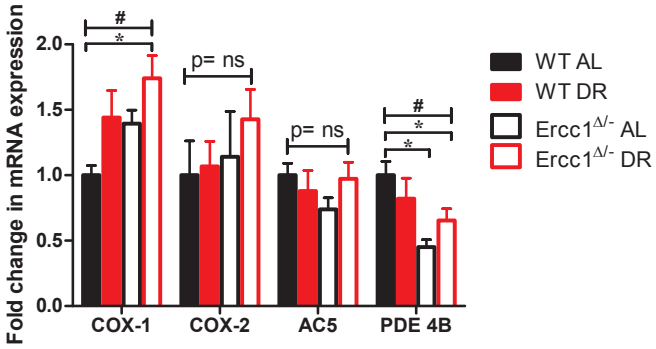
Lungs were snap frozen in liquid nitrogen and then stored at -80°C. Frozen tissues were homogenized in ice cold RIPA buffer (50 mmol/L HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate and 1 mmol/L EDTA) containing protease and phosphatase-

inhibitors (1mmol/L PMSF, 1 mmol/L $NaVO_4$, 1 mmol/L NaF, 1 µg/mL aprotinin, 1 µg/mL pepstatin and 1 µg/mL leupeptin) using a stainless-steel ultraturrax (Polytron). Homogenized tissues were centrifuged and protein concentration was measured in the supernatants using the BCA method (Thermo Scientific, USA). The samples were run on a criterion bis/tris gradient gels, and blotted to nitrocellulose membranes.

For eNOS, membranes were blocked with 5% milk TBS-T; for pSer1177- eNOS, COX-1, COX-2, PDE4B and SIRT-1 membranes were blocked with 5% BSA TBS-T. After blocking, membranes were incubated overnight with the primary antibodies was as follows:

eNOS (Santa Cruz, SC-654 1:500 in 5% milk TBS-T), pSer1177- eNOS (Santa Cruz, SC-21871-R 1:500 in 5% BSA TBS-T), COX-1 (Cell Signalling, #9896 1:1000 in 5% BSA TBS-T), COX-2 (Cell Signalling, #4842 1:1000 in 5% BSA TBS-T), PDE4B (Santa Cruz, SC-25812 1:1000 in 5% BSA TBS-T) and SIRT-1 (Abcam, ab28170 1:500 in 5% BSA TBS-T).

We used an HRP- conjugated antibody (Bio-Rad 1:2000 in 1% milk-TBS-T) to detect the primary antibodies. For visualization we used an enhanced chemiluminiscent substrate for detection of HRP (Pierce ECL Western Blotting Substrate, Thermo Scientific). All protein expression levels were normalized to Actin.



Supplementary figure 1. mRNA expression levels in lung tissue of COX-1, COX-2, AC 5, and PDE4B in 16-wk-old *Ercc1*$^{\Delta/-}$ mice and 20-wk-old wild-type (WT) littermates from the diet intervention study. All mRNA values are corrected for β-actin and normalized to WT-AL expression levels. Error bars: S.E.M. #P<0.05, one way- ANOVA; *P<0.05, Bonferroni's post-hoc tests.

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Chapter 6

Development of protein kinase G Iα-dimerizing antihypertensive drugs: are we standing at the shore of the Rubicon?

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Blood pressure control is largely dependent on the vasodilator capacity of blood vessels, which for the major part requires the release of endothelium-derived relaxing factors, i.e., NO and endothelium-derived hyperpolarizing factor (EDHF). In conduit vessels, NO is most relevant, while in resistance vessels EDHF is believed to have a more prominent role.

NO interacts with soluble guanylyl cyclase (sGC) to convert guanosine-5′-triphosphate (GTP) to cGMP, which subsequently binds to protein kinase G (PKG). Two PKG genes (I and II) exist, and vascular smooth muscle cells express only PKGI. The *PKGI* gene expresses as 2 separate isoforms (α and β), which are splice variants of the same gene and differ only in sequence of their N-terminal leucine zipper (LZ) interaction domain.[1] They occur as homodimers, and their LZ domains mediate PKG binding to specific substrates, via LZ–LZ co-interaction. For instance, cGMP-activated PKGIα phosphorylates vasodilator-stimulated phosphoprotein (VASP) (Figure). NO donors (nitrates), sGC activators/stimulators (riociguat, cinaciguat and vericiguat) and phosphodiesterase (PDE) 1 and 5 inhibitors (which block cGMP degradation by PDE1 and PDE5, e.g., ITI-214 and sildenafil) target this pathway (Figure 1). Yet, their indications include angina pectoris, erectile dysfunction, pulmonary arterial hypertension, and neurological disorders, but not systemic hypertension. This might be because central blood pressure is mainly regulated through resistance vessels, requiring drugs to exclusively target EDHF.

EDHF-mediated relaxant pathways display great diversity, and multiple candidates have been proposed to act as EDHF, possibly depending on the vascular bed and species that are investigated. The list includes cytochrome P450 metabolites (epoxyeicosatrienoic acids, EETs), cyclo-oxygenase products, K^+, H_2O_2, gap junctions and *S*-nitrosothiols.[2] Interestingly, H_2O_2 activates PKGIα in an cGMP-independent manner, by inducing the formation of a disulfide bond between the cysteine 42 (C42) residues of 2 adjacent chains in PKGIα homodimers.[3] This covalent bond can be formed by virtue of the LZ keeping the two chains together for oxidant substances to establish the disulfide bond (Figure). The covalently coupled PKGIα homodimers phosphorylate large-conductance Ca^{2+}-dependent K^+ (BK_{Ca}) channels, thus leading to smooth muscle hyperpolarization. Since C42 is unique to PKGIα, it might be a promising target for the development of novel antihypertensive drugs predominantly acting in resistance vessels. No such drugs exist until now.

In this edition of *Hypertension*, Burgoyne et al. report their quest to identify compounds capable of inducing PKGIα oxidation.[4] Using an *in vitro* assay with recombinant PKGIα and dibromobimane, which fluoresces when binding to reduced PKGIα, they screened around 300 electrophilic small molecules. Twelve promising compounds emerged and were screened for bioactivity in mouse mesenteric arteries in *ex-vivo* myograph experiments. The 5 compounds that displayed the highest relaxation efficacy underwent selectivity screening in mesenteric arteries of C42S PKGIα knock-in

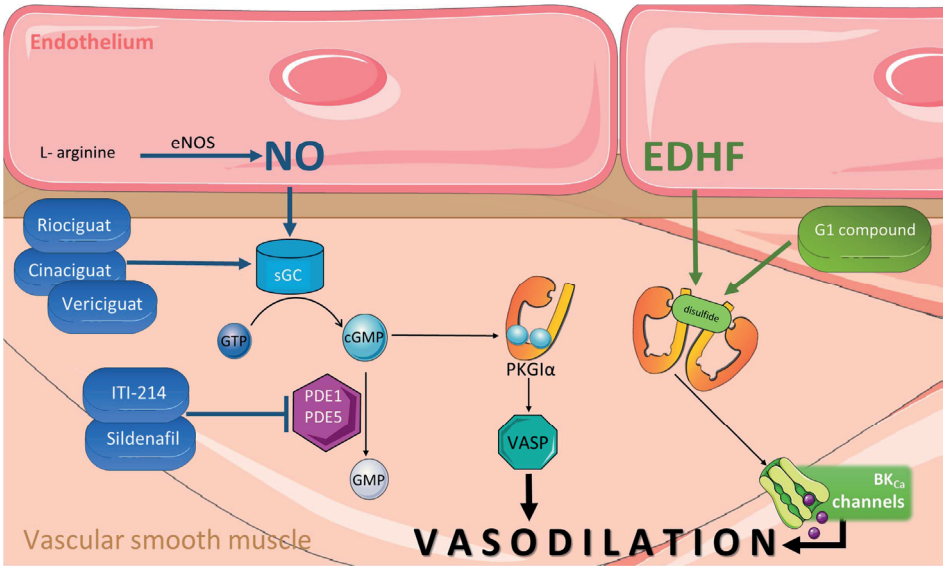

Figure 1. The vasodilator capacity of blood vessels depends on NO and endothelium-derived hyperpolarizing factor(s) [EDHF(s)] released from the endothelium. NO is generated by endothelial NO synthase (eNOS) from L-arginine, and activates soluble guanylate cyclase to generate cGMP from GTP. Next, cGMP binds to protein kinase G Iα (PKGIα), capable of phosphorylating vasodilator-stimulated phosphoprotein (VASP). EDHFs like H_2O_2 induce the formation of a disulfide bond between the cysteine 42 residues of 2 adjacent chains in PKGIα homodimers, and this results in the opening of potassium channels, leading to hyperpolarization. Since cGMP is degraded by phosphodiesterases 1 and 5 (PDE1, PDE5), novel drugs are aimed at blocking PDE, to enhance NO-cMP signaling. In addition, sGC activators/stimulators will also upregulate this pathway. Finally, compounds capable of inducing PKGIα oxidation might selectively upregulate the EDHF pathway.

(KI) mice, i.e., arteries that cannot be activated by oxidants. This yielded one candidate lead compound, named G1, capable of relaxing wild-type (WT), but not KI arteries. As a comparator, the sGC activator cinaciguat relaxed both arteries equally well. *In vivo*, intraperitoneal bolus injection of G1 (7.4 mg/kg) in WT mice did not alter mean arterial pressure (MAP) due to a compensatory heart rate increase. However, in mice rendered hypertensive by infusion with angiotensin II, intraperitoneally administered G1 (3.7 and 14.8 mg/kg) acutely lowered blood pressure. Also, oral intake of G1 (20 mg/kg) lowered MAP in angiotensin II-infused WT mice, with a swift onset of the antihypertensive effect. This effect appeared to wane off gradually over the 4 days that G1 was administered. G1 was without effect in KI mice in the latter protocol (oral application), but did lower MAP modestly in angiotensin II-infused KI mice when administered intraperitoneally at 7.4 mg/kg.

