Vascular Aging
from DNA Damage to Protection

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van DNA schade tot bescherming

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Introduction

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Introduction

The social and medical advancements made over the recent centuries have led to substantial lengthening of life expectancy, allowing aging to become a common feature within human society. This resulted in complex multifactorial diseases becoming the main causes of deaths in developed countries.[1] Prime examples are diseases of the cardiovascular system, which are the leading cause of death in many economically advanced countries as well as in emerging economies. Incidence and prevalence of coronary disease, hypertension, congestive heart failure, and stroke are highly associated with biological age. This association is partly a consequence of the prolonged exposure to risk factors, e.g. an unfavourable lipid profile, smoking and diabetes, during which accumulation of internal damage increases the risk to develop vascular dysfunction and associated diseases. However, as evidenced by several epidemiological studies, aging remains the strongest risk factor for cardiovascular disease (CVD), even after correction for classical cardiovascular risk factors. It is therefore an independent risk factor, whose mechanism of action remains unknown.[2]

The mechanism of aging

Aging is often defined as the random progressive loss of function leading to loss of homeostasis, accompanied by decreasing fertility and increasing mortality with advancing age.[3] It is a complex, multifactorial process, which is not well understood. One important question still to be answered is if the cause of aging is consistent in all cell types, organs and organisms and if therefore one unifying mechanistic explanation of aging can exist. Another problem when addressing aging is that it often results in the manifestation of some age-related diseases and that treatment of such diseases might lead to prolonged life span, although this treatment may not necessarily address the mechanisms behind aging and therefore will not affect the process of aging itself. Understanding and ensuing modulation of the aging process could be a new avenue for preventing age-related diseases and achieving healthy aging.

Several theories of aging have been generated. Among those best supported by evidence so far are the ones that state that damage to macromolecules and to DNA nucleotides in particular, is the driving principle of the decline in organismal function.[4-5] Sources of damage to DNA come in great variety, and include radiation (IR, UV, ionizing), reactive oxygen species and endogenous metabolic products.[6-8] The various types of DNA nucleotide damage affect genomic integrity, causing chromosomal structural defects and mutations. DNA damage may also lead to impairment of transcription, and replication and cause cell death and senescence, and impaired cellular and organ function. Organisms are equipped to deal with these causes of genomic instability by utilizing antioxidant molecules and enzymes, proteins restoring biomolecules to their native conformation (DNA repair) and enzymes degrading non-functional proteins.

An important indicator that DNA damage greatly contributes to aging stems from research in human syndromes of premature aging. In most of these syndromes, the cause can be traced back to a mutation in genes related to DNA repair or to other functions that

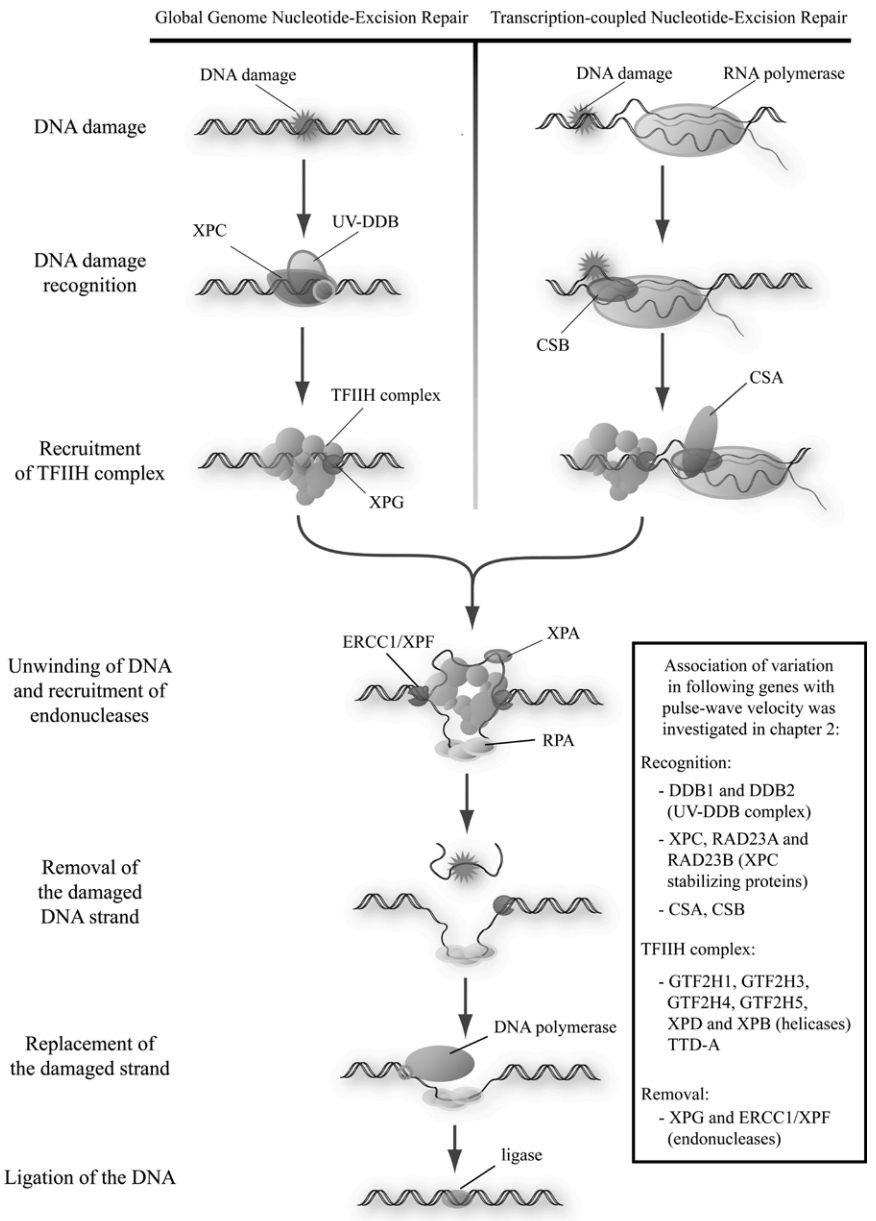

Figure 1. Mechanisms of nucleotide excision repair (NER).

Lesions distorting the shape of DNA double helix are recognized in the global genome NER by XPC protein and the UV-DDB protein dimer. In case of stalled transcription, this damage is recognized by proteins of transcription-coupled NER, CSA and CSB proteins. Recognition is followed by the recruitment of transcription factor IIH complex, opening of the DNA double helix by XPB and XPD helicases, and damage verification by XPA protein. Replication protein A (RPA), DNA binding protein complex, prevents reannealing of the single strand. The damaged strand is excised by ERCC1 /XPF and XPG endonucleases. The excised strand is then reconstituted by the replication machinery and sealed by ligase. This figure is based on a review by Hoeijmakers.[15]

prevent genomic instability.[8] Several mouse models harboring mutations in these genes were generated in recent years. They often exhibit either segmental progeria or increased incidence of cancer. An example of these models are mice with impaired nucleotide excision repair (NER, Figure 1), equivalent to human Cockayne syndrome and Trichothiodystrophy, showing a phenotype of premature aging.[9] Also specific mutations in the LMNA gene, encoding a protein vital for proper function of nuclear lamina, which leads in humans to development of Hutchison-Gilford progeria syndrome with high incidence of cardiovascular disease, was recapitulated in mice showing similar impairments.[10]

Conversely, one can consider the effect of metabolic adaptations that suppress genomic instability in relation to aging. A well-known example is caloric restriction, which in mammals leads to an increase in life span and was show to impede the development of cardiovascular disease in rodents.[11] Also mutations in genes leading to reduced levels of growth hormone, like in the case of Snell and Ames mice, can lead to an increase of life span.[12-13] These interventions influence a wide variety of metabolic changes, but it is considered that reduced metabolism leads to lower production of reactive oxygen species and other damaging metabolites, which in turn reduces damage to macromolecules, including DNA. Similarly when organisms have to deal with increased damage of DNA, they tend to react with a survival-response on the level of the whole organism, which shares many elements with longevity-regulating pathways and essentially shifts utilization of organismal resources from growth to maintenance, thereby greatly influencing the phenotype of the organism. Microarray expression profiles seen in NER impaired mice also show a reduced somatotropic axis. These mice have suppressed growth, metabolism and energy expenditure and also show upregulated antioxidant mechanisms.[14-15]

The aging of the cardiovascular system

The vasculature of healthy individuals undergoes several important changes with increasing age, which increase the chance to develop cardiovascular disease.

One of the most prominent traits is wall thickening and dilation in large elastic arteries, for example carotid intimal media (IM) thickness increases 2-3-fold between 20th and 90th year of age.[2] Excessive IM thickening indicates undetected atherosclerosis and IM thickness is predictive of future clinical cardiovascular disease events[16]

Another deleterious vascular change, highly associated with age, is vascular stiffening. The elasticity of the central conduit vasculature is often conveniently expressed as pulse wave velocity (PWV), the longitudinal propagation velocity over the carotid artery of a wave induced by a systolic pulse as measured with non-invasive techniques in humans. PWV increases with increasing stiffness of the vasculature and blood pressure.[17] PWV is increasing with age in healthy individuals, but diabetes or atherosclerosis cause further stiffening of the vessels.[18] These vascular changes have an impact on blood pressure. Systolic blood pressure is steadily increasing with increasing age of the population as a result of reduced vascular elasticity and compliance of conductive vessels.[19]

Role of endothelium in cardiovascular disease

It is speculated that the vascular blood pressure changes that emerge during aging are largely caused by endothelium-dependent mechanisms. This is probably due to the variety of functions endothelium provides. Besides the regulation of vasodilation (or vasoconstriction) endothelium fulfills an important barrier function, regulating the migration of blood elements and supplying underlying tissues with nutrients and regulating the fluid balance.[20] Endothelium reacts to many stimuli and adopts features which help to maintain the homeostasis. In response to inflammatory stimuli the endothelium increases expression of adhesion molecules and becomes more permeable. Moreover, as inflammation leads to increased generation of reactive oxygen species, it will also put the endothelium under constant stress.[21] Another important role of endothelium is its antifibrotic activity, which when disrupted can lead to clotting of blood elements.

In humans after 60 years of age, these physiological functions of the endothelium are clearly reduced.[2] As such, reduced endothelial function adds to this by increasing the tone, proliferation and migration of the smooth muscle in vasculature, leading to wall thickening, increased stiffness and increase blood pressure. Reduction of the endothelial function has also been observed to occur early on in the pathophysiology of atherosclerosis, and is prominently present in diabetes, an important risk factor for accelerated vascular aging.

It is theorized, that when endothelium gets damaged, either by stress related to inflammation, or in the progress of aging, the dysfunctional endothelial cells need to be replaced by circulating endothelial progenitor cells (EPC), to restore the function of the tissue. However, the continuous stress caused by endothelial damage and repair, along with CV risk factors, may detrimentally impact on the EPC.[22] Also during aging, the number and functionality of EPC is reduced[23-24] thus further contributing to the development of cardiovascular disease.[25]

Possible treatments of age-related deterioration of cardiovascular disease

As DNA damage leads to the deterioration of cellular and consequently tissue function, it can be assumed that preventing this damage, or improving its repair would be a useful intervention. Indeed it has been observed, that atherosclerotic plaques show increased levels of DNA damage of various types.[26-27] Widely used treatments in the cases of atherosclerosis are statins, which were also observed to reduce DNA damage in vivo and in vitro, to suppress senescence and to increase DNA repair.[28] This could further contribute to treatment of atherosclerosis, besides improving the lipid profile of the patient.

Reduction of DNA damage is also a property of a dipeptidyl-peptidase 4 inhibitor vildagliptin. This compound is used in treatment of diabetes mellitus type 2 and besides improving insulin release, it was shown to inhibit vascular cells senescence induced by reactive oxygen species. This was thought to be achieved via induction of antioxidant genes.[29]

Lifestyle differences can cause differential exposure to cardiovascular risk factors, but specific dietary habits can affect the aging of vasculature directly. Such a case is consumption of red wine, which has been shown to reduce incidence of cardiovascular

disease. This is partly mediated via flavonoids contained in red wine.[30] These compounds exhibit antioxidant effects,[31] increase activity of eNOS, antagonize endothelial dysfunction,[32] and improve VSMC sensitivity to NO via effect on soluble guanylate cyclase (sGC).[33]

Another potentially interesting agent, which was shown to reduce DNA damage after an oxidative insult, is bradykinin.[34] This endogenous peptide is degraded by angiotensin converting enzyme (ACE), and therefore its levels are importantly affected by ACE inhibitors (ACEi). Bradykinin acts on two receptors, the bradykinin type 1 and the bradykinin type 2 receptor, and as of yet, it is not clear if both of these receptors mediate its beneficial effects. This leaves space for the discovery of more selective DNA protecting agents.

Renin-angiotensin system

In relation to age-related cardiovascular problems the renin-angiotensin system (RAS) might harbour several interventional target molecules apart from the already mentioned ACE. The RAS importantly regulates the fate of the cardiovascular system. This hormonal system is involved in many physiological and pathophysiological processes, though its main action is considered to be regulation of blood pressure and fluid balance. The RAS is initiated by renin cleaving angiotensin (Ang) I from angiotensinogen. This is further converted by ACE into Ang II, which can exert its action through two receptors - Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors.

This system was originally conceived as a circulating system, but now it is widely accepted, that angiotensin is also generated at the tissue level.[35] This can be in close connection to, or quite independent from the circulating RAS. It was found in several organs, like heart, kidney, brain, pancreas and adipose tissue, which indicates that RAS-mediated effects are beyond mere regulation of vascular tone and fluid reabsorption. Based on its vasoconstrictive, profibrotic, prothrombotic, proinflammatory, antidiuretic and hypertrophic effects, mediated via AT_1 receptors in cardiovascular and renal tissues, the Ang II / AT_1 receptor axis has been implicated in hypertension, atherosclerosis, heart failure, stroke and renal failure.[36] Ang II / AT_1 receptors have also been implicated in aging of vascular smooth muscle cells (through DNA damage[37]) and of endothelial progenitor cells.[38] Although this possibility has not been investigated properly it is conceivable that these characteristics importantly contribute to vascular aging.

The action of AT_2 receptors in cardiovascular pathologies seems to be opposing the actions of AT_1 receptors, and AT_2 receptor activation was shown to cause vasodilation, natriuresis and aldosterone release. Also a part of the beneficial effects of AT_1 receptor blockade (ARB) could be ascribed to AT_2 receptor activation, as the elevated levels of Ang II during such treatment can bind to the non-blocked AT_2 receptor only.

It was hypothesized, that treatment with AT_2 agonist could have a further beneficial effect in cases of cardiovascular disease and therefore a search for specific AT_2 agonist began, leading to development of compound 21 (C21), the first selective, nonpeptide AT_2 agonist. This compound does not show a clear blood pressure lowering effect, however it

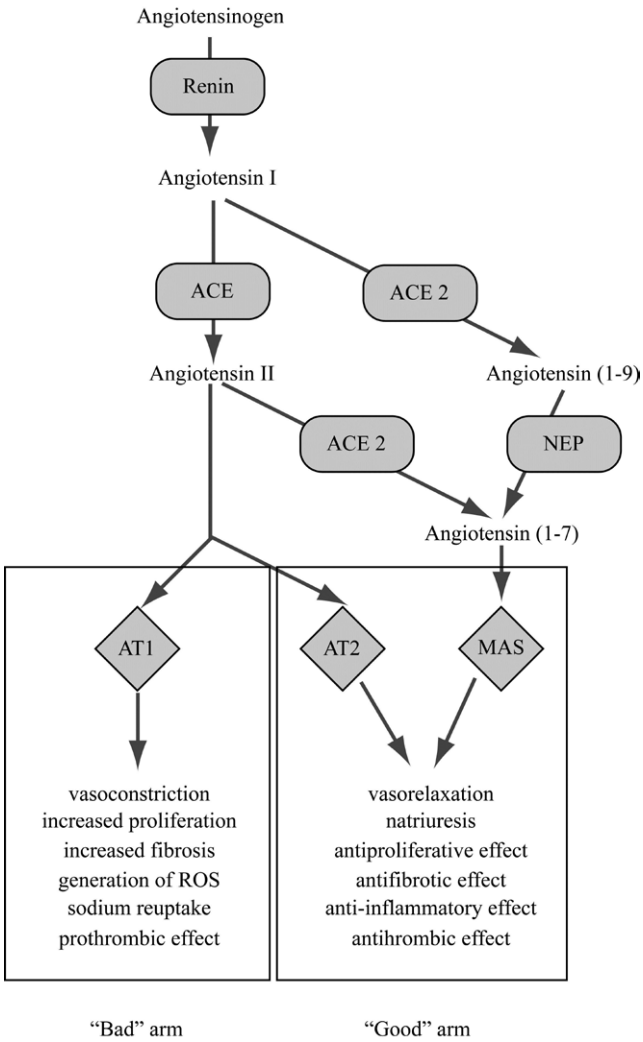

Figure 2. Dual effects of renin-angiotensin system in CVD.

Originally, the concept of RAS constituted the cascade leading to stimulation of angiotensine II type 1 (AT1) receptor. Overstimulation of these receptors causes a set of harmful effects aggravating cardiovascular disease. After the discovery of angiotensin-converting enzyme 2 (ACE2), Angiotensine II type 2 (AT2) and Mas receptor, the beneficial effects of the RAS system were uncovered. Balance between the activity of this "bad and good" arm of RAS can determine the progression of CVD pathology. This scheme doesn't containt other potentially important components in this signaling pathway (e. g. chymase, cathepsin C) whose exact role in pathology remains to elucidated.

was shown to have organ protecting effects. C21 reduced vascular injury and myocardial fibrosis, renal and neuronal damage in stroke-prone hypertensive rats, an animal model of hypertension.[39]

Within the last two decades, novel components of the RAS have been discovered, in particular angiotensin-(1-7) (Ang-(1-7)). Ang-(1-7) is mainly synthesized by angiotensin-converting enzyme 2 (ACE2) from Ang II, is degraded by ACE, and interacts with the G-protein-coupled receptor Mas. The axis formed by ACE2/Ang-(1–7)/Mas constitutes an endogenous counterregulatory pathway within the RAS, opposing the vasoconstrictor/proliferative arm of the RAS constituted by the ACE/Ang II/AT_1 receptor-dependent antifibrotic, antiproliferative, antihypertrophic and vasodilator actions.[40-41] AT_2 receptors

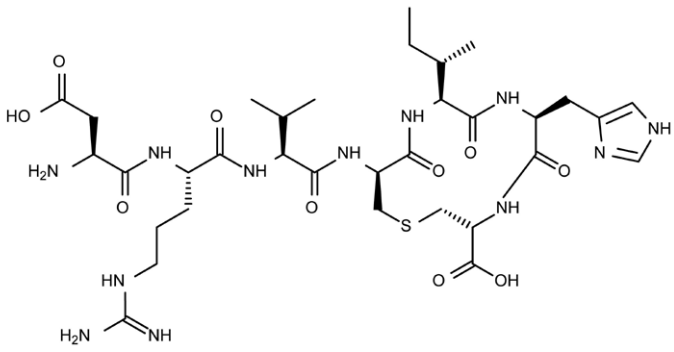

Figure 3. Cyclic angiotensin-(1-7).
The introduction of the thioether bridge between amino acid number 4 and 7 stabilizes the peptide against hydrolysis and abolishes its affinity to AT_1 receptors.

might also mediate various effects of Ang-(1-7).[42] (Figure 2)

Ang-(1-7) is implicated in affecting cardiac remodeling, as classical treatment with ACEi, ARB and aldosterone receptor blockers increases ACE2 activity and expression in the heart.[59] Also using animal models, it was shown that absence of ACE2 exacerbates cardiac hypertrophy and fibrosis and susceptibility to myocardial infarction (MI) development under different challenges, while ACE2 overexpression attenuates these changes in certain experimental models.[43] Ang-(1-7) treatment managed to improve coronary perfusion and cardiac function after MI and ischemia reperfusion injury[44-45] and exhibited antihypertophic and antifibrotic actions in the model of Ang II infusion.[46]

Ang-(1-7) was observed to play a role in establishment and progression of pulmonary diseases. Activation of this RAS axis brought improvement of pulmonary hypertension, fibrosis and even lung cancer. An important feature of the lung circulation is abundant expression of ACE2, which is now investigated as a target of XNT, an activator of ACE2. This approach could help to specifically treat pulmonary hypertension, or fibrosis without inducing the side effects seen with ACEi or ARB treatment.[43]

In the vasculature, the effects of Ang-(1-7) are directly opposing important changes seen during aging, e.g. it promotes vasorelaxation, it reduces oxidative stress and it exerts antiproliferative effects. These phenomena are mediated via the Mas receptors and not seen in Mas knockout mice[47] or when using the specific antagonist A-779.[48] Although Ang-(1-7) shows a high selectivity for the Mas receptor, it was shown to also interact with ACE, AT_1 and AT_2 receptors,[49] possibly causing negative effects at higher concentrations. Together with the short half-life this limits the usefulness of Ang-(1-7) use as a therapeutic drug. Therefore a derivate of Ang-(1-7) was developed, in which a thioether bridge was introduced into the structure (cAng-(1-7)), making it resistant to proteolytic degradation. (Figure 3) This modification is also causing a change in spatial orientation of amino abolishing the affinity of the compound for AT_1 and AT_2 receptors.

Endothelial progenitor cells and cardiovascular system repair

As stem and progenitor cells, like EPC, are implied in tissue regeneration, in particular of the vascular bed and the myocardium,[50-52] they might play an important role in aging and diseases of the cardiovascular system. This could be done either by physically replacing dysfunctional cells in the endothelial layer, or by exerting beneficial paracrine effects on the neighbouring cells.[53] The effect of the RAS on EPC has therefore been investigated. Ang II increases recruitment of EPC via AT_1 receptor stimulation, but longterm seems to have deleterious effects on proliferation and function.[54-55] Ang-(1-7) on the other hand seems to increase the levels of endothelial progenitor cells via its Mas receptor, without the deleterious long-term effects.[56]

Taken together these observations indicate that RAS modulation has a potential supportive role in regenerative treatments using stem cells of various origins

Aim of the thesis

DNA damage and repair thereof is regarded to play an important role in physiological aging. This is underlined by several human mutations in DNA repair systems, which lead to accelerated aging of the organism. This aging does not have the same rate in all the tissues and is therefore called segmental progeria. Rarely has the functioning of the cardiovascular system been addressed in detail in these patients, although it known that the incidence of CVD is different for different syndromes. The extreme example is Hutchinson–Gilford Progeria Syndrome, where 90% of the patients die from complications of atherosclerosis, such as heart attack or stroke. Other syndromes have a different common cause of death, for example most Xeroderma Pigmentosum patients die as a consequence of metastatic malignant melanoma and squamous cell carcinoma.

Also in animal models of this disease, the function of cardiovascular system has rarely been looked at.

In chapter 2 we studied the vascular function of mice harboring a mutation in the DNA repair genes XPD and ERCC1. In the latter case we explored possible mechanisms underlying this apparent dysfunction. We also investigated the possible associations of DNA repair genes variability with vascular stiffness, a parameter strongly associated with aging, in humans.

In chapter 3 we explored in detail the mechanisms of vascular smooth muscle dysfunction in $Ercc1^{d/-}$ mice.

As not only age but also prolonged exposure to other risk factors are important determinants for developing CVD, it is worthwhile to correlate lifestyle differences with the incidence of CVD. One such association was found in 1979 by St Leger, who observed an inverse correlation between mortality from coronary heart disease and red wine consumption. Later on, short term tests with dealcoholized red wine showed increased flow mediated dilation in healthy subjects, while red wine polyphenols decreased blood pressure in persons

with metabolic syndrome but not in normotensive subjects. As endothelial function is an important marker of CVD, we hypothesized that red wine polyphenols could positively influence cardiovascular aging.

In chapter 4 we investigated how the red wine extract (RWE) reduces ROS-induced senescence of endothelial cells. Among others, we focused on an important and widely studied RWE component, resveratrol.

New discoveries in RAS, namely the metabolite Ang-(1-7) and the AT_2 receptor, opened up possibilities for the development of new drugs. The AT_2 receptor was discovered to have effects opposing AT_1 receptor stimulation. Recently a non-peptide agonist with high affinity and specificity towards AT_2 receptors was developed: compound 21 (C21). Experimental results from in rat MI model showed that C21 can attenuate the deleterious remodeling after MI,[57] however this finding was recently challenged.[58]

In chapter 5 we investigated if the vasopressor actions of C21 are mediated by AT_2 receptor stimulation, making use of various human and rodent preparations that are known for the occurrence of AT_2 receptor-mediated effects.

Ang-(1-7) has been shown to be efficacious in the treatment of deleterious remodeling after MI in rats and also is a potent stimulator of hematopoiesis in humans. The major downsides of this drug are its low bioavailability, its affinity towards AT_1 and AT_2 receptors, and most importantly the vulnerability to the proteases in plasma, leading to a very short half-life. To circumvent these problems, the drug needs to be administered parenterally or in a special pharmaceutical formulation allowing the absorption from the GI system. Therefore a derivate of Ang-(1-7) was developed, in which a thioether bridge was introduced into the structure (cAng-(1-7)), making it resistant to proteolytic degradation. This modification is also causing a change in spatial orientation of amino acids in the peptide, which abolishes the affinity of the compound to AT_1 and AT_2 receptors and makes it therefore a specific Mas agonist.

In chapter 6 we explored the effects of cAng-(1-7) on the adverse cardiac remodeling and vascular function after MI in rats.

In chapter 7 we summarize the current knowledge on the effects of Ang II and Ang-(1-7) on hematopoiesis and mesenchymal stem cells. Further, we demonstrate that Ang-(1-7) might not only protect the vasculature through repair but also by preventing cellular aging.

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Based on:
Nucleotide Excision DNA Repair Is Associated With Age-Related Vascular Dysfunction
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Nucleotide excision DNA repair is associated with age-related vascular dysfunction

2

Abstract

Background

Vascular dysfunction in atherosclerosis and diabetes, as observed in the aging population of developed societies, is associated with vascular DNA damage and cell senescence. We hypothesized that cumulative DNA damage during aging contributes to vascular dysfunction.

Methods and results

In mice with genomic instability due to the defective nucleotide excision repair genes *ERCC1* and *XPD* (*Ercc1*$^{d/-}$ and *Xpd*^{TTD} mice), we explored age-dependent vascular function as compared to wild-type mice. *Ercc1*$^{d/-}$ mice showed increased vascular cell senescence, accelerated development of vasodilator dysfunction, increased vascular stiffness and elevated blood pressure at very young age. The vasodilator dysfunction was due to decreased endothelial eNOS levels as well as impaired smooth muscle cell function, which involved phosphodiesterase (PDE) activity. Similar to *Ercc1*$^{d/-}$ mice, age-related endothelium-dependent vasodilator dysfunction in *Xpd*^{TTD} animals was increased. To investigate the implications for human vascular disease, we explored associations between single nucleotide polymorphisms (SNPs) of selected nucleotide excision repair genes and arterial stiffness within the AortaGen Consortium, and found a significant association of a SNP (rs2029298) in the putative promoter region of DDB2 gene with carotid-femoral pulse wave velocity.

Conclusions

Mice with genomic instability recapitulate age-dependent vascular dysfunction as observed in animal models and in humans, but with an accelerated progression, as compared to wild type mice. In addition, we found associations between variations in human DNA repair genes and markers for vascular stiffness which is associated with aging. Our study supports the concept that genomic instability contributes importantly to the development of cardiovascular disease.

Introduction

Vascular and endothelial function deteriorates with age and is considered a key factor in the development and progression of age-related cardiovascular disease (CVD).[1-3] The high prevalence of CVD-related mortality due to increasing life expectancy highlights the necessity of understanding how aging influences vascular function. Currently, aging is viewed as a consequence of the prolonged exposure to risk factors e.g., an unfavourable lipid profile, smoking and diabetes, during which accumulation of damage increases the risk to develop vascular dysfunction and associated disease.[3] At the cellular level this could be related to the increased production of reactive oxygen species (ROS) and a resulting increase in lipid oxidation or interference with cellular metabolism. This can directly affect vascular function and/or lead to apoptosis or cellular senescence, a state in which the cell remains in cell cycle arrest and has lost its optimal function. In the case of endothelial cells (EC), this functional change results in a pro-vasoconstrictor and pro-inflammatory phenotype.[4]

Despite extensive research into oxidative stress-induced cellular damage and senescence, a main causative mechanism of aging and age-related CVD remains unknown. It is for instance unclear why vasomotor function declines with aging, even in the absence of apparent risk factors.[5] Moreover, in the vast majority of epidemiological studies, aging remains the most significant risk factor for CVD, even after correction for classical cardiovascular risk factors.[3]

The concept that unrepaired DNA damage has dramatic effects on the aging phenotype stems from multiple lines of evidence, including the fact that the majority of human progeroid syndromes are due to mutations in DNA damage repair and response genes.[6] One of the DNA repair systems that is very important in this regard is Nucleotide Excision Repair (NER), which removes a wide class of helix-distorting DNA lesions induced by UV, but also numerous man-made or natural chemical compounds and ROS. The process of DNA damage removal consists of 1) DNA damage recognition by XPC for some lesions assisted by the UV-DDB1/2 (XPE) complex, 2) local unwinding of DNA provided by the multi-subunit TFIIH complex, 3) damage verification by XPA, 4) excision of the damaged DNA section by endonucleases XPG and ERCC1/XPF and 5) replacement of the excised DNA using the intact strand as template. Mutation of factors involved in NER can have severe consequences for human health as demonstrated by several human progeroid syndromes. Examples are the rare autosomal recessive, genetic disorders Cockayne syndrome (CS), trichothiodystrophy (TTD) and the recently described XPF-ERCC1 (XFE1) syndrome.[7] For instance, TTD is caused by point mutations in the *XPD*, *XPB* or *TTDA* genes, affecting DNA repair function and stability of the dual functional NER/basal transcription initiation factor TFIIH.[8] This causes UV sensitivity and accelerated segmental aging symptoms, including early cessation of growth, cachexia, osteoporosis, progressive neurological abnormalities and premature death.[9] Likewise, several mouse models with NER defects show a segmental premature aging phenotype, where the severity depends on the extent to which the DNA repair system is affected.

To investigate whether DNA damage plays a role in age-related vascular dysfunction,

we studied vasomotor function and cellular senescence in two NER-defect mouse models, differing in type and severity of the DNA repair defect. In *Ercc1*$^{d/-}$ animals, one allele of the NER-DNA crosslink repair (XLR) endonuclease ERCC1 is mutated resulting in a truncated protein (lacking the C-terminal 7 amino acids) while the other allele is completely inactivated[10]. In *Xpd*^{TTD} mice, the XPD helicase of the TFIIH core complex carries a homozygous R^{722}W functional pointmutation as found in a TTD patient.[11] The NER-XLR defect in the *Ercc1*$^{d/-}$ animals is more severe, resulting in very early cessation of growth, very premature liver, kidney, bone marrow and neurological aging phenotype, and a reduced lifespan of approximately 5-6 months. The milder phenotype of *Xpd*^{TTD} mice results in retarded growth, cachexia, an age-related osteoporosis, and a slightly reduced lifespan.

To investigate if NER gene variations could have an impact on human vascular disease and in line with our murine phenotype, we performed genetic studies to examine the association of genetic variation in genes coding for proteins involved in NER with carotid-femoral pulse wave velocity (CFPWV). The associations between genetic variation in selected NER genes and the vascular phenotype were assessed within the framework of the ArotaGen Consortium.[12]

Materials and methods

For details of the experimental setup, see the Supplemental Material.

Animals

The animals used in experiments were 8- and 16-week-old *Ercc1*$^{d/-}$ mice, their wild-type littermates of the same age (WT), and 16-, 26- and 52-week-old mice of the same background - F1 hybrid between Fvb and C57Bl/6 and 26- and 52-week-old *Xpd*^{TTD} mice and their WT controls in a C57Bl/6 background. All animal studies were approved by an independent Animal Ethical Committee.

Isolation and culture of endothelial cells

Endothelial cells were isolated from 16-week-old *Ercc1*$^{d/-}$ mice and cultured under mouse lung endothelial cell medium under atmosphere of normal air enriched with 5% CO_2.

Senescence-associated β-galactosidase staining

Senescence was determined by senescence-associated β-galactosidase staining (SA-β-gal staining) at pH 6.0.

Quantitative real-time PCR

Relative expression of cyclin-dependent kinase inhibitor 1A (p21) and tumor protein 53 (p53) genes was measured in thoracic aortas of 16 week old *Ercc1*$^{d/-}$ and WT mice.

Assessment of blood pressure and vasodilator function in vivo

In vivo hindleg vasodilator function was measured by Laser Doppler perfusion imaging of reactive hyperemia after transient blood flow interruption, in 8-week-old *Ercc1*$^{d/-}$ and WT mice.

Simultaneously, blood pressure was measured in conscious *Ercc1*$^{d/-}$ mice and WT littermates using the tail cuff technique.

Organ bath experiments

The responses of aortic tissue of 8- and 16-week-old *Ercc1*$^{d/-}$ mice and their, 8-, 16-, 26- and 52-week-old WT littermates as well as 26- and 52-week-old *Xpd*^{TTD} mice and their WT littermates were measured in small wire myograph organ baths containing oxygenated Krebs-Henseleit buffer of 37^{o}C. Following preconstriction with 30 nmol/L U46619, relaxation concentration-response curves (CRCs) were constructed to acetylcholine, followed by an exposure to 100 µmol/L sodium nitroprusside. N$^{\omega}$-nitro-L-arginine-methyl ester (L-NAME) 100 µmol/L pretreatment was used to investigate the involvement of nitric oxide (NO). 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol; 100 µmol/L) and N-acetyl-cysteine (NAC, 30 µmol/L) were used as scavengers of reactive oxygen species (ROS). Tetrahydrobiopterin (BH4, 100 µmol/L) was used to prevent the uncoupling of eNOS. Vinpocetine (100 µmol/L) on was used to investigate phosphodiesterase (PDE) activity.

Measurement of mechanical properties of the vascular wall

Carotid arteries were explanted from 16-week-old *Ercc1*$^{d/-}$ and WT mice and mounted in the perfusion myograph. The vessel diameter – pressure relationship was determined and stress-strain relationships were constructed.[13]

Immunoprecipitation and immunoblotting of eNOS

Aortas and hearts were used to investigate the levels of eNOS and the fraction of eNOS phosphorylated at position Tyr-657. Ser-1177 eNOS phosphorylation was investigated in lungs, either at baseline or after 10 minutes of stimulation with 10 µmol/L acetylcholine.

Statistical methods of animal studies

Data are presented as mean±SEM. Statistical analysis between the groups of single values was performed by two-sided t-test (*), two-sided t-test after log transformation of the data (#), or Mann-Whitney U test (†) or 1-way ANOVA followed by Bonferroni's post-hoc test, where appropriate (*). To test the hypothesis that blood pressure would be increased in *Ercc1*$^{d/-}$ animals, we employed a one-sided t-test. Differences in dose-response curves were tested by ANOVA for repeated measures (sphericity assumed, *). Differences were considered significant at $p<0.05$.

Human studies

In accordance with the phenotype observed in mice, we investigated the association of SNPs in NER components with carotid-femoral pulse wave velocity (CFPWV), a measure of vascular stiffness. CFPWV is a well known marker of age-related vascular disease in humans and is strongly associated with increased risk for major CVD events. To investigate this association, we used the data from the AortaGen Consortium which consists of 20,634 participants from 9 cohort studies. A detailed description of the AortaGen Consortium is provided in the online-only Data Supplement.

Statistical methods of human studies

Genes coding for NER components that belong to the machinery that binds the DNA to either recognize or repair damage were selected. These were the following NER components: *ERCC8 (CSA) ERCC6 (CSB); DDB1; DDB2 (XPE); ERCC1; GTF2H1 (p62); GTF2H3 (p34); GTF2H4 (p52); GTF2H5 (TTDA, TFB5); RAD23A (hHR23A); RAD23B (hHR23B); ERCC3 (XPB); XPC; ERCC2 (XPD); ERCC4 (XPF);* and *ERCC5 (XPG)*. To test for association with CFPWV, we selected the tag SNPs that cover the variation in the genes of interest ± 50 kb region around and that were non-redundant at linkage disequilibrium (LD) threshold of $r^2 \geq 0.7$ using the Tagger program of Haploview. Our selection resulted in 310 SNPs. We decided *a priori* on a significance threshold of $p < 1.61 \times 10^{-4}$, which corresponds to the Bonferroni adjusted p value for the number of tested SNPs.

Results

Vascular cell aging in *Ercc1*$^{d/-}$ mice

SA-β-gal staining of 16-week-old *Ercc1*$^{d/-}$ mice versus their wild-type littermates (WT) aorta showed that, macroscopically, senescence staining clearly dominated in aortas from *Ercc1*$^{d/-}$ (Figure 1A). Microscopically, stained cells were detected in both the endothelium and the media of *Ercc1*$^{d/-}$ aortas. The quantity and visibility of senescent cells allowed reliable counting in the media, showing a marked increase in *Ercc1*$^{d/-}$ animals (Figure 1B). Similarly, RNA levels of the genes composing DNA damage-related CDK inhibition *p21 (Cdkn1a)* and *p53 (Trp53)* were increased in the aorta of *Ercc1*$^{d/-}$, however only p21 reached statistical significance (Figures 1C, 1D).

To further investigate the effect of defective NER on proliferative senescence in endothelial cells, we measured the percentage of SA-β-gal-positive cells after 20 days in culture. The levels of SA-β-gal-positive endothelial cells in the lung were on average 10.3 times higher in cultures from *Ercc1*$^{d/-}$ versus WT mice (Figure 1 E, F).

Based on the robust proliferation of freshly cultured lung endothelial cells, we decided to test endothelial-dependent angiogenic outgrowth potential in aortic explants of 16-week-old *Ercc1*$^{d/-}$ and WT mice. No significant difference in outgrowth was observed (Supplemental Figure 1).

