

Dietary restriction but not angiotensin II type 1 receptor blockade improves DNA damage-related vasodilator dysfunction

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ABSTRACT

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

INTRODUCTION

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

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

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

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

the vascular wall. Therefore, we additionally studied the vasoconstrictor responses of the *Ercc1*^{dl/-} mouse vasculature to Ang II under *ad libitum* (AL) feeding and DR. Also, we evaluated the effect of chronic AT₁ receptor blockade on endothelial function and blood pressure in AL-fed *Ercc1*^{dl/-} mice.

MATERIAL AND METHODS

Animals and interventions

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

Dietary restriction studies

Ercc1^{dl/-} mice and their wild-type littermates (WT) (B16/FVB F1 hybrids) underwent DR intervention from resp. 7 and 11 weeks after birth until sacrifice as described extensively in our previous publication, and in the Methods supplement.²

Losartan intervention study

From 5 weeks of age, *Ercc1*^{dl/-} and WT mice (B16/FVB F1 hybrids) were divided into two groups per strain, which were either treated with losartan (100 mg/kg/day) in drinking water, or drinking water only until the age of 12 weeks when the animals were sacrificed. Blood pressure was measured by tail cuff at the age of 11 weeks. The study rationale and animal numbers are described in the Methods supplement.

Organ bath experiments

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

In short, thoracic aorta and iliac arteries were collected and tested in small wire organ bath setups. Vasodilations to cumulative concentrations of acetylcholine (ACh) and sodium nitroprusside (SNP) were measured in vessels precontracted with U46619 to construct concentration-response curves (CRCs). When sufficient aortic tissue was available, the involvement of nitric oxide (NO) and prostaglandins in ACh responses was investigated by performing the experiments in the presence of the endothelial nitric oxide synthase (eNOS) inhibitor N^G-Methyl-L-Arginine acetate salt (L-NMMA, 10⁻⁵ mol/L), the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10⁻⁵ mol/L) or both inhibitors. In iliac arteries Ang II (10⁻¹⁰-10⁻⁷ mol/L) CRCs were constructed. PD123319 (10⁻⁷ mol/L) was used to test the involvement of Ang II type 2 (AT₂) receptors, and the guanylyl cyclase

inhibitor 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10^{-5} mol/L) to test the role of NO-cGMP signaling. Inhibitors were added 15 minutes prior to U46619 or Ang II.

Quantitative real-time PCR

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

Plasma renin concentration

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

Statistical methods

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

RESULTS

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

We first investigated the effect of genomic instability and DR on the diminished ACh response at different ages in *Ercc1*^{dl/-} and WT mice. As previously reported, AL-fed *Ercc1*^{dl/-} mutants showed a lifespan of 19 weeks (median age), which was extended by DR to a median age of 44 weeks.² ACh responses in the *Ercc1*^{dl/-} aorta of AL-fed animals age-dependently decreased between the age of 7 to 16 weeks (Fig. 1A), and at the latter age were significantly decreased compared to 20-wk old WT. WT aortas did not show any change in ACh response between 11 to 20 weeks (data not shown). To explore if DR would protect against endothelial dysfunction until an age at which AL-fed *Ercc1*^{dl/-} mice have already succumbed (predominantly occurring from neurodegeneration), we proceeded to an age of 30 weeks in DR-fed animals. In our initial publication on the effect of DR on general health² we demonstrated that DR improved the response to ACh in 16-wk-old *Ercc1*^{dl/-} mice. Here we show that the improvement of ACh responses persisted in 30-wk-old

DR-fed *Ercc1^{d/-}* mutants (Fig. 1B), well after the AL mice had died. In WT animals DR had no effect on ACh-induced relaxation (Fig. 1B). Thus, *Ercc1^{d/-}* mice showed decreased aortic relaxations to ACh with increasing age, which could partly be prevented by DR.