Although these results are exciting and might lead to the development of new antihypertensive therapies, the study is still very preliminary and several concerns should be addressed in future investigations. First, important pharmacological questions remain

to be answered. Unlike the NO-cGMP pathway, the EDHF field is full of uncertainties, and given the multitude of factors involved one should perhaps rather use the term endothelium-derived hyperpolarization (EDH), i.e., leaving out 'factor'. As a consequence of this complexity, after decades of work, no drug has emerged from this concept. Most studies agree on a role for endothelial small- and intermediate conductance Ca^{2+}-dependent K^+ (SK_{Ca}, IK_{Ca}) channels in EDH.[2] H_2O_2 is one of the many players in this field, and its activation of BK_{Ca} in vascular smooth muscle cells is mimicked by only a few other EDHFs, like the EETs.[5] PKG inhibition with KT5823 blocked the ex-vivo relaxant effects of G1 in mesenteric arteries by ≈50%, implying that potentially half of the effect of G1 is PKG-independent. The marginal G1-induced increase in PKGIα disulfide dimer % in aortic tissue (from 78 to 86%), combined with the G1-induced blood pressure-lowering effects in KI mice, support this view. In this regard, it is of interest to note that sGC activators might also act independent from sGC activity. For instance, during sGC inhibition with ODQ, the sGC activator BAY412272 still evoked vasodilation in porcine coronary arteries, by activating Na^+-K^+-ATPase.[6] To what degree this involved cGMP-independent PKG activation or non-PKG mechanisms is unknown. Clearly, future studies should investigate what non-PKG mechanisms are activated by G1, and how sGC activators might exert EDH, for instance via mechanisms involving Na^+-K^+-ATPase activation. Since G1 prevented VASP phosphorylation by the stable cGPM analogue 8-Br-cGMP, it is possible that the cGMP-activated PKGIα-VASP pathway and the oxidized PKGIα-BK_{Ca} pathway are mutually exclusive. If so, we need to know what exactly determines their respective contributions in conduit versus resistance vessels, since PKG occurs at both sites.

Second, at the organismal level, electrophilic drugs acting through formation of covalent binding, such as G1, could potentially be toxic, and provoke allergic or autoimmune responses.[7, 8] This fact, together with the observation that the G1 effect disappears slowly, awakens the concern that G1 and derivatives thereof should first undergo strict pharmacological, toxicological, and efficacy testing in chronic animal models before passing to patients. Despite a comprehensive discussion speculating on the putative non-toxicity of G1 and derivatives thereof, the authors admit that further lead optimization is necessary in particular to minimalize toxicity through widespread thiol modification: a point well-taken. The *in-vivo* disappearance of the effect is reminiscent of the tolerance phenomenon associated with nitrate use, which involves the loss of bioactivation of nitroglycerin. Does G1 require bioactivation, and/or does the G1 tolerance represent the upregulation of counterregulatory mechanisms (e.g., inactivation of oxidized PKGIα by thioredoxin)?

Finally, the question arises which models are appropriate to test G1 chronically. In particular, when aiming at treatment of essential hypertension, G1 should be additionally studied in models that do not rely on angiotensin II infusion, like the spontaneously hypertensive rat, and low-renin models such as the DOCA-salt rat. Here, the effects of

G1 on cardiac and vascular remodeling and kidney function might also be investigated, as PKGI activation is not limited to the vascular wall.[1] In such studies, the process of aging could simultaneously be taken into consideration, given the age-dependency of hypertension, and the fact that aging is associated with an increase of PDE1 activity, thereby decreasing NO signaling.[9, 10] Impaired NO signaling is often compensated for by upregulated EDHF responses, but this is not a uniform phenomenon, and thus drugs that improve EDHF signaling, like G1, could have great therapeutic potential even beyond hypertension. An obvious prerequisite is that such novel drugs are free of side-effects.

In summary, it seems that Burgoyne et al. have arrived with a fresh approach at the shore of the Rubicon, but are not yet ready to cross: before taking such a daring step we need more evidence!

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Chapter 7

Endothelium-derived hyperpolarizing factor and protein kinase G Iα activation: H2O2 versus S-nitrosothiols

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ABSTRACT

Protein kinase G Iα mediates the vasodilatory effects induced by nitric oxide. It has been suggested that other endothelium-derived hyperpolarizing factors (EDHFs), such as H_2O_2, can activate protein kinase G Iα by inducing the formation of a disulfide bond between the cysteine 42 (C42) residues of 2 adjacent chains in PKGIα homodimers in a cGMP-independent manner. However, it is unknown whether all EDHFs can activate PKGIα.

We investigated the contribution of PKGIα to NO- and EDHF-induced vasodilation in porcine coronary arteries (PCAs). PCAs were mounted in organ baths, and concentration response curves to bradykinin, H_2O_2 , L-*S*-nitrosocysteine (L-SNC) and polychromatic light were constructed in the presence or absence of inhibitors of *S*-nitrosothiols, thioredoxin reductase, PKG or soluble guanylyl cyclase (sGC).

Bradykinin fully relaxed preconstricted PCAs. The sGC inhibitor ODQ prevented this relaxation, inducing near complete blockade when administered together with the PKG inhibitor KT5823. Thioredoxin reductase inhibition with auranofin potentiated bradykinin ≈7-fold, both with and without ODQ, indicating that bradykinin-induced responses involve sGC and oxidized PKGIα. H_2O_2 concentration-dependently relaxed preconstricted vessels, and neither ODQ nor KT5823 blocked this effect. Thioredoxin reductase inhibition with 1-chloro-2,4-dinitrobenzene (DNCB) potentiated H_2O_2 2.2-fold. These data indicate that H_2O_2-induced relaxations involved oxidized PKGIα but not sGC. L-SNC fully relaxed preconstricted PCAs and this effect was only blocked by ODQ, indicating that the L-SNC-induced effects involve sGC, but not PKG. A 5-min light exposure relaxed PCAs by 64 ± 3.4%. ODQ fully prevented photorelaxation while KT5823 was without effect. Both DNCB and auranofin tended to enhance photorelaxation. Depletion of *S*-nitrosothiols diminished photorelaxation. Yet, after such depletion, the potentiating effect of DNCB (but not auranofin) became significant. This indicates that photorelaxation depends on sGC activation by *S*-nitrosothiols, while only after *S*-nitrosothiol depletion oxidized PKGIα comes into play.

In conclusion, both bradykinin- and light-induced relaxation of PCAs depend, at least partially, on oxidized PKGIα, and this does not involve sGC. H_2O_2 also acts via oxidized PKGIα in an sGC-independent manner. Yet, *S*-nitrosothiol-induced relaxation is PKGIα-independent. Clearly, PKG activation does not contribute universally to all EDHF responses, and targeting PKGIα may only mimick EDHF under certain conditions.

INTRODUCTION

Blood pressure control is largely dependent on the vasodilator capacity of blood vessels, involving the release of endothelium-derived relaxing factors, i.e., nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF).

NO interacts with soluble guanylyl cyclase (sGC) to convert guanosine-5'-triphosphate to cGMP, which subsequently binds to protein kinase G (PKG) Iα, thereby inducing dilation. Yet, EDHFs like H_2O_2 have also been suggested to activate PKGIα.[1-3] This occurs in an cGMP-independent manner, by inducing the formation of a disulfide bond between the cysteine 42 (C42) residues of 2 adjacent chains in PKGIα homodimers.[2] The covalently coupled PKGIα homodimers phosphorylate large-conductance Ca^{2+}-dependent K^+ (BK_{Ca}) channels, resulting in smooth muscle hyperpolarization. Oxidized PKGIα is extremely short-lived, as it is rapidly converted to the reduced state by thioredoxin. Thioredoxin is kept in the reduced state by thioredoxin reductase, and inhibitors of the latter enzyme, like auranofin and 1-chloro-2,4-dinitrobenzene (DNCB), will block thioredoxin activity, leading to an increase in the half-life of oxidized PKGIα.[4]

Given the great diversity of EDHF candidates,[5, 6] it is questionable whether all EDHFs act via PKGIα. Previous studies from our laboratory in porcine coronary arteries (PCAs) supported a role for *S*-nitrosothiols as EDHF in bradykinin-induced responses.[7-10] *S*-nitrosothiols are also major contributors to light-induced relaxation ('photorelaxation') of PCAs.[8, 9, 11] In the present study, we verified the contribution of PKGIα to NO- and EDHF-induced vasodilation in bradykinin- or light-exposed PCAs. For comparison, concentration-response curves (CRCs) to H_2O_2 and L-*S*-nitrosocysteine (L-SNC) were also constructed. Experiments were performed both in the absence or presence of inhibitors of *S*-nitrosothiols, thioredoxin reductase, PKG or sGC.

METHODS

Drugs

Bradykinin, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_2α$ (U46619), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), ethacrynic acid, auranofin, DNCB, and KT5823 were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Tissue Collection

PCAs were obtained from 49 slaughterhouse pigs. They were removed after the heart had been brought to the laboratory in cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. PCAs were stored in cold, oxygenated Krebs bicar-

bonate solution for 12-36 hours. They were then cut into segments of approximately 4 mm length, suspended on stainless steel hooks in 15 ml-organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 / 5% CO_2, and maintained at 37°C.

Organ bath studies

Vessel segments were allowed to equilibrate for at least 30 min, and the organ bath fluid was refreshed every 15 min during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, USA). The vessel segments, stretched to a stable force of about 15 mN, were exposed to 30 mmol/l KCl twice. Subsequently, the tissue was exposed to 100 mmol/l KCl to determine the maximal contractile response to KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: auranofin (3 µmol/l)[4], DNCB (4 µmol/l)[12], the PKG inhibitor KT5823 (1 µmol/l)[1], the sGC inhibitor ODQ (10 µmol/l), or the *S*-nitrosothiol-depleting agent ethacrynic acid (50 µmol/l)[8]. Vessels were then preconstricted with the thromboxane A2 analogue U46619 (1 µmol/l) and either exposed to bradykinin, H_2O_2, L-SNC or light. The light experiments involved six 5-min exposures to light using a halogen dissection lamp omitting polychromatic light in a dark room. Each exposure was followed by a period in the dark of 10 minutes.

Data Analysis

Data are given as mean±SEM. Relaxant responses are expressed as a percentage of the contraction to U46619. CRCs were analyzed as described using the logistic function described[13] to obtain pEC_{50} ($-^{10}logEC_{50}$) values. ODQ increased basal tone by maximally 15% of the maximum contractile response. U46619-induced preconstrictions were corrected for this increase in baseline. Statistical analysis versus control was obtained by two-way ANOVA for light experiments, and one-way ANOVA for agonist-induced relaxations. $P<0.05$ was considered significant.