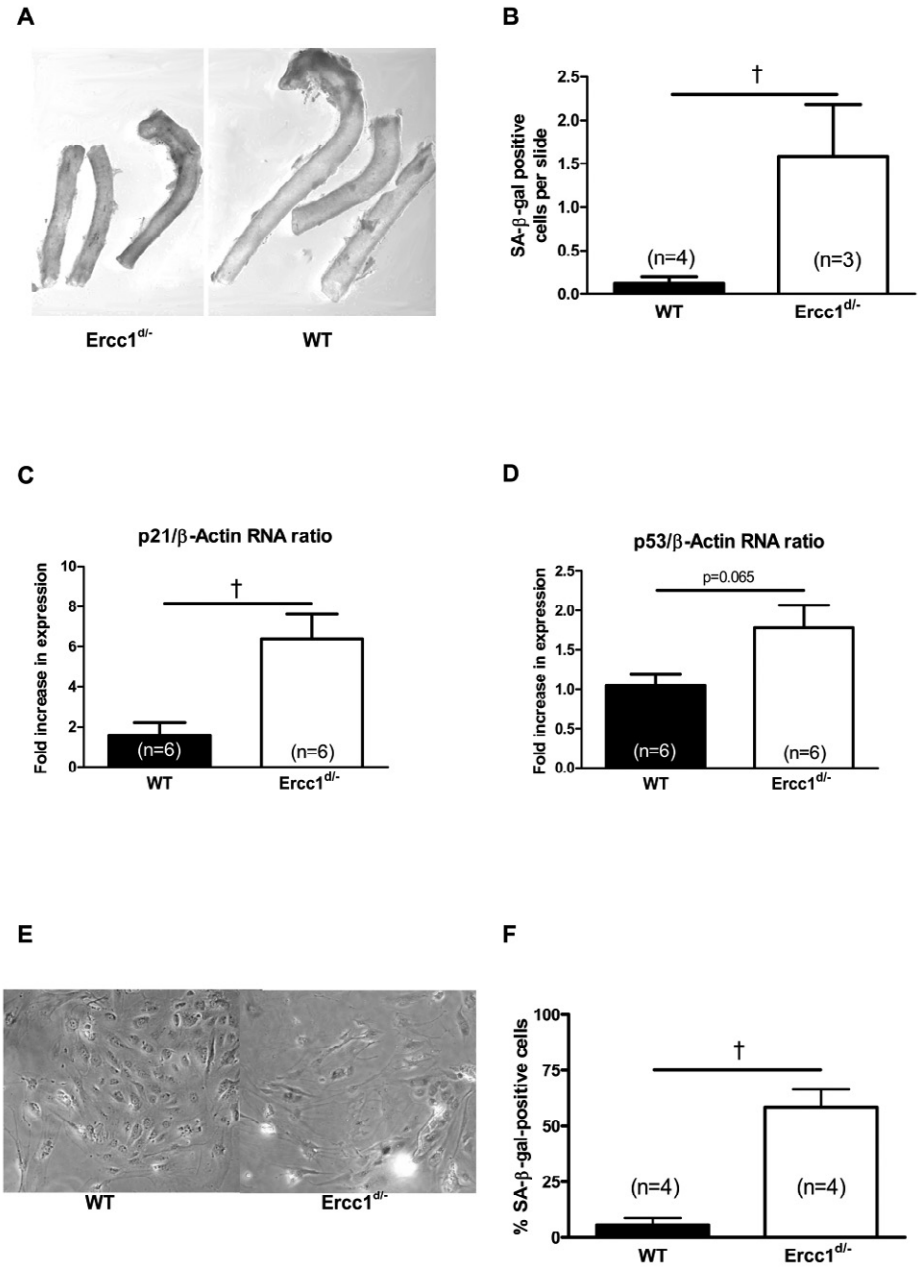


Figure 1. Senescence in *Ercc1*$^{d/-}$ mice vascular tissue

The thoracic aorta wall of *Ercc1*$^{d/-}$ and WT mice stained with SA-β-gal staining (A). Quantification of SA-β-gal positive cells in the lamina media of thoracic aorta (B). Aortic RNA levels of senescence markers p21 (C) and p53 (D). Percentage of SA-β-gal positive cells after prolonged culture of isolated lung endothelial cells in *Ercc1*$^{d/-}$ compared to WT mice (E, F). †= $p<0.05$ (Mann-Whitney U test). Images were adjusted for the purpose of black and white print, please refer to the article for the original images.

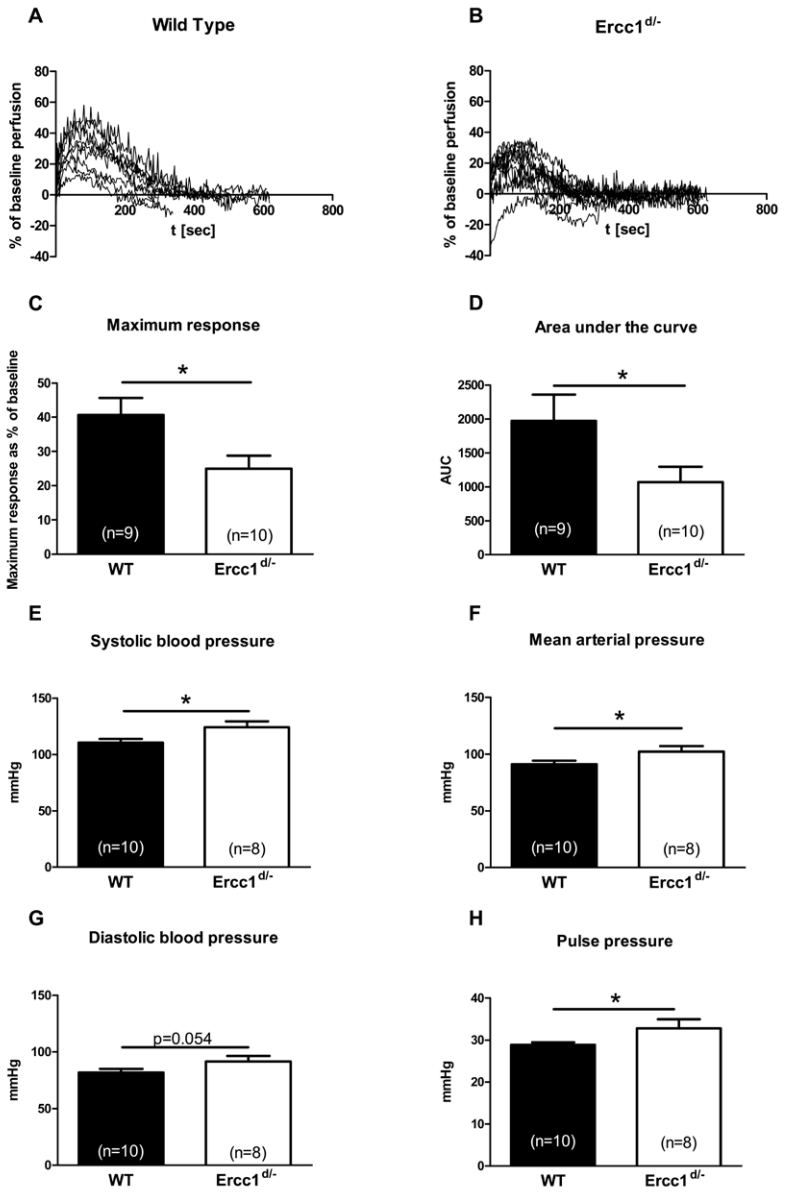


Figure 2. Measures of central and peripheral hemodynamics in 8 week old *Ercc1*$^{d/-}$ and WT animals

Functional Differences between skin reperfusion after 2 minutes of occlusion between WT (A) and *Ercc1*$^{d/-}$ (B), average maximum response (C), as well as area under the curve (D). Differences in systolic, mean, diastolic blood pressure (E, F, G) and pulse pressure (H) between 8 week old *Ercc1*$^{d/-}$ and their WT littermates. *= $p<0.05$ (t-test).

In vivo vascular function of NER-defective and WT mice

To determine possible functional changes in the vasculature we assessed vasodilator function in response to reactive hyperemia in the hindlimbs of 8-week-old WT (Figure 2A) and *Ercc1*$^{d/-}$ mice (Figure 2B). In 8-week-old *Ercc1*$^{d/-}$ animals we observed a decreased reactive hyperemia (Figure 2C, 2D). In addition we observed a significant increase in systolic pressure, mean arterial pressure (MAP) and pulse pressure in *Ercc1*$^{d/-}$ mice (Figures 2E-2H). Diastolic blood pressure tended to increase, albeit without statistical significance (Figure 2G).

Age-dependent change of ex vivo vascular function in WT and NER-defective mice

To reveal the mechanisms of vasodilator dysfunction in *Ercc1*$^{d/-}$ mice and to address the question of age-dependency, we compared vasodilator function in 8- and 16-week-old mice. At both ages, mice show signs of progeria, without obvious deterioration in general health. *Ercc1*$^{d/-}$ animals showed progressive reduction of acetylcholine-induced aortic relaxation at these ages (Figure 3A). Sodium nitroprusside respones were reduced in 16-week-old *Ercc1*$^{d/-}$ mice and tended to be decreased in 8-week-old *Ercc1*$^{d/-}$ mice (Figure 3B). To estimate the contribution of the endothelium to vasodilator dysfunction, acetylcholine-induced relaxations were corrected for sodium nitroprusside responses, revealing that the endothelial contribution to the response to acetylcholine was reduced in *Ercc1*$^{d/-}$ versus WT at both ages (Figure 3C).

DNA repair competent WT animals of 16, 26 and 52 weeks of age showed a much slower age-dependent reduction in acetylcholine responses than *Ercc1*$^{d/-}$, becoming statistically significant after 52 weeks (Figure 3D). Endothelium-independent responses to sodium nitroprusside did not change in WT (Figure 3E).

To determine whether or not a slower onset of progeria could delay the onset of vascular dysfunction, we assessed vascular function in the NER impaired *Xpd*^{TTD} mouse that displays a milder phenotype. Vasodilator responses to acetylcholine in U46619-precontracted aortic rings were significantly reduced in 52-week-old *Xpd*^{TTD} mice compared to those at 26 weeks, and more markedly than in WT littermates (Figure 3F). The noticeable, modest difference between 52-week-old *Xpd*^{TTD} and WT animals did not reach significance. Endothelial-independent responses to sodium nitroprusside were equal (Figure 3G).

Pretreatment with the endothelial nitric oxide synthase (eNOS) inhibitor, L-NAME, abolished all acetylcholine-induced relaxations in all animals (data not shown), indicating that acetylcholine responses entirely depended on eNOS/NO.

Mechanisms of endothelial vasodilator dysfunction in NER-defective mice

In aortas from 16 week old *Ercc1*$^{d/-}$ mice eNOS levels were reduced by approximately 67% compared to WT (Figure 4A). Phosphorylation of the tyrosine residue at position 657 (pTyr657-eNOS) inhibits eNOS,[14] and this tyrosine phosphorylation tended to be increased in *Ercc1*$^{d/-}$ hearts (Figure 4B). eNOS-activating phosphorylation of the serine residue at position 1177 (pSer1177-eNOS) was comparable in explanted WT and *Ercc1*$^{d/-}$ lungs at baseline, but 10 μmol/L acetylcholine only increased pSer1177-eNOS in WT (Figure 4E).

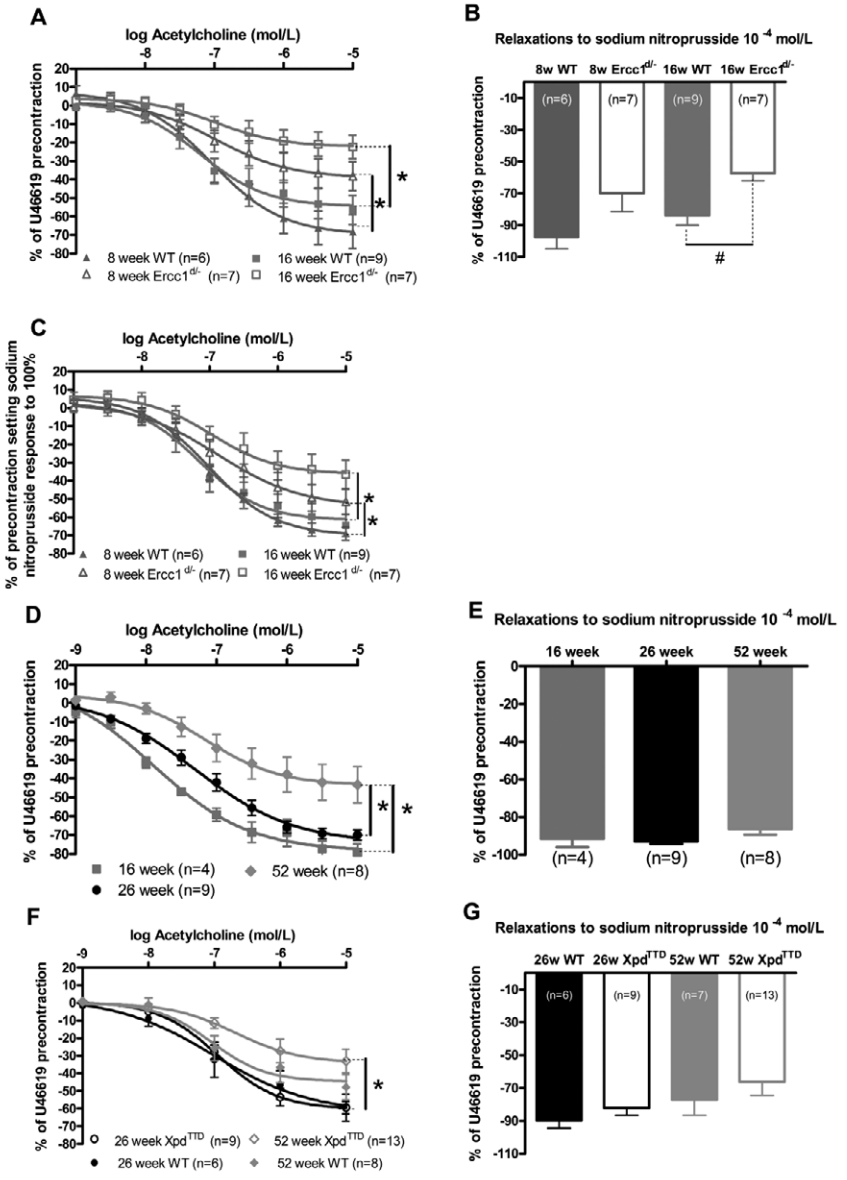


Figure 3. Ex-vivo vascular function in progeria models and aging models

Vasodilatations to (A) acetylcholine and (B) sodium nitroprusside in U46619-precontracted isolated aortic rings of 8 and 16 week old *Ercc1*$^{d/-}$ vs. WT, measured in organ bath setups. To express the contribution of the endothelium, acetylcholine responses were expressed as % of the response to sodium nitroprusside (C). Dilator responses to acetylcholine (D) and sodium nitroprusside (E) of WT mouse isolated aortic rings precontracted with 3x10^{-8} mol/L U46619, measured at 16, 26 and 52 weeks of age. Vasodilatations to (F) acetylcholine and (G) sodium nitroprusside in U46619-precontracted isolated aortic rings of 26 and 52 week old *Xpd*^{TTD} vs. WT. #,*= $p<0.05$ (t-test on log transformed values, general linear model – repeated measures).

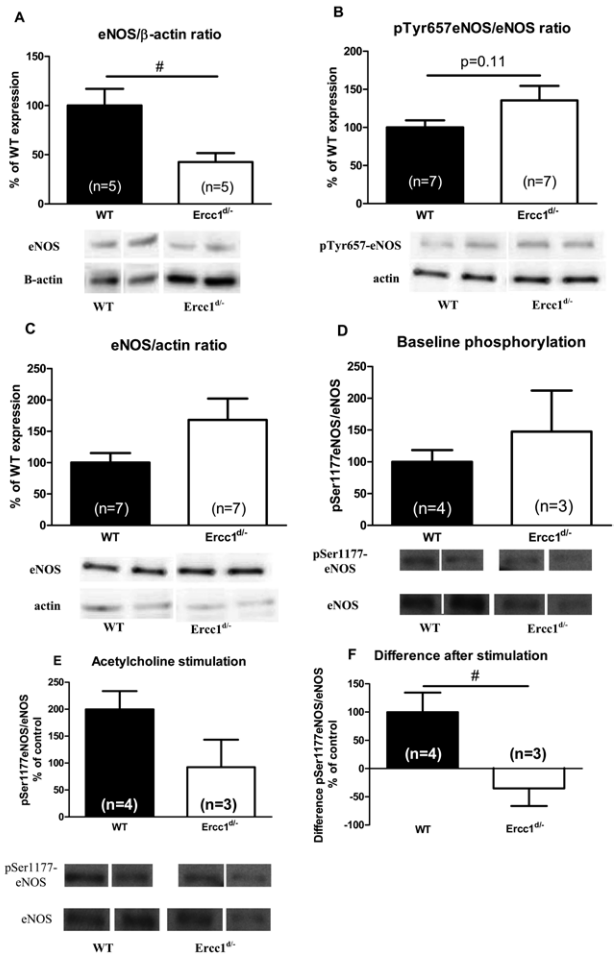


Figure 4. eNOS in aorta and myocardium of *Ercc1*$^{d/-}$ mice

Protein levels of eNOS in 16 week old *Ercc1*$^{d/-}$ animals and their WT littermates, in (A) aorta, and (B, C) cardiac ventricles. Phosphorylation of Ser1177-eNOS in lungs of 16 week old *Ercc1*$^{d/-}$ animals, and their WT littermates on baseline (D), and upon stimulation with 10^{-5} mol/L Ach (E). The difference in phosphorylation before and after stimulation (F). # = $p<0.05$ (t-test on log transformed values). Lanes were run on the same gel but are noncontiguous.

Uncoupling of eNOS results in a switch from NO to ROS production. It can be a consequence of the decreased availability of the essential co-factor tetrahydrobiopterin (BH4), and its reconstitution can restore NO production.[15] BH4 had no effect on the acetylcholine-induced relaxation of aortic rings from WT mice but restored that of rings from *Ercc1*$^{d/-}$ mice (Figure 5A). BH4 also increased endothelium-independent sodium nitroprusside responses (Figure 5B) in *Ercc1*$^{d/-}$.

ROS coming from various sources can scavenge NO, thereby leading to vasodilator dysfunction that can be rescued by ROS scavengers. Whereas tempol was without effect (Supplemental Figure 2A, B), NAC caused a modest and significant improvement of the acetylcholine and sodium nitroprusside responses in *Ercc1*$^{d/-}$ aortas (Figure 5C, D). After correction for sodium nitroprusside responses, acetylcholine responses were not changed by BH4 or by NAC (Figure 5E, F). Therefore, the BH4- and NAC-induced improvement of relaxations most likely represents improved VSMC sensitivity.

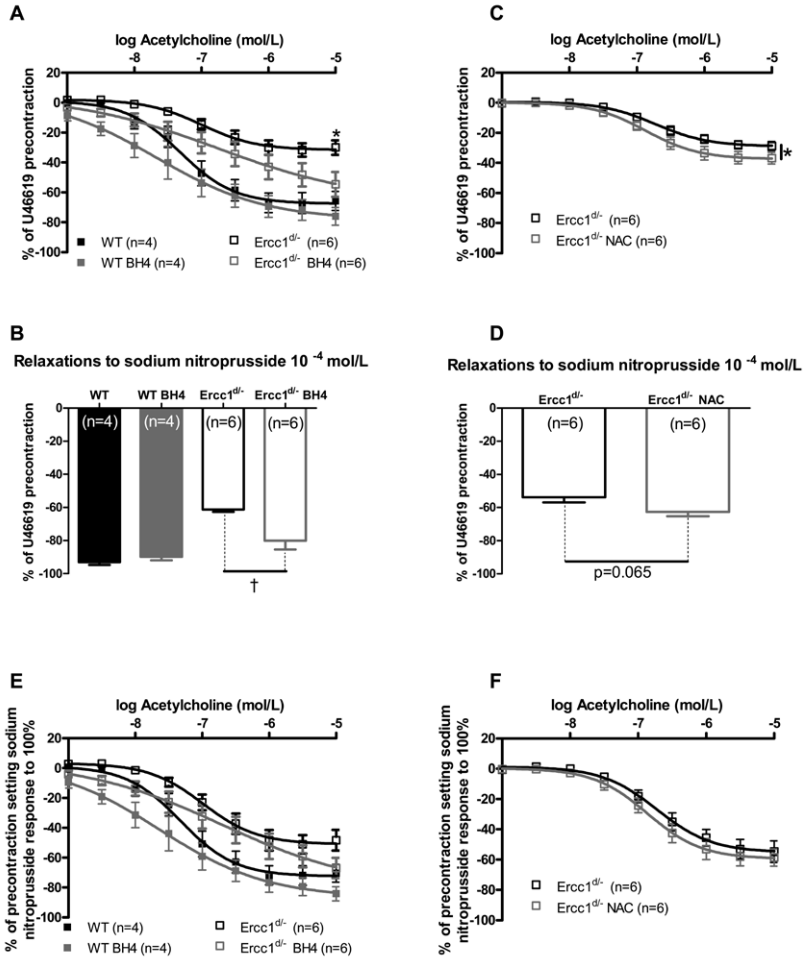


Figure 5.Effects of ROS scavenging and prevention of eNOS uncoupling on ex-vivo vascular function

Effects of acute BH4 supplementation on responses to acetylcholine (A) and sodium nitroprusside (B) in aortic tissue of 16 week old *Ercc1*$^{d/-}$ and WT mice. The acetylcholine responses after correction for individual responses to sodium nitroprusside (E). The effect of acute NAC supplementation on responses of *Ercc1*$^{d/-}$ to acetylcholine (C) and sodium nitroprusside (D). Acetylcholine responses expressed as % of the sodium nitroprusside response (F). †= $p<0.05$ (Mann-Whitney U test).

Mechanisms of VSMC vasodilator dysfunction in NER-defective mice

The phosphodiesterase inhibitor vinpocetine improved sodium nitroprusside responses in *Ercc1*$^{d/-}$ mice (Figure 6). PDE5-specific inhibitor sildenafil had similar effects (data not shown, n=5). The responses to an activator of protein kinase G (PKG) were identical in *Ercc1*$^{d/-}$ and WT littermates (n=9 per group; logarithm of concentration needed to reach 50% dilation: -5.268±0.2353 vs. 5.569±0.1957 respectively).

Mechanical properties of conductive vessels in *Ercc1*$^{d/-}$

Under similar perfusion pressure increments differential increases in the vessel lumen were observed in *Ercc1*$^{d/-}$ versus WT mice (Figure 7A). Recalculation of measured values demonstrated a significantly lower strain, indicating lower elasticity, in *Ercc1*$^{d/-}$ mice under comparable stress as in WT (Figures 7B, 7C). Morphometric analysis on microscopical sections shows that although the vascular wall thickness of *Ercc1*$^{d/-}$ is significantly lower, the wall-to-lumen ratio was equal (Supplemental Figure 3).

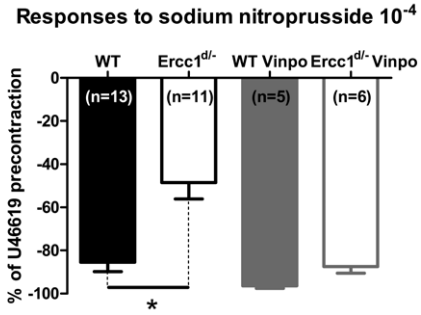


Figure 6. Effect of PDE inhibition vinpocetine on endothelium-independent vasodilatation

Ercc1$^{d/-}$ mice show reduced vasodilatory response to sodium nitroprusside 10^{-4} mol/L, when compared with WT littermates. PDE blockade abolishes this difference. * = $p<0.05$ (t-test).

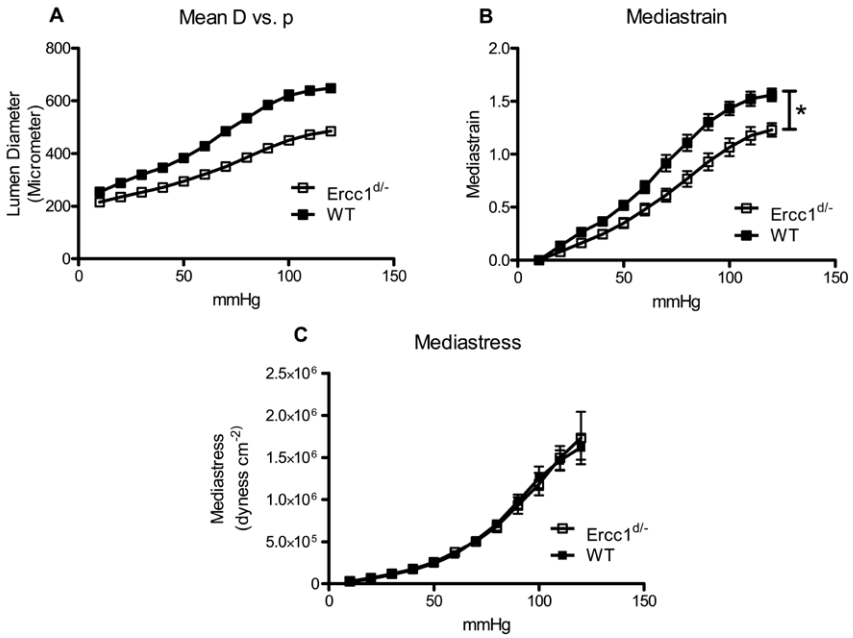


Figure 7. Mechanical properties of conductive vessels in *Ercc1*$^{d/-}$

Relationship of the diameter of carotid artery to the internal pressure (A). Reduced strain of the tissue of *Ercc1*$^{d/-}$ mice (B) is accompanied by no difference in stress on the wall (C). * = $p<0.05$ (general linear model – repeated measures)

Human studies

Previous human studies indicate that NER component SNPs influence the risk of developing cancer[16], suggesting that these SNPs modulate NER function. *Ercc1*$^{d/-}$ mice showed an increased vascular stiffness. We hypothesized that genetic variation in the NER components might also influence vascular stiffness in humans. Increased vascular stiffness is an important feature of human vascular aging, represented by the variable CFPWV. Therefore, we studied the effect of SNPs in NER components that, like *ERCC1* and *XPD*, belong to the machinery that binds the DNA to either recognize or repair damage on variation of CFPWV in the cohorts from the AortaGen Consortium.

The AortaGen Consortium used a sex-specific standardized regression residual for 1000/CFPWV, adjusted for age, age^2, height and weight for meta-analysis. Genome-wide association analyses were conducted using an additive gene-dose model. The results for the association of the genetic variants in the NER components with CFPWV in humans are presented in Table 1. The allele dose effect is expressed as standard deviations of inverse CFPWV per coded allele. After Bonferroni correction for multiple testing, one locus reached an association with CFPWV at the p-value threshold of 1.61×10^{-4}, namely SNP rs2029298 (Beta: -0.05, SE: 0.01, P-value: $1.04*10^{-4}$). The closest gene to this SNP is *DDB2 (XPE)*. Another SNP in this region, rs3781619 located within the *DDB2 (XPE)* gene, showed a suggestive association with the CFPWV measure (Beta: -0.03, SE: 0.02, p value=$3.80*10^{-2}$). In addition, we found suggestive associations for 8 other SNPs located within or near *ERCC5 (XPG), ERCC6 (CSB), GTF2H3, GTF2H1* and *ERCC2 (XPD)*. To summarize, *DDB2 (XPE)* was the most important gene associated with CFPWV.

Discussion

The present study shows that mice with an increased susceptibility to DNA damage due to a defect in NER as in *Xpd*^{TTD}, and NER and XLR as in *Ercc1*$^{d/-}$, display an increased number of senescent vascular cells, an increased susceptibility of EC to become senescent, and accelerated worsening of vasodilator function during aging. The latter involves both EC as well as VSMC dysfunction. Furthermore, *Ercc1*$^{d/-}$ mice display increased vascular stiffness, systolic blood pressure, and pulse pressure, and reduced reactive hyperemia, which are typical features of vascular aging in humans. In addition, genetic association studies from the AortaGen Consortium suggest that SNPs in NER genes coding for components that participate in DNA recognition and repair contribute to vascular stiffness, as measured by CFPWV, in humans. These results suggest that genomic instability is involved in the development of vascular aging, and warrant further investigations into the involvement of the DNA repair systems in age-related cardiovascular disease.

The involvement of DNA damage and repair in age-related vascular disease is also apparent from previous clinical studies showing that senescent cells and oxidative DNA damage are present in atherosclerotic plaques[17-18], the beneficial effects of statins on DNA repair[19], the increased levels of 8-hydroxy-2,9-deoxyguanosine in urine of hypertensive

Table 1. Association of the selected tag SNPs in the NER component with Pulse Wave Velocity.

	Chromosome		Allele		AortaGen Meta-analysis *			
SNP	Number	Position	Coded	Frequency	Beta	SE	P value	Closest Gene
Associations with P<1.61 x 10-4								
rs2029298	11	47191294	C	0.30	-0.05	0.01	1.04E-04	*DDB2*
Associations with 1.61 x 10-4<P<5.00 x 10-2								
rs4772511	13	102369013	T	0.19	0.04	0.01	1.07E-02	*ERCC5*
rs4253002	10	50417344	T	0.02	0.13	0.05	1.53E-02	*ERCC6*
rs2340693	12	122700941	G	0.03	-0.09	0.04	3.12E-02	*GTF2H3*
rs4150548	11	18304112	A	0.03	0.06	0.03	3.44E-02	*GTF2H1*
rs4253162	10	50361366	T	0.09	-0.04	0.02	3.51E-02	*ERCC6*
rs3781619	11	47211893	A	0.13	-0.03	0.02	3.80E-02	*DDB2*
rs50871	19	50554355	A	0.49	-0.03	0.01	3.80E-02	*ERCC2*
rs9586010	13	102375850	T	0.26	-0.03	0.02	3.90E-02	*ERCC5*
rs6488885	12	122685108	G	0.02	-0.09	0.04	4.92E-02	*GTF2H3*

Abbreviations: SNP, single nucleotide polymorphism; NER, Nucleotide Excision Repair; SE, standard error.
*.Coded allele is the minor allele. The analyses were adjusted for age, age2, sex, height and weight. The allele dose effect is the standard deviation of inverse carotid femoral pulse wave velocity (CFPWV) per coded allele. Because of the inverse transformation of CFPWV, a negative beta represents a higher CFPWV for each dose of the minor allele. For all of the SNPs in the table, N is 20634, except for rs2340693, rs9586010 and rs6488885 where N=16418.

subjects[20], and genome-wide association studies that report an association between SNPs in the 9p21 locus with risk for coronary artery disease, intracranial aneurysm and type 2 diabetes.[21] These SNPs, which are located in the ANRIL non-coding RNA, lie in the vicinity of the INK4A genes that code for the cyclin-dependent kinase inhibitors *p15/CDKN2B*, *p16/CDKN2A* and *p14/ARF*, which may affect induction of cellular senescence in response to DNA damage.[22] Deletion of ANRIL in mice reduces *Cdkn2a* and *Cdkn2b* expression, and increases cultured smooth muscle cell proliferation[23]. Vascular function has not been assessed in these mice. No previous studies examined vascular senescence in parallel to vascular function, but the studies cited here[17, 19-24] support our hypothesis that DNA damage and cellular senescence contribute the pathogenesis of age-related CVD.

Ercc1$^{d/-}$ mice exhibited reduced reactive hyperemia, suggesting vasodilator dysfunction in resistance vasculature. Increased peripheral resistance may lead to increased MAP, which was observed in *Ercc1*$^{d/-}$ mice. Since systolic blood pressure also increased, we suspected reduced compliance of conduit vessels. The observation that aortic dilator function was decreased in *Ercc1*$^{d/-}$ and *Xpd*^{TTD} supports this concept. However, *Ercc1*$^{d/-}$ dilator function seems to depend both on VSMC as well as endothelial function, whereas *Xpd*^{TTD} showed no impaired VSMC relaxation. Naturally aging mice also showed endothelial dysfunction without VSMC impairment. Thus, the observed VSMC dysfunction in *Ercc1*$^{d/-}$ might represent an extreme aging phenotype, as might be expected from a combined defect of NER as well as XLR. Although the senescence marker prelamin A is found in aortic VSMC of aged humans outside of atherosclerotic plaques [25], the role of VSMC senescence in human vasodilator function remains unclear, whereas a role in atherosclerosis has been proposed [26]. In contrast, the role of endothelial (progenitor) cell senescence has been amply addressed[4, 18, 27].

We explored the role of eNOS in vasodilator dysfunction and found that aortic NO function and eNOS levels are decreased in *Ercc1*$^{d/-}$ mice. This is in agreement with previous observations in 26- to 28-month-old mice, and with a diminished NO release and eNOS expression in cultured senescent endothelial cells and in atherosclerotic plaque samples.[18, 28-32] Our pharmacological studies with BH4, tempol and NAC show that ROS production due to eNOS uncoupling does not play a major role in the observed aortic endothelial dysfunction. At most, ROS modestly affected VSMC function. Interestingly, BH4 and NAC improved the vasodilator function to a similar extent. This might suggest a similar action of both compounds, which would be in line with ROS scavenging properties of BH4.[33]

Our observation that BH4 mainly improved VSMC instead of EC vasodilator function in aorta is in sharp contrast with the observation that the BH4 precursor sepiapterin improved dilations to acetylcholine but not to $NaNO_2$ in mesenteric arteries of 24-month-old mice,[34] and warrants exploration of vasodilator mechanisms in resistance vessels from our mice.

Exploration of the endothelium-independent vasodilator dysfunction revealed that PDE inhibition almost completely rescued VSMC vasodilator function. This strongly suggests the existence of a relative PDE overactivity in *Ercc1*$^{d/-}$ mice. Interestingly, the substrate of several PDE enzymes, cyclic guanosine monophosphate (cGMP), also regulates VSMC proliferation[35] and extracellular matrix composition[36]. Therefore, long-term changes in

cGMP levels could lead to vascular changes including increased stiffness and a higher number of senescent cells. Prolonged increase of PDE activity and reduction in NO production could thus lead to an increased vascular stiffness, as supported by the increased vascular stiffness, despite an increased sensitivity to NO, in eNOS knockout mice.[37] In *Ercc1*$^{d/-}$ mice the reduced sensitivity to NO will even further contribute to reduced cGMP levels.

Our human genetic study intended to explore if there is a possible relationship between genetic variants in NER genes and risk for increased vascular stiffness, a typical marker for human vascular aging that was recapitulated in the *Ercc1*$^{d/-}$ mice. Besides finding one SNP, rs2029298, upstream of the start codon of *DDB2 (XPE)* that passed the Bonferroni-corrected significance level, we found suggestive associations of SNPs in or close to *ERCC5, ERCC6, GTF2H3, GTF2H1,* and *XPD* for CFPWV. The finding that CFPWV is significantly associated with *DDB2* is compelling since CFPWV is the human equivalent of the mouse variables for vascular stiffness that were measured, and is negatively associated with vasodilator function in aging mice[32] and humans.[38] *DDB2* encodes the smaller subunit of a heterodimeric DNA binding protein with high affinity to UV-damaged DNA (UV-DDB), and has been studied both in rodents as well as human. XPE is a component of NER and its activity is not tied to transcription, but it acts in the whole genome. The role of XPE is to identify UV induced lesions, e. g. cyclobutane pyrimidine dimers and also polycyclic aromatic hydrocarbon adducts.[39] The function of the upstream region in which the SNP for *DDB2* was found has not been characterized, although the gene in humans has been shown to be under control of p53, the guardian of the genome, and to be inducible after genotoxic stress. Hence DDB2 (XPE) may at least in part control global genome NER.

As usual for common genetic variants, the effect sizes were small. The effect size of our top SNP, rs2029298, is -0.05 ± 0.01 SD/allele, after correction for age, age^2, sex, height, and weight. Extrapolation of this value of -0.05 SD/allele to an absolute age value for an individual with the use of CFPWV-age association graphs is hard since these relations are not linear. Moreover the impact of the SNP will depend on other risk factors that are present. It is, however, possible to compare the relative contribution of the risk allele of SNP rs2029298 to others found in GWA studies. In the AortaGen Consortium study, the locus, found in a *BCL11B* gene desert, that was strongest associated with CFPWV, had an effect size of -0.075±0.012 SD/allele. Clearly, the effect size of the *DDB2* SNP found in the present study is in a comparable range.

More detailed sequencing might pinpoint genetic variants with stronger associations. This is an important consideration in view of the evolutionary pressure against genetic variations that severely impair the function of DNA repair systems. Severe DNA repair dysfunctions are often lethal or lead to infertility, which makes them more likely to be present only as minor alleles. Furthermore, the involvement of a DNA repair defect in vascular damage might depend strongly on the presence of factors that induce DNA damage. Nonetheless, increased PWV is prominently present in Hutchinson-Gilford progeria, a very rare syndrome caused by genomic instability due to mutation of the *LMNA* gene, which is featured by premature cardiovascular death [12, 40].

In summary, we explored the possibility whether reduced efficiency of two DNA repair pathways, Nucleotide Excision and interstrand cross link repair, contributes to vascular aging in mice. We observed functional changes of the vasculature (worsened vasodilator function and increased vascular stiffness) and hypertension, which are reminiscent of changes in aging humans and animals. We also found an association between SNPs of genes that encode for relevant NER components with increased vascular stiffness. Based on these observations we conclude that DNA repair capacity is associated with accelerated vascular aging in mice, and that there may be implications for risk stratification in humans with respect to age-dependent cardiovascular disease. Whether this relates to oxidative stress, classical risk factors and/or local damage, or even extends beyond these limits, will be a central question in studies to come.

Supplemental Material

Expanded materials and methods

Animals

Ercc1$^{d/-}$ mice of 8 and 16 weeks in an F1 hybrid Fvb/C57Bl/6, background, their wild-type littermates (WT) of the same age, 16, 26 and 52 week old phenotypically equivalent WT and 26 and 52 week old *XPD*^{TTD} mice and their WT in a C57Bl/6 background, 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 (Dutch equivalent of the Institutional Animal Care and Use Committee).

Isolation and culture of endothelial cells

Endothelial cells of 16 week old *Ercc1*$^{d/-}$ mice were isolated according to previously reported methods.[41] After isolation the cells were seeded on 12 well culture plates (Costar) coated with 1% gelatine (225 bloom, Sigma Chemicals, the Netherlands) and cultured under mouse lung endothelial cell medium (MLEC; DMEM/F12 (Gibco), containing 20 % FCS (Invitrogen) and endothelial cell growth supplement (ECGS-H 8 µL/mL, Promocell, cat # C30120). The atmosphere in the culture cabinet consisted of normal air enriched with 5% CO_2. Cells were generally used after passage 1 or 2, and never beyond passage 3. Cells were passaged before they reach confluence.

Senescence-associated β-galactosidase staining

Senescence was determined by senescence-associated β-galactosidase staining (SA-β-gal staining) at pH 6.0 with previously described methods and reagents.[42-43] After staining cells were covered by Vectashield containing 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) to visualize the nuclei and pictures were taken under a microscope using normal and ultra-violet light. An overlay of these pictures was made and nuclei, combined with blue staining were counted as a positive cell.

SA-β-Gal-stained aortic tissue of 16 week old *Ercc1*$^{d/-}$ mice was embedded in paraffin, cut transversally and the number of SA-β-gal positive cells in the lamina media was counted under the microscope at 200x magnification.

Quantitative real-time PCR

Relative expression of cyclin-dependent kinase inhibitor 1A (p21) and tumor protein 53 (p53) genes was measured in thoracic aortas of 16 week old *Ercc1*$^{d/-}$ and WT mice. Quantitative real-time PCRs were conducted using a Step-One cycler Applied Biosystems (UK, Applied Biosystems), and the SYBR ® Green PCR Master Mix (UK, Applied Biosystems) as per manufacturer's recommendations. β-actin and HPRT-1 DNA quantitation was performed in parallel on all samples in order to determine the actual input amount of

cDNA and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency. The sense and antisense primers for mouse p21 were 5'-GCC-CAA-GGT-CTA-CCT-GAG-CCC-3'; 5'-TCT-TGC-AGA-AGA-CCA-ATC-TGC-GCT-3' , and the sense and antisense primers for mouse p53 were 5'-CTC-CAG-CTG-GGA-GCC-GTG-TC-3'; 5'-GCT-CCC-TGG-GGG-CAG-TTC-AG-3'respectively.