Endothelial vs. non-endothelial responses

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

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

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

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

Dilations in AL-fed vs. DR-fed WT mice did not differ and results were therefore pooled. ACh responses were almost completely dependent on NO in WT animals since adding the eNOS inhibitor L-NMMA blocked the response to ACh (Fig. 2A). As expected from our previous study⁴, NO also mediated a large part of the vasodilation to ACh in AL-fed *Ercc1^{d/-}* mice (Fig. 2B). The residual response suggests the emergence of an endothelium-derived hyperpolarizing factor (EDHF), which did not appear to be COX-dependent, since it was not affected by indomethacin (Fig. 2B). This result, together with the observation that

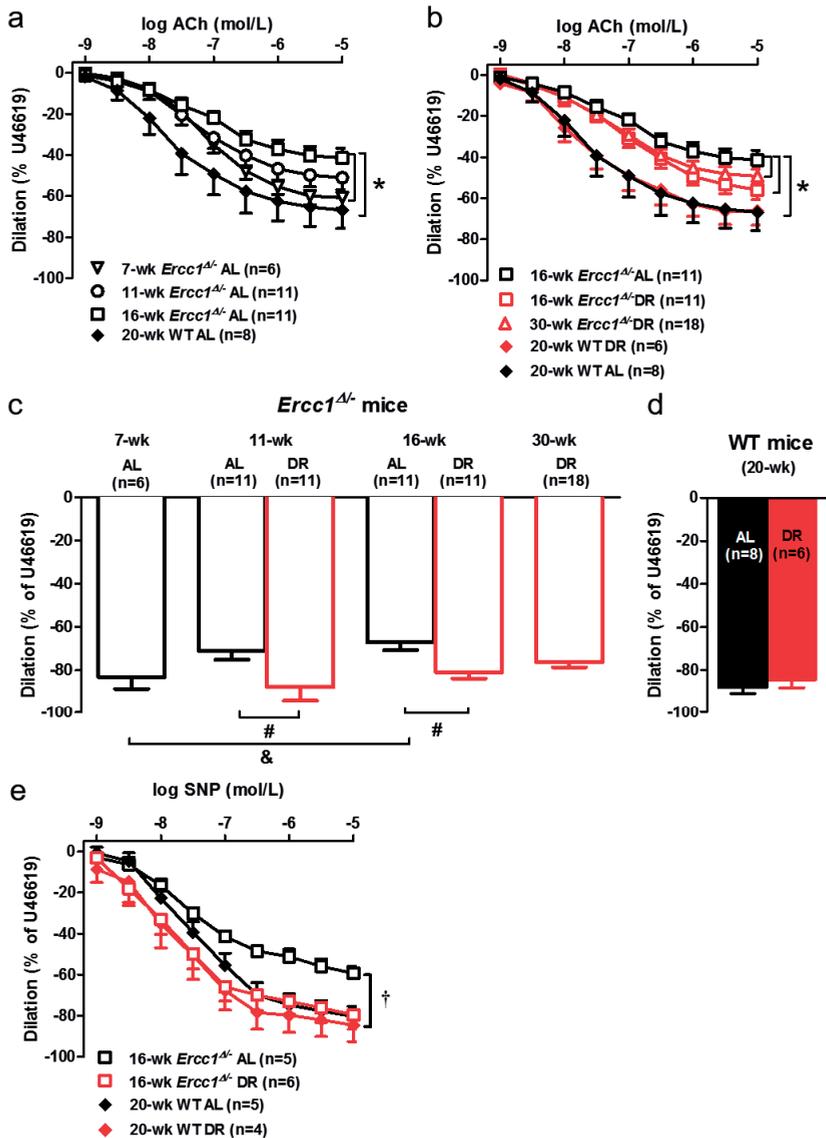


Figure 1. (A) Age-dependent acetylcholine (ACh)-induced vasodilation in aortic segments from *ad libitum* (AL) fed wildtype (WT) and *Ercc1^{Δ/Δ}* mice as measured *ex vivo* in small wire organ baths. (B) Effect of diet restrictions (DR) on the ACh responses. (C - E) age-dependent sodium nitroprusside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

inhibition of vasodilation by L-NMMA was much more pronounced in WT confirms the specific loss of NO signaling in *Ercc1^{dl/-}* aorta's. Remarkably, the DR-induced facilitation of the ACh response in *Ercc1^{dl/-}* mice appeared to be due to an upregulation of a vasodilator prostaglandin pathway, since now indomethacin did further reduce the response of ACh on top of L-NMMA, while the effect of L-NMMA alone was unaltered (Fig. 2C).