RESULTS

Auranofin and DNCB up to a concentration of 10 µmol/l did not relax preconstricted vessels (n=5; data not shown). Bradykinin fully relaxed preconstricted PCAs (pEC_{50} 7.9 ± 0.1; n=6). ODQ ($P<0.05$; n=6) and KT5823 (P=NS; n=6) prevented this relaxation, inducing near complete blockade when administered together (Figure 1A). DNCB non-significantly potentiated the effect of bradykinin, both with and without ODQ (Figure 1B), while auranofin potentiated bradykinin ≈7-fold, both with and without ODQ ($P<0.0001$

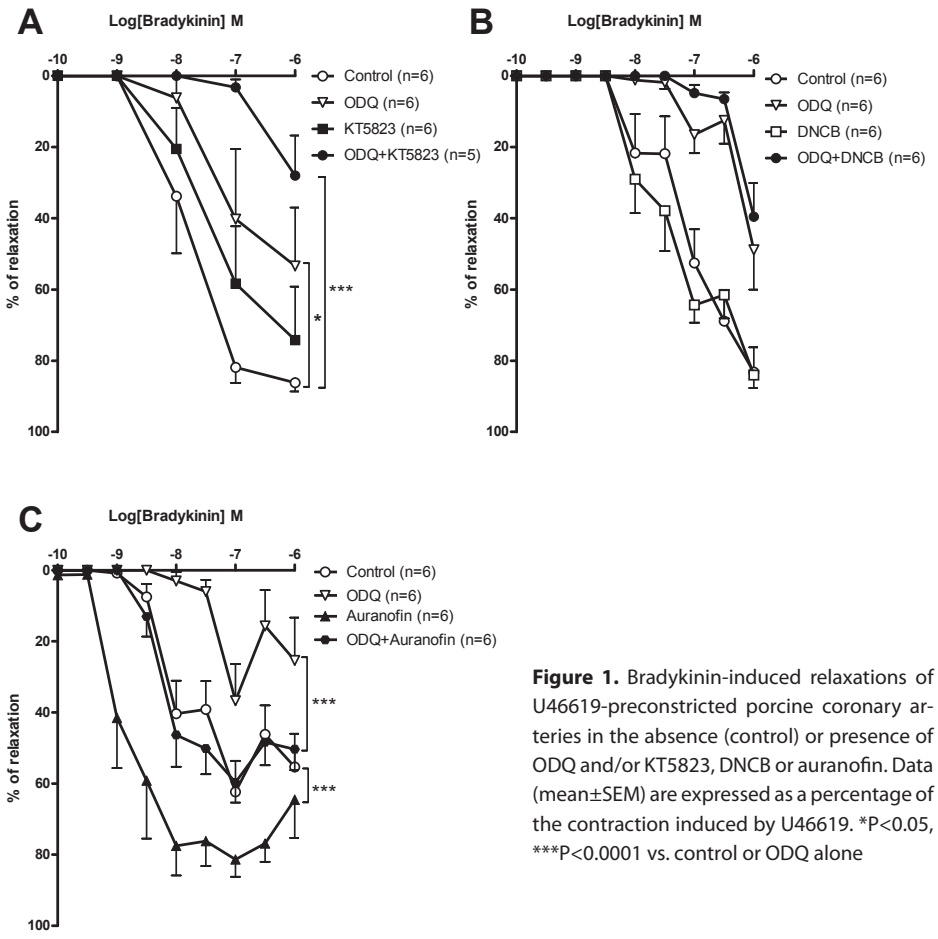

Figure 1. Bradykinin-induced relaxations of U46619-preconstricted porcine coronary arteries in the absence (control) or presence of ODQ and/or KT5823, DNCB or auranofin. Data (mean±SEM) are expressed as a percentage of the contraction induced by U46619. *P<0.05, ***P<0.0001 vs. control or ODQ alone

for both; n=6) (Figure 1C). This indicates that bradykinin-induced responses involve both sGC and oxidized PKGIα.

H_2O_2 concentration-dependently relaxed preconstricted vessels (pEC_{50} 4.1±0.1; n=6), and neither ODQ (n=6) nor KT5823 (n=5) blocked this effect (Figure 2A). DNCB potentiated H_2O_2 2.2-fold (pEC_{50} 4.4±0.1, n=6; P<0.0001 vs. control), and a similar tendency was observed for auranofin (pEC_{50} 4.3±0.1, n=6; P=NS) (Figure 2B). At concentrations above 0.1 mmol/l, H_2O_2 induced short-lasting (<60 seconds) constrictor responses (corresponding with <10% of the maximum contractile response to KCl) which preceded the relaxations (data not shown). These constrictor responses were not observed in the presence of DNCB (data not shown). These data indicate that H_2O_2-induced relaxations involved oxidized PKGIα but not sGC, and that enhancing these relaxations abolished the minor contractile responses to H_2O_2.

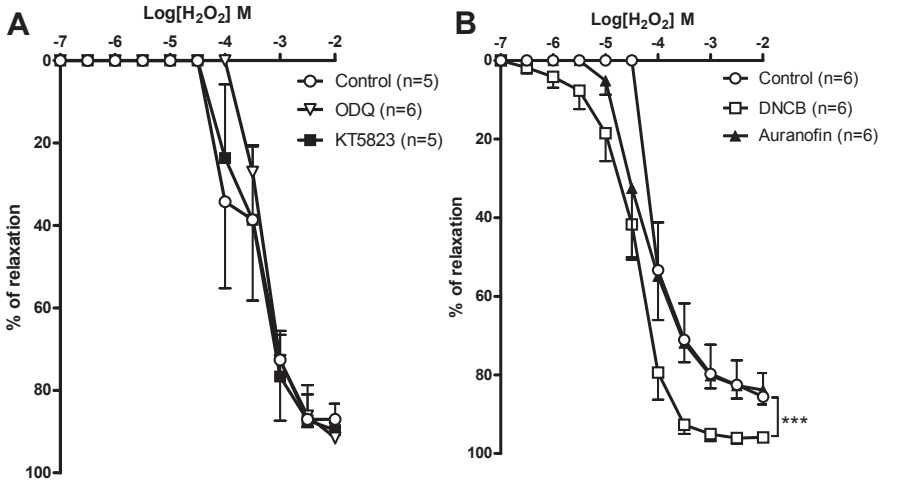

Figure 2. H_2O_2-induced relaxations of U46619-preconstricted porcine coronary arteries in the absence (control) or presence of ODQ, KT5823, DNCB or auranofin. Data (mean±SEM) are expressed as a percentage of the contraction induced by U46619. ***P<0.0001 vs. control.

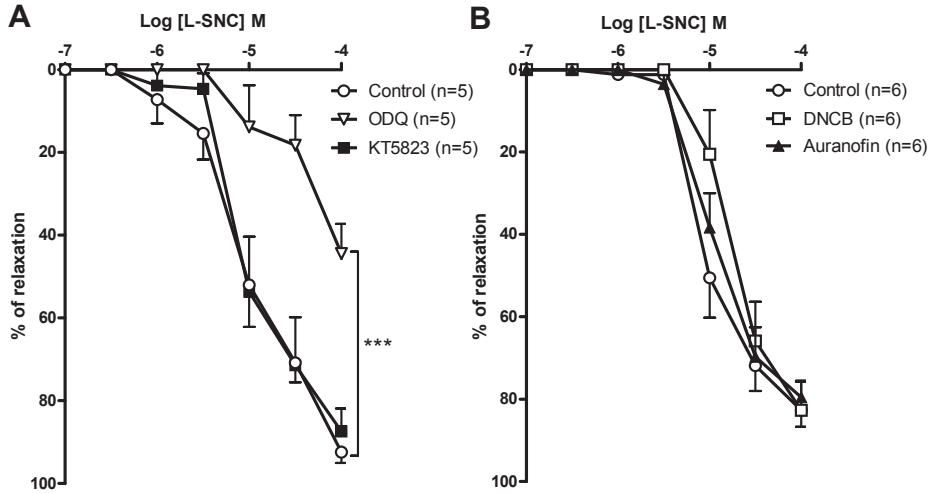

Figure 3. L-SNC-induced relaxations of U46619-preconstricted porcine coronary arteries in the absence (control) or presence of ODQ, KT5823, DNCB or auranofin. Data (mean±SEM) are expressed as a percentage of the contraction induced by U46619. ***P<0.0001 vs. control.

L-SNC fully relaxed preconstricted PCAs (pEC_{50} 5.0±0.1; n=5), and this effect was blocked by ODQ (pEC_{50} 4.5±0.2, n=5; P<0.001) (Figure 3A), but not KT5823 (n=5), auranofin (n=6), or DNCB (n=6) (Figure 3B). This indicates that the L-SNC-induced effects involve sGC, but not PKG.

A 5-min light exposure relaxed PCAs by 64 ± 3.4% (n= 7). Upon repetitive exposure to light, relaxations tended to get smaller, indicating depletion of the underlying relaxant factor(s) (Figure 4A). ODQ fully prevented relaxation (n= 3), while KT5823 (n=7) was without effect. Both DNCB (n=6) and auranofin (n=6) tended to enhance photorelaxation (Figure 4B and 4C). The *S*-nitrosothiol-depleting agent ethacrynic acid diminished photorelaxation (n=6; P<0.0001). Yet, after such depletion, the potentiating effect of DNCB (n=6) became significant (P<0.0001) while the enhancing effect of auranofin (n=6) remained non-significant (Figure 4B and 4C). This indicates that photorelaxation depends on sGC activation by *S*-nitrosothiols, while only after *S*-nitrosothiol depletion oxidized PKGIα comes into play.

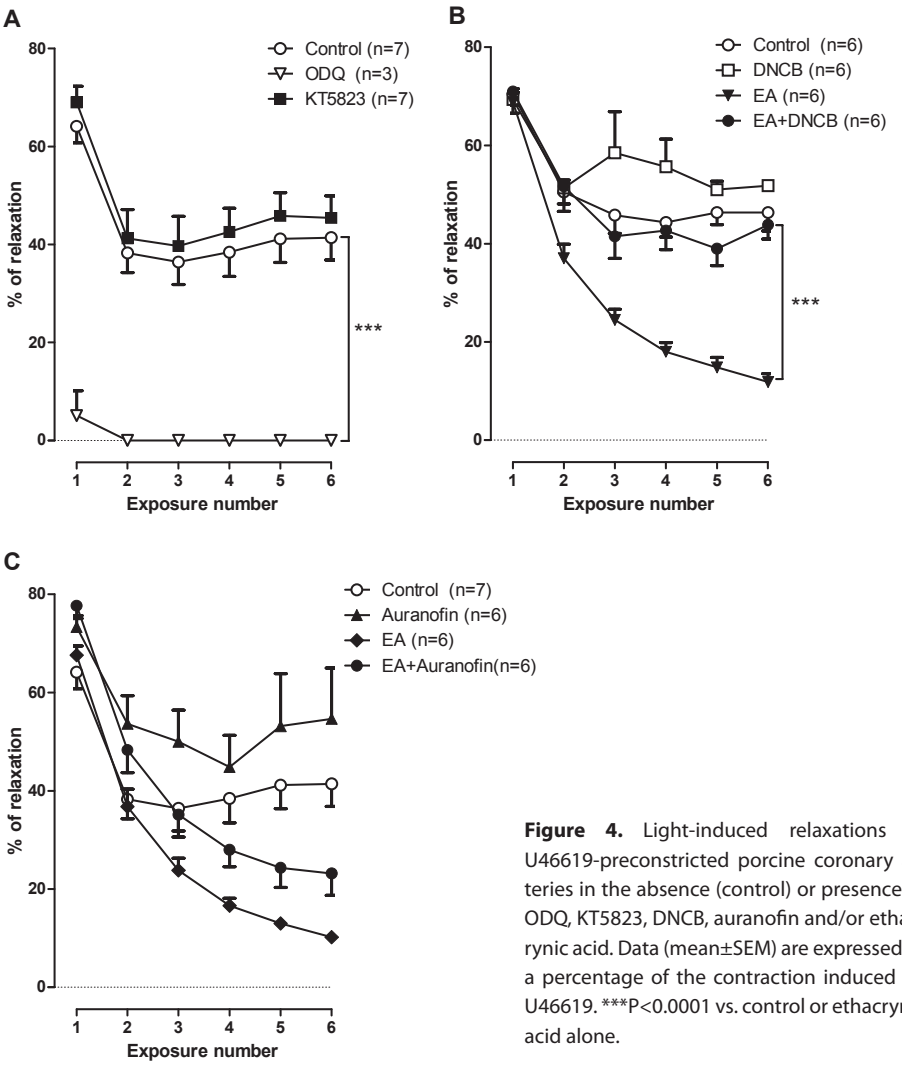

Figure 4. Light-induced relaxations of U46619-preconstricted porcine coronary arteries in the absence (control) or presence of ODQ, KT5823, DNCB, auranofin and/or ethacrynic acid. Data (mean±SEM) are expressed as a percentage of the contraction induced by U46619. ***P<0.0001 vs. control or ethacrynic acid alone.