A 2-fold dilution series was created from a random pool of cDNA from our sample groups. The PCR efficiency and correlation coefficients (R2) of each primer pair were generated using the slopes of the standard curves. The efficiencies were calculated by the formula: efficiency (%) = (10(−1/slope) − 1) * 100.

For a correct interpretation of the real-time PCR results all data has been normalized which is achieved by calculating the geometric mean of the two stable reference genes.[44-45]

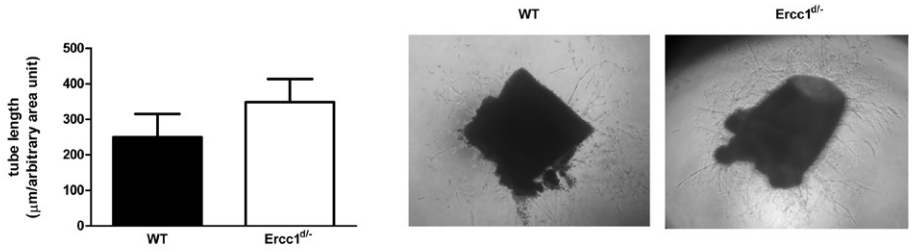
Ring outgrowth assay

For the ex vivo analysis of endothelium dependent sprouting of aortas, tissue of 16 week old *Ercc1*$^{d/-}$ and WT mice were harvested, cut into 2 mm thick segments, and placed in serum-reduced Matrigel in a 96 well plate (BD Biosciences, The Netherlands). Embedded aortic segments were then incubated with 200µL fibroblast-conditioned EGM2 medium per well for 4 days before evaluation by phase-contrast microscopy. Tube length was analysed with a commercial image analysis system (Impak C, Clemex Technologies, Canada).

Assessment of blood pressure and vasodilator function in vivo

To measure in vivo vasodilator function, a Laser Doppler perfusion imaging was used to determine the increase of the hindleg perfusion after transient occlusion of the blood flow (reactive hyperemia). This measurement was done at least 3 days and maximally 7 days after blood pressure measurement with tail cuff.

Twenty-four hours before measuring blood flow, the hair of the left hind leg was removed by a hair removal cream. At the day of measurement, mice were anesthetized by 2.8% isoflurane/O_2 ventilation, and kept on a heating pad regulated by a rectal thermometer to keep the body temperature stable between 36.4 and 37.0 °C. The hindleg in which the measurements were performed was kept in a steady position with the use of a fixation



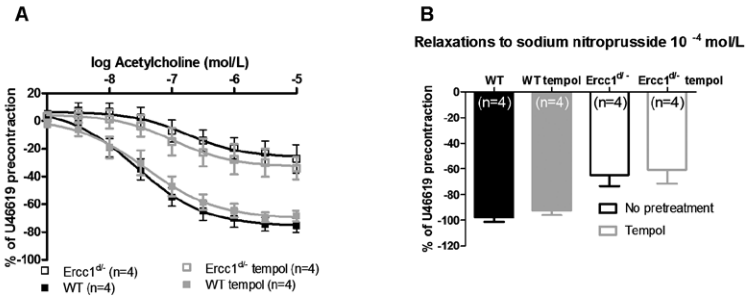
Supplemental figure 1. Tube outgrowth from aortic tissue under culture conditions.
The endothelium-dependent angiogenic potential is preserved in *Ercc1*$^{d/-}$ when compared with their WT littermates.

device that was designed for this particular purpose. After 20 minutes of equilibration, 5 minutes baseline perfusion was recorded. Then, 2 minutes of occlusion was applied with the use of an exogenous tourniquet. After release of the tourniquet, blood flow was monitored for 10 minutes during which hyperaemia and return of the blood flow to the post-occlusion baseline occurred. For analysis, the region of interest containing the whole of the leg was chosen. The results were expressed as relative values compared to the post-occlusion baseline. To compare the Ercc1$^{d/-}$ mice with WT mice the results were expressed as the maximum response to occlusion or the area under the response curve (only the area above the baseline was considered, values below the baseline were considered to be 0).

To address if impaired peripheral vasodilator function results in increased blood pressure we measured the blood pressure in conscious *Ercc1$^{d/-}$* mice and WT littermates using the tail cuff technique (using CODA High-Throughput device from Kent Scientific). Stress-induced responses were minimized by a 4-days period of acclimatization sessions. At day 5, 30 measurement cycles were recorded, and the mean of all correct measurements, as determined on the basis of pressure-flow relationships, were taken for each mouse.

Organ bath experiments

Ercc1$^{d/-}$ and WT mice were asphyxiated in a CO_2 chamber. The thoracic aortas were isolated and stored overnight in cold, oxygenated Krebs-Henseleit buffer solution. Sizes of the aortas were consistently lower in *Ercc1$^{d/-}$* than in WT. The following day, vessel segments were mounted in 6-mL organ baths (Danish Myograph Technology, Aarhus, Denmark) containing 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) 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. Preconstriction with 30 nmol/L U46619 resulted in 50-100% of 100 mmol/L KCl precontraction. Following preconstriction, relaxation concentration-response curves (CRCs) were constructed to acetylcholine, and responses to 100 µmol/L sodium nitroprusside were determined. L-NAME 100 µmol/L, given 10 minutes before U46619, was used to investigate the involvement of nitric oxide (NO)



Supplemental figure 2. Effects of acute tempol supplementation in *Ercc1$^{d/-}$*.

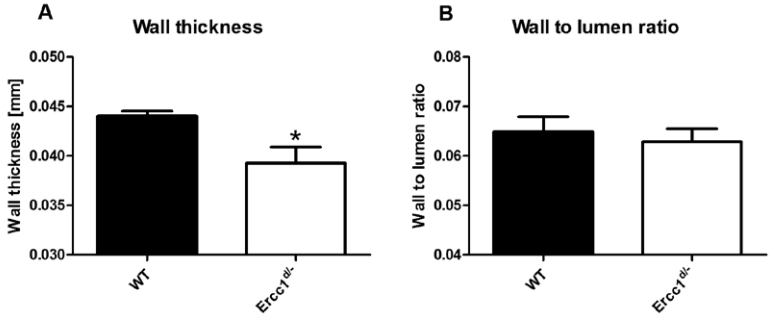
Responses to acetylcholine (A) and sodium nitroprusside (B) after pretreatment with tempol show no improvement.

in the relaxant responses. Similarly, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol), 100 µmol/L and N-acetyl-cysteine (NAC, 30 µmol/L) were used as scavengers of reactive oxygen species. Tetrahydrobiopterine (BH4, 100 µmol/L) was used to prevent the uncoupling of eNOS. To investigate the involvement of increased PDE enzyme activity in VSMC dysfunction of *Ercc1*$^{d/-}$ mice, 10 µmol/L vinpocetine was used. This PDE inhibitor displays selectivity towards PDE1.[46] vinpocetine was used together with 100 µmol/L L-NAME, to allow the measurement of the response to exogenous sodium nitroprusside (100 µmol/L) in the absence of endogenous NO. To test for the differences in protein kinase G (PKG) sensitivity, dose-response curves to 8-Bromo-ß-phenyl-1,N²-ethenoguanosine-3',5'-cyclicmonophosphate (8-Br-PET-cGMP) were constructed under L-NAME inhibition (100 µmol/L).

Measurement of mechanical properties of the vascular wall

The explanted thoracic aortas were fixed for 48 hours in formalin and consequently embedded in paraffin. Embedded tissue was transversally cut into 5 µm thick sections, and wall thickness and lumen diameter were measured.

The carotid arteries were explanted from 16 week old *Ercc1*$^{d/-}$ and WT animals. The region selected was between the aortic arch and first carotid branch, 2-3 mm of length. The carotid artery was 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), to measure the passive properties of the vessels. The intraluminal pressure of the vessel was increased stepwise by 10 mm Hg starting at 10 mm Hg and reaching 120 mm Hg. Each step was kept for 3 minutes for the vessel to equilibrate and at the end of the step, lumen and vessel diameter was measured. Using these data, mediastress and mediastrain were calculated and a stress-strain relationship was constructed.



Supplemental figure 3. Wall thickness and wall to lumen ratio.

Vessel dimensions are reduced in *Ercc1*$^{d/-}$ compared to WT mice. This can be also seen on the vessel wall thickness (A), however when related to its diameter (B), the ratio remains the same indicating, that the proportions are conserved and there is no relative hypertrophy in *Ercc1*$^{d/-}$ animals. * = $p<0.05$

Lung eNOS activation experiments

Freshly excised lungs from *Ercc1*$^{d/-}$ or WT mice were cut into approximately 1 mm^3 pieces, divided into equally sized portions and kept in the Krebs-Henseleit buffer at 37ºC and oxygenated with 95% O_2 and 5% CO_2. After 1 hour of equilibration, some portions of the lungs were exposed to 10 μmol/L acetylcholine for 10 minutes to determine the phosphorylation after stimulation, whilst others were not treated, to asses the baseline phosphorylation. After 10 minutes of treatment tissue was taken out of the buffer and snap-frozen in liquid nitrogen.

Immunoprecipitation and immunoblotting of eNOS

Aortas and pulverized hearts were lysed in Triton X-100 buffer. Tyr657-phosphorylated eNOS was immunoprecipitated with a specific antibody (Eurogentec, Seraing, Belgium, 1:1000).[47] Detergent-soluble proteins or immunoprecipitates were heated with SDS-PAGE sample buffer, separated by SDS-PAGE, blotted on nitrocellulose filters and incubated with specific antibodies against pSer1177-eNOS (sc-21871-R, Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000), eNOS (sc-654, Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000), β-actin (a1978, Sigma-Aldrich, 1:20,000) or pan-actin (DLN-07273, Dianova, Hamburg, Germany, 1:1000).

Statistical methods

Data are presented as mean±SEM. Statistical analysis between the groups of single values was performed by two-sided t-test or 1-way ANOVA followed by Bonferroni's post-hoc test, where appropriate. To test our assumption that blood pressure would be increased in *Ercc1*$^{d/-}$ animals, we employed a one-sided t-test. Differences in dose-response curves were tested by ANOVA for repeated measures (sphericity assumed). Differences were considered significant at $p<0.05$.

Human studies

To address the possible involvement of DNA repair capacity in development of CVD in humans, we investigated the association of SNPs in NER components to arterial stiffness. In accordance with the phenotype observed in mice, we examined the association of 310 selected NER SNPs with carotid-femoral pulse wave velocity (CFPWV) within the framework of the AortaGen Consortium. CFPWV is a measure of aortic stiffness that is strongly associated with increased risk for major cardiovascular disease events.

AortaGen Consortium

a. Consortium Organization: The AortaGen Consortium consists of 20,634 participants from 9 cohort studies that completed genome-wide genotyping and had measured carotid-femoral CFPWV[12]; including: the Age, Gene/Environment Susceptibility-Reykjavik Study (AGES-RS), the Baltimore Longitudinal Study of Aging (BLSA), the Erasmus Rucphen Family (ERF) study, the Framingham Heart Study (FHS), the Health, Aging and Body Composition (Health ABC) Study, the Heredity and Phenotype Intervention (HAPI) Heart

Study, the Rotterdam Study I (RS-I), the Rotterdam Study II (RS-II)[49], and the SardiNIA Study.

b. Carotid-femoral pulse wave velocity (CFPWV) as the phenotype: CFPWV is the gold standard method for assessment of arterial stiffness and is determined from the time taken for the arterial pulse to propagate from the carotid to the femoral artery. Different cohorts included in the AortaGen Consortium used different methods to ascertain carotid-femoral transit distance. To control for such differences, the AortaGen Consortium performed the genetic association analyses 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^2, height and weight. As a result of these transformations, the cohorts had a highly comparable distribution of CFPWV (mean of 0 and standard deviation of 1 with a normal distribution)

c. Genotyping and Imputation: Genotyping and imputation methods for all of the 9 cohorts included in the AortaGen Consortium have been described previously[12]. For genome-wide SNP sets, genotyping was carried out using commercially available arrays. Prior to imputation, quality control measures were applied. MACH was used by all cohorts for imputation of genotypes to the HapMap set of approximately 2.5 million SNPs.

d. Statistical Analyses: The AortaGen Consortium used a sex-specific standardized regression residual for 1000/CFPWV, adjusted for age, age^2, height and weight for meta-analysis. Genome-wide association analyses were conducted within each cohort using an additive gene-dose model. Linear mixed effects models were fitted to account for relatedness in pedigrees. Within-study associations were combined by prospective meta-analysis using inverse-variance weighting. Meta-analyses were performed using the software program MetABEL (http://www.genabel.org/packages/MetABEL). During meta-analysis, SNPs were excluded if weighted mean minor allele frequency was <1%, resulting in 2.41 million SNPs for analysis. The genomic control parameter was calculated to adjust each study and after meta-analysis, was recalculated to adjust for among-study heterogeneity.

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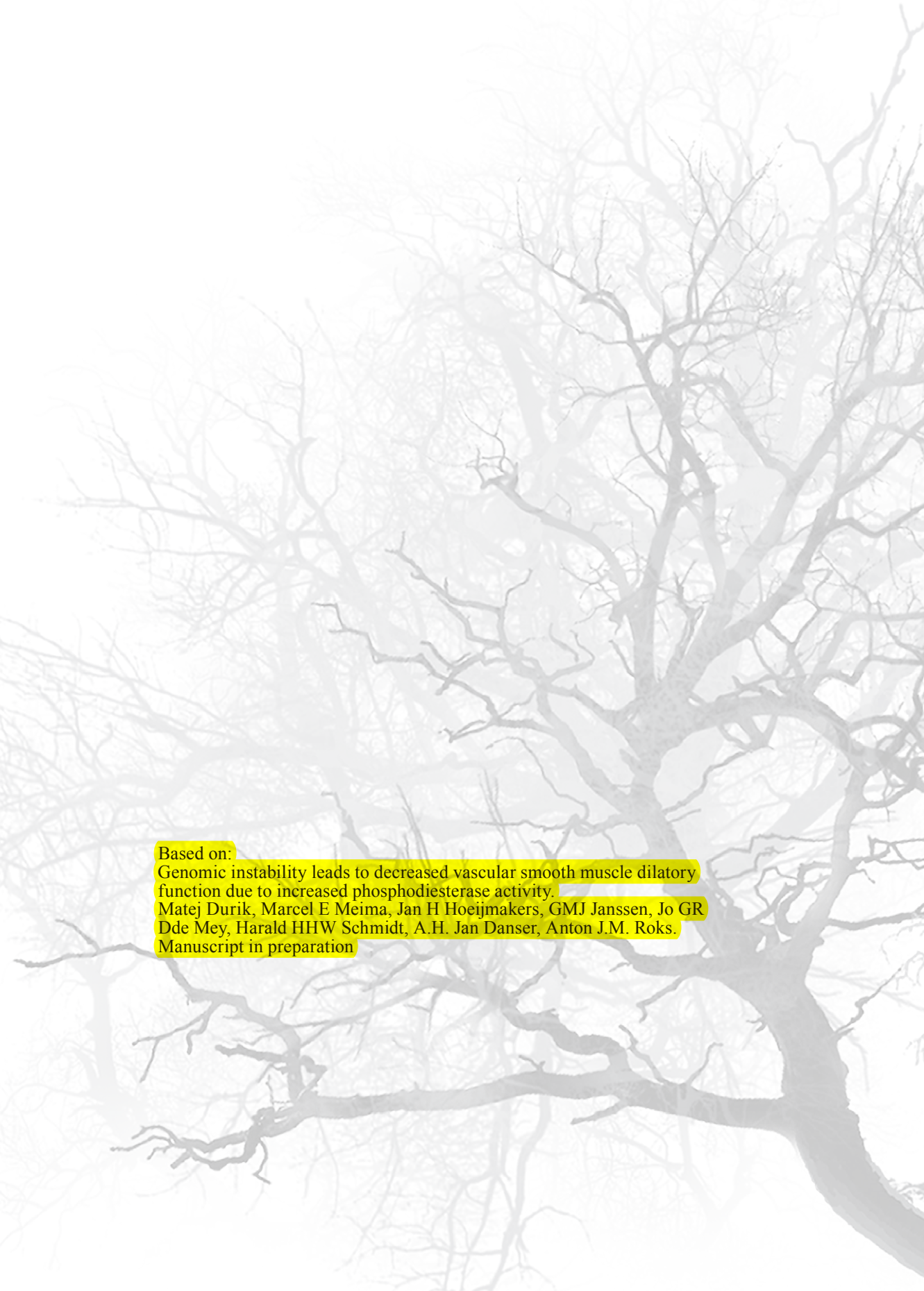

Based on:
Genomic instability leads to decreased vascular smooth muscle dilatory function due to increased phosphodiesterase activity.
Matej Durik, Marcel E Meima, Jan H Hoeijmakers, GMJ Janssen, Jo GR Dde Mey, Harald HHW Schmidt, A.H. Jan Danser, Anton J.M. Roks.
Manuscript in preparation

Genomic instability leads to decreased vascular smooth muscle dilatory function due to increased phosphodiesterase activity

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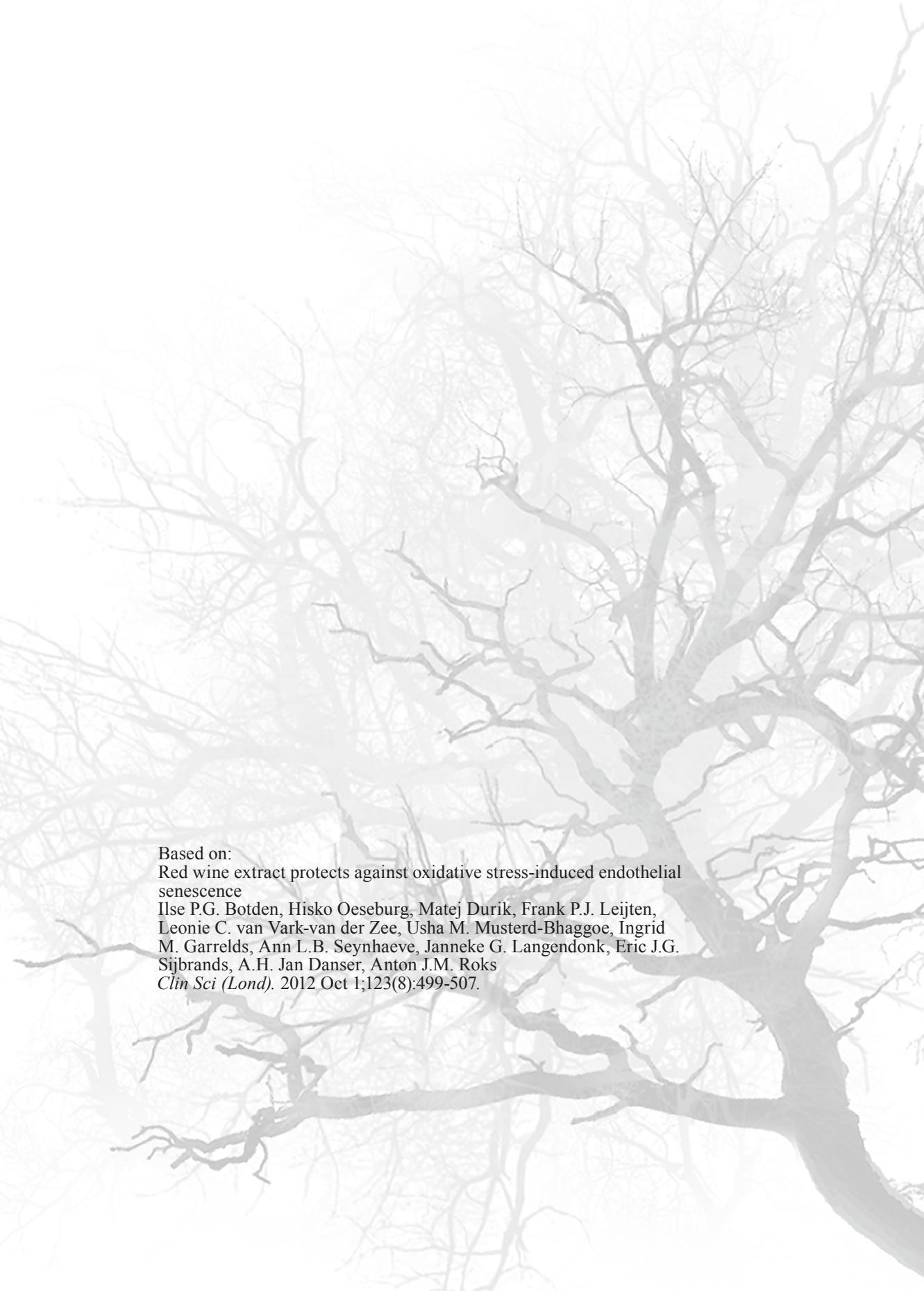

Based on:
Red wine extract protects against oxidative stress-induced endothelial senescence
Ilse P.G. Botden, Hisko Oeseburg, Matej Durik, Frank P.J. Leijten, Leonie C. van Vark-van der Zee, Usha M. Musterd-Bhaggoe, Ingrid M. Garrelds, Ann L.B. Seynhaeve, Janneke G. Langendonk, Eric J.G. Sijbrands, A.H. Jan Danser, Anton J.M. Roks
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Red wine extract protects against oxidative stress-induced endothelial senescence

4

Abstract

Objective

Red wine polyphenols may preserve endothelial function during aging. Endothelial cell senescence enhances age-related endothelial dysfunction. We investigated whether red wine extract (RWE) prevents oxidative stress-induced senescence in human umbilical vein endothelial cells (HUVECs).

Methods and Results

Senescence was induced by exposing HUVECs to *tert*-butylhydroperoxide (*t*BHP), and quantified by senescence-associated β-galactosidase staining. RWE (0-50 μg/mL) concentration-dependently decreased senescence by maximally 33±7.1%. RWE prevented the senescence-associated increase in p21 protein expression, inhibited *t*BHP-induced DNA damage of endothelial cells, and induced relaxation of porcine coronary arteries. Inhibition of SIRT1 by sirtinol partially reversed the effect of RWE on *t*BHP-induced senescence, whereas both the nitric oxide (NO) synthase inhibitor L-NMMA and the cyclo-oxygenase (COX) inhibitor indomethacin fully inhibited it. Furthermore, incubation of HUVECs with RWE increased eNOS and COX-2 mRNA levels as well as phosphorylation of eNOS at Ser1177.

Conclusions

RWE protects endothelial cells from *t*BHP-induced senescence. NO and COX-2, in addition to activation of SIRT1, play a critical role in the inhibition of senescence induction in human endothelial cells by RWE.

Introduction

Aging is an independent and important risk factor for cardiovascular disease.[1] Senescence of the vascular wall may be on the causal path to age-related endothelial dysfunction and atherogenesis, as it stimulates inflammation, raises blood pressure and promotes thrombosis.[2-3] DNA damage through excessive production of reactive oxygen species (ROS) is an important mechanism underlying endothelial senescence.[4] Currently, a large number of studies are ongoing that address the question if lifestyle or pharmacological intervention can inhibit this process.

Moderate consumption of red wine since long has been postulated to be part of a healthy life style.[5] Under controlled conditions in animal studies, red wine extract (RWE) prevented age-induced endothelial dysfunction.[6] We and others have shown that RWE elicits the release of endothelium-derived vasodilating factors and activation of SIRT1, a versatile deacetylase that has been implicated in endothelial cell aging.[7-12] Recently, we have also shown that the release of nitric oxide (NO), induced by the vasodilator peptide hormone bradykinin protects against ROS-induced endothelial cell senescence.[13] We hypothesized that RWE protects against ROS-induced endothelial cell senescence, and that this is due to the release of vasodilator signalling factors and SIRT1 activation.

In the present study, we investigated how RWE reduces ROS-induced endothelial cell senescence. Although only present in very small amounts, we also explored a possible role for the most studied red wine polyphenol resveratrol, which was also implicated in SIRT1-mediated protection against endothelial senescence.[12, 14]

Materials and Methods

Composition of RWE

The alcohol-free RWE we used is Provinols (Seppic, France). This RWE is derived from red wine produced in the Languedoc-Roussillon regions in the South-East of France. RWE contained 632 mg polyphenols/g, determined as gallic acid equivalents using Folin Ciocalteu reagent. The specific polyphenol contents in the red wine extract were assessed by HPLC analysis and this revealed that 550 mg of wine solids contain 18.8 mg anthocyanins, 6.9 mg phenolic acids, 4.0 mg catechins, 0.4 mg flavonols and 0.1 mg stilbenes.

Cell culture studies

HUVECs were isolated by collagenase digestion as described by Jaffe et al.[15] HUVECs were cultured on 0.2% gelatin coated plates in HUVEC culture medium containing human endothelial-serum free medium and Dulbecco's modified Eagle's medium (Invitrogen), 10% heat–inactivated newborn calf serum, 5% heat-inactivated human serum (Lonza), 10 ng/mL human recombinant basic fibroblast growth factor, and 50 ng/mL human recombinant epidermal growth factor (Peprotech) in a humidified incubator at 37°C and 5% CO_2. Experiments were conducted on cells with a passage number between 3 and 9.

Primary bovine aortic endothelial cells (BAECs) were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Thermo Scientific) in a humidified incubator at 37°C and 5% CO_2. Experiments were conducted on cells with a passage number between 5 and 9.

Design of the pharmacological studies

HUVECs were seeded at a density of 5000 cells/cm^2. After 24 hours, cells were starved with DMEM + 0.5% fetal calf serum for at least 6 hours. Next, the medium was replaced by HUVEC culture medium with or without RWE (3.125–50 µg/mL) in the presence or absence of the cyclooxygenase (COX) inhibitor indomethacin, the NO synthase inhibitor N^G-Methyl-L-arginine acetate salt (L-NMMA; Sigma-Aldrich), or the SIRT1 inhibitor sirtinol (Calbiochem). After one hour, tert-butylhydroperoxide (*t*BHP; Sigma-Aldrich) was added to the medium for 2 hours to induce senescence. Subsequently, the medium was replaced with HUVEC culture medium with or without RWE, indomethacin, L-NMMA or sirtinol.

Apoptosis

Apoptosis was determined with the Caspase-Glo 3/7 Assay (Promega) 18 hours after treatment. In 96-well plates, a 50-µL sample of supernatant was mixed gently for 30 seconds with 50 µL of Caspase-Glo 3/7 reagent and incubated for 2 hours at room temperature (20ºC). Caspase-3 activity was determined by luminescence of the samples measured using a Victor Wallac Multilabel Counter 1420.

ROS production

2',7'-dichlorofluorescein (DCF), a fluorescent dye, was used to assess endothelial ROS production. BAECs were cultured in a 96-well plate. At least 8 hours before the measurements, medium was replaced by DMEM medium without serum. Subsequently, the medium was replaced by HBSS with our without RWE (25 µg/mL). After 30 minutes, cells were loaded with CM-H_2DCFDA (Invitrogen) at a concentration of 10 µmol/L at 37°C in the dark for 30 minutes. Next, the medium was replaced by HBSS and *t*BHP (55 µmol/L) was added. Fluorescence (excitation, 488 nm; emission, 515–530 nm) was measured after one hour using a fluorescence plate reader (Wallac Victor2; PerkinElmer).

Evaluation of the number of senescent cells

When cells reached confluency, they were fixated in 2% formaldehyde / 0.2% glutaraldehyde for 10 minutes and the number of senescent cells was determined by senescence-associated SA-β-gal staining (150 mmol/L of NaCl, 2 mmol/L of $MgCl_2$, 5 mmol/L of $K_3Fe(CN)_6$, 5 mmol/L of $K_4[Fe(CN)_6]$, 140 mmol/L of Na_2HPO_4, 40 mmol/L citric acid, and 1 mg/mL of 5-bromo-4-chloro-3indolyl-β-*D*- galactoside, pH 6.0 for 18 hours at 37°C).[16] Cells were counterstained with 4',6-diamidino-phenylindole (DAPI; 2 µg/mL) to allow total cell number counting. Light microscopic pictures were taken on an inverted microscope (Zeiss Axiovert 200M) and the absolute number of senescent cells

and the total number of cells were counted per microscopic field by ImageJ software. In each well, 4 random fields were evaluated.

DNA damage assay

DNA damage was determined by single nuclei electrophoresis, also called comet assay.[17] Cells were harvested and approximately 700 cells were placed on a Trevigen Cometslide in 0.7% low melting agarose (Serva). Cells were lysed for one hour in Trevigen lysis solution, followed by 30 minutes of denaturation by 300 mmol/L alkaline solution and 1 mmol/L EDTA at pH>13 which was followed by 30 minutes of electrophoresis at 1 volt/cm in 300 mmol/L alkaline solution and 1 mmol/L EDTA at pH>13. DNA was stained with 1x SYBR Green (Invitrogen) and photos were taken with a 10x objective (Zeiss Axiovert 200M). Olive tail moment (percentage of DNA in the tail x distance to center of gravity of tail) was determined with CASP 1.2.2 software.[18] Experiments were repeated 3 times and per experiment more than 100 comets were analysed per treatment group.

Western blot analyses

Cultured cells were lysed with 50 mmol/L TrisHCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L Igepal CA-630, 5 mmol/L deoxycholic acid, and 1 mmol/L sodium dodecyl sulfate, in the presence of protease inhibitor cocktail (Roche) and serine-threonine phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Lysates were analyzed by standard Western blotting techniques under denaturating conditions. The following antibodies were used: anti-p21 (12D1, Cell Signalling), anti-p53 (DO-1, Sigma-Aldrich), anti-acetylated p53 (Lys 382, Cell Signalling), anti-eNOS (C-20, Santa Cruz), anti-phosphorylated eNOS (Ser1177, Santa Cruz), and anti-actin (C4, Millipore) for normalization of the protein levels. Signals were detected by enhanced chemiluminescence detection method and quantified by densitometry. Ratios of acetylated or phosphorylated proteins versus total proteins were calculated as (acetylated or phosphorylated protein/actin)/(total protein/actin) for each separate experiment.

Real-time quantitative reverse transcription PCR

Total RNA isolation was performed with the NucleoSpin RNA II kit (Machery-Nagel). RNA was reverse transcribed by use of the Quantitect Rev. Transcription Kit (Qiagen). Four nanograms of cDNA was amplified by real-time polymerase chain reaction (qPCR) and normalized to 36B4 as an endogenous control. Each reaction was performed in duplo with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: SIRT1 forward 5'-AGGCCACGGATAGGTCCATAT-3, reverse 5'-CCAATCATAAGATGTTGCTGAAC-3; eNOS forward 5'-CTTCCGCTACCAGCCAGAC-3', reverse 5'-TCTCGGAGCCATACAGGATT-3'; COX-2 forward 5'-CCCAGCACTTCACGCATCAG-3', reverse 5'-AGACCAGGCACCAGACCAAAGACC-3'.

Prostaglandin E_2 and prostacyclin release

HUVECs were cultured in 12- or 24-well plates without serum as described above. Next, RWE (25 μg/mL) or interleukin-1β (1 ng/mL; positive control) were added to the medium for 6 hours. Subsequently, the medium was collected and stored at -80°C until the analysis of prostaglandin E_2 (PGE_2; KGE00B, R&D Research) and prostacyclin (PGI_2; ADI-900-025, Enzo Life Sciences) by ELISA.

Vascular reactivity studies

Methods were set up as previously described.[19] Briefly, porcine coronary arteries (PCAs) were obtained from 8 slaughterhouse pigs. The arteries were cut into segments and suspended in 15-ml organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 / 5% CO_2 at 37°C. The vessel segments were exposed to 30 mmol/L KCl twice, and subsequently to 100 mmol/L KCl to determine the maximal contractile response. The segments were then incubated for 30 min in the absence or presence of one or more of the following compounds: the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), the intermediate- and small-conductance Ca^{2+}-dependent K^+-channels inhibitors TRAM34 (10 μmol/L) and apamin (100 nmol/L), or indomethacin (10 μmol/L). Vessels were then preconstricted with 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin F2α (U46619; 0.1–1μmol/L) to »80% of the maximal constriction, and RWE-concentration–response curves were constructed. Apart from RWE, all compounds were from Sigma-Aldrich.

Statistical Analysis

Values are presented as mean values ± SEM in the text and figures. Differences between the groups of the cell culture experiments, in which treatments were always performed in parallel, were analyzed using two-tailed paired Student t-tests or 1-way analysis of variance (ANOVA) with correction for multiple comparisons, unless indicated otherwise. Differences between groups in vascular reactivity studies were analyzed using the general linear model for repeated measures. Probability values less than 0.05 (or corrected after post-hoc tests) were considered significant.

Results

RWE and oxidative stress-induced endothelial senescence

To investigate the efffect of RWE on endothelial oxidative stress-induced senescence we exposed HUVECs to 55 μmol/L *t*BHP for 2 hours. To ensure that apoptosis did not bias the results, we measured caspase-3 activity (an indicator of apoptosis) after *t*BHP exposure. Caspase-3 increased at *t*BHP concentrations above 55 μmol/L (Figure 1A), and significance for this effect was reached at 100 μmol/L. Therefore, we performed all further experiments with 55 μmol/L *t*BHP.

Exposure of BAECs to 55 μmol/L *t*BHP increased ROS production almost 1.5-fold

(P<0.05, n=4), and RWE prevented this effect (Figure 1B). Exposure of HUVECs to 55 µmol/L *t*BHP increased the percentage of senescent cells 3.8 fold (from 4.8±1.2% to 18±2.1%, P<0.05, n=6). Treatment with RWE concentration-dependently decreased endothelial senescence by maximally 33±7.1% (Figure 1C and D). Removing RWE immediately before the application of *t*BHP yielded a similar effect (n=5; data not shown). To exclude the possibility that the effect of RWE on the percentage of senescent cells was due to an increase in cell proliferation, we counted the absolute number of cells. RWE had no effect on the total number of cells, but decreased the absolute number of senescent cells from 26±4.5 cells/microscopical field without RWE to respectively 20±4.1 and 15±2.5 cells/microscopical field for 25 and 50 µg/mL RWE (P<0.05, linear regression for trend, n=6).

*t*BHP induced DNA damage, as evidenced by the increased olive tail moment of the comet assay (Figure 2A), and upregulated p21 protein expression (Figure 2B), and RWE clearly reduced these effects. We did not find an effect of RWE on cells not exposed to *t*BHP.

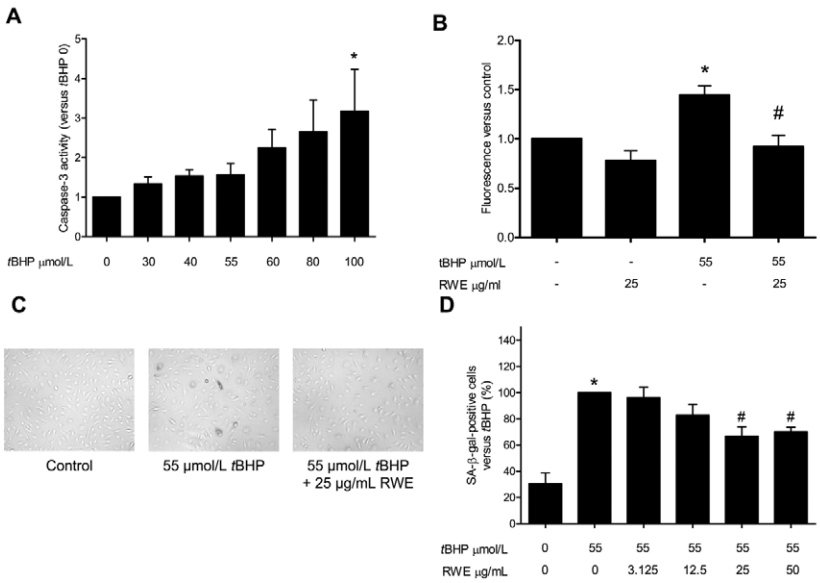

Figure 1. RWE and oxidative-stress-induced endothelial senescence

A, The effect of different concentrations of *t*BHP on apoptosis in HUVECs as analyzed by caspase-3 activity at 24 hours after addition of *t*BHP (**P*<0.05 versus control; n=3-5). B, Effect of RWE on ROS-production induced by *t*BHP, as measured by fluoresecence at one hour, after no treatment (control), 25 µg/mL RWE, 55 µmol/L *t*BHP or 55 µmol/L *t*BHP + 25 µg/mL RWE (**P*<0.05 versus control, #*P*<0.05 versus *t*BHP; n=4). C and D, Examples of SA-β-gal staining (C) and the effect of increasing concentrations of RWE (0-50 µg/mL) on *t*BHP-induced senescence in HUVECs as judged by SA-β-gal staining at 48 hours after addition of *t*BHP. D, Percentage SA-β-gal positive cells expressed relative to the 55 µmol/L *t*BHP group (**P*<0.05 versus control, #*P*<0.05 versus 55 µmol/L *t*BHP; n=5-6).

RWE and oxidative stress-induced endothelial senescence: role of SIRT1

RWE increased SIRT1 gene expression (by 14±10% at 10 minutes of exposure, n=8, P<0.05; data not shown). However, the SIRT1 activator resveratrol did not prevent *t*BHP-induced senescence (Figure 3A), although it did cause the typical morphological elongations described earlier [20]. In fact, resveratrol in a concentration of 50 µmol/L even increased senescence (P<0.05). The SIRT1 inhibitor sirtinol, like *t*BHP, increased the number of senescent cells, although their effects were not additive (Figure 3B). Sirtinol, at least partly, reversed the effect of RWE on *t*BHP-induced senescence. This indicates that SIRT1 activation by factors other than resveratrol may underlie the protective effect of RWE.

Sirtinol increased the levels of acetylated p53 (Figure 3C), and RWE fully reversed this effect. This finding supports the concept that SIRT1 decreases senescence through p53 acetylation. Yet, sirtinol decreased total p53 by »50% (P<0.05; Figure 3D), and RWE did not alter this decrease. As a consequence, the ratio of acetylated/total p53 increased after sirtinol (from 2.8±0.6 to 9.7±2.0; P<0.05). RWE reversed this increase (to 5.2±1.6; P=NS vs. control).

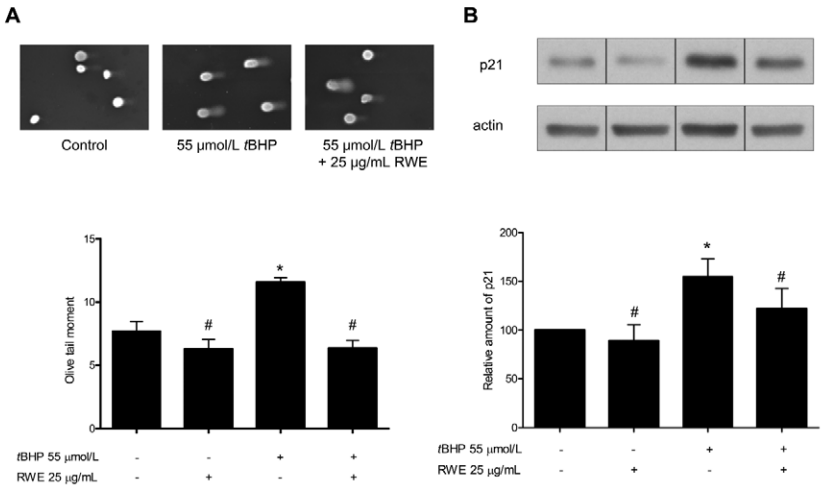

Figure 2. DNA damage protecting effects of RWE

A, Effect of RWE on DNA damage induced by *t*BHP, as assessed by comet assay. Top panel shows examples of single cell electrophoresis 24 hours after no treatment (control), 25 µg/mL RWE, 55 µmol/L *t*BHP or 55 µmol/L *t*BHP + 25 µg/mL RWE (**P*<0.05 versus control, #*P*<0.05 versus *t*BHP; n=3). B, Expression of p21 protein levels 24 hours after no treatment (control), 25 µg/mL RWE, 55 µmol/L *t*BHP or 55 µmol/L *t*BHP + 25 µg/mL RWE, as measured by western blot. P21 protein levels are corrected for actin protein levels (**P*<0.05 versus control, #*P*<0.05 versus *t*BHP; n=6-7).

RWE and oxidative stress-induced endothelial senescence according to the activation of eNOS and prostaglandins

RWE upregulated eNOS and COX-2 in HUVECs (Figure 4A), and both L-NMMA (Figure 4B) and indomethacin (Figure 4C) abolished the protective effect of RWE in *t*BHP-induced senescence, whereas these drugs were without effect when given alone. RWE increased levels of Ser1177-phosphorylated eNOS, and addition of *t*BHP did not significantly decrease this effect (Figure 4D).

The medium of HUVECs cultured for 6 hours under serum-free conditions contained 123±56 pg/mL PGE_2 and 100±10 pg/mL PGI_2 (n=3 for each). RWE lowered PGE_2 0.3±0.2 fold (n=3; P=0.09), whereas interleukin-1β increased it 2.7±0.3 fold (n=3; P<0.05). PGI_2 levels (92±5 and 101±9 pg/mL, respectively) remained unaffected. Results were identical in the presence of 55 µmol/L *t*BHP (n=3; data not shown).

RWE relaxed preconstricted PCAs in an NO- and endothelium-derived hyperpolarizing factor (EDHF)-dependent manner, as evidenced by the blockade obtained with L-NAME and/or TRAM34 + apamin (Figure 5). Indomethacin did not block vasorelaxation, but rather seemed to increase the RWE-induced vasodilation.

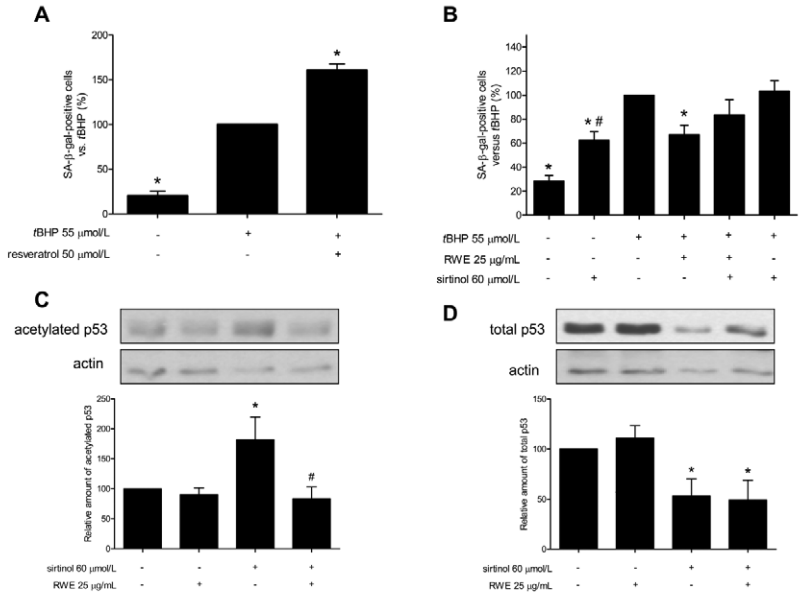

Figure 3. Interaction of RWE, resveratrol and SIRT1

A, The effect of resveratrol (50 µmol/L) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (**P*<0.05 versus *t*BHP; n=3). B, The effect of sirtinol (60 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. The percentage of SA-β-gal positive cells are expressed relative to 55 umol/L *t*BHP group (**P*<0.05 versus *t*BHP, #P<0.05 versus control; n=5-6). C and D, levels of p53 acetylation (C) and total p53 (D) protein levels 24 hours after either no treatment, 60 µmol/L sirtinol, 60 µmol/L sirtinol + 25 µg/mL RWE or 25 µg/mL RWE, as measured by western blot. Both acetylated and total p53 levels are corrected for actin levels (**P*<0.05 versus control, #*P*<0.05 versus sirtinol; n=4-8).

Discussion

Our results show that RWE protects endothelial cells from *t*BHP-induced oxidative senescence. The protective effect of RWE was associated with a decrease in p21, which is a DNA damage-related cyclin-dependent kinase inhibitor. Consistent with these findings, RWE protected endothelial cells from DNA damage. This protective effect appeared to be dependent on eNOS, COX-2 and SIRT1.

Our data provide first evidence that RWE is able to decrease the number of senescent cells and to reduce DNA damage in endothelium. Although to the best of our knowledge no other study has investigated the effect of RWE on endothelial senescence before, RWE intake by old rats was found to protect against deterioration of endothelium-dependent relaxations[6]. Since endothelial cellular senescence leads to diminished release of vasodilator substances, we here introduce a novel protective effect of RWE that might be closely

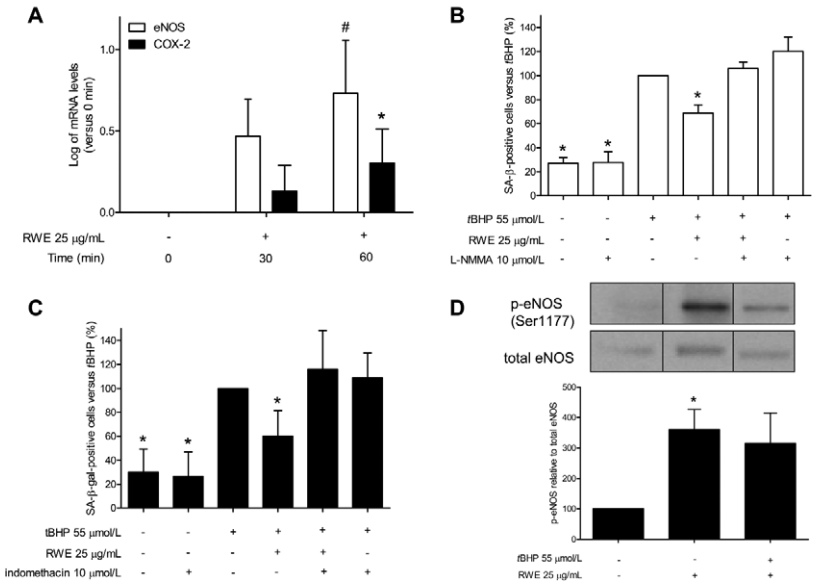

Figure 4. The role of eNOS and prostaglandins in RWE effects

A, eNOS and COX-2 mRNA levels in HUVECs after treatment with 25 µg/mL RWE for different time periods, as analyzed by real-time polymerase chain reaction. RNA is normalized to the internal control 36B4 (mean values are expressed as logarithmic values; **P*<0.05 versus control, #*P*=0.058 versus control; n=5-8). B, The effect of L-NMMA (10 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs as judged by SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (**P*<0.05 versus *t*BHP; n=6-7). C, The effect of indomethacin (10 µmol/L) and RWE (25 µg/mL) on *t*BHP-induced senescence in HUVECs according to SA-β-gal staining at 48 hours after addition of *t*BHP. Percentage SA-β-gal positive cells are expressed relative to 55 µmol/L *t*BHP group (**P*<0.05 versus *t*BHP; n=6-7). D, Levels of phosphorylated eNOS (p-eNOS) at Ser1177 2 hours after no treatment, 25 µg/mL RWE, or 55 µmol/L *t*BHP + 25 µg/ml RWE, as measured by Western blot. P-eNOS levels are corrected for total eNOS levels (**P*<0.05 versus control; n=4-5).

associated with this previous finding in aged rats.

Based on studies with the red wine product resveratrol,[12, 14] we expected that the protective pathway would involve SIRT1 activation. Overexpression of SIRT1 antagonizes cellular senescence through deacetylation of the DNA damage-related cell cycle regulator p53[21] and by promoting eNOS activity.[22] Moreover, resveratrol-containing red wine decreases the levels of assymetric dimethylarginine, an endogenous inhibitor of NO, in a SIRT1-dependent manner. [23] In line with these observations, we observed that the SIRT1 inhibitor sirtinol increased p53 acetylation and endothelial senescence. In addition, RWE modestly upregulated SIRT1 expression, and sirtinol, at least partially, reversed the effect of RWE on *t*BHP-induced senescence. Finally, RWE counteracted the effect of sirtinol on acetylated p53. Yet, resveratrol increased *t*BHP-induced senescence. The latter observation is complementary to recent studies[24-25] disputing the claim that resveratrol activates SIRT1 and thereby increases longevity.[10, 26] Taken together, our data indicate that SIRT1 activation by RWE does act protective, but is not induced by resveratrol. In fact, the actual resveratrol content of our RWE preparation (when applied at a concentration of 25 μg/mL) is estimated to result in a medium concentration of ≈0.2 μmol/L (analysed by Nutrinov Lab, Rennes, France), i.e., far below the resveratrol concentrations (10-50 μmol/L) that have been claimed to exert protective effects in vitro.[14]

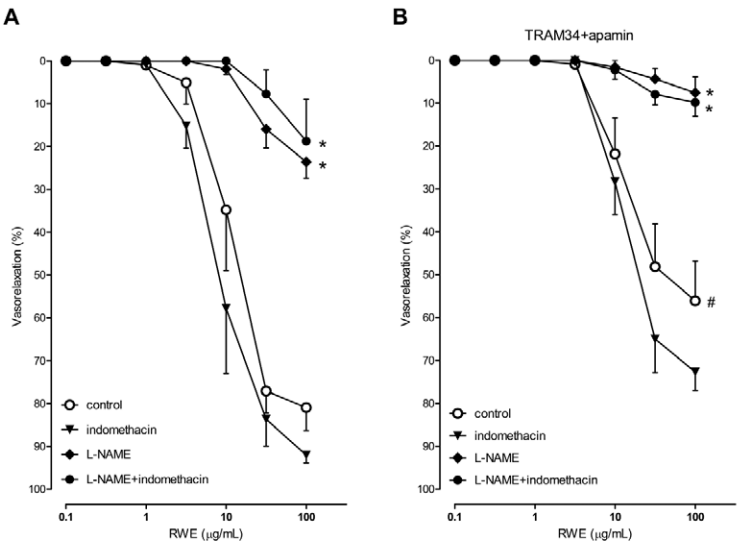

Figure 5. The role of endothelial vasodilating factors in RWE-induced relaxation

Concentration–response curves of U46619-preconstricted PCAs to cumulative doses of RWE, in the absence (A) and presence (B) of 10 μmol/L TRAM34 in combination with 100 nmol/L apamin (TRAM34+apamin), with or without 100 μmol/L L-NAME and 10 μmol/L indomethacin. Data are expressed as percentage of the contraction induced by U46619 (*P< 0.05 vs. control, #P<0.05 vs. control without TRAM34+apamin; n=6-8).

The protective effect of red wine against endothelial dysfunction was shown to be dependent on ROS scavenging.[27] This may also explain the effect on *t*BHP-induced senescence in our experiments. Indeed, RWE diminished ROS induced by *t*BHP, and protective effects were even observed when removing RWE shortly prior to the addition of *t*BHP. Clearly therefore, these effects do not depend on direct ROS scavenging by RWE, but rather on ROS inactivation by a secondary factor induced by RWE, possibly NO. In support of the latter, red wine upregulates eNOS (mRNA and protein) expression[28] and NO production[9] in endothelial cells. Consistent with these findings, we found that RWE increased eNOS mRNA levels and augmented eNOS phosphorylation in HUVECs. Moreover, RWE relaxed PCAs largely in an NO-dependent manner. Importantly, NOS inhibition reversed the senescence-inhibitory effect of RWE. Thus, the RWE-induced protection against senescence indeed depends on functional eNOS.

Alternatively, given the diminishing production of PGI_2 during *in vitro* aging of endothelial cells, a decreased production of (protective) prostaglandins might underlie senescence.[29] In line with this observation, the non-selective COX inhibitor indomethacin, similar to NOS inhibition, reversed the senescence-inhibitory effect of RWE. In addition, RWE upregulated COX-2, in full agreement with previous results in rats treated with red wine polyphenols.[30] However, RWE did not increase endothelial PGI_2 production, and even tended to decrease the production of PGE_2. Furthermore, indomethacin did not block the relaxant effects of RWE in PCAs, which argues against the possibility that RWE stimulates the production of vasodilator prostaglandins like PGE_2 and PGI_2. Thus, if COX-2-mediated prostaglandin production protects against senescence, this does not involve PGE_2 and PGI_2. It might involve thromboxane A_2 (TXA_2), since Diebolt et al.[30] demonstrated that red wine polyphenols increase endothelial, COX-2-dependent TXA_2 release. In fact, the latter study proposed that the NO-TXA_2 balance underlies the beneficial effects of red wine polyphenols. Other possible candidates include PGD_2, PGJ_2 and electrophile oxo-derivative (EFOX) molecules. The latter are of particular interest since they regulate nuclear factor erythroid-2-related factor-2 (Nrf2), which confers protection against oxidative stress.[31]

Because of the complex composition of RWE, it is difficult to determine the RWE bio-availability in vivo. Using the Fiolin-Ciocalteau method, Duthie et al.[32] found that intake of 100 mL of red wine by healthy volunteers increased the plasma concentration of polyphenolic monomers by ≈2-3 μg/mL. Since RWE contains 632 mg polyphenols/g, the RWE concentrations of 25 μg/mL we used in most experiments will yield polyphenol concentrations in the range expected in blood after drinking 336 mL (around 2 glasses) of red wine. Given that resveratrol is unlikely to be the protective constituent of our RWE extract, the question remains what is/are the responsible candidate(s). Identification of the specific RWE constituents that protect endothelial cells is important, because wine consumption and RWE may have large variability in composition. Different red wines showed different effects on vascular function.[33] Therefore, rational use of RWE protective effects can only be established by isolation of the specific relevant constituent(s). Since RWE contains at least 200 different polyphenols,[34] and because it may not be a single constituent that confers the protective effect, such a search demands high throughput

screening systems. Our present study suggests that such screening assays could use eNOS and COX-2 activation as read-out variables.

In summary, we have shown that RWE inhibits oxidative stress-induced endothelial senescence, and that activation of eNOS and COX-2, in addition to SIRT1, plays a critical role in the inhibition of a senescent phenotype in human endothelial cells. Our results indicate that RWE could exhibit a beneficial effect on the vasculature by protecting endothelial cells against senescence. Identification of the responsible components and testing them in clinical trials may provide novel therapeutic opportunities to counteract oxidative stress and age-associated cardiovascular diseases.

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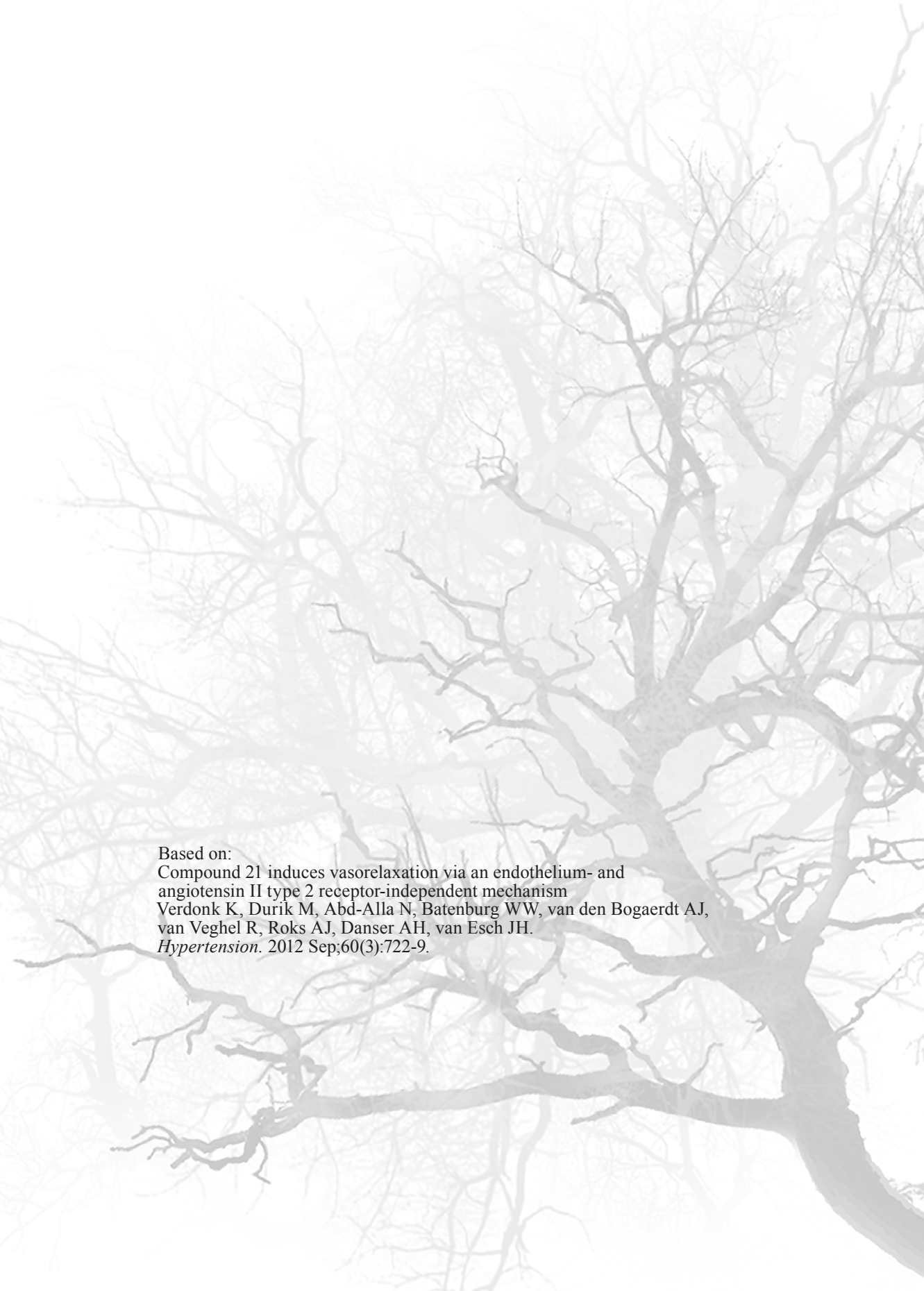

Based on:
Compound 21 induces vasorelaxation via an endothelium- and angiotensin II type 2 receptor-independent mechanism
Verdonk K, Durik M, Abd-Alla N, Batenburg WW, van den Bogaerdt AJ, van Veghel R, Roks AJ, Danser AH, van Esch JH.
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Compound 21 induces vasorelaxation via an endothelium- and angiotensin II type 2 receptor-independent mechanism

5

Abstract

Angiotensin II type 2 (AT_2) receptor stimulation has been linked to vasodilation. Yet, AT_2 receptor-independent hypertension and hypotension (or no effect on blood pressure) have been observed in vivo after application of the AT_2 receptor agonist compound 21 (C21). We, therefore, studied its effects in vitro, using preparations known to display AT_2 receptor-mediated responses. Hearts of Wistar rats, spontaneously hypertensive rats (SHRs), C57Bl/6 mice, and AT_2 receptor knockout mice were perfused according to Langendorff. Mesenteric and iliac arteries of these animals, as well as coronary microarteries from human donor hearts, were mounted in Mulvany myographs. In the coronary vascular bed of Wistar rats, C57Bl/6 mice, and AT_2 receptor knockout mice, C21 induced constriction followed by dilation. SHR hearts displayed enhanced constriction and no dilation. Irbesartan (angiotensin II type 1 receptor blocker) abolished the constriction and enhanced or (in SHRs) reintroduced dilation, and PD123319 (AT_2 receptor blocker) did not block the latter. C21 relaxed preconstricted vessels of all species, and this did not depend on angiotensin II receptors, the endothelium, or the NO-guanylyl cyclase-cGMP pathway. C21 constricted SHR iliac arteries but none of the other vessels, and irbesartan prevented this. C21 shifted the concentration-response curves to U46619 (thromboxane A2 analog) and phenylephrine (α-adrenoceptor agonist) but not ionomycine (calcium ionophore) to the right. In conclusion, C21 did not cause AT_2 receptor-mediated vasodilation. Yet, it did induce vasodilation by blocking calcium transport into the cell and constriction via angiotensin II type 1 receptor stimulation. The latter effect is enhanced in SHRs. These data may explain the varying effects of C21 on blood pressure in vivo.

Introduction

Stimulation of the angiotensin (Ang) II type 2 (AT_2) receptor mediates vasorelaxant,[1-6] natriuretic,[7] growth-suppressing[8] and antifibrotic[9] effects. As such, it seems to counteract Ang II type 1 (AT_1) receptor-mediated effects.[10] However, opposite findings have been reported as well, and according to some studies, AT_2 receptor effects mimic those of the AT_1 receptor (e.g., inducing vasoconstriction[11,12] and hypertrophy[13]). Our knowledge on AT_2 receptor function is largely based on the use of the AT_2 receptor antagonist PD123319, AT_2 receptor-deficient ($AT_2R^{-/y}$) animals and the peptidic AT_2 receptor agonist CGP42112A. The use of the latter is hampered by its partial agonistic properties. In 2004, Wan et al reported the synthesis of compound 21 (C21), the first selective non-peptide AT_2 receptor agonist.[14] C21 has an oral bioavailability of 20 to 30% and an estimated half-life of 4 hours in plasma. Administration of C21 in various cardiovascular disease models, including the post-myocardial infarction Wistar rat,[15] the stroke-prone hypertensive rat (SHR-SP)[16,17] and the 2-kidney, 1-clip (2K1C) hypertensive Sprague-Dawley (SD) rat,[18] resulted in beneficial organ-protective effects.

The vasorelaxant properties of C21 are less straightforward. C21 lowered MAP by ~25 mmHg in anesthetized spontaneously hypertensive rats (SHRs) but not in SD rats.[14] Remarkably, this response was not affected by AT_2 receptor blockade, suggesting that it did not involve AT_2 receptor stimulation. Bosnyak et al 19 reported a vasodepressor response after administration of C21 (300 ng/kg per minute) in conscious SHR (but not Wistar-Kyoto rats) on top of low-dose AT_1 receptor antagonism, which could be blocked by the AT_2 receptor antagonist PD1233319.[19] In the absence of AT_1 receptor blockade, no blood pressure-lowering effects were observed in stroke-prone hypertensive rats, 2-kidney, 1-clip hypertensive SD rats, post-myocardial infarction Wistar rats and C57BL/6 mice.[15-18,20] When infused over a short time period, 300 ng/kg per minute C21 induced a modest rise in mean arterial pressure (~4 mm Hg) in male SD rats which was not seen in combination with PD123319.[21] A 3.3-fold higher dose increased MAP by 20 mm Hg in male SHR in an AT_1 receptor-dependent manner.[19] Similarly, C21 transiently increased systolic blood pressure by ~20 mm Hg in an AT_1 receptor-dependent manner when given orally to stroke-prone hypertensive rats at a dose of 1 mg/kg per day.[17] Yet, in the latter study, C21 did not lower blood pressure on top of AT_1 receptor antagonism – in fact, C21 exerted no effect at all on blood pressure, possibly because the applied AT_1 receptor blocker dose had already resulted in maximum blood pressure-lowering effects.

Taken together, these in vivo data demonstrate C21-induced relaxation via AT_2 receptors or unknown mechanisms, as well as constriction via both AT_2 and AT_1 receptors, the latter requiring high doses. AT_1 receptor blockade appeared a prerequisite to observe AT_2 receptor-mediated hypotensive effects in vivo, but this is not a universal finding and may depend on the degree of AT_1 receptor blockade.[17,19,20] In vitro data on C21-induced relaxation are scarce, despite the wide range of in vitro studies supporting AT_2 receptor-induced relaxation in multiple vascular beds. No human data are available. Therefore, it was the aim of the present study to investigate C21-induced vasodilation/constriction in

vitro, taking into consideration species- (including humans), pathology- (hypertension) and concentration-related effects, and carefully considering its blockade by AT receptor antagonists or in AT_2 receptor-deficient ($AT_2R^{-/y}$) mice. We made use of preparations that in previous studies displayed clear AT_2 receptor-mediated vasodilation.[1,2,11,12,22]

Materials and Methods

Animal studies

Male Wistar rats (337±5 gram; n=45), male spontaneously hypertensive rats (SHR, 320±3 gram; n=18) and male C57BL/6 mice (29±1 gram; n=10) were obtained from Harlan. Male $AT_2R^{-/y}$ (28±1 gram; n=10) bred on a C57BL/6 background were obtained from the animal facilities of the Charité, Campus Benjamin Franklin, Berlin, Germany. C57BL/6 and $AT_2R^{-/y}$ mice were genotyped to verify AT_2 receptor expression. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Animals were anesthetized with pentobarbital (60 mg/kg i.p.). Rat hearts were excised and placed in ice-cold Tyrode buffer,[22] whereas mouse hearts were placed in modified Krebs-Henseleit (KH) buffer,[23] both gassed with 95% O_2/5% CO_2. Subsequently, iliac and mesenteric arteries were removed and either used directly or after overnight storage in cold, oxygenated KH solution. Such storage does not affect responsiveness.[24,25]

Human studies

Human coronary micro-arteries (HCMAs) were obtained from 5 heart-beating donors (2 men, 3 women, age 41±7 years), who died of non-cardiac causes (2 cerebrovascular accident, 1 head trauma, 1 subarachnoid bleeding and 1 suicide) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. HCMA were isolated and stored in KH as described before.[1]

Langendorff preparation

Rat and mouse hearts were perfused according to Langendorff as described before.[22,23] Gassed perfusion buffer was used to superfuse the mouse hearts to prevent temperature fluctuations. Coronary flow (CF) was measured with a flow probe (Transonic systems). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 mL) of perfusion buffer were applied three times to determine injection-induced changes in CF. Subsequently, concentration-response curves (CRCs) to C21 (kindly provided by Vicore Pharma) were constructed via bolus injections, in the absence or presence of the AT_1 receptor antagonist irbesartan (provided by Sanofi-Synthelabo) or PD123319. Blockers were present in the perfusion buffer starting 15 min before the first bolus injection.

Mulvany myograph

HCMAs (diameter ~750mm), rat iliac arteries (diameter ~800 mm), rat mesenteric arteries (diameter ~150 mm), mouse iliac arteries (diameter ~350 mm), and mouse abdominal aortas (diameter ~550 mm) were cut into ring segments of ~2 mm length. In some rat iliac artery segments, the endothelium was removed by gently rolling the vessel after insertion of the tip of a small-angled forceps into the lumen. Segments were mounted in a Mulvany myograph with separated 6-mL organ baths containing gassed KH buffer at 37°C as described previously, and tension was normalized to 90% of the estimated diameter at 100 mmHg effective transmural pressure. Following a 30-min stabilization period, the maximal contractile response was determined by exposing the vessels to 100 mmol/L KCl. Thereafter, vessels were pre-incubated for 30 min in fresh buffer in the absence or presence of 1 mmol/L irbesartan, 1 mmol/L PD123319, 100 mmol/L N^G-nitro-L-arginine methyl ester (L-NAME), 10 mmol/L 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 200 mmol/L hydroxocobalamin, 10 mmol/L Y27632 or 0.1 to100 mmol/L C21, and CRCs were constructed to C21, phenylephrine, U46619 or ionomycin. C21-induced relaxation was studied after preconstriction with U46619 (10-100 nmol/L) or 30 mmol/L KCl.

AT_2 receptor binding studies

HEK 293 cells stably expressing rat AT_2 receptors under geneticin selection (kindly provided by dr. W.G. Thomas, University of Queensland, Brisbane, Australia) were grown in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin/streptomycin, and 200 μg/mL geneticin. For binding studies, the cells were trypsinized and seeded in 48-well plates (Corning) at a density of $5x10^4$ cells per well. Cells were allowed to attach for 48 hours. The plates were then placed on ice, washed once with ice-cold Hanks' balanced salt solution, followed by another wash with cold Hanks' balanced salt solution supplemented with 0.1% BSA (binding buffer). After removal of binding buffer, C21, vehicle, or PD123319 (to determine non-specific binding) were added in 100 μL cold binding buffer and allowed to incubate for 20 minutes. Next, 50 μL binding buffer containing 25,000 cpm ^{125}I-Ang II was added. After 4 hours of incubation, binding buffer was removed, and wells were washed twice with Hanks' balanced salt solution. Subsequently, cells were lysed with 0.1 mol/L NaOH, and radioactivity was counted in a gamma counter.

Spectral analysis

To determine the molecular interaction between C21 and hydroxocobalamin, solutions containing hydroxocobalamin (200 μmol/L) and C21 (0.1-1 mmol/L) or NaCl (0.1-1 mmol/L; negative control) were prepared. Absorption spectra (300-560 nm) were determined using an UV mini-1240 spectrophotometer (Shimadzu). The pH values of individual solutions were measured afterward and were within the range of 6.7-7.0.

Data analysis

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments) and Labchart software (AD

Instruments). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc.) and Labchart. CRCs were analyzed as described before,[26] using Graph Pad Prism 3.01 (Graph Pad Software Inc.), to determine the maximum effect (E_{max}) and pEC_{50} (=-^{10}logEC_{50}) values. Statistical analysis was performed by one- or two-way ANOVA, followed by post-hoc evaluation according to Bonferroni. $P<0.05$ was considered significant.

Results

AT_2 receptor binding studies

C21 concentration-dependently prevented ^{125}I-Ang II binding to AT_2 receptor-transfected HEK-293 cells (K_i = 1.02±0.14 nmol/L, n=3; Figure 1). In addition, 1 µmol/L PD123319 displaced ^{125}I-Ang II binding to these cells by 96.9±1.6% (n=4).

Langendorff preparation

At concentrations >1 µmol/L (in the injection fluid), C21 induced a biphasic response in the coronary circulation of the Wistar rat (n=4): a CF decrease (constrictor phase) of maximally 14±3% (Figure 2A) followed by a CF increase (relaxant phase) of maximally 32±10% (Figure 2B). Irbesartan and PD123319 (n=4-5) abolished ($P<0.05$) the CF decrease, and enhanced the CF increase ($P<0.05$).

In SHRs, the constrictor effects of C21 were greatly enhanced (E_{max}: 48±4%; $P<0.05$ vs. Wistar rat; Figure 2C), whereas its relaxant effects were abolished (n=7; Figure 2D). Irbesartan (n=4) fully abolished the C21-induced coronary constriction ($P<0.05$) in SHR, and allowed the return of the relaxant response to C21 ($P<0.05$). PD123319 (n=4) partially reduced the constrictor response ($P<0.05$), but did not induce relaxation.

Results in C57BL/6 and $AT_2R^{-/y}$ mice (n=4, Figures 2E and 2F) mimicked those in Wistar rats.

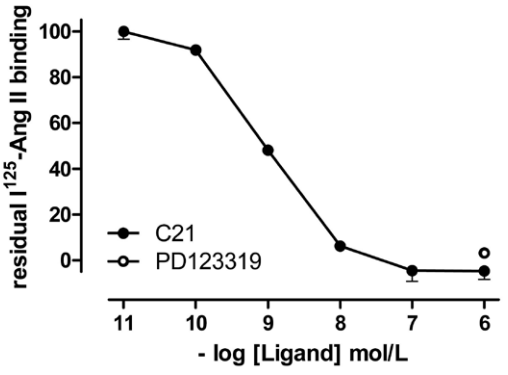

Figure 1.

Displacement of AT_2 receptor-specific ^{125}I-Ang II binding in AT_2 receptor-transfected HEK-293 cells by C21 and PD123319 (mean±SEM of triplicate measurement).

Figure 2

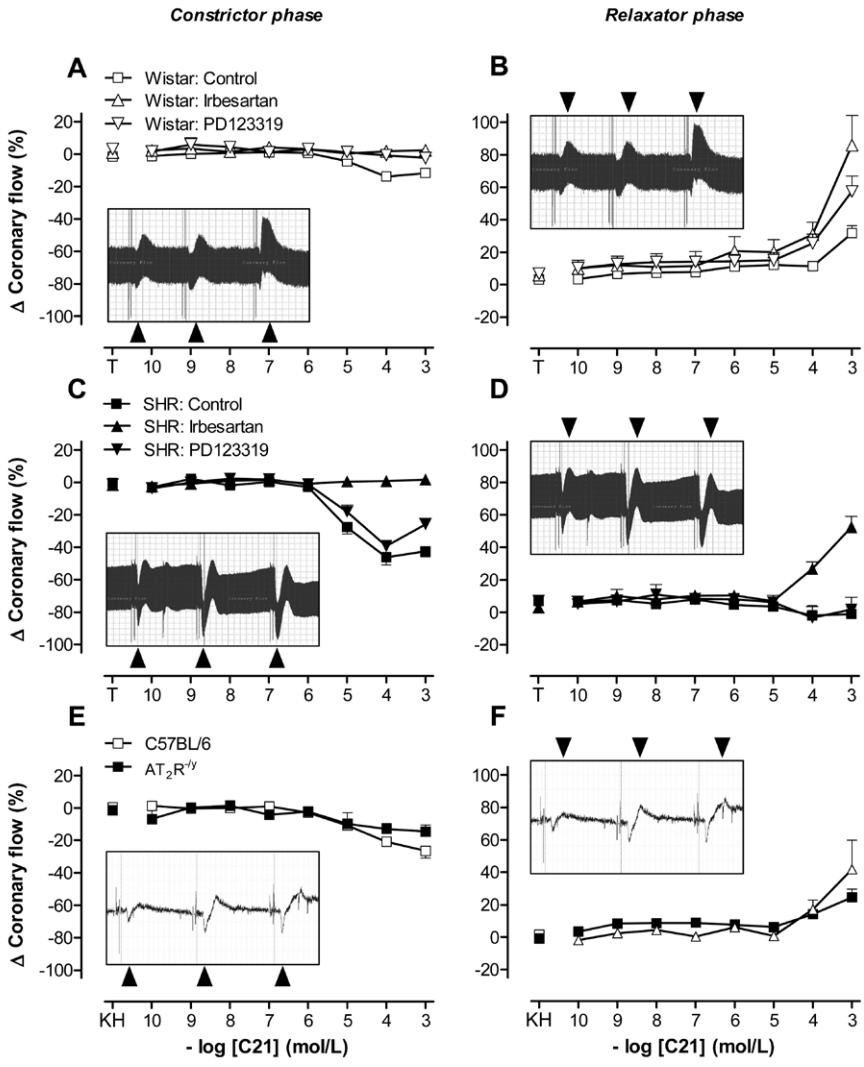

Figure 2.

Biphasic effect of C21 bolus injections (100 mL) on coronary flow in Wistar rats (A and B), SHR (C and D), and C57BL/6 versus $AT_2R^{-/y}$ mice (E and F) in the absence or presence of irbesartan or PD123319. Baseline flow values were 9.2±0.4, 11.3±0.7, 1.3±0.1 and 1.7±0.3 mL/min in Wistar rats, SHR, C57BL/6 and $AT_2R^{-/y}$ mice. Inlay, original tracing showing the effect of the highest C21 concentration. Arrows indicate the constrictor and relaxant phases. The x-axis displays the concentration of the agonist in the injection fluid. Data are mean±SEM of n=4-7, and represent percentage change from baseline. T and KH represent a bolus injection of Tyrode's (A-D) or KH (E and F) buffer, respectively. *P<0.05 vs. control.

Mulvany myograph

C21 concentration-dependently relaxed preconstricted HMCAs (pEC_{50} 4.8±0.3, E_{max} 83±4.2%, n=7; Figure 3A), Wistar rat iliac arteries (pEC_{50} 5.6±0.2, E_{max} 93.2±4.0%, n=7; Figure 3B), Wistar rat mesenteric arteries (pEC_{50} 5.8±0.3, E_{max} 93.8±6.6%, n=3; Figure 3C), , and SHR mesenteric arteries (pEC_{50} 6.6±0.2, E_{max} 93.2±2.6%, n=2; Figure 3D). C21 did not constrict human coronary microarteries (n=2, data not shown) or Wistar rat iliac arteries (n=4; Figure 3E). It did, however, constrict SHR iliac arteries (n=4; Figure 3F), and both irbesartan (n=4) and PD123319 (n=3) blocked this constrictor effect (Figure 3F).

To study the mechanism underlying the C21-induced vasorelaxation, we focused on AT receptors, the NO pathway and calcium entry. The NO scavenger hydroxocobalamin shifted the C21 CRCs in Wistar rat iliac arteries (pEC_{50} 4.7±0.2, E_{max} 85.5±8.4%, n=4; Figure 3B) and rat mesenteric arteries (pEC_{50} 5.1±0.2, E_{max} 89.5±7.0%, n=3; Figure 3C) ~5-10-fold to the right ($P<0.05$), whereas irbesartan, PD123319, the endothelial NO synthase (eNOS) inhibitor L-NAME or the guanylyl cyclase (GC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one were without effect. Irbesartan and PD123319 also did not affect the C21 responses in SHR iliac arteries (n=4; Figure 3D).

C21 relaxed preconstricted mouse iliac arteries (pEC_{50} 5.7±0.1, E_{max} 97.8±1.4, n=8; Figure 3G) and mouse abdominal aortas (pEC_{50} 5.7±0.1, E_{max} 98.4±0.3%, n=9; data not shown). Like in the rat, hydroxocobalamin, but not L-NAME, shifted the C21 CRCs in these arteries 4-10-fold to the right ($P<0.05$). Results in iliac arteries (n=6-10; Figure 3H) and abdominal aortas of $AT_2R^{-/y}$ mice (n=5-10; data not shown) were identical to those in C57BL/6 mice.