DISCUSSION

The present study supports the concept that oxidized PKGIα contributes to EDHF-mediated responses following bradykinin exposure of PCAs. The potentiating effect of the thioredoxin reductase inhibitor auranofin on top of sGC inhibition with ODQ illustrates the sGC-independency of this phenomenon. Results obtained with the thioredoxin reductase inhibitor DNCB did not reach significance. This may relate to the fact that thioredoxin reductase inhibition also prevents thioredoxin-dependent denitrosylation. Consequently, DNCB augments *S*-nitrosylation and may thus simultaneously impair relaxation.[12]

Bradykinin-induced, EDHF-mediated relaxation of PCAs involves endothelial small- and intermediate conductance Ca^{2+}-dependent K^+ channels.[7] Their activation, e.g., by *S*-nitrosothiols, results in K^+ release and smooth muscle cell hyperpolarization.[10, 14] In addition, *S*-nitrosothiols activate smooth muscle Na^+-K^+ ATPase in a sGC-dependent manner,[10] and they have been reported to activate BK_{Ca} channels via *S*-nitrosylation of cysteine residues.[15] Their mechanism of action involves binding to *S*-nitrosothiol recognition sites, resulting in the *S*-nitrosylation of cysteine residues.[15] This process occurs in a stereoselective manner.[16-18] Since BK_{Ca} channel activation also occurs after exposure to covalently coupled PKGIα homodimers,[1] one possibility is that *S*-nitrosothiols interfere with this phenomenon via PKGIα.

Yet, PKG inhibition was without effect towards L-SNC, and thioredoxin reductase inhibition tended to block L-SNC-induced relaxation (as opposed to its potentiating effects towards bradykinin). This suggests that oxidized PKGIα is not responsible for L-SNC-induced relaxation. Thioredoxin reductase inhibition did potentiate H_2O_2, confirming that at least this EDHF acts via oxidized PKGIα in PCAs.

In agreement with the concept that *S*-nitrosothiol-induced relaxation does not involve PKGIα activation, thioredoxin reductase inhibition non-significantly potentiated photorelaxation. Such relaxation largely depends on *S*-nitrosothiols, as illustrated by the suppression of this effect following *S*-nitrosothiol depletion by ethacrynic acid. Yet, after depletion, the effect of thioredoxin reductase inhibition with DNCB became significant. This implies that photorelaxation does involves additional factor(s) that apparently rely on oxidized PKGIα, and that can be distinguished only when suppressing the *S*-nitrosothiol component of photorelaxation. To what degree this factor is identical to the factor that is responsible for the EDHF response after bradykinin exposure remains to be determined.

Of interest, H_2O_2-induced relaxation, although depending on oxidized PKGIα, could not be blocked by the PKG inhibitor KT5823. It therefore seems that PKG activity (blocked by KT5823) does not necessarily have the same effect as the formation of a disulfide bond between the C42 residues of 2 adjacent chains in PKGIα. In our experimental setup,

millimolar concentrations of H_2O_2 induced modest, short-lasting contractions. Such contractions at high (supraphysiological) H_2O_2 concentrations have been noted before, and are most likely due to superoxide and/or thromboxane A2 upregulation.[19] Enhancing relaxation (with DCNB), most likely through physiological antagonism, abolished these contractile responses.

Finally, given the fact that L-SNC-induced relaxation and photorelaxation can be blocked by ODQ, but not KT5823, while both phenomena do involve Na^+-K^+ ATPase activation in smooth muscle cells,[7, 8] our data imply that sGC links to Na^+-K^+ ATPase activation in a PKG-independent manner.

In summary, both bradykinin- and light-induced relaxation of PCAs depend, at least in part, on oxidized PKGIα, and this does not involve sGC. H_2O_2 also acts via oxidized PKGIα in an sGC-independent manner. Yet, *S*-nitrosothiol-induced relaxation is PKGIα-independent. Clearly therefore, PKG activation does not contribute universally to all EDHF responses, and targeting PKGIα may only mimic EDHF under certain conditions.[20]

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Chapter 8

Summary and discussion

Dutch summary

Resumen en español

SUMMARY

Age remains the greatest independent risk factor for all major life-threatening disorders, such as cardiovascular and neurological diseases. While lifespan has increased steadily over the last decades, healthspan has not increased at the same pace.[1] Aging used to be considered a non-modifiable process but in the past decade it has been shown that it is a complex and highly regulated biological process, and that DNA damage is a main causative factor.[2] The evidence supporting a role for DNA damage in the age-related vascular dysfunction in general population and in mouse models is reviewed in **Chapter 1**.

Mice with defective DNA repair are excellent exploration tools because they rapidly reproduce hallmarks of human aging.[3] It is known that in a mouse model of genomic instability due to defective DNA repair (*Ercc1*$^{\Delta/-}$ mice), accelerated age-related vascular dysfunction takes place, which is characterized by decreased NO/cGMP responsiveness and increased cellular senescence in the vascular smooth muscle cell (VSMC) layer.[4] In **Chapter 2** we showed that increased metabolism of cGMP by phosphodiesterases (PDE) was responsible for the reduced NO/cGMP signaling in VSMC of *Ercc1*$^{\Delta/-}$ mice (Figure 1B), since PDE inhibition fully restored endothelium-independent vasodilation to the level of WT mice. Our data also suggest that in *Ercc1*$^{\Delta/-}$ mice, the contribution of PDE1 relative to PDE5 is increased. Similar observations were made in human senescent VSMCs. In addition, in our candidate gene look-up using GWAS data, single nucleotide polymorphisms in the PDE1A gene were associated with increased diastolic blood pressure and carotid intima media thickness, two hallmarks of human age-related vascular dysfunction. PDE1 has also been implicated in atherosclerosis and malignant cardiac remodeling. Hence, PDE1 plays a central role in age-related cardiovascular disease.

Despite previous findings and the evidence presented in Chapter 2, one important question that remains is whether the observed accelerated vasodilator dysfunction in *Ercc1*$^{\Delta/-}$ mice arises from defective local vascular DNA repair or if it is a consequence of the systemic deterioration from which these mice suffer. To answer this, cardiovascular function was investigated in mouse models with defective DNA repair in either vascular endothelial cells or in smooth muscle. In **Chapter 3** we studied this in a mouse model with specific loss of *Ercc1* in vascular endothelial cells (EC-KO). We found that local endothelial genomic instability causes macrovascular and microvascular vasodilator dysfunction due to specific loss of endothelium-dependent NO signaling, i.e., not endothelium-dependent hyperpolarization (EDH) or prostaglandins. Endothelium-independent vasodilation was not affected (Figure 1C). The preserved endothelial cell layer, as confirmed by scanning electron microscopy, normal von Willebrand factor levels and normal EDH excludes the loss of NO through reduced EC numbers. The reduction of vasodilator response was only associated with a temporary blood pressure increase.

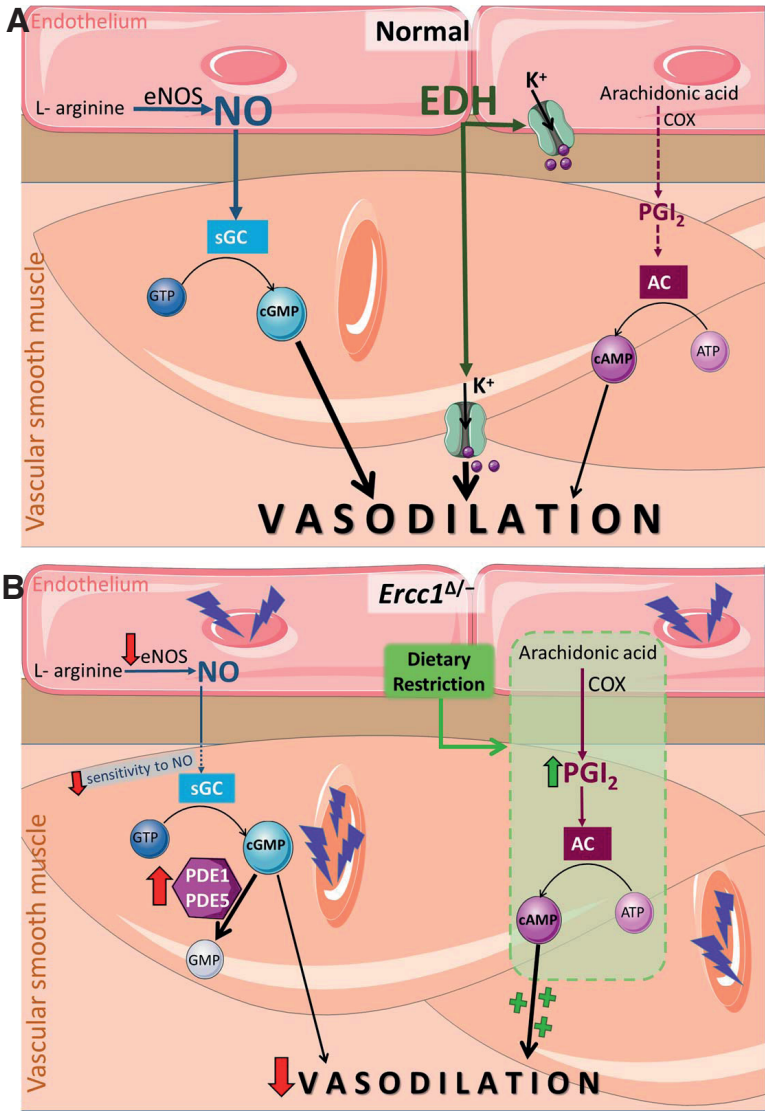

Figure 1. Summary of our findings on vasodilation in mouse models of vascular aging.
In normal conditions NO and EDH mediate most of the endothelium-dependent vasodilation. Contribution of prostaglandins varies depending on vessel type (A). In *Ercc1*$^{\Delta/-}$ mice NO signalling is impaired due to decreased eNOS expression and increased metabolism of cGMP by PDE1 and 5. Dietary restriction improves vasodilation in *Ercc1*$^{\Delta/-}$ mice by recruiting prostaglandins (B). When DNA-damage is restricted to endothelial cells NO signalling is impaired while EDH is unaffected (C). When DNA damage is restricted to smooth muscle NO contribution to endothelium-dependent vasodilation and NO sensitivity in VSMC is decreased. This is compensated by EDH and prostaglandins (D).
The weight of the arrows are indicative of the contribution of that pathway. AC, adenylyl cyclase; COX, cyclooxigenase; EDH, endothelium-derived hyperpolarization; eNOS, endothelial NO synthase; PDE, phosphodiesterase; PGI2, prostaglandin I2 or prostacyclin; sGC, soluble guanylyl cyclase.