U46619 concentration-dependently constricted rat iliac arteries (n=21; Figure 4A). C21 (at a concentration of 1, 10 and 100 mmol/L, respectively) shifted the U46619 CRCs ~8, ~25 and >80-fold to the right ($P<0.05$ for all), and hydroxocobalamin, but not endothelium removal, irbesartan, PD123319, L-NAME or 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one prevented this shift (Figures 4B-4D). Similar observations were made for phenylephrine (n=3-4, Figure 4E). In contrast, 10 mmol/L C21 did not affect contractions induced by the calcium ionophore ionomycin, neither alone nor on top the RhoA-kinase inhibitor Y27635 (n=7; Figure 4F). As expected, Y27635 did suppress the ionomycin-induced contraction (n=7, $P<0.05$; Figure 4F).

Spectral analysis

Spectral analysis (300-560 nm) of solutions containing 200 μmol/L hydroxocobalamin demonstrated a peak absorption at a wavelength of 349.5 nm. In presence of 100 μmol/L and 1 mmol/L C21 the peak absorption was shifted to 354.5 nm and 358.0 nm respectively, whereas this was unaffected by the presence of equimolar NaCl concentrations (Figure 5). Spectral analysis of solutions containing 100 μmol/L C21 or water did not reveal any absorption peaks within this wavelength range.

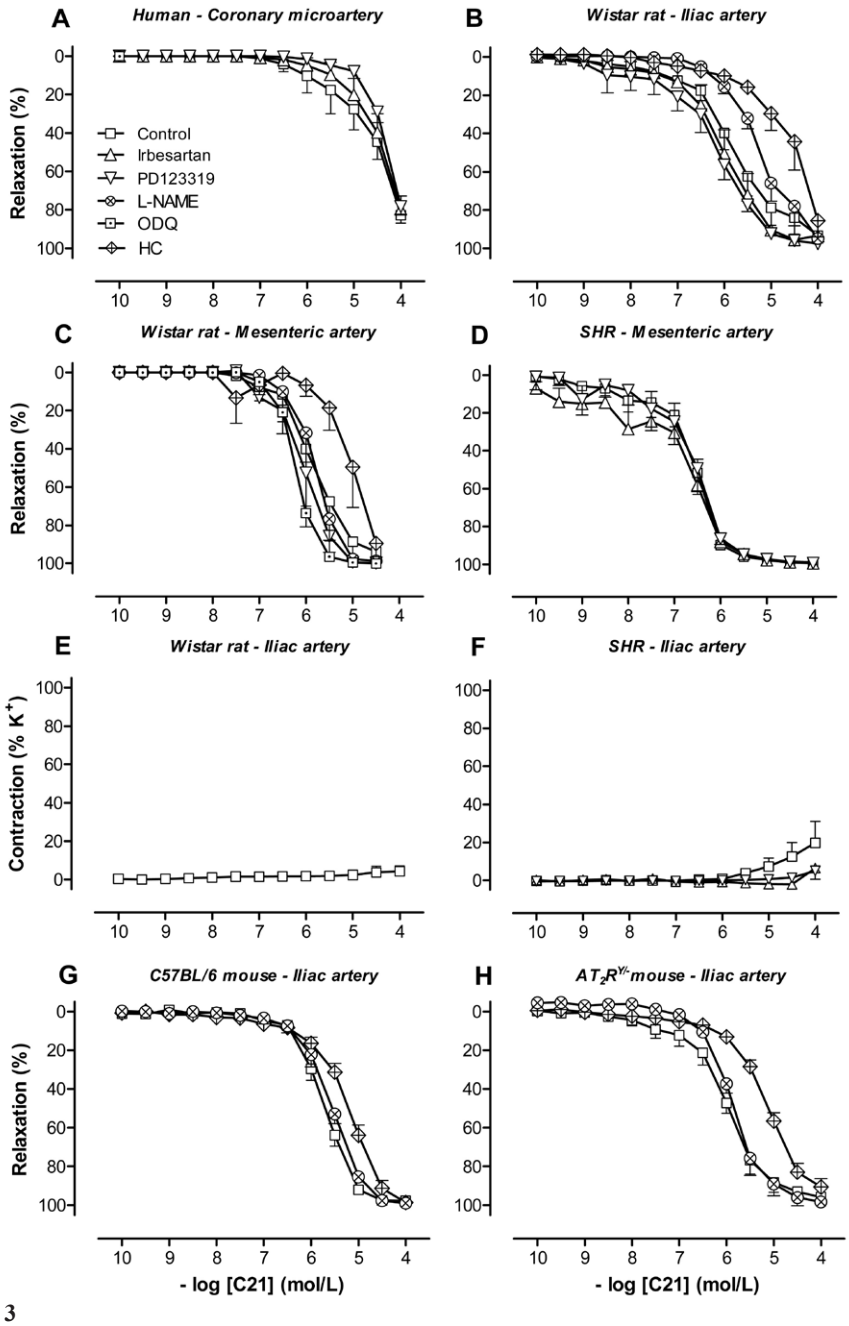

Figure 3

Effect of C21 in KCl-preconstricted HMCAs (A), in U46619-preconstricted Wistar rat iliac arteries (B), Wistar rat mesenteric arteries (C) and SHR mesenteric arteries (D), and in U46619-preconstricted iliac arteries of C57BL/6 (G) and $AT_2R^{-/y}$ mice (H), in the absence or presence of irbesartan, PD123319, L-NAME, ODQ or hydroxocobalamin (HC). Panels E and F show the C21-induced constrictor responses (or absence thereof) in iliac arteries of Wistar rats and SHR. Data are mean±SEM of n=6-10, and have been expressed as a percentage of the maximum contraction induced KCl or U46619.

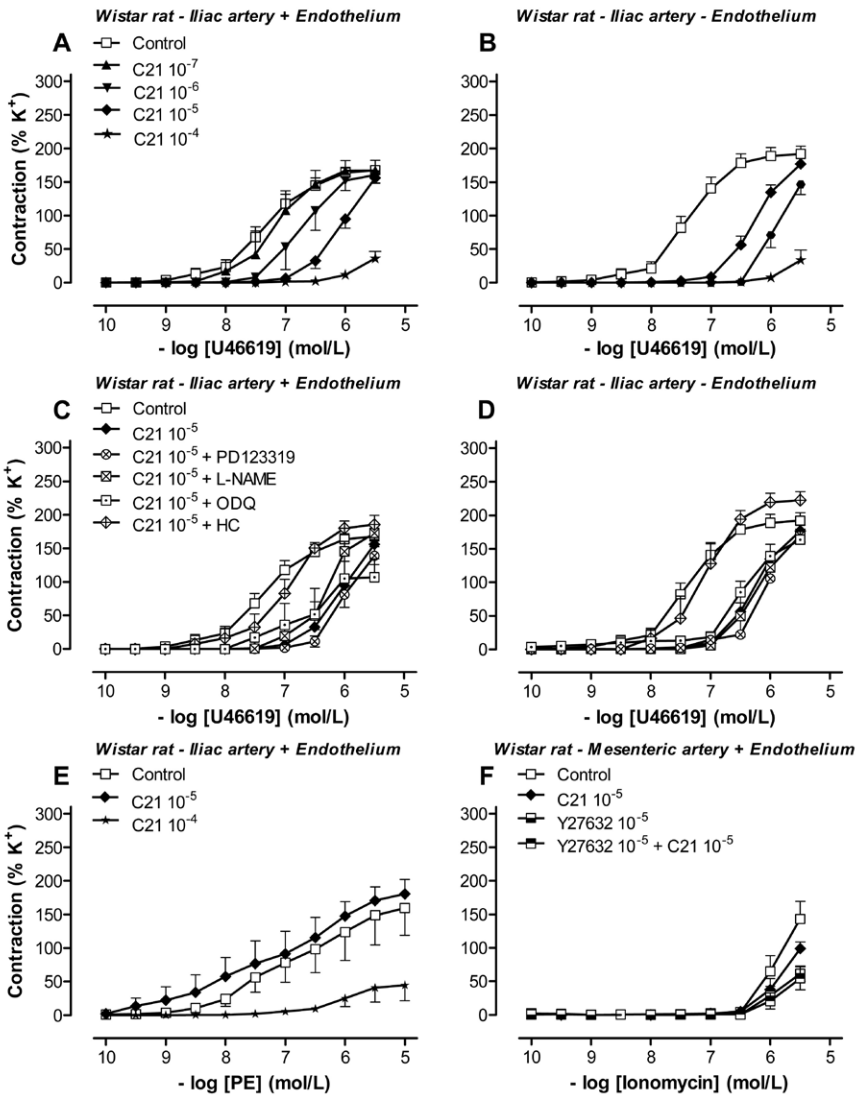

Figure 4.

Effects of U46619 (A-D), phenylephrine (E) or ionomycin (F) in the absence or presence of increasing C21 concentrations with or without endothelium, PD123319, L-NAME, ODQ, hydroxocobalamin (HC) or Y27632 in iliac (A-E) and mesenteric (F) arteries of the Wistar rat. Data are mean±SEM of n=3-19 and have been expressed as a percentage of the contraction induced by 100 mmol/L KCl.

Discussion

The present study does not reveal C21-induced, AT_2 receptor-mediated vasodilation in any of the models tested, despite the fact that such vasodilation has been demonstrated previously in these models.[1,2,11,12,22] Simultaneously, we were able to confirm that C21 binds with high affinity to AT_2 receptors.[14,27] Yet, C21 did induce relaxant, as well as constrictor effects, in full agreement with the diversity of C21 effects on blood pressure in a wide range of models, ranging from decreases to increases of ≤25 mm Hg.[14,19-21] Our study now shows at what concentrations these effects occur, and provides the mechanisms that potentially underlie these phenomena. Remarkably, the constrictor effects appeared to be enhanced under pathological conditions, because they were best observed in the coronary vascular bed and iliac artery of the SHR. Irbesartan blocked these constrictor effects, suggesting that they were AT_1 receptor-mediated. This agrees with the well-established AT_1 receptor upregulation in SHR.[28] Clearly therefore, C21 is capable of stimulating AT_1 receptors, as has also been suggested based upon in vivo studies.[19,20]

Interestingly, in both Wistar rats and SHR, PD123319 partially blocked the coronary constrictor effects of C21 (Figure 2), and partial blockade was also observed in C21-constricted iliac arteries of the SHR. Moreover, in male SD rats Hilliard et al[21] reported inhibition of a C21-induced rise in MAP by PD123139. A unifying explanation of these findings is the existence of AT_1/AT_2 receptor heterodimers, coupling to net dilatory/constrictor effects, depending on their ratio and/or location. Alternatively, it should be considered that PD123319, at the applied concentration of 1 mmol/L, exerted a modest degree of AT_1 receptor blockade in our studies.

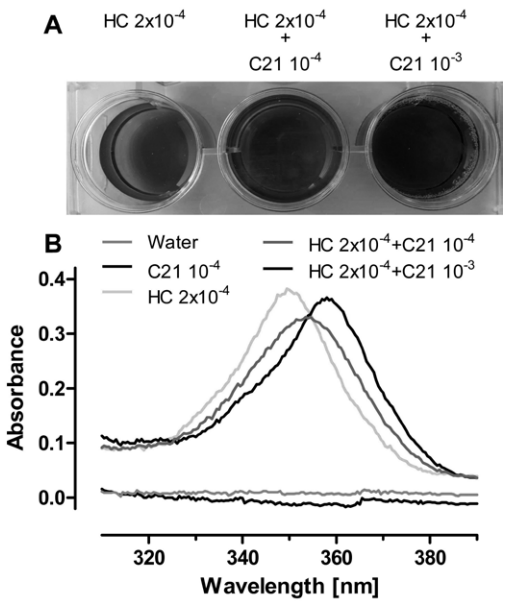

Figure 5.
C21-induced alteration of the absorption spectrum of hydroxocobalamin (HC). Please note that NaCl has no such effect.

The biphasic coronary effects of C21 in Wistar rats were mimicked in C57BL/6 mice. In SHR, following the enhanced coronary constrictor response to C21, a dilator phase was virtually absent. Blocking the initial constrictor effect with irbesartan enhanced the subsequently occurring vasodilation in Wistar rats and reintroduced coronary vasodilation in SHRs. At first sight, this supports an unmasking of AT_2 receptor-mediated coronary vasodilation. However, PD123319 did not block the C21-induced coronary vasodilation in Wistar rats, and a similar vasodilation occurred in $AT_2R^{-/y}$ mice. Moreover, as discussed above, PD123319, if anything, blocked vasoconstriction and enhanced coronary dilation. In addition, in a previous study we were unable to detect AT_2 receptor-mediated vasodilation in the SHR coronary vascular bed.[11] Thus, a non-AT receptor-dependent mechanism must underlie the coronary relaxant effect of C21.

Indeed, all of the responses in isolated vessels, including HCMAs, in our study support a C21-induced, AT_2 receptor-independent vasorelaxation. The concentrations at which this effect occurred were in the micromolar range, that is, well above its nanomolar affinity for AT_2 receptors. Complete relaxation of preconstricted vessels required a C21 concentration of ~1-10 mmol/L in rodents and 10-100 mmol/L in humans. Concentrations of 1 mmol/L were sufficient to shift the constrictor curves to U46619 ~10-fold to the right. Most in vitro studies investigating the effects of C21 applied concentrations of ≥0.1 mmol/L.[14,19,29] The single (to the best of our knowledge) previous study investigating C21-induced effects in rodent vessels reported C21-induced relaxations that are comparable to those observed here. However, this study stopped its CRCs at 1 mmol/L C21, and thus no E_{max} or pEC_{50} could be determined.[19] Remarkably, this study observed larger effects (~25 to 30% relaxation at 1 mmol/L C21) in the SHR aorta than in the mouse aorta or rat mesenteric artery (~10 to 20% relaxation).[19] This is unexpected because of the AT_2 receptor phenotype shift reported for SHR, allowing AT_2 receptors to induce constriction instead of relaxation.[11,12] Unfortunately, no studies with PD123319 were performed to confirm that the relaxant effects of C21 in the SHR aorta truly involved AT_2 receptor stimulation.

In vivo, C21 has been infused at doses ranging from 0.05-5 mg/kg per minute, or was applied IP or orally at doses ranging from 0.3 to10 mg/kg per day.[14-21,30] Given its distribution volume of 3 times total body water, its half life of ≈ 4 hrs and a bioavailability of ≈ 30%,[14] this is expected to result in C21 plasma levels ranging from 0.1 to 5 mmol/L, that is, well within the range applied here. Such levels (up to >10.000-fold above the reported inhibition constant for the AT_2 receptor) are also in agreement with the fact that C21 induced AT_1 receptor-mediated effects in vivo, because its inhibition constant for AT_1 receptors is >10.000 times above that for AT_2 receptors.

C21-induced relaxation occurred in an endothelium-independent manner and could be blocked by the NO scavenger hydroxocobalamin but not the NO synthase inhibitor L-NAME or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (Figures 3 and 4). This initially suggested a role for nonendothelial NO synthase– derived NO-like factors like S-nitrosothiols.[31] However, an alternative explanation is that the cobalt group of hydroxocobalamin inactivated C21 through interaction with its imidazole ring. Indeed, spectral analysis subsequently confirmed this concept. Thus, most likely, the effect

of hydroxocobalamin is due to its capacity to bind/inactivate C21, and does not involve NO scavenging.

C21 concentration-dependently shifted the CRCs to both the thromboxane A_2 agonist U46619 and the α-adrenoceptor agonist phenylephrine to the right, and fully relaxed U46619- and KCl-preconstricted vessels, which demonstrates that its relaxant effects are not related to a specific receptor. Importantly, C21 did not alter the constrictor response to the calcium ionophore ionomycin. This raises the possibility that C21, instead of directly interfering with contractile (Ca^{2+}-dependent) responses, blocks calcium transport into the cell, thus preventing responses that depend on extracellular calcium, like vasoconstriction. C21 did not block the RhoA-Rho kinase pathway, which has been reported previously to underlie AT_2 receptor-mediated vasodilation.[32]

Perspective

Despite overwhelming data supporting AT_2 receptor-mediated vasodilation,[3-6,33-35] for instance in the preparations investigated in these studies,[2,11,12] we were unable to demonstrate such vasodilation in response to the AT_2 receptor agonist C21 in rat, mouse and human vessels. Yet, our current study does support C21-induced vasorelaxation, albeit in an AT receptor-independent manner, possibly involving blockade of Ca^{2+} transport into the cell. Simultaneously, C21 is capable of activating AT_1 receptors, thereby causing vasoconstriction. Taken together, this combination of both relaxant and constrictor effects can help explaining the hyper- and hypotensive effects of C21 in vivo, including even the absence of such effects.[15-17,20,36] It rules out the application of C21 as an antihypertensive agent. Clearly, studies observing organ-protective effect of chronic C21 application should now carefully determine to what degree these effects truly depend on AT_2 receptor activation, by using either selective AT_2 receptor antagonists or by simultaneously studying the effects in AT_2 receptor knockout models. To guarantee AT_2 receptor selectivity, it appears that C21 infusion rates should stay well below 0.5 mg/kg per minute.

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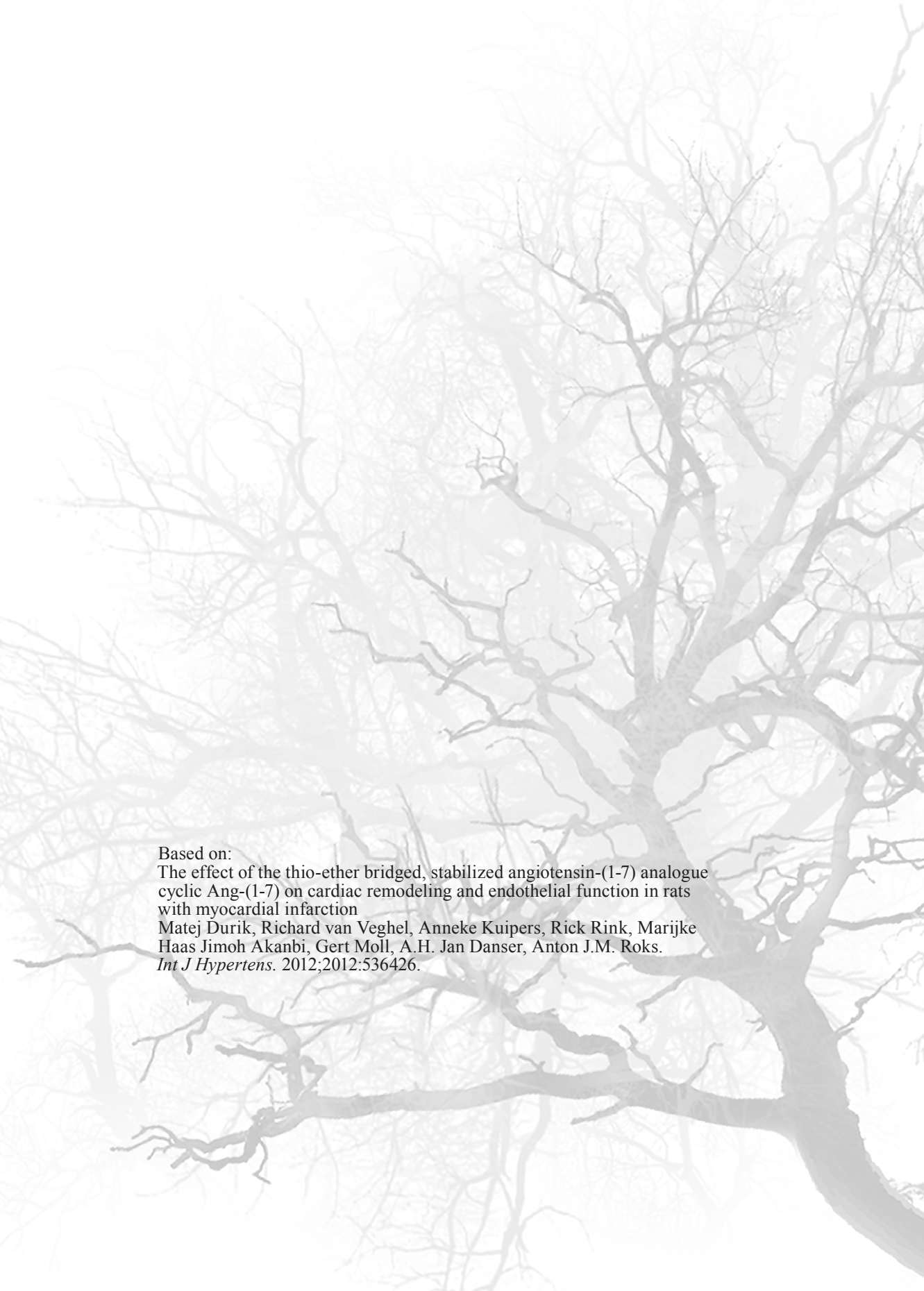

Based on:
The effect of the thio-ether bridged, stabilized angiotensin-(1-7) analogue cyclic Ang-(1-7) on cardiac remodeling and endothelial function in rats with myocardial infarction
Matej Durik, Richard van Veghel, Anneke Kuipers, Rick Rink, Marijke Haas Jimoh Akanbi, Gert Moll, A.H. Jan Danser, Anton J.M. Roks.
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The effect of the thio-ether bridged, stabilized angiotensin-(1-7) analogue cyclic Ang-(1-7) on cardiac remodeling and endothelial function in rats with myocardial infarction

6

Abstract

Modulation of renin-angiotensin system (RAS) by angiotensin-(1-7) (Ang-(1-7)) is an attractive approach to combat the detrimental consequences of myocardial infarction (MI). However Ang-(1-7) has limited clinical potential due to its unfavorable pharmacokinetic profile. We investigated effects of a stabilized, thioether-bridged analogue of Ang-(1-7) called cyclic Ang-(1-7) in a rat model of myocardial infarction. Rats underwent coronary ligation or sham surgery. Two weeks thereafter infusion with 0.24 or 2.4 μg/kg/hour cAng-(1-7) or saline was started for 8 weeks. At the end of the experiment cardiac morphometric and hemodynamic variables as wells as aortic endothelial function were measured. The average infarct size was 13.8 % and was not changed by cAng-(1-7) treatment. MI increased heart weight and myocyte size, which were restored by cAng-(1-7) to sham levels. In addition, cAng-(1-7) lowered left ventricular end-diastolic pressure and improved endothelial function. These results suggest that cAng-(1-7) is a promising new agent in treatment of myocardial infarction and warrant further research.

Introduction

Myocardial infarction is a leading cause of mortality and morbidity in western society. Current intervention relies on prevention of myocardial hypertrophy, fibrosis and of thrombosis. Since these processes are partially mediated by an increase of the renin-angiotensin system (RAS) hormone, angiotensin (Ang) II, inhibition of this hormone through drugs, that decrease its production or its signaling via the Ang II type 1 (AT_1) receptor, forms an important part of the applied pharmacotherapy. The ever culminating knowledge of RAS brought about by relentless research of a vast group of scientists; has raised the awareness that there is more to achieve than with classical RAS intervention only. Possible novel intervention strategies have emerged, of which those based on stimulation of angiotensin-(1-7) (Ang-(1-7)) pathway is one of the most appealing.[1-2]

Angiotensin-(1-7) (Ang-(1-7)) is a hormone that in general counteracts Ang II through its own signaling pathways, which involves the Mas receptor.[3] Studies in animal models have shown that it has ample therapeutic potential in cardiovascular disease, in particular diseases that are featured by malignant remodeling of the heart. We showed that chronic infusion of Ang-(1-7) in rats or mice after myocardial infarction improves cardiac and endothelial function.[4-5] The beneficial effect of Ang-(1-7) infusion after myocardial infarction relies on the versatile effects of the hormone, which comprises antihypertrophic, antifibrotic, and antithrombotic function, improvement of eNOS function, blockade of Ang II-induced ROS production, and stimulation of endothelial progenitor cell-mediated angiogenesis.[1-2,5-9] In spite of being a therapeutic prodigy, Ang-(1-7) does not offer ideal prospects for clinical use because of its pharmacokinetical and pharmacodynamical properties (as is also elaborated in 1). Firstly, the peptide is rapidly metabolised in plasma and tissue. Secondly, beneficial effects of Ang-(1-7) take place at low concentrations at which Mas receptors are stimulated. At higher concentrations, Ang-(1-7) binding becomes unspecific, being a partial Ang II type 1 receptor agonist and an Ang II type 2 receptor agonist. Thus, overdosing might interfere with its Mas receptor-associated functions.

To improve the pharmacological profile we have developed cyclic Ang-(1-7) (cAng-(1-7)), an Ang-(1-7) analogue in which amino acid residues 4 and 7 have been linked with a thioether bridge, thus forming a lanthionine.[10] The strategy of thioether bridging is used by bacteria to stabilize peptides, and we previously showed that enzymatically synthesized cAng-(1–7) was fully resistant against degradation by angiotensin-converting enzyme, and had enhanced resistance against breakdown by other proteases. It displayed 34-fold enhanced presence in the blood circulation in Sprague–Dawley (SD) rats during continuous intravenous infusion. The thioether ring did not prevent cAng-(1–7) from agonistically interacting with the Mas receptor, the receptor of native angiotensin-(1–7). cAng-(1–7) even induced a two-fold larger relaxation of precontracted SD rat aorta rings than native Ang-(1–7). Moreover, it is a specific agonist for Ang-(1-7) receptors. Therefore, cAng-(1-7) holds promise for use in cardiovascular therapy. In this study we have tested the effect of chronic cAng-(1-7) infusion on hemodynamic function after myocardial infarction in the rat.

Methods

Animals

Male Sprague-Dawley rats weighing 280-300 grams were obtained from Harlan (Horst, the Netherlands). Animals were put on standard rat chow and water, available ad libitum. Housing was at room temperature with a 12 h light - 12 h dark cycle. After at least one week of acclimatization in the caretaking facility, the rats were operated to induce left ventricular myocardial infarction (MI) or underwent a sham procedure.

Surgery to induce MI and surgical procedures

Prior to surgery 0.01 mg/kg buprenorphine was given subcutaneously for post-operative analgesia, which was repeated after surgery for 2 days, 2 times daily. Operations were performed under 2.5 % isoflurane in air ventilation anesthesia for which the rats were intubated. Through an opening in the left 4th intercostal space of the chest, MI was induced by ligation of the left coronary artery with a 6/0 silk suture. After induction of MI, as witnessed by bleaching of the myocardium, the chest was closed and animals were withdrawn from anesthesia. Sham-operated animals (SHAM) underwent an identical procedure, however, without tying the silk suture to close the coronary artery. MI surgery was performed in 106 animals, 8 animals were sham-operated. Perioperative mortality was 45% in the MI group.

Treatment with cAng-(1-7)

Two weeks after induction of MI, rats were randomly allocated to intravenous infusion of either 0.24 (low dose or low cAng-(1-7)) or 2.4 μg/kg/h (high dose or high cAng-(1-7)) of cAng-(1–7) (n=12 for each dose), or saline (n=25) by 4-week osmotic minipumps (Alzet model 2004). Sham-operated controls (n=8) received saline or high dose of cAng-(1-7) (2.4 μg/kg/h). Animals were infused for 8 weeks, changing pumps at week 4. For intravenous infusion a polyethylene tube was implanted in the left jugular vein. cAng-(1-7) was made by BiOMade / LanthioPep, Groningen.

Measurements of hemodynamic and vascular function

After 8 weeks of treatment animals were weighed (body weight: BW) and hemodynamic studies were performed under isoflurane anesthesia (2.5 % in air) with a 2F catheter-based, microtip pressure transducer (Millar, Houston, USA) that was introduced into the left ventricle via intraluminal passing through the right carotid artery. Rats were anesthetized for 20 minutes before the start of the measurement.

After measurement of hemodynamic function, the heart was excised for histological studies. After removal of ventricular blood, the heart was weighed to obtain total heart weight (HW). The thoracic aorta was isolated to perform functional studies. To this end the aorta was kept in Krebs solution 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. Surrounding periaortic adipose tissue was carefully removed with small scissors. Rings of 2 mm length were cut and mounted

in small wire organ baths containing Krebs at 37ºC. To investigate the contribution of dilator signaling factors, nitric oxide (NO) production was blocked using L-NAME (100 μmol/L), and endothelium-derived hyperpolarizing factor (EDHF) was blocked with apamin (0.5 μmol/L) and charybdotoxin (0.1 μmol/L). Subsequently, in the absence or presence of these inhibitors, concentration-response curves were constructed to metacholine and SNP after preconstriction with phenylephrine. All chemicals were from Sigma-Aldrich, the Netherlands.

Histology

Midventricular slices of the heart were fixed with 4% formaldehyde, embedded in paraffin and processed for histochemical analysis. Infarct size was determined on picrosirius red/fast green–stained sections and was expressed as the percentage of scar length of the average of left ventricular internal and external circumference. Rats with all infarct sizes were included in the analysis. The cross-sectional area of the individual cells was measured on gomori-stained sections. Myocyte density was determined by assessment of the number of cells per tissue area for each slide and subsequent conversion to mm^2. Fibrosis was measured on picrosirius red/fast green–stained sections from three randomly selected regions of the surviving myocardium.

Statistical Analysis

Data are presented as mean ±SEM. Statistical differences between the groups were evaluated by t-test or by 1-way ANOVA for hemodynamic and histological variables, using Dunnett's t-test or Bonferroni correction where appropriate. One-sided testing was applied in all bar graphs as the effects were in the expected direction. For testing of trend linear regression analysis was applied. Differences in concentration-response curves to metacholine were tested by general linear model ANOVA for repeated measures. Differences were considered significant at $P<0.05$.

Results

Weight and histological characteristics

General parameters at the end of treatment are shown in Table 1. No differences were observed in body weight between the 4 groups. Infarct sizes were in general small, and did not differ significantly between the cAng-(1–7) and saline-treated group. Similarly, fibrosis did not differ between the groups (Table 1) . Despite the small infarct sizes total heart weight to body weight ratio was modestly but significantly increased in saline-treated MI group compared with SHAM (Figure 1A). Both doses of cAng (1-7) abolished the significant difference between MI and SHAM, however, only the higher dose of 2.4 μg/kg/h cAng-(1-7) resulted in a lower heart weight compared to saline, though not significantly different.

To further determine the cause of the weight differences, the effect of cAng-(1-7) on

myocyte size was measured. Myocardial infarction increased myocyte cross-sectional area and decreased myocyte cell density (Figure 1B and C). Treatment with both doses of cAng-(1-7) restored myocyte cross-sectional area to the level of saline-treated sham (Figure 1B). Myocyte density was only restored by the higher dose of cAng-(1-7) (Figure 1C). In sham-operated animals, cAng-(1-7) treatment showed a trend towards a decrease in myocyte size, but this effect did not reach a statistical significance (Figure 1B and C).

Hemodynamics

After 8 weeks of treatment, cardiac function was measured *in vivo* in anesthetized rats. In accordance with the small infarct size, cardiac function was not significantly impaired in untreated MI rats as compared with SHAM (Table 1). In agreement with the absence

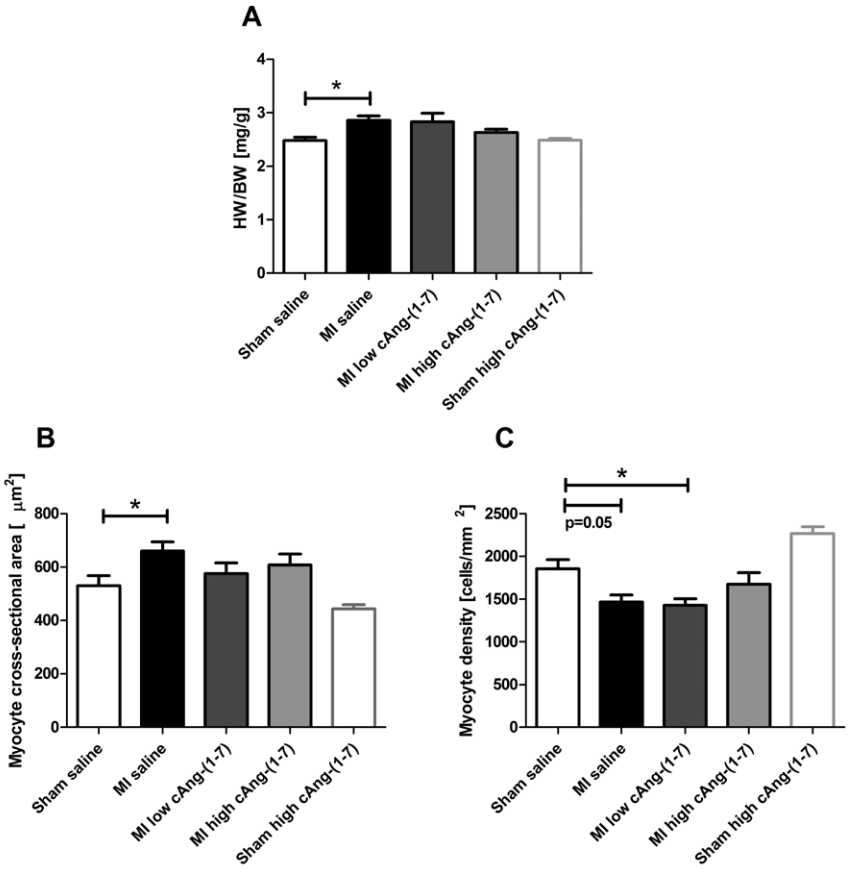

Figure 1.

Comparison of heart weight/ body weight ratios between the different treatments (A), variables of cardiac hypertrophy: myocyte cross-sectional area (B) and myocyte density (C). (* $p<0.05$, One way ANOVA, Dunnett's post hoc testing)

of systolic or diastolic heart failure MI did not significantly change left ventricular end diastolic pressure (LVEDP) or left ventricular minimal pressure (Pmin) (t-test, p = 0.199 for LVEDP; p = 0.090 for Pmin), and therefore the effect of cAng-(1-7) was tested within the MI and sham group respectively (Figure 2). In the MI group, cAng(1-7) treatment lowered LVEDP which was significant at the highest dose (Figure 2A). Since there seemed to be a dose-dependent effect we tested for a trend line, which resulted in a significance for trend. Pmin seemed also to be lowered in MI animals, but this effect did not reach statistical significance (Figure 2B). In sham animals, cAng-(1-7) given at a dosis of 2.4 µg/kg/hour lowered both LVEDP and Pmin (Figure 2 A, B). All other measured pressure variables were not changed by cAng-(1-7) treatment as compared to MI saline (Table 1).

Endothelial function

Endothelial dysfunction is a key feature in the development of heart failure after MI since it contributes to the increase of peripheral vascular resistance. This leads to increased cardiac workload resulting in hypertrophy and contractile dysfunction of the myocardium.

	Sham saline	MI saline	Sham high cAng-(1-7)	MI low cAng-(1-7)	MI high cAng-(1-7)
BW, g	477.1±6.6	475.3±6.4	484.6±7.4	458.6±10	492.3±9.5
Infarct size, %	0±0	15.853±3.17	0±0	9.721±2.89	14.535±4.10
Fibrosis, %	3.896±0.57	3.992±0.39	4.289±0.54	4.695±0.65	4.479±0.34
HR beats/min	255.12±16.0	260.3±5.5	240.4±9.8	274.7±6.2	266.7±6.2
MaxP, mmHg	107.8±3.8	103.5±2.9	98.6±8.3	93.0±4.7	96.4±4.7
ESPress, mmHg	103.6±4.2	100.1±3.0	93.5±9.3	89.4±5.3	92.4±5.1
dpdtMax, mmHg/sec	5572.0±188.2	5456.8±198.7	5533.0±530.7	5026±269.3	5111.3±345.1
dpdtMin, mmHg/sec	-5189.1±178.8	-4919.1±265.2	-6123.5±1268.9	-4615.4±361.8	-4742.6±404.9
N	8	25	5	12	12

Table 1. Weight, basic histological and cardiac parameters.

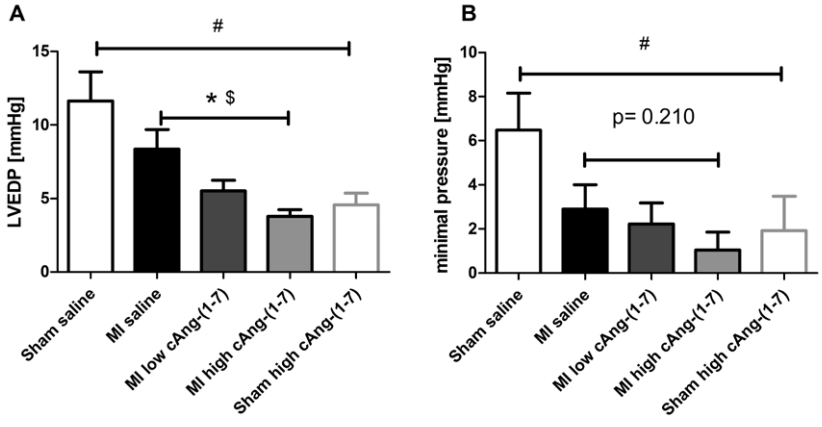

Figure 2.

Effects of cAng-(1-7) on left ventricular end diastolic pressure and minimal pressure in both sham-operated rats and rats with myocardial infarction. (# $p<0.05$ t-test sham saline vs. sham cAng-(1-7); * $p<0.05$, One way ANOVA for MI groups, Dunnett's post-hoc testing; $ $p<0.05$ for linear trend for MI groups)

Therefore, we investigated endothelium-dependent relaxation in isolated aortic rings.

Phenylephrine (1 μmol/L) caused similar contractile responses in all groups (data not shown). The responses of aortic rings to endothelium independent vasodilator SNP were not changed between groups (Data not shown). Responses to the endothelium-dependent vasodilator metacholine were unchanged in saline-treated MI animals when compared to SHAM (data not shown). However, both doses of cAng-(1-7) showed increased responsiveness to metacholine when compared to saline-treated MI group, which was most pronounced and only significant in the higher dose (Figure 3A). After blocking the NO production of endothelium with L-NAME, the response to metacholine was greatly suppressed in all the groups, however the increased responsivity of high dose cAng-(1-7) treated animals remained present (Figure 3B). After blocking both NO and EDHF, leaving prostaglandins as the remaining dilator factor, the difference between saline and cAng-(1-7)-treated animals disappeared (Figure 3C) indicating that cAng-(1-7) works via EDHF.

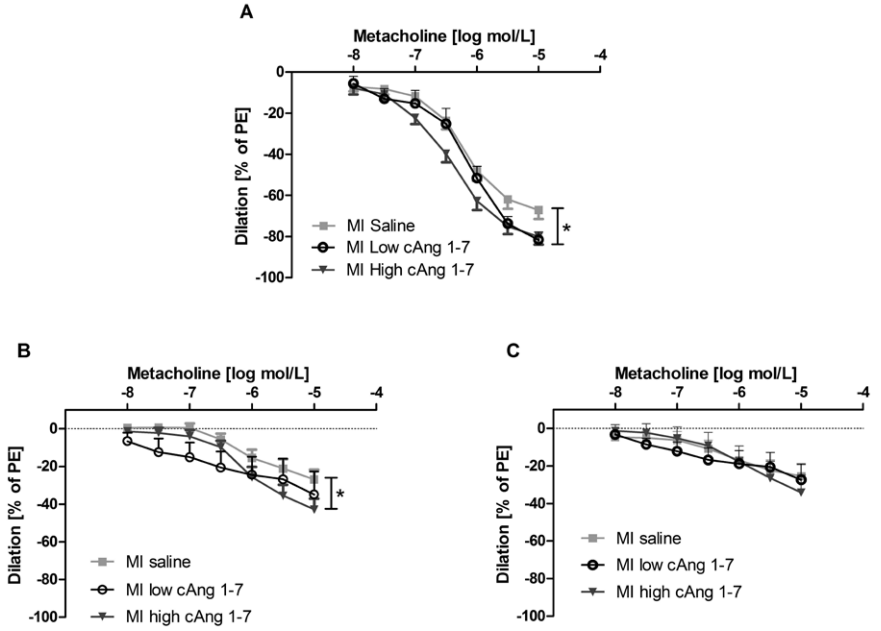

Figure 3.

Endothelial-dependent dilator function of rat aorta to metacholine (A), after blockade of eNOS/NO signaling (B) and after combined blockade of eNOS/NO and EDHF vasodilator mechanisms (C). (* = $p<0.05$, GLM-RM)

Discussion

Stimulation of the Ang-(1-7) / Mas receptor axis is a promising therapeutic strategy for treatment of MI and prevention of heart failure. For this purpose we tested the effect of the metabolically protected and Mas receptor-specific compound cAng-(1-7). Given at doses that were respectively 10 and 100 times lower than the minimally effective dosis of native Ang-(1-7),[11] cAng-(1-7) dose-dependently lowered left ventricular weight and diastolic pressure in an MI model in which no contractile failure had yet occurred. The effect on cardiac weight seemed to depend at least partially on reduction of cardiomyocyte hypertrophy, as evidenced by the decrease in myocyte dimensions. The effects on the heart morphology and function were independent from the presence of an infarction since they also occurred in sham animals. In addition to effects on the heart, cAng-(1-7) improved peripheral endothelium-dependent vasodilation, as measured in isolated aortic rings; an effect that predominantly involved EDHF. cAng-(1-7) therefore shows favorable characteristic with regard to improvement of cardiovascular function after MI.

The present results with respect to cardiac improvement are in accordance with previous results in the MI model obtained after infusion of native Ang-(1-7).[4-5] A limitation of the present study, however, is the fact that infarct sizes were relatively small as compared to the previous studies, thus not allowing us to study possible beneficial effects of the compound on systolic function and cardiac fibrosis.[4, 12-13] Nevertheless, the implications of the present study are relevant since patient populations also comprise subjects with relatively small infarct sizes but who will eventually develop heart failure, albeit after a relatively longer period. The full potential of cAng-(1-7) as an experimental drug can be appreciated from evaluation in a model of heart failure or cardiac fibrosis. The present data warrant such studies.

Endothelial dysfunction is an important hallmark in heart failure caused by MI and believed to be pivotal in malignant cardiac remodeling due to increased afterload. Ang-(1-7) was shown to restore endothelium-dependent vasodilator function in heart failure, after stent placement, after a high salt diet and in the atherosclerosis-prone ApoE knockout mouse when infused chronically.[4, 11, 14-15] Vascular upregulation of ACE2, which increases Ang-(1-7) levels, improves endothelial function in hypertensive rats.[16] Conversely, Mas receptor knockout or chronic treatment with A779, an antagonist of Mas receptor-associated effect diminishes endothelial function.[17-19] In accordance with the suggested role of Mas receptor signaling in improvement of endothelial function cAng-(1-7) infusion led to improved endothelial function in our rats with small MI. The improvement that was observed by us appears to be mainly caused by an increase of endothelium-derived hyperpolarizing factor (EDHF), and not through prostaglandin release. In a previous study, which involved relatively older rats that developed endothelial dysfunction after stent placement, chronic infusion of native Ang-(1-7) mainly increased prostaglandin.[20] Furthermore, short term infusion of the native peptide improves the hypotensive response to acetylcholine through NO signaling, whilst Mas receptor knockout results in impaired NO bioavailability.[19, 21] Thus, the model that is used for studying the effect of Ang-(1-7)-

mediated vasodilations seems to determine the signaling pathway that is improved. Our present results are to our knowledge the first to show an increased contribution of EDHF and emphasizes the versatility of the therapeutic potential of the Ang-(1-7)/Mas receptor axis towards endothelial function.

As noted above, cAng-(1-7) was intravenously administered by osmotic minipump in a dose that was 10 to 100 times lower than in previous studies the lowest efficacious dose for native Ang-(1-7). This approach allowed us to make comparisons with these previous studies and indicate that the pharmacological properties of cAng-(1-7) seem to be superior to those of native Ang-(1-7). To provide conclusive evidence it will be necessary to test cAng-(1-7) in a model of heart failure. Furthermore, a clinically relevant method of drug delivery will have to be developed. Most commonly, clinically applicable peptides are administered subcutaneously where the peptide is not degraded and which allows manipulation of the rate of peptide release, such as in the case of insulin formulations. In a recent study it was shown that subcutaneous cAng-(1-7) resulted in a 98% bioavailability. Although less efficient, oral and especially pulmonary delivery (28% bioavailability) of cAng-(1-7) appeared possible too. Therefore translation to the clinic, is feasible.[22] There are other approaches to design a clinically relevant delivery method to exploit the Ang-(1-7)/Mas axis. These designs fall into four main categories: local delivery of the native peptide, non-peptide analogues, protective incapsulation of the native peptide, and upregulation of the Ang-(1-7)-synthesizing enzyme ACE2. Local delivery is an elegant way to circumvent loss of bio-availability of Ang-(1-7). This approach has been explored to counteract problems that are associated with stent placement, and has led to prevention of endothelial dysfunction.[23] Theoretically, this strategy should also be applicable for solid tumors. Peptide incapsulation includes PEG-liposome complexes that can be delivered intravenously,[23] but most promising appears to be the use of hydroxypropyl β-cyclodextrin, which has led to successful cardioprotection after infarction or chronic isoproterenol infusion in rats when delivered orally.[24] Non-peptide analogues include AVE 0991 and CGEN-856S, which show vasodilatory and cardioprotective properties (less arrythmias during recovery from I/R) in vitro, and antihypertensive effects in vivo.[23, 25] However, oral delivery has not been attempted with these compounds. Lastly, upregulation of ACE2 has been successfully attempted as intervention in cardiac and pulmonary fibrosis models, and in Ang II-dependent renal fibrosis. Of particular interest is the use of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT), an ACE2 ligand and activator of the enzyme. Until present, XNT was shown effective against cardiac and pulmonary fibrosis and against pulmonary hypertension when administered subcutaneously with minipumps.[26-27]

In summary, we present the first data showing that lanthionine-bridged Ang-(1-7), shortly cAng-(1-7), holds promise as a therapeutic agent after MI, as it improves cardiac remodeling and endothelial function and since it has previously[22] been demonstrated that it can be delivered orally and pulmonarily. Our present results warrant further testing of this compound in various models of heart failure and possible other diseases that can be a target of beneficial Ang-(1-7)/Mas receptor axis signaling.

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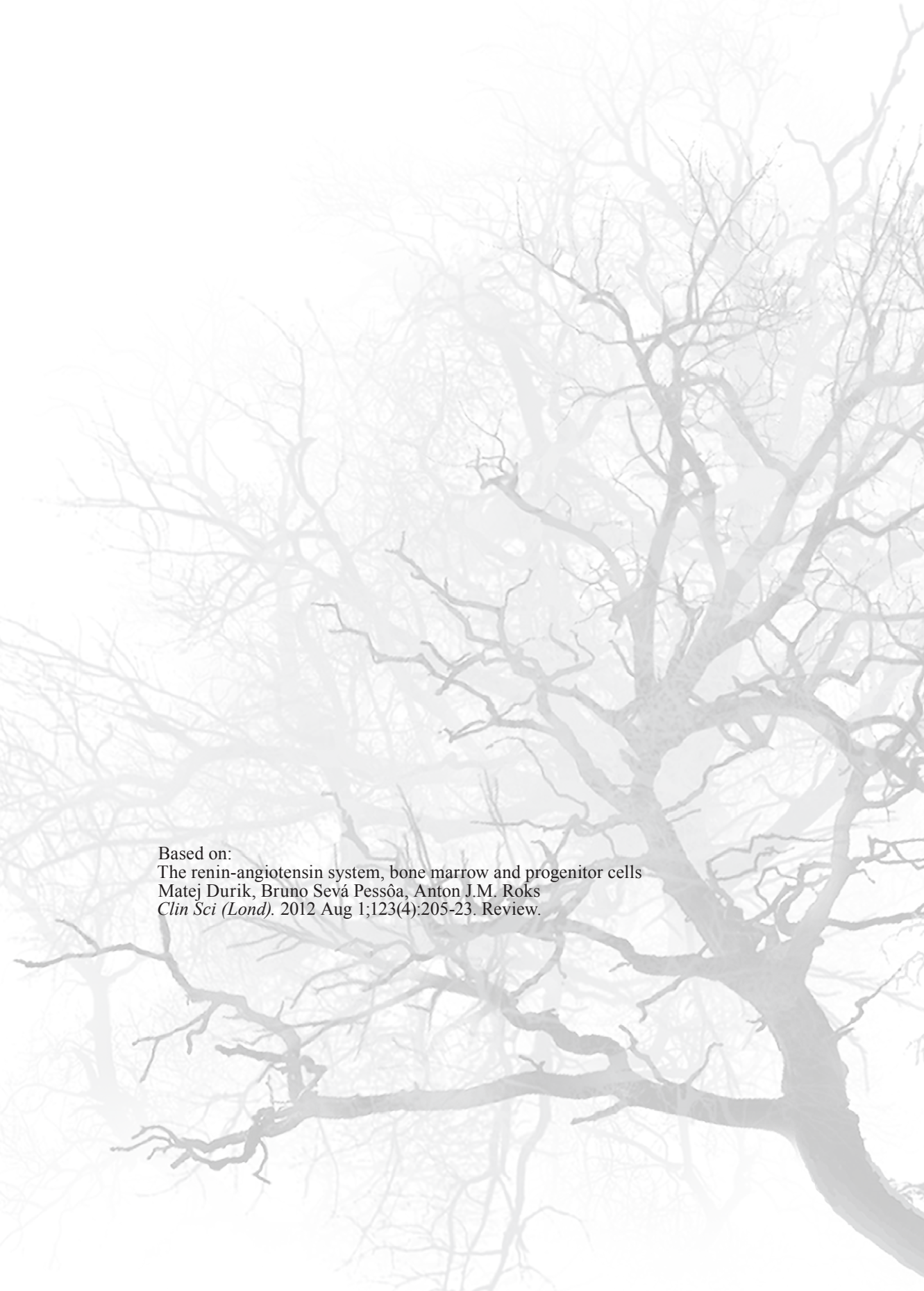

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The renin-angiotensin system, bone marrow and progenitor cells
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The renin-angiotensin system, bone marrow and progenitor cells

7

Abstract

Modulation of the RAS (renin-angiotensin system), in particular of the function of the hormones AngII (angiotensin II) and Ang-(1-7) [angiotensin-(1-7)], is an important target for pharmacotherapy in the cardiovascular system. In the classical view, such modulation affects cardiovascular cells to decrease hypertrophy, fibrosis and endothelial dysfunction, and improves diuresis. In this view, excessive stimulation of AT_1 receptors (AngII type 1 receptors) fulfils a detrimental role, as it promotes cardiovascular pathogenesis, and this is opposed by stimulation of the AT_2 receptor (angiotensin II type 2 receptor) and the Ang-(1-7) receptor encoded by the Mas proto-oncogene. In recent years, this view has been broadened with the observation that the RAS regulates bone marrow stromal cells and stem cells, thus involving haematopoiesis and tissue regeneration by progenitor cells. This change of paradigm has enlarged the field of perspectives for therapeutic application of existing as well as newly developed medicines that alter angiotensin signalling, which now stretches beyond cardiovascular therapy. In the present article, we review the role of AngII and Ang-(1-7) and their respective receptors in haematopoietic and mesenchymal stem cells, and discuss possible pharmacotherapeutical implications.

Introduction

Angiotensin II versus angiotensin-(1-7)

The renin-angiotensin system (RAS) is a major regulator of renal and cardiovascular function and plays a central role in the homeostasis of the cardiovascular system and of the hydro-electrolyte balance.[1] For a long period, research was focussed on production and signaling of angiotensin II (Ang II), highlighting angiotensin-converting enzyme (ACE) and renin with respect to production, and the angiotensin II type 1 receptor (AT_1 receptor) with respect to signaling of Ang II. Since Ang II is an important player in unfavourable remodeling of cardiovascular tissue, ACE inhibitors (ACEi), AT_1 receptor blockers (ARB) and renin inhibitors are successfully used in treatment of cardiovascular disease, and novel tools to further optimize intervention in the RAS are being developed.[1]

In the past two decades these optimization efforts resulted in the identification of novel therapeutic targets within the RAS, including the Ang II type 2 receptor (AT_2 receptor)[2], angiotensin-(1-7) (Ang-(1-7)) and its G-coupled receptor Mas,[3] and angiotensin-converting enzyme 2 (ACE2),[4-5] which is important for Ang-(1-7) formation. An increasing amount of evidence shows that Ang-(1-7) is one of the most important hormones of the RAS.[6-8] In the cardiovascular system, Ang-(1-7) mainly has opposite actions as compared to Ang II, leading to a dichotomy in the RAS, namely the proliferative, prothrombotic and vasoconstrictor actions of Ang II versus the anti-proliferative, anti-thrombotic and vasodilator actions of Ang-(1-7).[6] Ang-(1-7) can be formed directly from Ang II by ACE2, but also from Ang I. As ACE metabolizes Ang-(1-7), ACEi treatment increases Ang-(1-7) levels, and this is believed to play an important role in beneficial cardiovascular effects of ACEi.[6-7] As an alternative, improvement of Ang-(1-7) formation by ACE2 is now under consideration as a novel cardiovascular therapy.[8] Similarly, stimulation of AT_2 receptor, a receptor for both Ang II and Ang-(1-7), often opposes AT_1 receptor signaling, and this principle is being explored for cardiovascular therapy with specific AT_2 receptor agonists.[9]

Stem cells: general features and clinical use

At the same time that novel pharmacological tools within the RAS are being found for cardiovascular therapy, a novel target tissue for RAS intervention has been identified, namely the bone marrow (BM) and other sources of stem cells and progenitor cells. These cells can either be hematopoietic stem cells (HSC) or hematopoietic progenitor cells (HPC), or can be mesenchymal stem cells (MSC), also called multipotent stromal cells. Many articles use the term HSC for cells that are in a very early stage of phenotypic development, still allowing differentiation towards diverse lineages, and that may have a potential for self-renewal. HPC is then used for cells that are further differentiated, and have lost the potential for self-renewal. HSC and HPC form cells of the erythroid, myeloid, or lymphoid lines (Figure 1). Since the boundaries between these two hematopoietic populations are not always clear, we will here use the term HSC for both populations. Endothelial progenitor cells (EPC) and fibrocytes are special HPC types that are involved in angiogenesis and

fibrosis respectively. MSC have been identified as cells that can form a plethora of non-hematopoietic cells, including cardiomyocytes, smooth muscle-like cells, renal cell types, connective tissue cells, neural cells etc. (Figure 3). MSC are most abundantly present in BM, fat tissue and dental pulp, but can also be present in tissues such as the myocardium and the subintima or adventitia of large arteries. Accordingly, these cells are under investigation as a tool for tissue regeneration of any imaginable kind. Since both HSC, MSC and isolated whole bone marrow are interesting for tissue repair, all three sources have been used for cardiac and vascular repair. Other precursor cells that may potentially be used for this purpose are embryonal stem cells or induced pluripotent stem cells.[10] These cells can either serve as sources for novel cardiomyocytes or angiogenic cells, or act as paracrine cells that favourably affect cardiac remodelling or vascular repair. Several clinical studies have been performed with autologous BM-derived stem cells showing moderately improved cardiac performance in cardiac ischemic diseases.[11-12] As to yet, it is not clear which stem cell type and which preparation and infusion method gives the best results, and this is an important research goal. A second important goal is upgrading of the stem cells' abilities to perform its reparative function. For this purpose, diverse strategies are being studied,[10] and, as will become clear from the present review, pharmacological intervention in the RAS may be one of them.

RAS and stem cells: general implications

The notion that the RAS is involved in regulation of bone marrow and stem cells, and in particular hematopoiesis and erythropoiesis, was generated already shortly after the introduction of ACE inhibitors in the clinic when in 1982 two independent groups observed that a high dose of ACE inhibition causes anemia and leucopenia.[13-14] Later, the presence of all major RAS components in BM cells, including stromal cells, HSC / HPC and MSC, was confirmed. This led to the concept of a potential autocrine-paracrine mechanism for local RAS-mediated regulation of hematopoiesis.[15] Another important reason to study effects of angiotensins and RAS-modulating medicines on BM-derived cells is that in the case of cardiovascular disease where RAS modulation is indicated as a treatment, stem or progenitor cells are under investigation for application in tissue regeneration, in particular of the vascular bed and the myocardium.[16-26] Furthermore, inflammation and fibrosis have been identified as important targets for RAS modulation, and these processes may be related to regulation of HSC and MSC, as will be explained later in the article. Dedicated studies have now provided clear evidence that ACE-, Ang II/AT_1 receptor and Ang-(1-7)/Mas receptor activity is involved in hematopoiesis as well as in the formation of cardiovascular cells and other somatic cell types from progenitor cells.[27]

In the present article we will review the role of Ang II, Ang-(1-7) and their respective receptor / signaling pathways in propagation and differentiation of HSC and MSC, and discuss possible clinical implications. Furthermore, for more extensive discussions regarding ACE substrates and metabolites other than Ang II and Ang-(1-7) we refer to previous reviews.[28-29] The present review contains two summarizing figures:

Figure 1 depicts effects of Ang II and Ang-(1-7) on hematopoietic stem cells and derived progenitors; Figure 3 summarizes effects on mesenchymal stem cells. In addition, we provide a table (Table 1) that summarizes beneficial vs. detrimental effects of the main angiotensin receptors with respect to cardiovascular disease.

The role of Ang II and Ang-(1-7) in hematopoiesis

Erythropoiesis

Soon after the initial studies that uncovered that manipulation of ACE activity interfered with erythropoiesis, it became clear that the role of the RAS in erythropoiesis is very complex, being involved in virtually every step between the hematopoietic stem cell and the fully differentiated erythrocyte. When looked at in further detail it was shown that the stimulation of AT_1 receptor increased formation of early erythroid progenitors; an effect that requires the presence of erythropoietin.[30] Also, genetic manipulation leading to overactivity of AT1a receptors in mice results in an increase of hematocrit.[31] Conversely, AT_1 receptor knockout mice show a decrease of hematocrit values when compared with wild-type animals.[32] The stimulatory role of AT_1 receptors in erythropoiesis has clinical implications: like ACEi, ARB treatment was reported to reduced erythropoiesis in healthy persons, and also in patients undergoing hemodialysis.[33]

The mechanism of Ang II-mediated regulation of erythropoesis is largely unclear. Most of its effects are exerted in the early phases of erythropoesis.[30, 33] Some authors imply that Ang II acts indirectly via its influence on erythropoietin levels,[34] or erythropoietin sensitivity,[35] while others do not observe a link between erythropoietin and Ang II in erythropoiesis.[36-37] A possible second messenger system via which Ang II could be affecting erythropoiesis is the Jak-STAT pathway, which is known to be stimulated by Ang II,[38] and to be vital in the erythrocyte-stimulating action of erythropoietin.[39]

Since Ang-(1-7) is degraded by ACE, the anemic effect of ACEi might be due to changed Ang-(1-7) levels. However, it was shown that Ang-(1-7) stimulated erythroid burst-forming units (BFU-E) cultured from mice that were treated with the myelosuppressive agent 5-fluorouracil, and reduced anemia in breast cancer patients after chemotherapy.[40-41] These findings suggest that Ang-(1-7) stimulate erythropoiesis, and should counteract the anemic effect of ACEi. Apparently, this does not happen, and the question remains how important Ang-(1-7) is for erythropoiesis.

General effects of Ang II on hematopoiesis of white blood cells

Leucopenia induced by a high dosage of ACE inhibitors as observed early after introduction of ACEi[14] was an indication that the RAS may be involved in the formation of white blood cells (WBC) by HSC. In agreement, genetic ablation of ACE in mice leads to perturbations in myelopoiesis, which can be reliably recapitulated with ACE inhibition.[42] When focusing the attention on Ang II, it has been shown that Ang II induces the proliferation of mouse bone marrow and human cord blood HSC *in vitro*. This Ang II

	HSC		MSC	
	beneficial	**detrimental**	**beneficial**	**detrimental**
AT_1 receptor	Improves HSC proliferation under hematopoietic stress[45] Pro-angiogenic EPC stimulation [73-74]	EPC apoptosis and senescence [76-78] Fibrocyte-related fibrosis [135, 137-138]		Neointima formation or inflammation by VSMC-like progenitor cells [169-171] Promotes adipocyte formation [161] Inhibits cardiomyocyte formation [143] Inhibits neural repair by MSC after brain ischemia [146]
AT_2 receptor	Counteracts fibrocytes [135]		Promotes cardiomyocyte formation [142-143] Inhibits adipocyte formation [161] Improved neural repair by MSC after brain ischemia [146]	
Ang-(1-7)/Mas receptor	Improves HSC proliferation under hematopoietic stress [40-41, 45, 50] Increases early EPC [53]			

Table 1. Summary of beneficial or detrimental effects of angiotensin receptor signalling effects in bone marrow stem cells.

effect is elicited in part through stimulation of Lin^- BM stromal cells and partly mediated by the direct stimulation of HSC in the presence of colony stimulating factor (CSF).[43] AT_1 receptors mediate this Ang II effect because losartan blocks it, and the presence of AT_1 receptor in both HSC and stromal cells is compatible with the dual pathway.[43] Furthermore, Ang II/AT_1 receptor signaling promotes the monocyte colony stimulating factor (M-CSF)–mediated differentiation / proliferation of BM monocyte-lineage cells.

Through these general mechanisms Ang II can contribute to regulation of WBC hematopoiesis. Strictly spoken, however , AT_1 receptor signaling doesn't seem to be of great importance for hematopoiesis under normal physiological conditions: ACE-KO mice show a block in terminal granulopoiesis which leads to a reduction of segmented neutrophils. Monocytes and macrophages are at normal levels in ACE-KO and AT_1-KO mice, although they are to be functionally immature.[29, 42, 44] This would correspond to the fact that ARB treatment does not reduce WBC levels. However, as observed recently,[29] under hematopoietic stress Ang II / AT_1 receptor signaling has readily visible effects. This is noticed for instance after chemotherapy or irradiation, where Ang II infusion improves the repopulation of bone marrow with HSC cells, and thus accelerates restoration of WBC counts. It is with these circumstances in mind that the next section, in which we deliberate specific HSC subtypes, has to be read.

Role of Ang II in specific subtypes of white blood cell precursors

As already indicated, important data on the role of Ang II in regulation of HSC comes from studies that explored the restoration of WBC populations after irradiation or chemotherapy in animal models. Under such circumstances, *in vivo* Ang II infusion leads to accelerated restoration of total blood leucocytes, lymphocytes and platelets, and to increased granulocyte macrophage colony forming units (GM-CFU), granulocyte erythroid megakaryocyte macrophage colony forming units (GEMM-CFU) and BFU-E in *in vitro* expanded bone marrow cells.[45] Therefore, Ang II has a rather broad spectrum when it comes to stimulation of HSC. This is in accordance with the observation that in the early development of HSC to WBC, i.e. in CFU cultured under pan-lineage conditions, Ang II has a proliferative effect.[29]

This proliferative effect is lost when cells are further differentiated due to culturing in lineage-specific media, containing granulocyte-macrophage colony-stimulating factor (GM-CSF) with macrophage colony-stimulating factor (M-CSF) or granulocyte colony-stimulating factor (G-CSF).[42] When the HSC has passed the stage of myeloblast, pharmacological or genetically-induced interruption of AT_1 receptor or ACE activity delays myeloid differentiation rather than proliferation, as witnessed by an increase in the myeloblast / early myelocyte marker CD11b and a decreased development of macrophage and neutrophil markers.[42] This was observed *in vivo* in the absence of hematopoietic stress. Furthermore, AT_1 receptor blockade also leads to the decreased differentiation towards dendritic cells of human monocyte or murine BM cell cultures.[46] AT_2 receptor stimulation counteracts this AT_1 receptor effect. Accordingly, the summed effect of these Ang II functions is an accelerated general increase in leucocytes during hematopoietic stress, and

more subtle qualitative changes in populations of mature leucocytes, which can be seen in the absence of hematopoietic stress.

Similar to erythropoiesis, Ang II does not seem to have a stand-alone effect in development of WBC, but rather plays a facilitating role, as observed during CFU-GM and CFU-GEMM formation. In absence of colony stimulation factors (SCF, GM-CSF, IL-3 and EPO) no growth effect of Ang II on these colonies was observed, but in their presence, Ang II dose-dependently increased CFU-GM and CFU-GEMM formation.[43] The factors that are needed to allow an Ang II effect are released by Lin$^-$ BM stromal cells.[43] Also, in the absence of M-CSF, cultured BM cells of AT_1 receptor KO and WT mice showed no difference in myeloid progenitors and promonocytes.[44] However, the increase of these cell types that finds place upon stimulation with M-CSF was attenuated in AT_1 receptor KO vs. WT. The facilitating role of Ang II on M-CSF was shown to be due to TNF-alpha release from

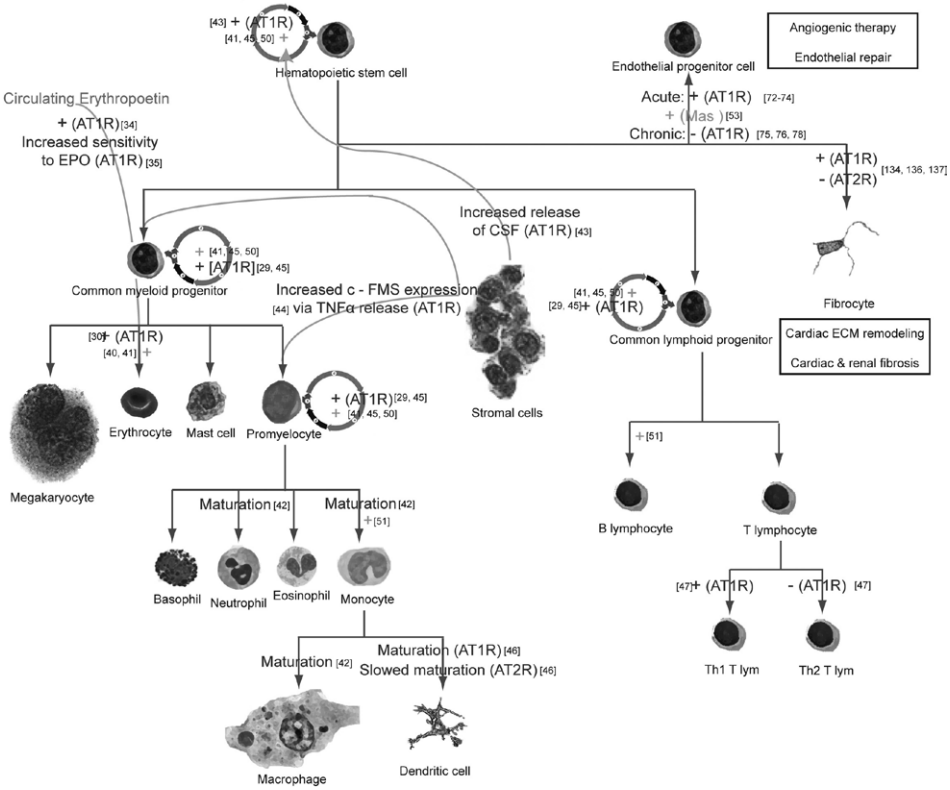

Figure 1: Schematic representation of the effects of AngII (black lettering) and Ang-(1–7) (gray lettering) on haematopoietic cells

The direction of the effect of AngII, Ang-(1–7) or angiotensin receptor stimulation is indicated, with '+' being stimulatory and '−' being inhibitory. The receptor involved, if known, is indicated in parentheses (AT1R, AT_1 receptor signalling; AT2R, AT_2 receptor signalling). Straight black arrows indicate effects on differentiation or, when specifically indicated, on maturation. Cell-cycle diagrams indicate a proliferative effect. Curved arrows originating from stromal cells represent paracrine effects. Reference numbers are to reports that directly show an effect of the angiotensin. Th T lym, T-helper cell.

stromal cells which increases expression of C-Fms, the receptor for M-CSF, in HSC.[44]

Apart from stimulating the myeloid path of hematopoiesis Ang II affects the lymphoid development. AT_1 receptor stimulation appears to promote inflammatory activation of lymphocytes. In addition, Ang II infusion leads to a shift of CD4+ T cells (T helper) from Th2 to Th1, which leads to increased production of pro inflammatory cytokines (IFN-gamma, IL-2, and tumor necrosis factor-beta). Blockade of AT_1 receptors decreased this shift and led to reduced infiltration of tissues by activated macrophages and T-cells.[47]

Based on the mild effects of ACE/AngII/AT_1 receptor on WBC population in the absence of hematopoietic stress, it seems that the major part of the effect of RAS modulation on inflammation is mainly caused by its effects that do not directly relate to hematopoiesis. Ang II is known to induce monocyte recruitment to the vascular wall and to stimulate these monocytes to release various inflammatory cytokines. It also increases production of reactive oxygen species (ROS) which in turn stimulate NF-kappaB signaling leading to a proinflammatory phenotypes in various cell types. These processes are reviewed in detail in other publications.[48-49]

The role of Ang-(1-7) in HSC regulation

The effects of Ang-(1-7) on hematopoiesis are documented in several articles and are, in short terms, similar to the effect of Ang II. Ang-(1-7) stimulates recovery from irradiation and chemotherapy by increasing proliferation of HSC and multi-lineage hematopoietic progenitors.[41, 45, 50] In NOD/SCID mice the engraftment and proliferation of human mononuclear cells was increased in mice receiving Ang-(1-7) treatment, which also increased numbers of differentiated cells of myelomonocytic and B cell lineages.[51] Like for Ang II, Ang-(1-7) effects are readily visible during hematopoietic stress, but not so evidently under normal physiological circumstances. This is reflected in a recent study in which toxicological studies with Ang-(1-7) infusion did not show apparent effects on blood variables.[52]

The role of angiotensin receptor subtypes for Ang-(1-7) effects on hematopoiesis has not been investigated. However, our own studies in which we used isolated BM mononuclear cells (BM-MNC) from rat and mice, suggest that Mas receptors are mediating the proliferative effect of Ang-(1-7) on HSC.[28, 53] Since these tests were done under conditions that promote the development of endothelial progenitor cells, they do not provide conclusive evidence.

Endothelial progenitor cells

Whereas a modest number of studies have addressed the relationship between HSC and RAS, some more intensive research has been done on endothelial progenitor cells (EPC).[27-28] EPC are a special type of HSC or MSC-derived progenitor cells that develop endothelial-like features in specific culturing conditions, and that have been implicated in endothelial repair and vasculogenesis.[54-55] Vasculogenesis is the formation of new blood

vessels from progenitor cells such as hemangioblasts, as opposed to angiogenesis, in which new vessels originate from sprouting of pre-existing ones. Vasculogenesis takes place during embryogenesis, but it has been proposed that it can also occur during adulthood, which would involve EPC.[56]

EPC starting as HSC in the bone marrow are being intensively studied. Their recruitment as HSC from the bone marrow to the circulation involves stress-induced stromal-derived factor-1 (SDF1), which activates proteases that degrade the adherence proteins that bind HSC to endosteal cells. Subsequently, HSC can migrate to the circulation. Once arrived in the vascular lumen they can home as angiogenic monocytes and macrophages that pre-process the tissue that requires neovascularization or endothelial repair, or as EPC that will form the new endothelium.[57] Thus, the process of vasculogenesis involves various hematopoietic cell types with varying progenitor, myeloid and endothelial cell markers, complicating the identification of "true EPC".[27] This identification can either be based on immunohistological staining for stem cell and endothelial membrane markers, followed by flow cytometry, or by culturing of BM- or blood-derived MNC in specialized endothelial culture medium and subsequent colony observation or (immuno)histochemical staining. A simple histochemical staining for cultured angiogenic cells is combined acetylated low density lipoprotein uptake (Ac-LDL uptake; a feature of phagocytotic monocytes, macrophages and endothelial cells) with binding to lectin from Bandeiraea (Griffonia) simplicifolia-1 (BS1) or Ulex europaeus (Ulex europaeus agglutinin-I: UEA-1).[54] More specific EPC markers, for cultured as well as freshly obtained cells processed for flow cytometry, combine stem cell surface markers c-Kit, Sca-1 and CD133 with an endothelial cell (EC) surface marker (e.g. CD34 or Flk-1 (mouse equivalent for human kinase domain receptor (KDR) or VEFG receptor 2 (VEGFR-2)). With respect to culturing methods, in vitro propagation of blood-derived MNC distinguishes early EPC and colony-forming unit endothelial cells (CFU-EC/CFU-Hill) on the one hand from late EPC (comprising outgrowth endothelial cells (OEC) and endothelial colony forming cells (ECFC)) on the other hand.[56, 58-62] Early EPC and CFU-Hill are found from 2 days until 3 weeks of culture and might be more related to angiogenic monocytes and macrophage since they show low proliferation and tube formation capacity. Late EPC, appearing from 3 weeks up until 12 weeks of culture could represent true EPC, displaying highly proliferative and tube formation capacity. For further critical reviewing of EPC selection criteria we refer to previous publications.[27, 63-64]

Although the identity of true EPC and their permanent incorporation into repaired and newly formed vessels is still a matter of debate, it is evident that the various "EPC" contribute to the vascular regeneration and repair processes, and thus bear clinical relevance.27-28 In addition, EPC plasma levels have been used as a biologic marker for vascular function and cumulative cardiovascular risk. It has been shown that the number of CFU-Hill correlate with brachial endothelial function, measured as reactive hyperemia, arterial calcification, with Framingham risk score, and with several cardiovascular risk factors.[60, 65-66] Quantification of cultured EPC might be a rather laborious method for prognostic purposes. Instead, circulating EPC can be measured with flow cytometry,

using CD34 alone of in combination with a marker expressed by endothelial cells, often KDR. As such, CD34+KDR+ cell levels were shown to be associated with coronary artery disease, outcome after angioplasty, and traumatic brain injury, although peripheral arterial calcification only correlated with colony number of cultured EPC.[66-69] In a direct comparison between cultured EPC and quantitation with flow cytometry, both methods show association of EPC levels with coronary artery disease, but only cultured EPC are predictive for progression of the disease.[70] Also, an increase of these markers is associated with beneficial effects of RAS intervention, as reviewed earlier.[27] The use of EPC as a standard risk marker in cardiovascular disease is however still remote for the daily practice. Hence, EPC remain under investigation for regenerative, angiogenic therapy in organs after ischemic events and as a prognostic marker during pharmacotherapy directed against vascular disease. Since RAS intervention is often used in those cases it is important to characterize the effects of angiotensins on EPC.

Role of Ang II, AT1 receptor and AT2 receptor in EPC

Although the identity of true EPC is still a question[27] many studies have confirmed the role of angiogenesis-associated progenitor cells, whether they are mobilized from BM to peripheral blood or isolated, cultured and re-injected, in endothelial regeneration, and neovascularization.[71] The fundamental parts of these processes bear relationship with Ang II signaling through AT_1 and AT_2 receptors. AT_1 receptor stimulation can lead to pro-angiogenic effects and recruitment of EPC, but on the other hand the stimulation can reduce EPC proliferation and function. These paradoxical effects can be explained from acute as compared to chronic AT_1 receptor signaling. Acute Ang II signaling is pro-angiogenic. In EPC, this pro-angiogenic effect depends on NADPH oxidase activation and enhanced VEGF anti-apoptotic function through upregulation of VEGF type 2 receptors and improved NO release, as well as on PI3K/Akt signaling.[72-74] The deleterious effects of Ang II arises from chronic stimulation, and consists of two consecutive phases.[75] In the first phase, taking place between day 2 and 5 of stimulation of EPC with Ang II, AT_1 receptor-mediated increase of NADPH oxidase activity leads to ASK-1 – JNK/p38 MAPK – Bax/Bcl2 signaling-induced apoptosis involving caspase 3.[76] The second phase, which is noticed from day 5 and onward, involves production of cytotoxic levels of ROS leading to cellular senescence.[77-78] As a result, chronic treatment with Ang II decreases human and mouse EPC numbers and function.[67, 76-79] Employing AT_1 receptor KO MNC and BM transplant in wild type and ApoE KO mice, it was shown that AT_1 receptor signaling affects vascular repair function and thus promotes atherogenesis.[76] As commented in detail earlier, it is still a question if these *in vivo* vascular effects solely depend on EPC, or involve an interplay with inflammatory cells or even BM stromal cells.[75]

The protective role of Ang-(1-7) / Mas receptor signaling in endothelium and EPC

Ang-(1-7) was shown to improve endothelial vasodilator and eNOS function in various studies,[80-93] pointing to effects that are opposite to Ang II with respect to vasomotor function. We have expanded this contrast with Ang II by studying the effect of Ang-(1-7)

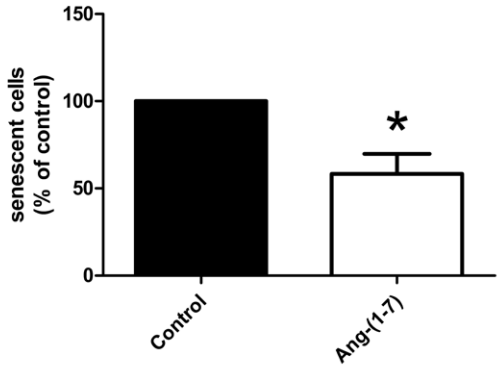

Figure 2: Effect of Ang-(1-7) on stress-induced senescence.