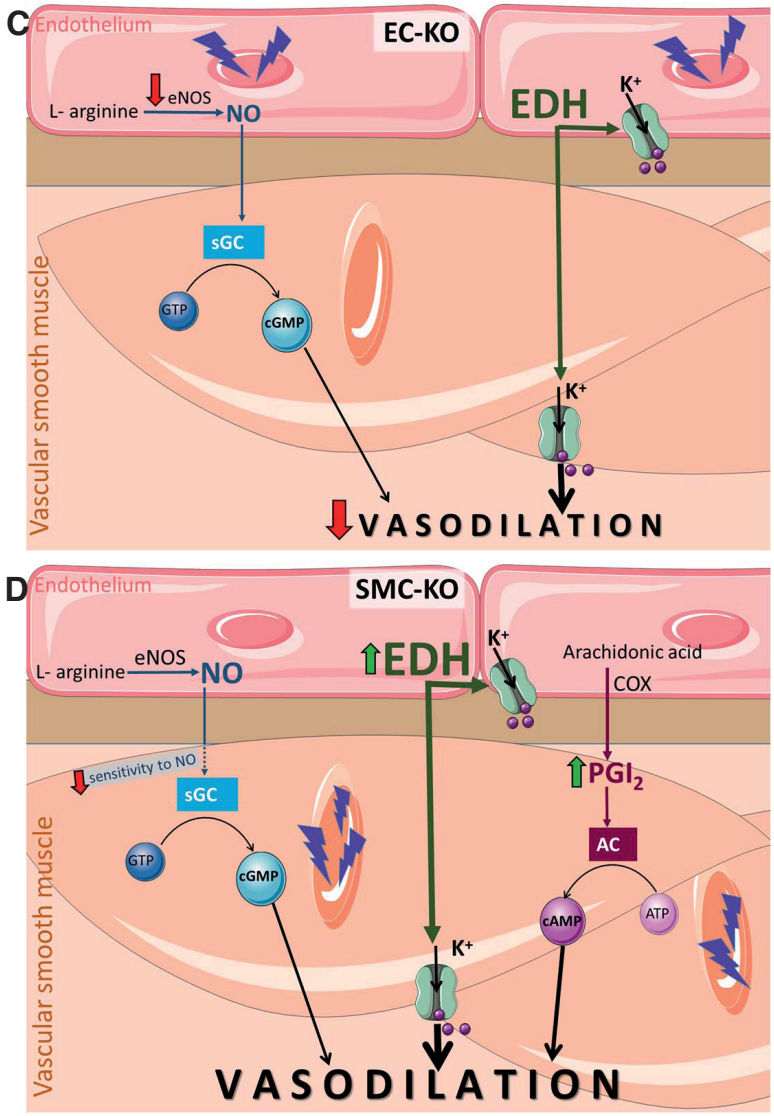

Figure 1. (continued)

Apart from vasodilator dysfunction, EC-KO mice showed a severely compromised microvascular barrier function in the kidney, potentially leading to premature death. Furthermore, cardiac output was affected at 5 months of age and aortic distensibility was reduced, suggesting increased vascular stiffness.

In **Chapter 4** we studied the effects of local DNA damage in VSMC in a mouse model with specific deletion of the *Zmpste24* gene (SMC-KO). This causes accumulation of prelamin A, leading to impaired recruitment of DNA damage repair proteins and check-

point activation, and thus results in defective DNA damage repair, apoptosis and cell senescence.[5-7] The findings in SMC-KO mice contrast with those in EC-KO mice. EC-KO mice showed a specific decrease of endothelium-dependent NO-mediated relaxation, whereas SMC-KO mice display a specific decrease of endothelium-independent NO-mediated relaxation. EC-KO mice do not compensate for the loss of acetylcholine (ACh)-induced dilation, whereas SMC-KO mice do, namely by increasing prostaglandin-induced dilatation and recruiting EDH (Figure 1D). Another striking finding is that EC-KO mice do not show increased carotid stiffness, whilst SMC-KO mice do. It may be that endothelial dysfunction does not lead so swiftly to increased stiffness as VSMC dysfunction. The observations in SMC-KO mice are in agreement with observations in *Ercc1*$^{\Delta/-}$ mice, and suggest that DNA damage in VSMC contributes to the increase of vascular stiffness that is seen in aging. Thus, both DNA damage in EC and VSMC, in what seems to be a cell-autonomous way, lead to specific changes that are relevant for aging-related vascular disease. This answers an important question remaining from our previous study in the *Ercc1*$^{\Delta/-}$ mouse model, showing that NO signaling was disturbed both in EC and VSMC.

In **Chapter 5** we examined whether the vasodilator dysfunction present in *Ercc1*$^{\Delta/-}$ mice could be prevented with dietary restriction (DR) or chronic blockade of the angiotensin (Ang) II type 1 (AT_1) receptor with losartan. DR improved vasodilation up to an age of 30 weeks by enabling aortic tissue to recruit endothelium-derived vasodilatory prostaglandins, which are normally absent (Figure 1B). A possible explanation for the emerging prostaglandin response is the increase in COX-2, combined with a decrease in PDE4B, which together should lead to improved vasodilator cAMP signaling. In addition, DR improved the responsiveness of VSMC to NO. Chronic losartan treatment reversed the enhanced Ang II-induced vasoconstriction in *Ercc1*$^{\Delta/-}$ mice. This reduction could not be explained by physiological antagonism through effects on endothelial function, since the endothelium-dependent vasodilation was not improved by losartan. Also, blood pressure itself is not a driving mechanism in the observed vasodilator dysfunction, since blood pressure-lowering did not affect this dysfunction.

As shown in Chapters 3, 4 and 5, in our aging mouse models the eNOS-NO-cGMP pathway is specifically affected, and under dietary restriction its impaired signaling is compensated for by prostaglandins. Disturbed eNOS-NO-cGMP signaling is observed not only in aging, but also in essential hypertension and hypercholesterolemia.[8] Although several available therapies increase eNOS-NO-cGMP signaling, none of them are currently indicated for the treatment of systemic hypertension. Therefore, interventions that improve EDH signaling could have great therapeutic relevance not only for hypertension but also in other conditions associated with impaired endothelial function. In **Chapter 6** we discuss findings from a recent study presenting proof of principle for a new class of antihypertensive drugs.[9] Although protein kinase G Iα (PKGIα) mediates the vasodilatory effects induced by NO, it has been suggested that other EDH factors, such as

H_2O_2, can also activate PKGIα by inducing the formation of a disulfide bond between the cysteine 42 residues of 2 adjacent chains in PKGIα homodimers in a cGMP-independent manner.[10] The candidate lead compound discovered in the discussed study targets EDH based on the mechanism of PKGIα activation. Because it is unknown whether all EDHs can activate PKGIα, in **Chapter 7** we investigated the contribution of PKGIα to NO- and EDH-induced vasodilation in porcine coronary arteries (PCAs). Our results suggest that bradykinin- and light-induced relaxation of PCAs depends, at least in part, on oxidized PKGIα, and this does not involve sGC. H_2O_2 also acts via oxidized PKGIα in a sGC-independent manner. Yet, *S*-nitrosothiol-induced relaxation is PKGIα-independent. Clearly therefore, PKGIα activation does not contribute universally to all EDH responses, and targeting PKGIα may only mimick EDH under certain conditions.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Utility of the mouse models of vascular aging and how DNA damage leads to vascular aging

The present results in cell-specific models also open novel opportunities to develop more accurate disease models. In general it is hard to include aging in rodent models because of the costs and/or an incomplete human-like aging phenotype in old wild type (WT) animals. The introduction of DNA repair mutants such as *Ercc1*$^{\Delta/-}$ mice have led to opportunities to explore aging and anti-aging interventions very rapidly.[3, 11] A potential limitation of these models for cardiovascular research is their relatively more prominent neurodegenerative phenotype, general frailty and small size, which will lead to premature death and restrictions to surgical interventions and organ size. Cell type-specific models provide a solution to a certain extent, and from the results in this thesis interesting opportunities are generated. For example, atherosclerosis, another important feature of vascular aging, is difficult to investigate on an aged background.[12, 13] Cell type-specific knockout models have already generated useful knowledge on the involvement of DNA damage response proteins in plaque progression and vulnerability.[14] Our present models might help to assess if DNA damage itself is involved in plaque formation. Especially the role of PDE1, which is specifically increased in plaques, and the potential of PDE1 inhibitors as a new class of medicines against aging-related cardiovascular diseases can be investigated properly in *Ercc1*-mutant mice. The role of PDE1 in SMC-KO mice still needs to be addressed.

Also in research regarding the effect of aging-related end-organ function our present models offer interesting possibilities. Indeed, the models show very typical vascular aging features encountered in humans, thus demonstrating their potential clinical relevance. The strong renal phenotype of EC-KO, possibly even leading to mortality,

might give novel insight in the alleged role of the DNA damage response in renal function.[15, 16] This topic remains virtually unexplored despite the general awareness that the kidney strongly suffers from the aging process.[17] Regarding cardiac disease, EC-KO mice showed lowered cardiac output, but normal ejection fraction (EF), whereas SMC-KO mice showed lowered EF. Thus, the models might respectively represent the strongly aging-related heart failure with preserved EF vs. heart failure with reduced EF. At the point of changes in cardiac function we cannot exclude that these changes, especially in EC-KO, are benign adaptations. More long-lived models might provide answers. To this end, instead of *Ercc1*$^{f/-}$ or *Zmpste24*$^{f/-}$, a milder model might be created, e.g. by making use of *Ercc1*$^{f/\Delta}$ mice.