Bovine arterial endothelial cells pretreated with 10-7 mol/L Ang-(1-7) (Ang-(1-7)) or not (Control) were treated with 25 mmol/L superoxide radical donor (tBHP(n=3) or H_2O_2 (n=2): pooled data, n=5, are shown). After 48 hours senescence-associated b-galactosidase staining was performed. The amount of senescent cells in Ang-(1-7)-pretreated cells relative to Control was calculated. Controlis the difference between numbers at baseline and after treatment with superoxide donor. *: $p<0.05$ vs. 100%, one value t-test.

on endothelial cell senescence. As discussed earlier, chronic exposure of EPC to Ang II increases senescence of EPC in a later state of differentiation. In a pilot study, we tested the effect of Ang-(1-7) on exogenous ROS-induced senescence in adult bovine aortic endothelial cells. Ang-(1-7) pretreatment inhibited H_2O_2-induced increase of senescent cells (Figure 2). This finding again shows the opposite, protective role of Ang-(1-7) as compared to Ang II, and the result is in agreement with the observation that Ang-(1-7) is diminishing nuclear ROS formation induced by Ang II.[94]

Although the protective effects of Ang-(1-7) in adult endothelial cells depend on the acute activation of eNOS, inhibition of NADPH oxidase.[95-97] or protection against exogenously administered ROS, others may point to a potential role of Ang-(1-7) in endothelial regeneration. Such a role was suggested by the observation that Ang-(1-7) improved the recovery of HSC,[40-41, 45] from which EPC are derived. Therefore, dedicated studies to explore Ang-(1-7) effects on EPC were performed.[53] In adherent rat or mice BM-MNC cultures, which most likely resemble early EPC, 7 days of treatment with Ang-(1-7) increased AcLDL/lectin-positive cells, which were found to be positive also for VEGF type 2 receptors. Mas receptor signaling is an important mediator of the stimulatory effect of Ang-(1-7) on BM-MNC and EPC because the effect disappeared when Mas receptor signaling was prohibited by genetic ablation of Mas or treatment with A779. AT_2 receptors did not seem to play a role.[28] The *in vitro* stimulatory effect of Ang-(1-7) on EPC can explain why we found that *in vivo* infusion of the peptide in mice with myocardial infarction led to an increase in VEGF+ and c-kit+ cells in the heart.[53] Since local cardiac overexpression of Ang-(1-7) peptide did not lead to such an effect, we hypothesized that BM-derived, angiogenesis-related progenitor cells were recruited to the heart. This potential mechanism as well as the consequences for myocardial angiogenesis remains to be characterized.

Several other questions regarding angiogenesis need to be clarified as well. Firstly, Ang-(1-7) can also inhibit angiogenesis *in vivo*[98-101] and tends to inhibit *in vitro* tube formation by human umbilical cord vene endothelial cells (HUVEC).[102] This principle is now under investigation in the context of application of Ang-(1-7) as an anti-cancer therapy.[100, 103] There can be several explanations for such a discrepancy with our findings on EPC. First, it can relate to differences in cell types, i.e. EPC versus adult endothelial cells. Second, the inhibitory Ang-(1-7) effect on tube formation by HUVEC was not dose-dependent, and involved Mas receptors as wells as AT_1 receptors.[102] Since these were acute responses, the inhibitory effect of Mas receptors on AT_1 receptor signaling[104] might have inhibited angiogenesis. However, this explanation only works if one presumes the presence of spontaneous AT_1 receptor signaling or a paracrine / autocrine RAS. Third, in the sponge model for angiogenesis and after tumor implantation the anti-angiogenic response might involve effects on surrounding cells that produce angiogenic factors. The myocardium in our experiments may respond differently to Ang-(1-7) with respect to the release of angiogenic factors as compared to the tissue that surrounds sponges or tumours. In fact, there is evidence that tumour angiogenesis markedly differs from that in normal tissues, as extensively reviewed elsewhere.[105] Fourth, the Ang-(1-7) concentration and its relation to the duration of the stimulus may be important. In acute studies, such the mentioned study in HUVEC,[102] Ang-(1-7) might simply display the usual opposite effect Ang II. Instead, our studies in cultured MNC and EPC employed chronic stimulation, and again Ang-(1-7) appear to be opposite of the Ang II effect, i.e. stimulatory vs. inhibitory. Looking in better detail, we found a bell-shaped concentration-dependent effect of a 7-day Ang-(1-7) treatment, given every 2 days, with a maximal response between 10^{-9} and 10^{-8} mol/L.[53] Similarly, pretreatment with 10^{-8} mol/L Ang-(1-7) stimulated porcine BM-MNC to increase tube formation, whilst higher concentrations seem to reduce this ability.[106] This observation prompts another explanation. Recently, it was discovered that Ang-(1-7) leads to Mas receptor internalization.[107] A continuous presence of Ang-(1-7) concentration of 1 nM and higher might induce permanent Mas receptor internalization, thus abolishing Mas receptor signaling. This could in turn even promote chronic AT1 receptor signaling, thus reducing EPC. Alternatively, Ang-(1-7) might be diverted to AT_2 receptors, which would suppress angiogenesis.[108] Since Ang-(1-7) is rapidly degraded in the presence of serum,[109] the presence of Mas receptors at the cell membrane might be warranted when intermittent administration of the peptide is used, even at concentrations that are slightly higher than 1 nmol/L.

Regardless of the here mentioned reasons for the paradoxical effects of Ang-(1-7) on EPC as compared to angiogenesis, it is clear that in the development of pharmacotherapy based on EPC stimulation by Ang-(1-7) it will be important to dissect the diverse signaling pathways. This concerns both the exploration of the differential function of these pathways as well as the development of optimal pharmacotherapy.

Improvement of EPC recruitment with ARB and ACEi.

The *in vivo* effects of ARB on EPC generally correspond well with the effects that would be predicted from the functions of Ang II and Ang-(1-7) as observed in animal or cell culture studies. ARB treatment in animal studies raises EPC levels during hypertension,[110-112] after nephrectomy[113] or myocardial infarction,[114] in atherosclerosis models,[115] and in the ischemic hindlimb model.[116-117] Although the situation in animal models may quite differ from the clinical presentation of patients, the effects of ARB on EPC from animals and patients correlate rather well. In patients with type II diabetes, ARB treatment alone or as a part of multiple drug therapy was proposed to exerts its beneficial cardiovascular effects by increasing the number of regenerative EPCs.[118-119] In accordance with this idea, ARB treatment increased EPC counts, angiogenic factors and endothelial vasodilator function in normotensive patients with coronary artery disease.[120] Similarly, ARB increase EPC in patients with acute coronary syndrome.[121] The ARB treatment also promotes an increase in EPC levels in kidney transplant patients.[122] A special role might be performed by telmisartan, a combined ARB / PPAR-γ agonist. The PPAR-γ might have additional value when stimulation of EPC is concerned,[123-124] as was discussed in detail in previous reviews.[27-28] Together, the data suggest that in animal models as well as in patients ARB treatment increases EPC levels, thus preserving the endothelium. It seems logical to ascribe these beneficial effects to a decrease of EPC apoptosis and senescence caused by excessive Ang II / AT_1 receptor signaling.

ACEi have been shown to have beneficial effects in cardiovascular disease that are believed to be related to EPC function.[60, 67, 125-130] However, the actual number of patient studies that explored effects of ACEi on EPC is very limited. ACEi increased the levels, proliferation, migration, adhesiveness, and tube formation of EPC that were cultured from blood of patients with coronary artery disease.[79] In patients with acute coronary syndrome ramipril increased circulating EPC as measured by flow cytometry, and its effect on EPC was nearly identical to that of ARB telmisartan.[121] Very recently, two studies appeared that claim a stimulatory effect of ACEi on EPC of patients with hypertension and acute coronary syndrome, although the characterisation of EPC in these studies is limited.[131-132] In agreement with clinical studies, ACEi increase EPC in several animal models.[114, 116-117, 133-134] These effects may involve various factors other than angiotensin metabolism, as amply discussed elsewhere.[27-28]

Differentiation of HSC into fibrocytes

Fibrocytes are CD34+, CD45+, or CD133+ bone marrow-derived circulating cells that co-express collagen I or smooth muscle actin.[135] They are derived from hematopoietic cells and related to monocytes, and infiltrate organs to establish fibrosis. In models of renal fibrosis AT_1 receptor mediates increased bone marrow fibrocytes, increased renal infiltration and stimulates collagen I production in cultured fibrocytes. AT_2 receptor counteracts these effects. Recruitment of fibrocytes to the kidney involves CXCL16, a ligand for the receptor CXCR6 [136]. However, it is not certain if this relates to Ang II signaling.

With respect to cardiac remodeling, it was shown that Ang II infusion promotes cardiac

fibrosis through the recruitment of fibrocytes in mice.[137-138] Since chemokine (C-C motif) receptor 2 (CCR2) KO abrogated the Ang II effect, the chemokine (C-C motif) ligand (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), is apparently required for Ang II-induced fibrocyte recruitment. Blood pressure is not involved, since CCR2 knockout did not change this variable. MCP-1 is an important stimulator of monocytes, dendritic cells and T cells, and therefore these data show an important relationship between inflammatory response and Ang II-induced fibrosis. It should be noted, however, that the recruitment of fibrocytes by Ang II to the myocardium is not necessarily detrimental. CCR2 knockout, which ablates this Ang II effect, leads to less fibrosis, but also to an exaggerated ventricular dilatation and a worsened systolic function. The fibrotic response seems to provide the necessary matrix to support the hypertrophied myocardium.[137] Therefore, to obtain clinical benefit from suppression of Ang II-induced fibrosis one should also prevent myocardial hypertrophy to avoid myocardial dilation, which is detrimental.

Mesenchymal stem cells from bone marrow

Mesenchymal stem cells are non-hematopoietic progenitor cells that are able to form many diverse cell types, amongst which cells with features of adipocytes, cardiomyocytes,

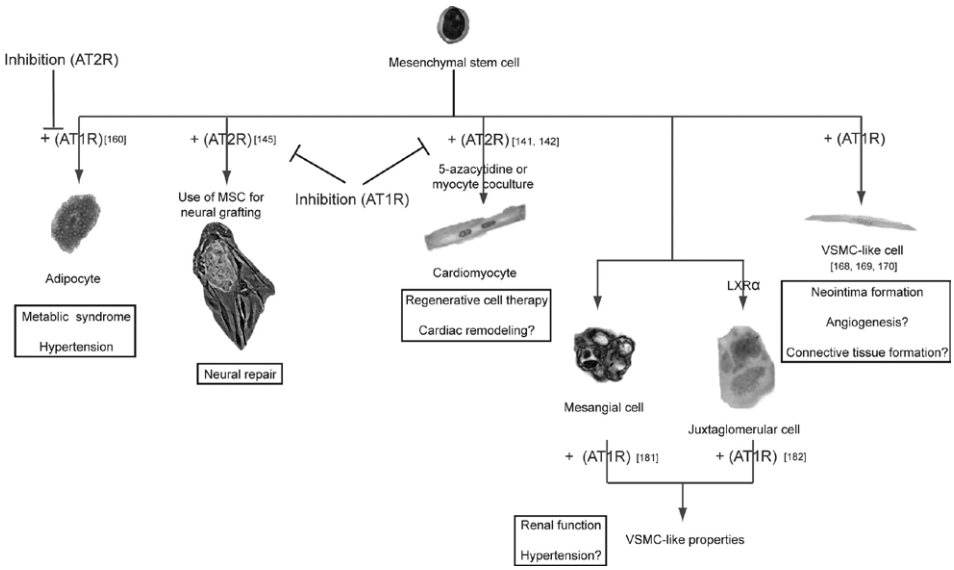

Figure 3 Effects of AngII on MSCs

The direction of the effect of AngII or angiotensin receptor stimulation is indicated, with '+' being stimulatory on differentiation, proliferation or use in regenerative therapy. The receptor involved, if known, is indicated in parentheses (AT1R, AT_1 receptor signalling; AT2R, AT_2 receptor signalling). Reference numbers are to reports that directly show an effect of AngII. Clinical implications or possible applications are indicated in the boxes.

fibroblasts, endothelial cells vascular smooth muscle cells and renal cells, which all bear relevance for cardiovascular disease and therapy. They are found in many different tissues, including the bone marrow. Given the pluripotency of MSC these cells may play a role in diverse physiological and therapeutical processes. We here outline the role of RAS in various of these processes.

Cardiomyocyte formation from MSC

BM-MSC were shown to be able to develop cardiomyocyte features through reprogramming of the genetic program by *in vitro* treatment with 5-azacytidine, a DNA-demethylating agent.[139] This finding was an important step towards the principle that autologous MSC transplantation could be used for cardiac repair, and led to clinical trials. Meta-analyses have shown that the small trials conducted thus far show only limited benefit, which has prompted novel research on optimization of stem cell therapy.[140-141] Optimization studies include issues like cell type that is used, number of grafted cells, moment of injection, but also the effect of pharmacological modulation, including RAS components. Ang II was shown to promote differentiation of rat BM-MSC to cardiomyocytes *in vitro*, and have an additive effects to 5-azacytidine.[142] Thus, Ang II treatment might reduce the culture time of BM-MSC allowing earlier grafting of the differentiated cultures.

Further characterisation of the involved receptor subtypes came from a study in which human BM-MSC (Yub623) were stimulated to differentiate to cardiomyocytes by co-culturing on murine cardiomyocytes for 2 weeks.[143] Various ARB's were shown to equally improve BM-MSC differentiation, apart from telmisartan that had additional effectiveness because of its pleiotropic actions as a PPAR-γ agonist. The effect was independent from the co-cultured murine cells. AT_2 receptor antagonist PD 123319 alone was without effect, but it reduced the effect of candesartan. Since also Ang II alone did not stimulate differentiation in this study, unless given together with candesartan, it was concluded that the differentiation of BM-MSC to cardiomyocytes was mediated through AT_2 receptors. Because ACE inhibition mimicked ARB effects a local RAS in BM-MSC was proposed, as was to be expected from previous PCR studies on rat BM-MSC.[15] However, the fact that renin inhibitor aliskiren was without effect in human BM-MSC suggests that not all RAS components may be present in relevant amounts in those cells. Therefore, it cannot be excluded that ARB's, when given in the absence of exogenously applied Ang II, might have unknown pleiotropic effects, especially when applying the high concentration (3 μmol/L) used in this particular study.

The Ang II-induced differentiation of BM-MSC to cardiomyocytes theoretically is in accordance with the observation that Ang II stimulates cardiac extracellular matrix remodelling through recruitment of fibrocytes.[137] In the context of physiological remodelling these effects of Ang II would be complementary to each other if there is a need for increased myocardial mass, for instance to improve physical performance, because they would provide a balanced increase of myocytes and extracellular matrix. Such a remodeling would beneficially influence cardiac performance. Whether Ang II-induced

BM-MNC differentiation into cardiomyocytes occurs, as well as the question if this is in balance with fibrocyte recruitment, remains to be investigated in models for physiological cardiac remodelling. An exaggerated AT_1 receptor-mediated response may, however, cause stiffening of the myocardium and relative ischemia, a concept that fits in the general paradigm of pathological remodelling due to high blood pressure or ischemia. Blockade of AT_1 receptor binding of Ang II and concurrent redirection of Ang II towards AT_2 receptors would inhibit this detrimental response, because it restores the equilibrium between differentiation of MSC into fibrocytes and cardiomyocytes.

ARB treatment can also be combined with BM-MSC grafting. Indeed, in the rat myocardial infarction model it was shown that pretreatment of BM-MSC with ARB improved the therapeutic effect of BM-MSC grafting on systolic heart function.[143] This was further improved by oral administration of ARB. However, oral ARB intake combined with non-pretreated BM-MSC grafting did not have an additive effect compared to the single ARB treatment. Therefore, pretreatment of BM-MSC with ARB or AT_2 receptor agonists may be the most optimal condition for cardiac repair. It should be noted however that BM-MSC that are injected for cell therapy in the myocardium are only transiently present, as observed in mice, and the fusion with resident cardiomyocytes might be poor.[144] This has favoured the concept that BM-MSC are therapeutic primarily due to a paracrine function.[145] Therefore, it is important to explore these paracrine factors and the impact of RAS modulation thereupon.

Neural repair

Similar to cardiac ischemia, BM-MSC grafting has been tested for brain ischemia.[146] In the mouse mid-cerebral artery occlusion (MCA) model, BM-MSC grafting improved survival, ischemic lesion volume, neurological score and cerebral edema. Cerebral blood flow and TNFα measurements suggest that reduction of inflammation rather than a pro-angiogenic effect is responsible for the effect of BM-MSC grafting. BM-MSC from AT_2 receptor KO mice, however, did not improve neurological variables, and even showed a trend to worsen survival. Pretreatment with the ARB valsartan restored the survival effect of BM-MSC grafting. These results imply that AT_1 receptor signaling, when not counteracted by AT_2 receptor, is detrimental for BM-MSC repair function. ROS-mediated pro-inflammatory AT_1 receptor signaling could be the provocative stimulus in the case where AT_2 receptor KO cells seem to worsen the outcome. Since AT_2 receptor signaling results in NF-kappaB-mediated neural repair and neural differentiation of embryonic stem cells through stimulation of methyl methanesulfonate-sensitive 2 release,[147-148] it would be worthwhile to study if these mechanisms are also involved in BM-MSC grafting.

BM-MSC grafting is believed to represent a new intervention technique for patient with stroke,[149-150] and AT_1 receptor blockade might provide further improvement. Pretreatment of mice with valsartan in a dose without a blood pressure effect led to comparable improvement of neurological score and better blood flow than BM-MSC grafting.[146, 151] Other ARB show comparable results to valsartan.[152] Further neuroprotective effects of ARB were shown in hypertensive patients, who show less stroke-related events when ARB

treatment was applied, leading to a better prognosis.[153] In acute stroke, animal models also show improved recovery with ARB treatment.[154-156] However, in patient studies of acute stroke ARB treatment was not at all effective, showing even a tendency towards adverse effects, which might relate to a hypersensitivity of the patients towards blood pressure lowering.[156-157] Therefore, optimization of RAS treatment in acute ischemia might benefit from (pre)clinical research to interaction with endogenous as wells as grafted BM-MSC.

Adipogenesis

Adipogenesis, the formation of fat tissue, is importantly implicated in metabolic syndrome and cardiovascular risk. As extensively reviewed elsewhere.[158-159] Adipose tissue contains a complete RAS needed for Ang II production and signaling. The physiological importance of this local RAS extends to the entire organism, contributing to both local and systemic regulation of RAS. This is believed to contribute to obesity, diabetes, metabolic syndrome and cardiovascular disease. The adipose tissue can be roughly divided in two compartments, the visceral and the subcutaneous adipose tissue. Adipose tissue can form part of normal physiology as an energy storage compartment. This healthy fat tissue contains small insulin-sensitive adipocytes that store fat until use as an energy source. These cells produce anti-inflammatory effectors, such as adiponectin. Adipose tissue can also be related to diabetes and metabolic syndrome, and in this case is featured by the occurrence of large insulin-resistant adipocytes.[160] These large adipocytes release pro-inflammatory factors amongst which TNF-α, a key player in insulin resistance, diabetes and metabolic syndrome. Obesity is associated with large adipocytes and an activated adipose tissue RAS, and it is believed that obesity thus contributes to cardiovascular disease.[158]

Adult adipocytes may arise from two sources: pre-adipocytes in the fat tissue and MSC from various sources, including the bone marrow. In pre-adipocytes the role of Ang II and its respective receptors is matter for a complicated debate that involves culture conditions, type of adipogenic stimulus, phase of differentiation, etcetera.[161-162] A feasible concept of Ang II-mediated dysregulation of adipogenesis in diabetes and metabolic syndrome is that AT_1 receptor stimulation prevents insulin-induced differentiation of pre-adipocytes into adult small adipocytes, and that as a consequence the pool of large adipocytes increases.[163] Compared to research in pre-adipocytes, research in MSC differentiation to adipocytes is relatively young. It was shown that human BM-MSC cultured under adipocyte-inducing conditions, all key Ang II-related RAS components are expressed. Thus, Ang II seems to enhance differentiation into adipocytes through AT_1 receptors in an autocrine fashion.[161] This effect is inhibited by AT_2 receptor. Since expression of AT_2 receptor is rather high in MSC that are cultured under the given conditions, the blockade of adipogenesis by AT_2 receptors was readily visible when the antagonist PD123319 was added. However, since exogenously given Ang II mimicked the AT_2 receptor-mediated blockade of differentiation, AT_1 receptor signaling is dominant when only the locally produced Ang II is present.

Given the complicated manner in which AT_1 receptor and AT_2 receptor signaling is involved in differentiation of MSC and pre-adipocytes, as well as the question what fate will fall upon MSC with respect to formation of small vs. large adipocytes, it is in this moment

hard to establish the importance of BM-MSC differentiation in therapeutic effects of RAS modulation. Further studies will require BM-MSC-specific RAS component knockout cell culture or mouse models, as was previously suggested also for adipose tissue.[158] Culturing conditions and metabolic status will be important issues in such studies.

The importance of Ang-(1-7)/Mas axis in adipose tissue is only beginning to be uncovered. Ang-(1-7) and ACE2 are present in adipose tissue,[164-165] Measurement of angiotensin metabolites in organ bath and cell culture experiments by liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS and ACE2 is regulated by dietary fat.[165] Also, Ang-(1-7) and the Mas receptor were reported to have an impact on fat and glucose metabolism, adiponectin levels and on insulin sensitivity of adipocytes.[166-167] Moreover, the peri-aortic fat may play a role in Ang-(1-7)-induced vasodilations.[168] Therefore, research to the role of Ang-(1-7) in BM-MSC differentiation into adipocytes is warranted.

MSC with vascular smooth muscle cell traits

Vascular smooth muscle-like cells can be derived from both BM stem cell pools as well as MSC populations present in the adipose tissue. In mouse studies it was shown that Ang II stimulates the expression of typical smooth muscle cell markers in cultured adherent BM-MNC and MSC from adipose tissue through AT1 receptors.[169-170] Similar results were obtained with MNC derived from peripheral blood of rabbits.[171] In adipose tissue MSC it was shown that this effect is mediated by TGF-β receptor stimulation and subsequent Smad2 and ERK activation.[170] Replacement of wildtype with GFP-labeled BM in mice showed that GFP-positive α-smooth muscle cell actin (α-SMA) -positive cells incorporated in the neointima of damaged femoral arteries of wild-type C57bl/6 mice.[169] The recruitment of such cells was stimulated by Ang II infusion, and inhibited by AT_1 receptor blockade. Since neointima formation followed a similar pattern it was concluded that Ang II-induced differentiation of BM progenitors to vascular smooth muscle cells (VSMC) contributes to neointima formation. Indeed, BM-derived cells that express typical VSMC markers had been implicated in neointima formation before.[172-173] In ApoE KO mice with LacZ-positive BM it was shown that local vascular production of SDF1α, a ligand for chemokine (C-X-C motif) receptor type 4 (CXCR4) that is important for chemoattraction of various BM-derived cells, plays a central role in homing of the VSMC-like cells to the neointima. Recently, a study that explored the effect of replacement of normal with AT_1 receptor KO BM in wild-type mice showed a connection between Ang II and SDF1α signalling.[174] AT_1 receptor KO led to diminished plasma levels and neointimal incorporation of Lin^- BM-derived progenitor cells, and decreased neointima formation. Although no specific VSMC markers were used in this study it was proposed that lack of AT_1 receptor signaling leads to decreased VSMC progenitor recruitment. This seemed to depend on decreased SDF1α release by local platelets at the site of injury due to the absence of AT_1 receptor signaling in those platelets. Therefore, AT_1 receptor signaling may affect neointimal homing of VSMC-like cells from BM in two ways: through increased differentiation of progenitor

cells into VSMC-like cells and attraction of progenitor cells through stimulation of platelet-derived SDF1α.

It should be noted, however, that VSMC-like progenitor cells may not undergo complete differentiation into adult vascular smooth muscle cells. Some studies show that, when injected in the ischemic myocardium, BM stem cells/BM-MSC with VSMC traits are not incorporated in vessels.[175-176] Indeed, in earlier studies it was suggested that mesenchymal stem cells develop a smooth muscle cell-like contractile apparatus to form myoid cells that use this apparatus in their function to support hematopoiesis of inflammatory cells.[177] Interestingly, it was recently shown that BM-derived cells that incorporate into neointima and atherosclerotic lesions and express α-SMA are also expressing monocyte/macrophage markers.[178] Therefore, VSMC-like cells originating from the BM, and perhaps also from adipose tissue, may play a pro-inflammatory role, rather than that they represent a pool of progenitor cells that will permanently differentiate into adult VSMC. This might explain why we found that all BM-derived cells in neointima after stenting expressed inflammatory cell markers.[179] The importance of such cells may of course be significant: the effect of Ang II on stimulation of the development of the VSMC-like cells that support or possess traits of inflammatory cells may be another example of the versatility of this peptide as a pro-inflammatory factor. Exploration of the role of AT2 and Mas receptors on these cells would be of great interest. Also of importance is further exploration of the link with SDF1α-CXCR4 signaling in pro-atherogenic environments, since blockade of this pathway in inflammatory cells leads to increased plaque formation in ApoE KO mice,[180] which is in sharp contrast with the earlier discussed decrease of neointima formation in injured arteries of wild-type mice.[174] It is essential to discover how these contrasting findings would translate to AT_1 receptor blockade effects in a clinical setting.

Finally, α-SMA expression in MSC might be part of a transition towards development of connective tissue.[181]

Renal progenitor cells

BM-derived progenitor cells may play a role in the remodeling of renal tissue. About a decade ago it was shown, with the help of replacement of wild-type BM with GFP+ BM in mice, that BM-derived cells incorporate into the glomerular mesangium of the kidney.[182] GFP+ mesangial cells increased over time after BM replacement, suggesting a long lasting, if not permanent residence of these cells. Although some of these cells express macrophage and lymphocyte markers, a large part of these cells do not appear to be leucocytes. Like MSC these cells express α-SMA, and therefore might represent pools of mesangial cells that contribute to glomerular vasomotor control. In accordance with this idea, isolated and cultured GFP+ cells contracted upon exposure to Ang II.[182]

Another α-SMA-expressing cell type that is associated with the glomerulus is the juxtaglomerular cell, present at the intersection of the distal convoluted tubule and the afferent arteriole of the glomerulus. These cells contain renin granules that can be release in reaction to low blood pressure or low filtrate osmolarity. The resulting increase in Ang II production causes normalization of blood pressure and filtrate osmolarity. It was recently

shown that BM-MSC can develop into renin-expressing granular cells that resemble juxtaglomerular cells, and that subsequently start to increase α-SMA-expression.[183] This process is under the control of liver x receptor α (LXRα) stimulation. This observation has shifted the paradigm of the origin of juxtaglomerular cells from being a VSMC-derived cell towards a MSC-derived cell that actually develops VSMC-like features in a later phase of differentiation. Perhaps even, a part of the BM-derived GFP+ glomerular cells that were observed in the fate-tracking experiment in mice might have developed in juxtaglomerular cells, but this was not explicitly explored.[182] Since the VSMC-like features develop relatively late during MSC differentiation it is tempting to speculate that this phenotype is a result of autocrine Ang II signaling following renin upregulation. Thus, Ang II might contribute to the development of cell types that release renin, control glomerular blood flow or both. This in turn would implicate an involvement in hypertension and renal disease.

General conclusions and future perspectives

There is a growing body of evidence that Ang II and Ang-(1-7) can affect proliferation and differentiation of BM-HSC and BM-MSC. The observed effects imply that RAS modulation in these cells can be used to inhibit cardiac, vascular and renal fibrosis as well as neointima formation, to improve renal function, to improve blood pressure control and organ perfusion, and to induces cardiac and renal repair. Beyond the cardiovascular system, RAS modulation in these cells can beneficially influence adipogenesis, neural repair, connective tissue formation, and wound healing, but foremost, stimulation of the Ang-(1-7)/Mas signaling axis is now under evaluation as an anti-cancer therapy with the combined benefit of improving hematological recovery after chemotherapy or irradiation. For a large part, signaling mechanisms remain to be investigated, especially in relation to the stage of differentiation of HSC and MSC, to the culture conditions (*in vitro*) or surrounding tissue (*in vivo*), and to the pathophysiological context. Such information might lead to more refined pharmacotherapy, while novel drugs for stimulation of specific angiotensin receptor subtypes are already being developed.

With respect to these novel drugs, the emerging ACE2/Ang-(1-7)/Mas receptor axis-oriented drugs are of particular interest. Since Ang-(1-7) promotes post-chemotherapy or –irradiation hematopoiesis in the absence of a pressor effect, the peptide is now under evaluation for hematological recovery of cancer patients. This already involves clinical trials with TXA127, an Ang-(1-7)-containing drug formulation.[184] The paradoxical effect of Ang-(1-7) on angiogenesis and its potential dependency on a delicate balance in stimulation of specific receptor subtypes was discussed. Several attempts have been made to specifically improve Ang-(1-7) signaling, for example through infusion of cyclodextrin-enveloped Ang-(1-7)[185] or increased ACE2 expression.[186] Even more specific are the non-peptide analogue CGEN-856S[187] or the thioether-bridged Ang-(1-7) analogue called cyclic Ang-(1-7)[188-189], which are specific Mas receptor agonists. Infusion of cyclic Ang-(1-7) has shown promise in rat models as an intervention for acute respiratory syndrome or after myocardial infarction.[190-191] It will be of major interest to perform comparative studies on

native Ang-(1-7) versus these specific Mas receptor agonists, and to unravel their effects on EPC and other progenitor cells.

As discussed, the AT_2 receptor is importantly involved in Ang II effects on progenitor cells. Through its effect on HSC and MSC, AT_2 receptor stimulation may have beneficial effects on fibrosis by inhibiting fibrocyte development, prevention of heart failure by promoting myocardial regeneration, and improvement of neural repair. Therefore, the design of AT_2 receptor agonists may be an important development. Currently, two agonists are being explored, the non-peptide drug Compound 21 (C21) and Ang II with single β-amino-acid substitutions.[9, 192] C21 has shown promise in the rat model of myocardial infarction by improving systolic and diastolic function, and by anti-apoptotic and anti-inflammatory effects [193]. Also it was found that C21 alone or in combination with losartan may improve endothelial function and vascular composition; by reducing oxidative stress, collagen content, fibronectin, and inflammatory cell infiltration in stroke-prone spontaneously hypertensive rats.[194] The development of β-amino-acid Ang II analogues awaits further *in vivo* characterisation, with respect to pharmacokinetic aspects as well as therapeutic effects. Interaction with stem cell therapy is certainly one of the most relevant issues that can be addressed in further studies with these AT_2 receptor agonists.

Another relevant goal for future research is further characterisation of α-SMA-expressing cells types formed by MSC in response to Ang II. It will be important to study the relation between tissue localization and culture conditions and the final phenotype and function that such cells will assume, which comprises VSMC, connective tissue and neointimal cell types, pro-inflammatory cell types, mesangial cells or juxtaglomerular cells. Without any doubt this feature of Ang II-AT_1 receptor signaling will play a role in clinical effects of RAS modulation on arterial and renal remodelling, and perhaps on blood pressure control. Futhermore, the formation of connective tissue cells from MSC is being explored for application in tissue engineering related to wound healing and repair of cartilage.[195] This may represent a novel research field in which to explore the role of RAS. It will be a major challenge to chart the versatile effect of Ang II, and possibly also other angiotensins, such as Ang-(1-7), on this route of MSC differentiation.

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Summary and discussion

8

Summary

Genomic instability and cardiovascular disease

Vascular dysfunction and related cardiovascular disease (CVD) are highly associated with increasing age. The mechanisms that cause aging in general and their influence on the pathophysiology of CVD in particular are not clear; however the causal role of genomic instability in the aging process is generally recognized.[1] We set out to investigate the hypothesis, as stated in **Chapter 1**, that genomic instability, as e.g. created by defective DNA repair, is causing accelerated vascular aging. In **Chapter 2** we used our severe progeroid mouse model of impaired NER and crosslink repair (*Ercc1*$^{d/-}$ mice), and milder progeroid model with NER impairment (XpD^{TTD}) to explore whether this leads to accelerated vascular aging. The DNA repair impairment in mice led to rapid development of several features resembling the aged human cardiovascular phenotype, reflected in worsened endothelium-dependent and -independent vasodilator function, as established *ex vivo*, and occlusion-reperfusion-mediated vasodilator function *in vivo*, increased vascular stiffness and hypertension. Endothelial dysfunction in our model is partially mediated via reduced levels and activation of nitric oxide synthase (eNOS). Interestingly, the vasculature of these mice showed increased levels of p21 mRNA which is a DNA-damage-related cyclin-dependent kinase inhibitor and a high number of senescent cells, a cellular phenotype found in atherosclerotic lesions of humans. We also investigated possible associations of human single nucleotide polymorphisms (SNPs) of genes that encode for relevant NER components with increased vascular stiffness, as determined by pulse wave velocity. We found a significant association in the DDB2 gene, which encodes a protein that recognizes DNA damage and initiating the NER, with pulse wave velocity. Based on these observations we conclude that DNA repair capacity is associated with accelerated vascular aging in mice, and that there may be implications for risk stratification in humans with respect to age-dependent cardiovascular disease.

Part of the decreased vasodilator reponse in the progeroid mouse *Ercc1*$^{d/-}$ was due to vascular smooth muscle cell (VSMC) dysfunction. Impairment of VSMC vasodilator function is observed in the aging vasculature of animals and humans less often than endothelial dysfunction, and is usually associated with higher age of the animals and presence of diabetes mellitus or chronic heart failure in humans.[2-3] Therefore VSMC impairment could be an indicator of a progressive vascular aging phenotype. We explored the VSMC function of *Ercc1*$^{d/-}$ mice with defective nucleotide NER in further detail (**Chapter 3**). Reduced vasodilator responses to endothelium-independent nitric oxide / soluble guanylyl cyclase / cyclic guanine monophosphate (NO/sGC/cGMP) -mediated vasodilation as compared to WT animals were observed, confirming findings in Chapter 2. To investigate the mechanisms of impairment, we explored the functionality of the individual components of NO/sGC/cGMP signaling pathway in tissues of *Ercc1*$^{d/-}$ animals. The levels and activity of soluble guanylate cyclase, an enzyme producing cGMP, were found to be comparable to those in WT animals. PDE inhibition improved the vasodilatations to SNP by increasing the maximal responses in *Ercc1*$^{d/-}$ mice, and in agreement with this observation

we observed increased PDE activity in lungs of *Ercc1*$^{d/-}$ animals *versus* WT. The levels and activity of cGMP-dependent protein kinase 1α were unchanged. Therefore, genomic instability due to defective NER and crosslink repair leads to increased PDE activity, thus hindering VSMC relaxation.

Towards prevention and repair of cardiovascular damage

The problems caused by genomic instability might in principle be countered by three general strategies, i.e. prevention of genomic instability, counterregulation of the unfavorable vascular signaling pathways, and replacement of the dysfunctional apoptotic and senescent vascular cells with new, functional cells by tissue repair mechanisms. These issues were addressed in the remaining part of the thesis.

The presence of senescent vascular cells, as brought forth by genomic instability, is observed in atherosclerosis[4] and some models of vascular dysfunction such as diabetic rats. Due to their pro-inflammatory, prothrombotic, mineralizing and vasoconstrictor phenotype, which contributes to elevated blood pressure, increased stiffness and atherosclerosis, senescent cells are likely to play a causative role in the development of CVD. Several pharmacological interventions and dietary habits, including consumption of red wine, were shown to improve vascular function and reduce the incidence of CVD.[5] Red wine polyphenols are established anti-oxidants. Since oxidative free radicals cause DNA damage red wine extract should promote genomic integrity and prevent vascular cell senescence. In **Chapter 4** we investigated the mechanism of beneficial effects of red wine extract (RWE) consisting of flavonoids, largely polyphenols, on senescence of vascular cells caused by oxidative stress. RWE protected endothelial cells from tBHP-induced senescence. The protective effect of RWE was associated with a decrease in p21. Consistent with this effect, RWE protected endothelial cells from DNA damage and this protective effect appeared to be dependent on eNOS and COX-2. RWE furthermore increased eNOS mRNA levels, increased the activation of eNOS by phosphorylation, and in the porcine coronary arteries led to relaxation. RWE increased expression of SIRT1 and SIRT1 inhibition partially attenuated the DNA damage protective effect of RWE. Resveratrol, a polyphenol that is often regarded as a main protective polyphenol within red wine, did not prevent tBHP-induced senescence.

Next, a novel pharmacological pathway to counter-regulate unfavorable vascular signaling was explored, namely angiotensin II type 2 (AT_2) receptor stimulation. The discovery of this "beneficial arm" of renin-angiotensin system (RAS) and its utilization could confer additional improvement to the classical RAS interventions. In previous studies, AT_2 receptor agonism was shown to mediate NO-dependent vasodilator, natriuretic, antiproliferative, and antifibrotic effects, all of which are beneficial in CVD.[6-7] Therefore, the non-peptide AT_2 receptor agonist compound 21 (C21) was developed. In **Chapter 5** we investigated the mechanisms of the vasodilator effects of C21. We did not observe C21-induced, AT_2 receptor-mediated vasodilation in any of the experimental

models employed. This is in contrast with previous studies of other groups that showed AT_2-mediated relaxations to C21. However, we observed AT_2-independent relaxant effects of C21 as well as constrictor effects. Irbesartan pretreatment abolished these constrictor effects and the concentrations needed to evoke them indicate that the agonism of angiotensin II type 1 receptor is involved. As to the vasorelaxant properties of C21, these were not inhibited by AT_2 antagonist treatment or interference with NO/sGC/cGMP pathway. Pretreatment with C21 also reduced constricting potency of the constrictors U46619 and phenylephrine, but did not affect the constrictions of the calcium ionophore ionomycin, suggesting its possible effect on inward calcium transport into the cell.

Alternatively to AT_2 receptor stimulation, angiotensin-(1-7) (Ang-(1-7)), the endogenous agonist of the Mas receptor, has been implicated as a causal element in the beneficial effects of common RAS interventions.[8] Ang-(1-7)/ Mas receptor signaling has been proposed as a novel target for counter-regulation of harmful cardiovascular signaling next to AT_2 receptor stimulation. The possibility of utilizing a Mas receptor agonist in the treatment of myocardial infarction (MI) and prevention of heart failure, which in humans are highly associated with age, was investigated in **Chapter 6**. For this purpose we tested the effect of the proteolysis-resistant and Mas receptor-specific compound cAng-(1–7) in a rat MI model. This compound dose-dependently lowered left ventricular weight and end-diastolic pressure in an MI model in which no contractile failure had yet occurred. The effect on cardiac weight was associated with reduction of cardiomyocyte hypertrophy, as evidenced by the decrease in myocyte dimensions. The effects on the heart morphology and function were independent from the presence of an infarction since they also occurred in sham animals. In addition to effects on the heart, cAng-(1–7) improved peripheral endothelium-dependent vasodilation, as measured in isolated aortic rings. cAng-(1–7) therefore shows favorable characteristic with regard to improvement of cardiovascular function after MI.

An interesting aspect of RAS modifying agents, besides their direct effect on vascular cells is the ability to modulate vascular regeneration on the level of bone marrow-derived stem cells. Stimulation of tissue repair by stem cells from the bone marrow is a novel, experimental strategy to combat age-related cardiovascular problems. As the application of stem cells in the treatment of MI is already being investigated,[9] this gives the currently used RAS modulators important potential as a supplementary treatment. In **Chapter 7** we reviewed the current knowledge of the role of the RAS and modulation thereof in hematopoietic and cardiovascular stem cells. The effects of RAS intervention that are most interesting for cardiovascular application are stimulation of the proliferation and differentiation of, and inhibition of Ang II/AT_1 receptor/ROS-induced senescence in endothelial progenitor cells (EPC), which are importantly implicated in vascular and cardiac regeneration. The observed effects imply that RAS modulation in these cells can be used to beneficially alter cardiac, vascular and renal fibrosis, neointima formation, and vascular repair to improve cardiac and renal repair, BP control and organ perfusion. Moreover, pilot results show that Ang-(1-7) prevents stress-induced endothelial cell senescence.

Beyond the cardiovascular system, modulation of the RAS in stem cells can beneficially influence adipogenesis, neural repair, connective tissue formation and wound healing, but, foremost, stimulation of the Ang-(1–7)/Mas signaling axis is now under evaluation as an anti-cancer therapy with the combined benefit of improving hematological recovery after chemotherapy or irradiation.

General discussion and future perspectives

Chapters 2 and **3** show that genomic instability leads to premature vascular aging, as is reflected by the functional assays and the observation of cell senescence. Endothelial cells as well as VSMC were affected. As explained earlier, vascular cell senescence can be induced *in vitro* with excessive oxidative stimuli, as also shown for endothelial cells in **Chapter 4**, and cause a phenotype that strongly brings up associations with the vascular phenotype found in aging vessels It is therefore tempting to assume, that cellular senescence, as caused by genomic instability, is an integral part of the aging process. Implicitly, strategies that reduce the number of senescent cells could lead to improvement of age-related phenotype. Indeed, transgenic mice in which cells that express the senescence marker p16, a cyclin-dependent kinase coded by the *INK4A* gene, are deleted by activation of a suicide gene, extension of lifespan was observed.[10] The cardiovascular phenotype was not yet studied in these animals. However, human epidemiological studies show that the 9p21 locus, a putative regulatory site of *INK4A* genes, contains the most strongly associated polymorphisms found until now in relation to age-related CVD.[11] Strikingly, deletion of this locus in mice leads to a profound effect on vascular smooth muscle cell proliferation as it attenuates proliferation-induced senescence.[12] This could be explained by reduced expression of the neighbouring genes that express cell cycle-inhibiting proteins. Also an INFgamma-mediated route to interfere with the cell cycle was proposed.[13] All together, this finding places regulation of proliferation and senescence cells in an important position in CVD pathology.

Furthermore, mitochondrial and telomeric DNA damage have been associated with age-related cardiovascular dysfunction. Animals with attenuated mitochondrial DNA repair leading to mutations and deletions in mitochondrial DNA have a shortened lifespan and a progeroid phenotype with marked cardiomyopathy.[14] This phenotype was partially rescued by transgenic expression of mitochondrially located catalase, thereby reducing the burden of oxidative stress in these organelles.[15] Furthermore, the impairment of telomere maintenance by genetic ablation of the telomerase RNA component also leads to a cardiovascular phenotype including hypertension, heart failure and increased inflammation.[16] Telomere attrition has been proposed as an important mediator of CVD, has been implicated in beneficial effects of statin therapy, and is currently being investigated as a predictive marker.[17-19]

Altogether, there is growing evidence that genomic instability, in nuclear as well as mitochondrial DNA, contributes to cardiovascular aging. Prevention of this process will be an important target for prevention.

An important consideration with respect to our observations is whether the observed vascular phenotype stems from the consequences of genomic instability within the vascular cells themselves or if it is caused by the global phenotype of the animals, characterized by vast systemic changes,[20] which would include unfavorable cardiovascular adaptation. Although it is true that DNA damage causes vascular cell dysfunction and that local DNA

damage and senescence is observed in atherosclerotic plaques or blood vessels of diabetic animals, this alternative hypothesis has not yet been tested. It is not an unlikely possibility, as is e.g. illustrated by the fact that in NER defective mice there is a pro-inflammatory gene expression pattern,[21] which might contribute to vascular aging.[22] The issue could be addressed by generation of tissue-specific conditional knockouts of NER component genes like *Ercc1*. This approach was already successfully utilized to investigate the effect of *Ercc1* gene ablation in the excitatory forebrain neurons of mice.[23] In this case, neuron-specific *Ercc1* knockouts developed a similar neuronal phenotype as global *Ercc1* functional knockdowns.

Another important issue that may be solved with a similar approach is the question what is the contribution of genomic instability in atherosclerosis. In the presently used mouse models we did not observe any sign of atherosclerosis. However, this is anyway uncommon in mice, even when aged, and normally requires the mutation of genes that are critical for appropriate lipid metabolism, such as apolipoprotein E and Factor V Leiden.[24-25] Therefore, hybrid mice resulting from cross-breeding of atherosclerotic with (cell-specific) DNA repair defective mice would be models to address this question. In relation to clinical applicability these models will help to predict what would be the best approach to develop interventions against cardiovascular aging: either local vascular targeting or affecting the aging process in general.

In **Chapters 4** to **7** some possible strategies to prevent vascular aging due to genomic instability were studied, namely red wine consumption leading to eNOS and COX2 stimulation and treatment with Mas receptor agonist Ang-(1-7). Stimulation of the Ang-(1-7) / Mas receptor axis seems particularly beneficial as it addresses all three main strategies to intervene in cardiovascular aging, resulting in improved vasodilator signaling, preventing cellular aging and stimulation of repair by stem cells. This is also an example of how the three interventional strategies might show an overlap, which is in this case explained by the opposite effect that NO/sGC/cGMP signaling and inhibition of AT_1 receptor/ROS signaling has on vasomotor function and EPC recruitment on the one hand *versus* cellular aging on the other.

Very attractive treatment possibilities for cardiovascular aging are classical RAS modulation and stimulation of the NO/sGC/cGMP system since these are within reach of the existing pharmaceutical armament. AT_1 receptor antagonists, ACE inhibitors, PDE inhibitors are already in clinical use. sGC activators or stimulators are medicines that are passing phase II and III clinical studies for pulmonary hypertension and decompensated heart failure.[26] However, to find optimal application in vascular aging one should go beyond simple functional normalization of blood vessel function and find a protocol to slow the aging process in vascular cells. This is likely achievable with RAS modulation, as the AT_1 receptor blockade and ACE inhibition was shown to slow endothelial aging in rodents[27-28] and the deletion of AT_1a receptor in mice increases their longevity by ≈28%, compared to WT animals.[29]

Based on the vascular signaling induced by AT_2 receptor agonism one might expect that

C21 might have a similar effect with respect to vascular aging as Ang-(1-7), ACEi or AT_1 receptor antagonists. Unfortunately, we were not able to find any AT_2 receptor-mediated response in the vascular specimens that were investigated in **Chapter 5**. On the contrary: C21 showed AT_1 receptor-mediated vasoconstrictory effects. Since these seem to be AT_1 receptor-mediated and might relate to ROS signaling, it seems unlikely that C21 is an ideal compound for prevention of vascular aging. Despite its apparent lack of AT_2 receptor-specific vasorelaxant action in several animal models and AT_2 receptor-unspecific actions, its relatively high affinity for AT_2 receptors should allow its use in concentrations without precipitating these unspecific effects. Even then the signaling behavior of AT_2 receptor itself is currently not fully understood and findings are occasionally contradictory. An important case is the apparent constrictory effect of AT_2 stimulation in resistance vasculature of aged rats[30] or other pathological conditions such as hypertension.[31] However, the map of the intricate signaling behavior of AT_2 receptors begins to unfold, and ultimately the optimal conditions for AT_2 agonist treatment might be known. The option of AT_2 receptor agonism to target vascular aging is therefore still interesting.

There is one important issue that was not addressed in the previous chapters. Identification of possible treatment strategies is not the only challenge that hallmarks the campaign against vascular aging. Another major challenge that awaits scientists in their attempt to translate preclinical studies into studies in humans will be the identification of early biomarkers of age-related cardiovascular disease. As to yet, the clinical cardiovascular markers that are used (e. g. forearm blood flow, pulse wave velocity, lipid profile, C-reactive protein and homocysteine plasma levels) are a reflection of the already derailed homeostasis and currently no novel early-stage markers are known. Even so challenging is the quest for markers that will indicate whether the therapy will be successfully slowing the vascular aging process. An interesting option might be to use a similar strategy as is applied for telomeres, namely the measurement of genomic damage in blood borne cells such as white blood cells and EPC. Alternatively, and relating to our proposal in Chapter 2, one could determine the genetic variability or even measure the efficiency of the DNA repair system.

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Samenvatting

Genetische instabiliteit en cardiovasculaire ziekten

Vasculaire dysfunctie en gerelateerde cardiovasculaire ziekten (CVZ) hangen sterk samen met veroudering. De algemene mechanismen die veroudering veroorzaken en hun invloed op de pathofysiologie van CVZ in het bijzonder zijn niet duidelijk, maar de causale rol van genetische instabiliteit in het verouderingsproces wordt in toenemende mate duidelijk.[1] In **Hoofdstuk 1** is de hypothese dat genetische instabiliteit in vasculaire veroudering resulteert geponeerd. In **Hoofdstuk 2** is dit onderzocht in een muizenmodellen voor versnelde veroudering, ofwel progeria, door een verslechterd DNA reparatie systeem. In muizen met een ernstige (*Ercc1*$^{d/-}$ muizen) en een relatief mildere (*XpD*^{TTD} muizen) progeria is gekeken of een dit reparatie defect leidt tot versnelde vasculaire veroudering in vergelijking tot normale, ofwel wildtype (WT), muizen. Het verslechterde DNA herstel in muizen leidde tot snelle ontwikkeling van verschillende kenmerken die lijken op het cardiovasculaire fenotype van verouderde mensen, wat zichtbaar was als verslechterde endotheel-afhankelijke en –onafhankelijke vaatverwijdende functie (endotheeldisfunctie), zoals *ex vivo* vastgesteld is, en occlusie-reperfusie-gemedieerde vaatverwijdende functie *in vivo*, verhoogde vaatstijfheid en hypertensie. Voorts werd getoond dat endotheeldisfunctie in *Ercc1*$^{d/-}$ muizen deels gereguleerd werd via verlaagde hoeveelheden en activatie van stikstofoxide-synthase (eNOS), een enzym dat de belangrijke vaatverwijdende signaalstof stikstofmonoxide (NO) produceert in de endotheelcellen. Opmerkelijk is dat het vatenstelsel van deze muizen een hogere hoeveelheid mRNA bevatte voor het eiwit p21, wat een aan DNA schade gerelateerde cel cyclus remmer is. Ook werd er een verhoogd aantal verouderde cellen gezien, gedetecteerd middels de aankleuring van verouderings-gerelateerd β-galactosidase (SA-β-Gal), een fenomeen dat bij mensen in atherosclerotische laesies te vinden is. We hebben ook mogelijke associaties van menselijke genvarianten, zogenaamde single nucleotide polymorphisms (SNPs), en verhoogde vaatstijfheid, in genen die relevant zijn voor de aanmaak van DNA reparatie componenten die tot het nucleotide excissie reparatie (NER) systeem behoren. Verhoogde stijfheid is een belangrijk onderdeel van vaatveroudering, en werd gemeten aan de hand van de klinische variabele Pulse Wave Velocity (PWV). We vonden een significante associatie tuseen PWV waarde en een variate in het gen dat codeert voor het eiwit DDB2 dat DNA schade detecteert en de NER initieert. Op basis van deze bevindingen concluderen we dat de capaciteit voor DNA herstel samenhangt met versnelde vasculaire veroudering in muizen, en dat er implicaties kunnen zijn voor het risico en de voorspelling daarvan in mensen met betrekking tot leeftijdsgerelateerde cardiovasculaire ziekten.

Een groot deel van de verminderde respons in de *Ercc1*$^{d/-}$ muis werd veroorzaakt door disfunctie in de vasculaire gladde spiercellen (VSMC). Vermindering van de vaatverwijdende functie van de VSMC wordt minder vaak beschreven in de verouderde vaatstelsels van dieren en mensen dan een verminderde endotheelfunctie, en wordt voornamelijk geassocieerd met de hogere leeftijd van de dieren en de aanwezigheid van diabetes mellitus of chronisch hartfalen.[2-3] Daarom zou verminderde VSMC functie een

indicator kunnen zijn van een meer progressieve vorm van vasculaire veroudering. In **Hoofdstuk** 3 is de VSMC functie van *Ercc1*$^{d/-}$ muizen nader bekeken. Normaal wordt door endotheelcellen NO geproduceerd die een relaxatie van VSMC veroorzaakt door de stimulatie cyclisch guanine monofosfaat productie (cGMP) door het enzym cytosolair guanylyl cyclase (sGC), de zogenaamde NO/sGC/cGMP-as. In vergelijking met WT dieren bleek deze as in VSMC niet goed te functioneren, wat enkele bevindingen in Hoofdstuk 2 bevestigt Het mechanisme achter de verminderde functie van de NO/sGC/cGMP-as werd uitgebreider gekarakteriseerd door verder betrokken componenten te onderzoeken. De hoeveelheden en activiteit van sGC en van cGMP-afhankelijke proteïnekinase 1α (ofwel) proteine kinase G, een enzym dat het relaxerende signaal van cGMP binnen de VSMC ten uitvoer brengt, bleek vergelijkbaar te zijn in *Ercc1*$^{d/-}$ en WT dieren. Remming van fosfodiesterase (PDE), een enzyme dat cGMP afbreekt, normaliseerde de relaxatie van VSMC in *Ercc1*$^{d/-}$ muizen nagenoeg tot het niveau van WT muizen. Ook was er in *Ercc1*$^{d/-}$ muizen een verhoogde PDE activiteit ten opzichte van WT muizen, gemeten in longweefsel. Uit deze bevindingen blijkt dat genetische instabiliteit veroorzaakt door een defecte DNA reparatie systeem leidt tot verhoogde PDE activiteit, wat de relaxatie van VSMC verhindert.

Naar preventie en reparatie van cardiovasculaire schade

De problemen die veroorzaakt worden door genetische instabiliteit kunnen in principe aangepakt worden met drie algemene strategieën: preventie van genetische instabiliteit, counterregulatie van ongunstige vasculaire signaaltransductie en vervanging van de slecht functionerende apoptotische and verouderde vasculaire cellen met nieuwe, functionele cellen door middel van weefselherstellende mechanismen zoals recrutering van stamcellen. Deze zaken worden besproken in de resterende hoofdstukken van het proefschrift.

De aanwezigheid van verouderde vasculaire cellen, zoals voortgebracht wordt door genetische instabiliteit, is te zien in atherosclerose[4] en sommige modellen van vasculaire dysfunctie zoals diabetische ratten. Door hun pro-inflammatoire, prothrombotische, mineraliserende en vaatvernauwende fenotype, die bijdragen aan verhoogde bloeddruk, verhoogde vaatstijfheid en atherosclerose, zullen verouderde vaatcellen mogelijk een causale rol spelen in de ontwikkeling van CVZ. Meerdere farmacologische interventies en dieetgewoonten, waaronder consumptie van rode wijn,verbeteren de vasculaire functie en remmen CVZ.[5] De beschermende factoren in rode wijn zouden de polyfenolen kunnen zijn. Deze neutraliseren superoxiden. Superoxiden zijn zeer reactieve stoffen, zogenaamde oxidatieve vrije radicalen (ROS), die door de cel kunnen worden geproduceerd en daar normaliter een belangrijke signaalfunctie hebben. Bij een te hoge hoeveelheid van deze ROS, tot stand gekomen door een te hoge cellulaire productie of toevoer van buitenaf (bijv. tijdens het roken), wordt DNA schade veroorzaakt. Rode wijn extract zou door het neutraliseren van ROS dus de genomische integriteit moeten bevorderen en vasculaire celveroudering moeten voorkomen . In **Hoofdstuk 4** onderzochten we het mechanisme van de positieve effecten van rode wijn extract (RWE), bestaande uit voornamelijk polyfenolen, op de veroudering

van vasculaire cellen veroorzaakt door oxidatieve stress. RWE beschermde endotheelcellen tegen verouderingen veroorzaakt door de ROS verhogende stof *tert*-butylhydroperoxide (tBHP). Dit was zichtbaar als een afname van SA-β-Gal kleuring en een afname in p21. In overeenstemming met dit effect verminderde RWE DNA schade, en dit beschermende effect leek afhankelijk te zijn van eNOS en cyclo-oxygenase 2 (COX-2), een enzym dat vasodilaterende prostaglandine, een andere groep van signaalfactoren naast NO, produceert. RWE verhoogde bovendien de hoeveelheden eNOS mRNA, verhoogde de activatie van eNOS door fosforylatie van het enzym, en leidde tot relaxatie in de kranslagaders van varkens. RWE verhoogde de expressie van SIRT1, een belangrijke regulator van vele intracellulaire processen tijdens celveroudering, en SIRT1-remming zorgde voor een gedeeltelijke daling van het beschermende effect van RWE op DNA schade. Resveratrol, een polyfenol dat vaak als voornaamste beschermende polyfenol in rode wijn beschouwd wordt, voorkwam tBHP-geïnduceerde veroudering niet.

Vervolgens is een nieuw farmacologisch mechanisme onderzocht om ongunstige vasculaire signaaltransductie tegen te gaan, namelijk angiotensine II type 2 (AT2) receptor stimulatie. De ontdekking van deze "gunstige arm" van het renine-angiotensine systeem (RAS), een belangrijke bloeddrukregulerend hormoonsysteem, en haar gebruik zou een toegevoegde verbetering zijn van farmacologische RAS interventies die reeds in de kliniek worden toegepast. In voorgaande studies werd aangetoond dat AT2 receptor agonisme NO-afhankelijke vaatverwijding veroorzaakt, en natriuretische, antiproliferatieve, and antifibrotische effecten teweegbrengt, hetgeen een gunstig effect zou moeten hebben bij CVZ.[6-7] Om dat uit te buiten is de AT2 receptor agonist compound 21 (C21) ontwikkeld. In **Hoofdstuk 5** onderzochten we de mechanismen van de vaatverwijdende effecten van C21. We zagen geen C21-geïnduceerde, door AT2 receptor veroorzaakte vaatverwijding in alle gebruikte experimentele modelen. Dit contrasteert met voorgaande studies van andere onderzoeksgroepen, die door AT2 veroorzaakte relaxaties in respons op C21 aantoonden. We zagen echter wel AT2-onafhankelijke relaxerende effecten van C21, evenals vaatvernauwende effecten. Voorbehandeling met Irbesartan, een remmer van de vasoconstrictoire angiotensine II type 1 (AT1) receptor, maakte deze vaatvernauwende effecten ongedaan. Bovendien waren de concentraties van het niet volledig AT2 receptor-specifieke C21 die benodigd waren om constrictie te induceren dusdanig hoog dat inderdaad ook de AT1 receptor geactiveerd zou kunnen worden. Wat de vasorelaxerende eigenschappen van C21 betreft: deze werden niet geremd door de behandeling met een AT2 antagonist of door remming van de NO/sGC/cGMP-as. Voorbehandeling met C21 verminderde ook de vaatvernauwende potentie van de constrictors U46619 en phenylephrine, maar had geen invloed op de vaatvernauwing met ionomycine, een niet-lichaamseigen stof die in experimenten gebruikt wordt om calcium naar de binnenkant van de cel te transporteren. Omdat constrictie van VSMC afhankelijk is van hoge intracellulaire calcium concentraties en dilatatie van lage concentraties, wekken de resultaten de indruk dat C21 het calcium transport richting de binnenkant van de VSMC naar aanleiding van fysiologische prikkels remt.

Naast AT2 receptor stimulatie, is vermoedelijk ook angiotensine-(1-7) (Ang-(1-7)), de endogene agonist van de Mas receptor, betrokken bij gunstige klinische effecten van farmacologische RAS interventies.[8] Ang-(1-7)/ Mas receptor signalering wordt geopperd als een nieuw farmacologisch doel voor tegenregulatie van schadelijke cardiovasculaire signaaltransductie naast AT2 receptor stimulatie. De mogelijkheid om een Mas receptor agonist te gebruiken bij de behandeling van myocardiale infarcten (MI) en preventie van hartfalen, wat bij mensen sterk gerelateerd is aan leeftijd, is onderzocht in **Hoofdstuk 6**. Hiervoor hebben we de effecten getest van de proteolyse resistente en Mas receptor-specifieke stof cAng-(1–7) in een rat model voor het myocard infarct (MI). Deze stof liet een dosis-afhankelijke verlaging van het gewicht van de linker ventrikel zien en van de eind-diastolische druk na een MI in een stadium waarin er nog geen ernstige pompfunctieverlies was waar te nemen. Het effect op het gewicht van het hart was gerelateerd aan de vermindering van cardiomyocyt hypertrofie, wat duidelijk werd uit de afname van de afmetingen van de myocyten. De effecten op de morfologie en de functie van het hart waren onafhankelijk van de aanwezigheid van een infarct, aangezien ze ook voorkwamen in dieren zonder MI. Naast de effecten op het hart, verbeterde cAng-(1–7) de perifere endotheel-afhankelijke vaatverwijding, zoals gemeten is in geïsoleerde aorta ringetjes. De conclusie is dat cAng-(1–7) gunstige eigenschappen laat zien wat betreft de verbetering van de cardiovasculaire functie na een MI.

Een interessant aspect van RAS modulerende stoffen is dat ze naast hun directe effect op vasculaire cellen ook stamcellen uit beenmerg beïnvloeden die betrokken zijn bij regeneratie van beschadigd cardiovasculair weefsel. Stimulatie van weefsel regeneratie door stamcellen uit het beenmerg is een nieuwe, experimentele strategie om leeftijdsgerelateerde cardiovasculaire problemen te lijf te gaan. Daar de interventies met stamcellen bij de behandeling van MI al onderzocht wordt,[9] geeft dit de nu gebruikte RAS modulatoren belangrijke potentie als een toegevoegde behandeling. In **Hoofdstuk 7** is een overzicht gegeven van de huidige kennis over de rol van het RAS en de modulatie ervan in regulatie van bloedvormende en cardiovasculaire stamcellen. De effecten van RAS interventie die het meest interessant zijn voor cardiovasculaire toepassingen zijn stimulatie van de proliferatie en differentiatie in endotheelstamcellen (EPC), waaraan een belangrijk rol worden toegedicht in vasculaire en cardiale regeneratie. Tevens zou RAS interventie Ang II/ AT1 receptor/ROS-geïnduceerde veroudering van EPC kunnen remmen. De in de literatuur beschreven effecten lijken te tonen dat RAS modulatie in deze cellen gebruikt kan worden ter voorkoming van cardiovasculaire bindweefselvorming en vaatwandverdikking, en verbetering van bloeddrukregulatie en orgaanperfusie. Bovendien laten de pilot resultaten zien dat Ang-(1-7) ROS-geïnduceerde endotheelcelveroudering voorkomt. Naast het cardiovasculaire systeem, kan modulatie van de RAS in stamcellen een gunstige invloed hebben op vetweefselvorming, herstel van neuraal weefsel, en wondgenezing. Tevens wordt stimulatie van de Ang-(1–7)/Mas signaling axis nu geëvalueerd als een therapie voor kanker met het bijkomend voordeel van de verbetering van het hematologisch herstel na chemotherapie of bestraling.

Zhrnutie

Genomická nestabilita a kardiovaskulárne ochorenia

Zhoršená funkcia ciev a s ňou spojené kardiovaskulárne ochorenia (KVO) sa vyskytujú častejšie so stúpajúcim vekom človeka. Mechanizmy spôsobujúce starnutie a ich vplyv na patofyziológiu KVO nie sú jasné, ale genomická nestabilita je všeobecne považovaná za jednu z príčin starnutia. Rozhodli sme sa preskúmať hypotézu, uvedenú v Kapitole 2, že genomická nestabilita, napríklad zapríčinená zníženou efektivitou opravy DNA, spôsobuje zrýchlené starnutie ciev. Na tento účel sme použili dva experimentálne rýchlo starnúce modely, myši s vážnym zlyhaním opravy DNA *Ercc1*$^{d/-}$ a s miernejším zlyhaním opravy DNA *XPD*^{TTD}. Znížená efektivita opravy DNA u týchto myší viedla k rýchlemu rozvinutiu niekoľkých príznakov pripomínajúcich kardiovaskulárny fenotyp u starých ľudí: zhoršená relaxačná funkcia ciev meraná *ex vivo* čiastočne závislá od endotelu a čiastočne od hladkého svalu ciev, zhoršená vazorelaxačná funkcia meraná *in vivo*, znížená elasticita ciev a zvýšený krvný tlak. Dysfunkcia endotelu v *Ercc1*$^{d/-}$ myšiach je čiastočne spôsobená zníženým množstvom a aktiváciou proteínu syntázy oxidu dusnatého (eNOS). Je taktiež zaujímavé, že tieto myši vykazovali zvýšené množstvá mRNA p21, inhibítoru kinázy závislej od cyklínu, ktorého hladina je úzko spätá s poškodením DNA, a taktiež vykazovali zvýšené množstvo senescentných buniek, bunkového fenotypu nachádzajúceho sa v aterosklerotických plakoch u ľudí. Taktiež sme skúmali možnú spojitosť genetických variácií, takzvaných polymorfizmov na jednom nukleotide (SNP), so zníženou elasticitou ciev v génoch kódujúcich proteíny opravy DNA - NER. Znížená elasticita ciev je významnou zúčasťou cievneho starnutia a určuje sa na základe klinickej premennej: rýchlosť vlny pulzu (PWV). Našli sme signifikantnú spojitosť medzi PWV a polymorfizmom v géne DDB2, ktorý kóduje proteín rozoznávajúci poškodenie DNA a spúšťajúci celý proces opravy – NER.

Na základe týchto pozorovaní vyslovujeme záver, že kapacita opravy DNA je spojená s rýchlym cievnym starnutím u myší a že individuálna kapacita opravy DNA môže pomôcť predvídať riziko kardiovaskulárnych ochorení spojených so zvyšujúcim sa vekom u ľudí.

Časť zníženia relaxačnej funkcie ciev u myší *Ercc1*$^{d/-}$ je spôsobená zníženou relaxačnou fukncIou cievnych buniek hladkého svalu (VSMC). Toto zníženie funkcie VSMC je menej často pozorovateľné v starnúcom cievnom systéme zvierat a ľudí ako zníženie endotelovej funkcie a u ľudí je obvykle sprevádzané vyšším vekom a prítomnosťou diabetes mellitus alebo srdcového zlyhania. Preto by mohla byť znížená funkcia VSMC považovaná za charakteristiku fenotypu pokročilého cievneho starnutia. V kapitole 3 sme do väčšej hĺbky skúmali funkciu VSMC u myší *Ercc1*$^{d/-}$. U týchto myší sme v porovnaní so zdravými zvieratami z rovnakého vrhu (WT) pozorovali znížený relaxačnú odpoveď ciev na od endotelu nezávislé relaxačné stimuly, mediované signálnou osou oxid dusnatý/rozpustná guanylát cykláza/cyklický guanín monofosfát (NO/sGC/cGMP), čo potvrdzuje naše závery v Kapitole 2. Aby sme odhalili mechanizmus tohto zhoršenia, skúmali sme funkcionalitu jednotlivých komponentov signálnej osi NO/sGC/cGMP v tkanivách týchto zvierat. Množstvo a aktivita rozpustnej guanylát cyklázy, enzýmu produkujúceho cGMP,

boli porovnateľné so zvieratami WT. Inhibícia enzýmu phosphodiesterázy (PDE) zlepšila relaxačnú odpoveď na nitroprussid sodný (SNP), látku ktorá je donorom oxidu dusnatého, zvýšením maximálnej odpovede u myší *Ercc1*$^{d/}$ a zároveň sme pozorovali zvýšenú aktivitu PDE v pľúcach myší *Ercc1*$^{d/}$ v porovnaní s myšami WT. Množstvo a aktivita proteín-kinázy 1α závislej od cGMP, enzýmu prenášajúceho relaxačný signál osi NO/sGC/cGMP na svalový aparát bunky, bol u oboch typov myší porovnateľné. Na základe týchto pozorovaní sme došli k záveru, že genomická nestabilita vedie k zvýšenej aktivite PDE a tým bráni relaxácii VSMC.

Prevencia a oprava poškodenia kardiovaskulárneho systému

Problémom spôsobeným genomickou nestabilitou by sa v princípe mohlo čeliť tromi všeobecnými prístupmi: prevenciou genomickej nestability, reguláciou nepriaznivých signálnych mechanizmov v cievach a nahradením apoptotických a senescentných buniek so zníženou funkciou novými, funkčnými bunkami využívajúc mechanizmy opravy tkanív. Táto problematika bola preskúmaná vo zvyšných kapitolách tejto práce.

Prítomnosť senescentných cievnych buniek, ako bolo nami pozorované v prípade genomickej nestability, je možné taktiež nájsť pri ateroskleróze a v niektorých zvieracích modeloch zníženej funkcie ciev, ako napríklad u diabetických potkanov. Senescentné bunky sú pravdepodobne jedna z príčin rozvoja KVO, vďaka svojim vlastnostiam podporujúcim zápal, zrážanlivosť krvi, mineralizáciu a zužovanie ciev, ktoré sa spolupodieľajú na zvýšenom krvnom tlaku, zníženej cievnej elasticite a urýchlení procesu rozvoja aterosklerózy. Existujú viaceré farmakologické prístupy a stravovacie návyky, vrátane požívania červeného vína, ktoré preukázateľne zlepšujú cievnu funkciu a znižujú častosť výskytu KVO.

Polyfenoly obsiahnuté v červenom víne sú známe svojimi antioxidačnými vlastnosťami. Keďže voľné radikály spôsobujú poškodenie DNA, extrakt červeného vína by mal napomáhať integrite a predchádzať senescencii cievnych buniek. V Kapitole 4 sme skúmali mechanizmus prospešného účinku extraktu z červeného vína (ECV), pozostávajúceho z polyfenolov, na senescenciu cievnych buniek spôsobených oxidatívnym stresom. ECV ochránil endotelové bunky pred senescenciou spôsobenou tert-butyl hydroperoxidom (tBHP), látkou spôsobujúcou oxidačný stres. Ochranný účinok ECV bol spojený so znížením hladiny p21. V súlade s týmto účinkom ECV ochránil endotelové bukny pred poškodením DNA a tento ochranný účinok bol závislý od funkcie proteínov eNOS a cyklooxygenázy 2 (COX-2). Okrem toho ECV zvýšil mRNA proteínu eNOS, zvýšil aktiváciu eNOS pomocou fosforylácie a v koronárnych artériách ošípaných vyvolal relaxáciu. ECV zvýšil expresiu proteínu SIRT1 a blokovanie tohoto enzýmu čiastočne znížilo DNA ochraňujúce účinky ECV. Resveratrol, polyfenol ktorý je často považovaný za hlavnú ochrannú zložku červeného vína, nedokázal predísť senescencii spôsobenej tBHP.

Stimulácia angiotenzín II typ 2 receptoru (AT_2) patrí medzi nové farmakologické spôsoby ako regulovať signalizáciu nepriaznivými signálnymi mechanizmami v cievach.

Využitie tejto „prospešnej vetvy“ renín-angiotenzínového systému (RAS) by mohlo priniesť dodatočné zlepšenie klasických liečebných postupov modifikujúcich RAS. Agonizmus na AT_2 receptore v predchádzajúcich štúdiách preukázal cievno-relaxačné účinky ktoré boli závislé od NO, zvýšil vylučovanie sodíka obličkami, mal antiproliferatívne a antifibrózne účinky. Všetky tieto účinky sú priaznivé v prípade KVO. Za účelom stimulácie AT_2 receptorov bol vyvinutý ich nepeptidový agonista – compound 21 (C21).

V kapitole 5 sme skúmali mechanizmus ciev relaxujúceho účinku C21. Narozdiel od predošlých štúdií iných vedeckých skupín sme v žiadnom experimentálnom modeli nepozorovali relaxačné účinky C21 na cievy, ktoré by boli závislé od AT_2 receptora. Napriek tomu sme pozorovali relaxačné účinky C21 nezávislé od AT_2 receptorov a takisto sme pozorovali aj cievy sťahujúce účinky. Inkubácia s Irbesartanom úplne odstránila tieto sťahujúce účinky a koncentrácia C21 potrebná na ich dosiahnutie napovedá, že v tomto prípade dochádza k agonizmu na angiotenzín II typ 1 receptoroch (AT_1). Čo sa týka relaxačných účinkov C21, tieto neboli inhibované antagonizmom na AT_2 receptoroch ani blokovaním signálnej osi NO/sGC/cGMP. Preinkubácia s C21 tiež znížila potenciu ciev sťahujúcich látok U46619 a fenylefrínu, ale nemala vplyv na kalciový ionofór ionomycín, čo nasvedčuje pravdepodobnému úcinku C21 na transport kalcia dovnútra bunky.

Ďalšou súčasťou „prospešnej vetvy“ RAS je angiotenzín-(1-7) (Ang-(1-7)), endogénny agonista Mas receptorov, ktorý môže byť podľa niektorých štúdií dôležitým faktorom zabezpečujúcim pozitívne účinky bežnej liečby modifikujúcej RAS. Signálna os Ang-(1-7)/Mas receptor je spolu s agonizmom na AT_2 receptore niektorými skupinami navrhovaná ako nový farmakologický cieľ na reguláciu nepriaznivých signálnych mechanizmov v KVO. Možnosť využitia agonistov Mas receptora v liečbe infarktu myokardu (IM) a prevencii srdcového zlyhania, ktoré sú u ľudí spojené so zvyšujúcim sa vekom, bola preskúmaná v Kapitole 6. Za týmto účelom sme testovali látku cAng-(1-7) ktorá je odolná voči proteolýze a špecificky aktivuje Mas receptory. Táto látka znížila hmotnosť ľavej komory a tlak počas diastoly v závislosti od dávky v modeli IM, v ktorom ešte nenastalo srdcové zlyhanie. Účinok na hmotnosť ľavej komory je spojený so znížením hypertrofie buniek srdcového svalu, čo sa ukazuje na menších rozmeroch týchto buniek. Tento účinok na morfológiu srdca je nezávislý od prítomnosti infarktu, keďže bol takisto pozorovaný aj v kontrolnej skupine zvierat. Okrem účinku na srdce, cAng-(1-7) zlepšil relaxáciu ciev závislú od endotelu, meranú v izolovanej aorte. cAng-(1-7) vykazuje priaznivé charakteristiky vzhľadom na zlepšenie srdcovo-cievnej funkcie po IM.

Zaujímavým aspektom látok modifikujúcich RAS, mimo ich priamy účinok na bunky ciev, je ich schopnosť modulovať cievnu regeneráciu na úrovni kmeňových buniek pochádzajúcich z kostnej drene. Stimulácia regenerácie tkanív kmeňovými bunkami je nová experimentálna stratégia na potlačenie srdcovo-cievnych problémov spojených so zvyšujúcim sa vekom. Fakt, že použitie kmeňových buniek na liečbu IM je intenzívne skúmanou oblasťou, dáva látkam modifikujúcim RAS potenciál podpornej liečby v tomto procese. V kapitole 7 sme urobili súhrn znalostí úlohy RAS a jeho modulácie

vo vývoji hematopoetických a srdcovo-cievnych kmeňových buniek. Účinky liečby využívajúcej RAS, ktoré sú najzaujímavejšie pre aplikáciu v prípadoch srdcovo-cievnych chorôb, sú stimulácia proliferácie, stimulácia diferenciácie a inhibícia senescencie spôsobenej signálnou osou Ang II/AT_1 receptor/reaktívne formy kyslíka, v endotelových kmeňových bunkách (EPC). Tieto bunky pravdepodobne hrajú dôležitú úlohu v cievnej a srdcovej regenerácii. Pozorované účinky napovedajú že modifikácia RAS systému v týchto bunkách môže byť použitá na zlepšenie stavov, v ktorých dochádza ku srdcovej, cievnej a obličkovej fibróze, zhrubnutiu cievnej neointimy; a taktiež na zlepšenie opravy ciev, srdca a obličiek, kontrolu krvného tlaku a prekrvenia orgánov.

Navyše prvotné výsledky naznačujú, že Ang-(1-7) dokáže predchádzať senescencii endotelu spôsobenej oxidatívnym stresom. Modulácia RAS v kmeňových bunkých môže okrem srdcovo-cievneho systému pozitívne ovplyvniť proces adipogenézy, opravy neurónov, tvorby spojovacieho tkaniva a liečby rán. No predovšetkým je stimulácia signáglnej osi Ang-(1–7)/Mas testovaná ako nová terapia na potláčenie rakoviny majúca pozitívny účinok rýchlejšej obnovy krvných buniek po chemoterapii alebo iradiácii.

Curriculum Vitae

Matej Ďurík was born on July 4th 1984 in Trstená, Slovakia. In 2002 he started his study at Faculty of Pharmacy, Commenius University, Bratislava. After defending his Master thesis titled: „Computer aided design of lipoxygenase inhibitors “ and graduating in 2007 he worked as a Pharmacist in a public pharmacy in Bratislava. In 2008 he came to The Netherlands and started his work as a PhD candidate at the department of Pharmacology at the Erasmus MC. Under the supervision of Dr. A.J.M. Roks and Prof. A.H.J. Danser he did his research on the relationship of DNA damage with vascular aging and with novel treatments in age-related cardiovascular disease.

Publications

Nucleotide excision DNA repair is associated with age-related vascular dysfunction
Matej Durik, Maryam Kavousi, Ingrid van der Pluijm, Aaron Isaacs, Caroline Cheng, Annemarieke E. Loot, Usha Musterd-Bhaggoe, Frank Leijten, Richard van Veghel, René de Vries, Goran Rudez, Renata Brandt, Yanto Ridwan, Elza van Deel, Martine de Boer, Dennie Tempel, Ingrid Fleming, Andre Uitterlinden, Albert Hofman, Henricus J. Duckers, Cornelia M. van Duijn, Ben A. Oostra, Jacqueline Witteman, Dirk J. Duncker, A.H. Jan Danser, Jan Hoeijmakers, Anton Roks
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PhD Portfolio

Vascular Aging from DNA Damage to Protection
M. Ďurík

Erasmus MC Department:	Internal Medicine, Division of Pharmacology and Vascular medicine
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
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PhD training	**year**
Research skills (19.5 ECTS)	
Biostatistical Methods I: Basic Principles	2011
COEUR Courses and research seminars	2008-2011
Principles of laboratory animal science	2008
Symposia and conferences (15.2 ECTS)	
High Blood Pressure Research 2011 Scientific Sessions, Orlando, United states *	2011
European Meeting on Hypertension and Cardiovascular Research, Milan, Italy *	2011
High Blood Pressure Research 2010 Scientific Sessions, Washington DC, United states **	2010
European Meeting on Hypertension and Cardiovascular Research, Milan, Italy **	2009
High Blood Pressure Research 2009 Scientific Sessions, Chicago, United States	2009
NHG/MIVAB days, Bizenmortel, The Netherlands	2008
Wetenschapsdagen Inwendige Geneeskunde, Antwerpen, Belgie * **	2009-2012
FIGON Dutch Medicine Days, Lunteren, The Netherlands * **	2008-2011
Teaching (2.4 ECTS)	
Supervising Practicals	2008-2011

Prizes and grants

Accomodation Grant – European Meeting on Hypertension and Cardiovascular research	2011, 2009
New Investigator Award for European Fellows – High Blood Pressure Research 2009 Scientific Sessions	2009
Presentation prize - Dutch Medicines days 2010	2010
Poster prize - Dutch Medicines days 2009	2009

* poster presentation, ** oral presentation

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