A central question that remains is how DNA damage leads to features of aging. An important consideration for solving this question was unveiled in this thesis: the *effects* of DNA damage that can also be found in aging human vasculature are generated locally within the affected vascular cells (despite the fact that the harmful agents, e.g. inflammatory cells producing free radicals, might be acting systemically). Why is this important? The answer lies in the approach that is needed to explore how the moment of DNA damage connects to the moment when vasomotor control derails.

Parts of the puzzle are in place: in EC eNOS expression and its ACh-induced phosphorylation decrease with aging. Decreased Ca^{2+} responsiveness in EC as a possible cause should be explored as a potential cause of this phenomenon. In VSMC PDE1 is increased, hampering cGMP signaling. The role of PDE1 and the potential of PDE1 inhibitors as medicines in aging-related vascular disease can now be further elucidated. EDH might compensate for loss of NO signaling, and the question is then whether EDH acts through disulfide bridging of PKGI subunits. This is of clinical relevance now that novel drugs that activate this pathway are becoming available. Clearly, these drugs need to be tested under conditions of advanced aging.

A more appropriate approach might be to start treatment before vasomotor dysfunction develops. This requires a detailed insight in the chain of events that cause vascular aging. Here age-/time-resolved experiments might be of help. Candidate signal analysis as well as hypothesis-free methods are options, and the choice between these two is mostly a matter of personal taste of individual scientists working on this problem. Whatever the choice may be, it is of great importance that this thesis demonstrates that one has to look at vascular cell-specific changes. Hence, the use of aging WT or whole body DNA repair mutant mice in all likelihood will not be very efficient since it allows noise of unspecific signals. EC- and VSMC-specific models appear to be a much more effective tool. Since DNA damage effects are very local, even cell culture models might help. Especially 3D models have a high potential. Endothelial cells in such models better sustain their in vivo properties.[18] When using DNA repair-defective vascular cells in such setups, local cellular processes generated by DNA damage will not be disturbed

by systemic signals. Conversely, one could expose healthy young cells to serum of aged individuals. Thus, this approach allows a thorough dissection of the contribution of local DNA damage vs. external harmful signals that may act through DNA damage or not. Another strong advantage is that one could use human cells in this setting, allowing immediate translation. A limitation is the fact that only microvascular function can be addressed.

Angiotensin II blockade in mouse models of vascular aging

Ang II responses were not significantly affected in EC-KO or SMC-KO mice, although in SMC-KO mice they showed an unexpected (but non-significant) tendency to be lower. In contrast, when DNA repair is undermined in both cell types, such as in *Ercc1*$^{\Delta/-}$ mice, Ang II-induced constrictions were upregulated (Chapter 5).[19] Despite this paradox, both in *Ercc1*$^{\Delta/-}$ and SMC-KO mice the opposite function of AT_1 vs. Ang II type 2 (AT_2) receptors appeared to be lost. AT_2 receptors can be present both on EC and VSMC where they respectively fulfill a dilatory vs. an AT_1 receptor-inhibiting function, which is lost in disease conditions.[19, 20] The AT_2 receptor might even invert its function to one supporting constriction. How this occurs is not completely understood. Our results now suggest that at least DNA damage in the VSMC might prompt this change, providing a tool for further study, perhaps even in cell culture conditions in which DNA damage can be easily applied. Why this loss of AT_2 receptor activity does not lead to increased Ang II contractile responses in the present study is not clear. The strong loss of Ca^{2+} responses in VSMC leading to very low Ang II constrictions and the still present endothelial compensatory function might be responsible. If the latter is the case, only DNA damage in both EC and VSMC, such as occurs in *Ercc1*$^{\Delta/-}$ mice, would be sufficient to increase Ang II-induced constriction. The Ang II responses being very low in the SMC-KO, however, call for reservations in the interpretation of the current results. Further exploration in mice lacking DNA repair in both EC and VSMC might provide a conclusive answer.

Importantly, we were able to prevent the increase of Ang II constrictions in *Ercc1*$^{\Delta/-}$ mice by chronic treatment with AT_1 receptor antagonist losartan (Chapter 5). In these studies, the role of AT_2 receptors was unfortunately not assessed.[19] AT_1 receptors have been detected in the endothelium of some vessel types. In such vessels it is coupled to phospholipase C and phospholipase A signaling,[21] which might result in NO and prostaglandin release. EC can also contain NADPH oxidase (Nox) 1 and Nox 2, which are known to be activated by AT_1 receptors. ROS thus produced by Ang II can cause DNA damage, as was shown in VSMC.[22, 23] Since chronic losartan treatment did not prevent endothelial dysfunction in aortic tissue in *Ercc1*$^{\Delta/-}$ mice we must conclude that in the aortic endothelium Nox coupled to AT_1 receptors does not appreciably contribute to DNA damage in vivo. Responses of VSMC to exogenously given NO, however, were improved by losartan. The coupling of Nox to AT_1 receptors in VSMC is well known and ubiquitously present

throughout the vasculature.[24] It would therefore be interesting to test the effect of chronic losartan on DNA damage and vasodilator dysfunction in SMC-KO mice. Whether in EC in vivo Nox coupled to Ang II simply has no role of importance or whether it is a matter of the explored vessel type remains the question. The first option could however explain why RAS blockade may not be effective in some diseases, such as HFpEF, which is strongly dependent on coronary artery endothelial function.[25] This question could be addressed by submitting EC-KO mice to myocardial infarction or pressure overload and apply chronic losartan treatment, followed by measurement of coronary perfusion and coronary artery endothelial function.

Our observations suggest that effects of local DNA damage in EC and VSMC are likely to be cell-autonomous. Moreover, the effects display specificity towards Ang II since ET-1 signaling was not altered. Hence it is unlikely that DNA damage effects are simply due to the observed loss of medial cells or general transcriptional problems. The findings regarding changes in Ang II receptor signaling and resistance to losartan treatment lead to potential clinical implications. Therefore, markers of DNA damage could help to discern the patients that will benefit from Ang II blockade. Given the variability in treatment response to such intervention and the relentless but until now fruitless efforts of scientists to find markers identifying therapy-resistant patients,[26] exploration of this possibility is recommendable. Prelamin A levels, telomere length, genetic variations in DNA damage response genes and senescence markers are candidate markers for such studies.

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DUTCH SUMMARY

Leeftijd blijft de grootste onafhankelijke risicofactor voor alle belangrijke levensbedreigende ziekten, zoals cardiovasculaire en neurologische aandoeningen. Terwijl de levensduur de afgelopen decennia geleidelijk is gestegen, is de gezondheid niet evenredig en in hetzelfde tempo meegegroeid.[1] Veroudering werd altijd beschouwd als een onveranderbaar proces, maar de afgelopen 10 jaar is gebleken dat er een complex, gereguleerd biologisch proces aan ten grondslag ligt en dat DNA-schade een voorname drijvende kracht achter dit proces is.[2] Het bewijs voor de rol van DNA-schade in leeftijdsafhankelijke vasculaire disfunctie in de algemene populatie en in muismodellen, wordt besproken in **hoofdstuk 1**.

Muizen met een defect in hun DNA reparatie zijn een uitstekend onderzoeksmodel, omdat zij snel de kenmerken van menselijke veroudering vertonen.[3] Het is bekend dat in een muismodel met genetische instabiliteit door een defect DNA-herstel (*Ercc1*$^{\Delta/-}$ muizen), leeftijd-gerelateerde vasculaire disfunctie versneld optreedt, hetgeen gekarakteriseerd wordt door een afname in de NO afgifte door het endotheel, een afname van de NO/cGMP respons in de laag met vasculaire gladde spiercellen (VSMC), en een toename van celveroudering daarin.[4] In **hoofdstuk 2** toonden we aan dat een verhoogd metabolisme van cGMP door fosfodiesterasen (PDE) verantwoordelijk is voor de verslechterde NO/cGMP signalering in VSMC van *Ercc1*$^{\Delta/-}$ muizen (Figuur 1B), omdat PDE-remming de endotheel-onafhankelijke bloedvatverwijding volledig herstelde naar hetzelfde niveau als bij WT muizen. Onze data suggereert ook dat in *Ercc1*$^{\Delta/-}$ muizen de bijdrage van PDE1 in verhouding tot PDE5 toegenomen is. Men ziet hetzelfde in humane verouderende VSMCs. In onze kandidaatgenonderzoek met gebruik van GWAS data werden bovendien verbanden tussen single nucleotide polymorfismen in het PDE1A-gen en een verhoogde diastolische bloeddruk alsmede de wanddikte van de halsslagader, twee kenmerken van humane leeftijd-gerelateerde vasculaire disfunctie. PDE1 wordt ook in verband gebracht met aderverkalking en ongunstige ventrikelremodelering. Zodoende speelt PDE1 een centrale rol in leeftijdgerelateerde hart- en vaatziekten.

Ondanks eerdere bevindingen en het bewijs dat in hoofdstuk 2 gepresenteerd wordt, blijft er één belangrijke vraag onbeantwoord: komt de versnelde disfunctie in de bloedvatverwijding in *Ercc1*$^{\Delta/-}$ muizen voort uit defectieve lokale vasculaire DNA-reparatie, of is het een gevolg van de systemische verslechtering waar deze muizen aan lijden? Om deze vraag te beantwoorden, is de cardiovasculaire functie bestudeerd in muismodellen met een defect in de DNA-reparatie in vasculaire endotheelcellen of in gladde spiercellen. In **hoofdstuk 3** bestudeerden we dit in een muismodel met een specifieke verwijdering van *Ercc1* in vasculaire endotheleelcellen (EC-KO). We vonden dat lokale DNA schade in het endotheel tot gevolg heeft en dat er macrovasculaire en microvasculaire vaatverwijdingsdisfunctie optreedt als gevolg van het specifieke gemis

van endotheel-afhankelijke NO signalering, dat wil zeggen, er was geen effect op endotheel-afhankelijke hyperpolarisatie (EDH) of prostaglandines. Dit had geen effect op de endotheel-onafhankelijke vaatverwijding (Figuur 1C). De behouden endotheelcellaag, gecontroleerd door middel van scanning elektronenmicroscopie, bevat normale waarden van het transcript voor de Von Willebrand factor en normale EDH, hetgeen het verlies van NO door verminderde EC aantallen uitsluit. De verminderde vaatverwijdingsrespons was geassocieerd met een tijdelijke verhoging van de bloeddruk. Naast een slechtere vaatverwijdingsfunctie vertoonden EC-KO muizen een ernstig beschadigde microvasculaire barrièrefunctie in de nieren, wat mogelijk kan leiden tot een voortijdige dood. Bovendien was op de leeftijd van 5 maanden de pompfunctie van het hart aangetast en de elasticiteit van de aorta verminderd, wat duidt op een verhoogde vaatstijfheid. Zodoende is lokale endotheliale DNA schade voldoende om te leiden tot verschillende vasculaire afwijkingen die ook waarneembaar zijn bij veroudering.

In **hoofdstuk 4** bestudeerden we de effecten van lokale DNA schade in VSMC in een muismodel met een specifieke verwijdering van het *Zmpste24* gen in deze cellen (SMC-KO). Dit veroorzaakte een ophoping van prelamine A, wat leidde tot een verslechterde DNA schade reparatie, apoptose en celveroudering.[5-7] De bevindingen in SMC-KO muizen komen niet overeen met die in EC-KO muizen. EC-KO muizen vertoonden een specifieke afname van endotheel-afhankelijk NO-gemedieerde relaxatie, terwijl SMC-KO muizen een specifieke afname van endotheel-onafhankelijk NO-gemedieerde relaxatie vertonen. Dit verklaart waarom in onze recente studie met het *Ercc1*$^{\Delta/-}$ muismodel zowel de NO signalering vanuit EC als in VSMC verstoord was, en duiden op een zeer lokaal effect van de DNA schade. EC-KO muizen kunnen niet compenseren voor het verlies van acetylcholine (ACh)-geïnduceerde vaatverwijding, terwijl SMC-KO muizen dit wel doen, namelijk door een verhoging van prostaglandine-geïnduceerde vaatverwijding en werving van EDH (Figuur 1D). Een andere opmerkelijke bevinding is dat EC-KO muizen geen toegenomen stijfheid in de aorta laten zien, terwijl SMC-KO muizen die wel vertonen. Mogelijkerwijs leidt endotheel-disfunctie niet zo snel tot een verhoogde vaatstijfheid als VSMC disfunctie. De observaties in SMC-KO muizen komen overeen met observaties in *Ercc1*$^{\Delta/-}$ muizen, en suggereren dat DNA schade in VSMC bijdraagt aan de toename van vaatstijfheid die gezien wordt bij veroudering. Daarom leidt DNA schade in zowel EC als VSMC, op een ogenschijnlijke cel-autonome wijze, tot specifieke veranderingen die relevant zijn voor verouderings-gerelateerde vasculaire ziekten.

In **hoofdstuk 5** hebben we gekeken of het mogelijk is om de verstoorde vaatverwijdingsfunctie die gezien wordt in *Ercc1*$^{\Delta/-}$ muizen te voorkomen door de toepassing van dieetrestrictie (DR) of door permanente blokkade van de angiotensine (Ang) II type 1 (AT_1) receptor met losartan. DR verbeterde de vaatverwijding tot een leeftijd van 30 weken door weefsel in de halsslagader in staat te stellen om vaatverwijdende prostaglandines uit het endotheel te werven, welke normaal afwezig zijn (Figuur 1B). Een

mogelijke verklaring voor de ontstane prostaglandine respons is de toename in COX-2, gecombineerd met een afname in PDE4B, welke samen zouden moeten leiden tot een verbeterde vaatverwijdende cAMP signalering. Bovendien verbeterde DR de reactie van VSMC op NO. Doorlopende behandeling met losartan zorgde voor het terugdraaien van de verhoogde Ang II-geïnduceerde bloedvatvernauwing in *Ercc1*$^{\Delta/-}$ muizen. Deze afname kon niet verklaard worden door effecten op fysiologisch antagonisme door het endotheel, aangezien de endotheel-afhankelijke vaatverwijding niet verbeterd werd door losartan. Daarnaast is de bloeddruk zelf geen aandrijfmechanisme in de vaatverwijdingsdisfunctie die we zien, aangezien een verlaging van de bloeddruk geen invloed had op deze disfunctie.

Zoals we laten zien in hoofdstuk 3, 4 en 5, is in onze muismodellen voor veroudering de eNOS-NO-cGMP pathway in het bijzonder aangedaan, en bij dieetrestrictie wordt de verstoorde signalering gecompenseerd door prostaglandines. Verstoorde eNOS-NO-cGMP signalering wordt niet alleen bij veroudering gezien, maar ook bij hypertensie zonder aanwijsbare oorzaak en hypercholesterolemie.[8] Alhoewel er meerdere behandelingen voorhanden zijn om de eNOS-NO-cGMP signalering te verbeteren, is geen van deze behandelingen op dit moment geïndiceerd voor de behandeling van systemische hypertensie. Om deze reden zouden interventies die de EDH signalering verbeteren van grote therapeutische waarde zijn, niet alleen voor de behandeling van hypertensie maar ook van andere ziektebeelden waarin een verslechterde endotheelfunctie een rol speelt. In **hoofdstuk 6** bespreken we de bevindingen van een recente studie die de mogelijke bruikbaarheid aantoont voor een nieuwe groep anti-hypertensieve middelen.[9] Hoewel proteïne kinase G Iα (PKGIα) de vaatverwijdende effecten veroorzaakt door NO doorgeeft, wordt er geopperd dat andere EDH factoren, zoals H_2O_2, de PKGIα kunnen activeren door de stimulering van de vorming van een di-sulfideverbinding tussen de cysteïnes op positie 42 van 2 naastgelegen ketens in PKGIα homodimeren op een cGMP-onafhankelijke manier.[10] Het mogelijke kandidaat-geneesmiddel dat in de besproken studie ontdekt is, grijpt aan op de EDH, uitgaande van het mechanisme van PKGIα-activatie. Aangezien het onbekend is of alle EDHs PKGIα kunnen activeren, onderzoeken we in **hoofdstuk 7** de rol van PKGIα in NO- en EDH-geïnduceerde vaatverwijding in de coronair arterie van varkens (PCAs). Onze resultaten suggereren dat bradykinine- en licht-geïnduceerde relaxatie van PCAs, tenminste voor een deel, afhangt van geoxideerd PKGIα, en hier geen GC bij betrokken is. H_2O_2 stimuleert bovendien geoxideerd PKGIα op een sGC-onafhankelijke manier. Maar *S*-nitrosothiol-geïnduceerde relaxatie is PKGIα-onafhankelijk. Daarom is het duidelijk dat PKGIα-activatie niet universeel bijdraagt aan alle EDH responsen, en het aangrijpen op PKGIα alleen onder bepaalde omstandigheden EDH zou kunnen nabootsen.

RESUMEN EN ESPAÑOL

La edad sigue siendo el mayor factor de riesgo independiente para todas las enfermedades que causan más muertes en el mundo, tal como las enfermedades cardiovasculares y neurológicas. Aunque la esperanza de vida ha aumentado constantemente en las últimas décadas, la duración de los años de vida saludables no ha aumentado al mismo ritmo.[1] El envejecimiento solía ser considerado como un proceso no modificable, pero en la década pasada se ha demostrado que es un proceso biológico complejo, altamente regulado y que la acumulación progresiva de daño en el ADN es un factor causal importante.[2] En el **Capítulo 1** se revisó la evidencia, en población general y en modelos murinos, que respalda el rol del daño en el ADN en la disfunción vascular relacionada con la edad**.**

Los ratones con reparación defectuosa del ADN son excelentes herramientas de exploración porque reproducen rápidamente los signos distintivos del envejecimiento humano.[3] Previamente encontramos que en un modelo murino de inestabilidad genómica causada por reparación defectuosa del ADN (ratones *Ercc1*$^{\Delta/-}$), se produce disfunción vascular relacionada con la edad de manera acelerada y ésta se caracteriza por disminución de la capacidad de respuesta de la vía del óxido nítrico/GMP cíclico (NO/cGMP) y aumento de la senescencia celular en la capa de células de músculo liso vascular (VSMC).[4] En el **Capítulo 2** se demostró que en los ratones *Ercc1*$^{\Delta/-}$ el metabolismo aumentado de cGMP por parte de fosfodiesterasas (PDE) es responsable de la reducción de NO/cGMP en VSMC, ya que la inhibición de las PDEs restauró completamente la vasodilatación independiente del endotelio (Figura 1B). Nuestros datos también sugieren que en ratones *Ercc1*$^{\Delta/-}$, la contribución de PDE1 es mayor a la contribución de PDE5. Encontramos hallazgos similares en VSMC senescentes humanas. Además, polimorfismos de un solo nucleótido en el gen *PDE1A* se asocian con el aumento de la presión arterial diastólica y el grosor de la íntima media carotídea, dos características de la disfunción vascular relacionada con la edad en humanos. Varios estudios han reportado que PDE1 está implicada en aterosclerosis y en el remodelado cardiaco maligno. Por lo tanto, PDE1 juega un papel central en la enfermedad cardiovascular relacionada con la edad.

A pesar de los hallazgos previos y la evidencia presentada en el capítulo 2, una pregunta importante que permanece es si la acelerada disfunción vasodilatadora observada en ratones *Ercc1*$^{\Delta/-}$ es causada por la reparación defectuosa del ADN a nivel local en la pared vascular, o si es una consecuencia del deterioro sistémico del que sufren estos ratones. Para responder a esto, se investigó la función cardiovascular en modelos murinos con reparación defectuosa del ADN restringida a células endoteliales vasculares o al músculo liso. En el **Capítulo 3** estudiamos ésto en un modelo murino con pérdida específica de la proteína reparadora de ADN *Ercc1* en células endoteliales vasculares (ratones EC-KO). Encontramos que el daño en el ADN restringido al endotelio

causa disfunción vasodilatadora macro- y microvascular debido a la pérdida específica de la señalización de NO dependiente del endotelio; es decir, las otras vías vasodilatadoras derivadas del endotelio (hiperpolarización dependiente del endotelio(EDH) o prostaglandinas) no son afectadas. De igual manera, la vasodilatación independiente del endotelio no es afectada (Figura 1C). Análisis morfológicos con microscopía electrónica de barrido, niveles normales de factor de von Willebrand y la respuesta normal de la vía EDH confirman que la capa de células endoteliales está preservada; lo que excluye la posibilidad de que la pérdida de NO endotelial se deba a un número menor de células endoteliales. La reducción de la respuesta vasodilatadora sólo se asoció con un aumento temporal de la presión arterial. Aparte de la disfunción vasodilatadora, los ratones EC-KO mostraron una función de barrera microvascular gravemente comprometida en el riñón, que podría ser la causa de muerte prematura en estos ratones. Además, el gasto cardíaco se vio afectado a los 5 meses de edad y la distensibilidad aórtica se redujo, lo que sugiere una mayor rigidez vascular en ratones EC-KO.

En el **Capítulo 4** estudiamos los efectos locales del daño en el ADN en VSMC en un modelo murino con deleción específica del gen *Zmpste24* (ratones SMC-KO). La deleción del gen *Zmpste24* provoca la acumulación de prelamina A, lo que conduce al reclutamiento alterado de las proteínas que reparan el daño en el ADN y la activación defectuosa de los puntos de control en el ciclo de división celular, y por lo tanto, resulta en reparación defectuosa de los daños en el ADN, apoptosis y la senescencia celular.[5-7] Los hallazgos en ratones SMC-KO contrastan con los observados en ratones EC- KO. Los ratones EC-KO mostraron una disminución específica de la relajación mediada por NO dependiente del endotelio, mientras que los ratones SMC-KO muestran una disminución específica de la relajación mediada por NO independiente del endotelio. Los ratones EC-KO no compensan la pérdida de dilatación inducida por acetilcolina (ACh), mientras que los ratones SMC-KO lo hacen, aumentando la dilatación inducida por prostaglandinas y reclutando EDH (Figura 1D). Otro hallazgo sorprendente es que los ratones EC-KO no muestran mayor rigidez carotídea, mientras que los ratones SMC-KO sí. Puede ser que la disfunción endotelial no conduzca tan rápidamente a una mayor rigidez como la disfunción de la capa VSMC. Las observaciones en los ratones SMC-KO están en línea con las observaciones en ratones *Ercc1*$^{\Delta/-}$, y sugieren que el daño en el ADN en VSMC contribuye al aumento de la rigidez vascular que se observa en el envejecimiento. En conclusión, tanto el daño en el ADN en células endoteliales como en VSMCs, en lo que parece ser una manera celular autónoma, conduce a cambios específicos que son relevantes para las enfermedades vasculares relacionadas con el envejecimiento. Esto responde a una pregunta importante que quedaba de nuestro estudio anterior en el modelo de ratones *Ercc1*$^{\Delta/-}$, en donde encontramos que la señalización de la vía NO estaba perturbada tanto en células endoteliales como en VSMCs.

En el **Capítulo 5** se examinó si la disfunción vasodilatadora presente en ratones *Ercc1*$^{\Delta/-}$ podría prevenirse con restricción dietética (DR) o bloqueo crónico (con losartán) del receptor tipo 1 de la angiotensina (Ang) II. La DR mejoró la vasodilatación hasta una edad de 30 semanas permitiendo que la aorta reclutara prostaglandinas vasodilatadoras derivadas del endotelio que normalmente están ausentes (Figura 1B). Una posible explicación de la respuesta emergente a las prostaglandinas es el aumento de la enzima ciclooxigenasa COX-2, combinado con una disminución de la PDE4B, que conjuntamente deberían conducir a una mejora de la señalización vasodilatadora mediada por cAMP. Además, la DR mejoró la respuesta de VSMC a NO. El tratamiento crónico con losartán normalizó la vasoconstricción aumentada en respuesta a Ang II en ratones *Ercc1*$^{\Delta/-}$. Esta normalización no se explica por los efectos de losartán sobre la función endotelial, ya que la vasodilatación dependiente del endotelio no fue mejorada por este tratamiento. Además, la presión arterial en sí misma no es un mecanismo impulsor en la disfunción vasodilatadora observada, ya que en los ratones *Ercc1*$^{\Delta/-}$ la normalización de la presión arterial no afectó a esta disfunción.

Como se muestra en los capítulos 3, 4 y 5, en nuestros modelos murinos de envejecimiento la vía NO/cGMP es afectada específicamente, y bajo DR esta señalización alterada es compensada por prostaglandinas vasodilatadoras. La señalización perturbada de la vía NO/cGMP se observa no sólo en el envejecimiento, sino también en hipertensión esencial y en hipercolesterolemia.[8] Aunque varias terapias disponibles aumentan la respuesta de la vía NO/cGMP, ninguna de ellas está actualmente indicada para el tratamiento de la hipertensión sistémica. Por lo tanto, las intervenciones que mejoren la señalización de otras vías vasodilatadoras derivadas del endotelio, tal como la vía EDH, podrían tener una gran relevancia terapéutica no sólo en hipertensión, sino también en otras condiciones asociadas con alteración de la función endotelial. En el **Capítulo 6** se discuten los hallazgos de un estudio reciente que presenta una prueba de concepto para una nueva clase de fármacos antihipertensivos.[9] Aunque la proteína quinasa G Iα (PKGIα) media los efectos vasodilatadores inducidos por el NO, se ha reportado que otros factores EDH, como el peróxido de hidrogeno (H_2O_2), también pueden activar PKGIα al inducir la formación de un enlace disulfuro entre los residuos de cisteína 42 de dos cadenas adyacentes en homodímeros de PKGIα, de una manera independiente de cGMP.[10] El compuesto candidato descubierto en el estudio discutido, se basa en ese mecanismo de activación de PKGIα. Debido a que se desconoce si todos los EDH pueden activar PKGIα, en el **Capítulo 7** se investigó la contribución de PKGIα a la vasodilatación inducida por NO y EDH en las arterias coronarias porcinas (PCA). Nuestros resultados sugieren que la relajación de las PCAs inducida por bradicinina y por luz halógena depende, al menos en parte, de PKGIα oxidada, y dicha señalización no implica la guanililciclasa soluble. H_2O_2 también actúa vía PKGIα oxidada de una manera independiente de guanililciclasa soluble. Sin embargo, la relajación inducida por S-nitrosotioles es independiente de

PKGIα. Es claro, por lo tanto, que la activación de PKGIα no contribuye universalmente a todas las respuestas de EDH y la activación de PKGIα sólo puede imitar dicha EDH bajo ciertas condiciones.

Appendices

About the author

List of publications

PhD Portfolio

Acknowledgements

ABOUT THE AUTHOR

Paula Katherine Bautista- Niño was born on March 14th 1988 in Bucaramanga, Santander, Colombia. From 2004 until 2008, Paula studied microbiology and clinical laboratory at Universidad Industrial de Santander. In 2008, she obtained the highest national score in the Quality of Higher Education-ECAES test and was distinguished as the best student among all microbiology undergraduates in Colombia. From 2009 she worked as research assistant at the Genetic Study of Complex Diseases Group in Universidad Autonoma de Bucaramanga. In 2010 she was awarded a Young Researchers and Innovators "Virginia Gutiérrez de Pineda" grant.

In the summer of 2012 she came to the Erasmus MC to do the Master of Science on Health Sciences-Epidemiology at the Netherlands Institute for Health Science (NIHES) under the supervision of Prof. Oscar Franco. From 2013-2014 she studied a Doctor of Science in Genetic Epidemiology. After being awarded a grant from the Colombian government (Colciencias) she joined the department of Pharmacology-Internal Medicine to do her PhD under the supervision of Prof. Jan Danser and Dr. Anton Roks in collaboration with Prof. Oscar Franco and Dr. Mohsen Ghanbari from the department of Epidemiology.

LIST OF PUBLICATIONS

Phosphodiesterase 1 regulation is a key mechanism in vascular aging
Bautista Niño PK*, Durik M*, Danser AH, de Vries R, Musterd-Bhaggoe UM, Meima ME, Kavousi M, Ghanbari M, Hoeijmakers JH, O'Donnell CJ, Franceschini N, Janssen GM, De Mey JG, Liu Y, Shanahan CM, Franco OH, Dehghan A, Roks AJ. *Equal contributors
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Development of PKG Iα (Protein Kinase G Iα)-Dimerizing Antihypertensive Drugs: Are We Standing at the Shore of the Rubicon?
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Publications on other topics

Maternal fish consumption, fatty acid levels and angiogenic factors: The Generation R Study
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Voortman T, Vitezova A, Bramer WM, Ars CL, **Bautista PK**, Buitrago-Lopez A, Felix JF, Leermakers ET, Sajjad A, Sedaghat S, Tharner A, Franco OH, van den Hooven EH. *Br J Nutr*. 2015 Feb 14;113(3):383-402.

PHD PORTFOLIO

Name PhD student	Paula Katherine Bautista-Nino
Erasmus MC department:	Internal Medicine, Division of Pharmacology and Vascular medicine and Department of Epidemiology
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotors:	Prof. A.H.J. Danser and Prof. O.H. Franco
Co-promotors:	Dr. A.J.M. Roks and Dr. M. Ghanbari
PhD period:	2014-2017

	Date	ECTS
Master of Science in Health Sciences, Epidemiology (2012-2013)		
Principles of Research in Medicine	2012	0.7
Methods of Public Health Research	2012	0.7
Introduction to Global Public Health	2012	0.7
Primary and Secondary Prevention Research	2012	0.7
Social Epidemiology	2012	0.7
Markers and Prognostic Research	2012	0.7
Study Design	2012	4.3
Biostatistical Methods I: Basic Principles	2012	5.7
Clinical Epidemiology	2012	5.7
Methodologic Topics in Epidemiologic Research	2012	1.4
Courses for the Quantitative Researcher	2012	1.4
Biostatistical Methods II: Classical Regression Models	2012	4.3
Women's Health	2013	0.9
Principles of Epidemiologic Data-analysis	2013	0.7
Maternal and Child Health	2013	0.9
Repeated Measurements in Clinical Studies	2013	1.4
Missing Values in Clinical Research	2013	0.7
Topics in Meta-analysis	2013	0.7
Principles of Genetic Epidemiology	2013	0.7
Genomics in Molecular Medicine	2013	1.4
Genome Wide Association Analysis	2013	1.4
Doctor of Science in Health Sciences, Genetic epidemiology (2013-2014)		
Advances in Genome-Wide Association Studies	2014	1.4
Mendelian Randomization	2014	0.9

	Date	ECTS
Bayesian Statistics	2014	1.4
Logistic Regression	2014	1.4
Advances in Epidemiologic Analysis	2014	0.4
Causal Inference	2014	0.7
History of Epidemiologic Ideas	2014	0.7
Causal Mediation Analysis	2014	0.7
Molecular biology in cardiovascular research	2014	1.5
General academic and research skills		
Laboratory Animal Science	2014	3
Scientific Integrity	2017	0.3
Presentations at conferences and symposia		
COEUR symposium Current Cardiac and Vascular Aging Research at EMC	2014	
Physphar Days, Mastricht	2014	
Science days, Antwerp	2015-2017	
Ageing summit, London	2015	
Spring Day of the Dutch Pharmacological Society (NVF)	2015-2017	
COEUR – MiVaB seminar about cardiac, vascular and renal aging.	2016	
Keynote speaker "Barriers of the Brain in Ageing" meeting. Leiden	2017	
Teaching		
Lecturer at the COEUR Cardiovascular Pharmacology course	2015	
Teaching assistant in the Study Design course of the NIHES master of science in Health Sciences.	2016	
Teaching assistant in practical sessions of the "Pharmacological effects on the autonomic nervous system" course.	2014-2017	

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"As so often happens, the thing left undone tires you most of all, you only feel rested when it has been accomplished." J. Saramago