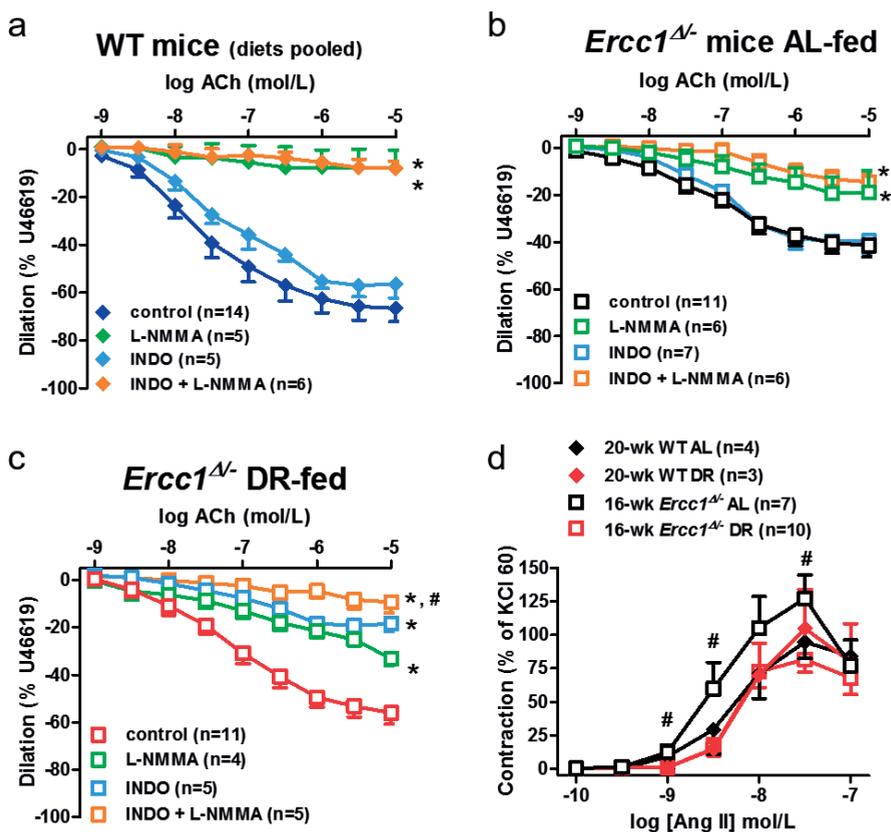


Figure 2. Contribution of nitric oxide (NO) and prostaglandins to ACh-induced vasodilation of aortic segments from 20-wk-old AL- and DR-fed wild-type (WT) (A), and 16-wk-old AL-fed (B) and DR-fed (C) *Ercc1^{dl/-}* mice measured in organ baths. L-NMMA (10^{-5} mol/L) and INDO (10^{-5} mol/L) resp. inhibit NO and prostaglandin synthesis, and were added to the organ baths 10 minutes before U46619. Responses are expressed as % relaxation of the U46619 precontraction. (D) Vasoconstriction to Ang II expressed as % of contraction to 100 mM KCl. Error bars: S.E.M. *: $p < 0.05$ vs. non-pretreated segments. #: $p < 0.05$ vs. L-NMMA-treated segments.

Prostaglandins are produced by COX-1 or 2, and exert their vasodilator effects through the IP receptor using adenylyl cyclase (AC) 5/6 – cAMP signaling as a second messenger system. cAMP is prone to degradation by phosphodiesterase type 4B/D (PDE4).¹³⁻¹⁵

To investigate which of these components could be responsible for the upregulated prostaglandin response we quantified their expression in blood vessel-rich lung tissue. Ct values for the IP receptor, PDE4D and AC6 mRNA levels were on average >34 , and therefore we considered these levels too low for reliable detection. COX-1 mRNA showed a trend to increase ($p < 0.05$ one-way ANOVA for all 4 groups) after DR in WT and in *Ercc1*^{Δ/Δ} mice, and did significantly increase in DR-fed *Ercc1*^{Δ/Δ} mice as compared with AL-fed WT mice (Fig. 3A). PDE4B mRNA was decreased in both *Ercc1*^{Δ/Δ} mouse groups compared with AL-fed WT mice (Fig. 3A). COX-2 and AC 5/6 mRNA did not show significant changes among the groups. The results suggest that both an increase of prostaglandin production by COX₁ and decreased metabolism by PDE4B underlie the improved vasodilation after DR in *Ercc1*^{Δ/Δ} mice.

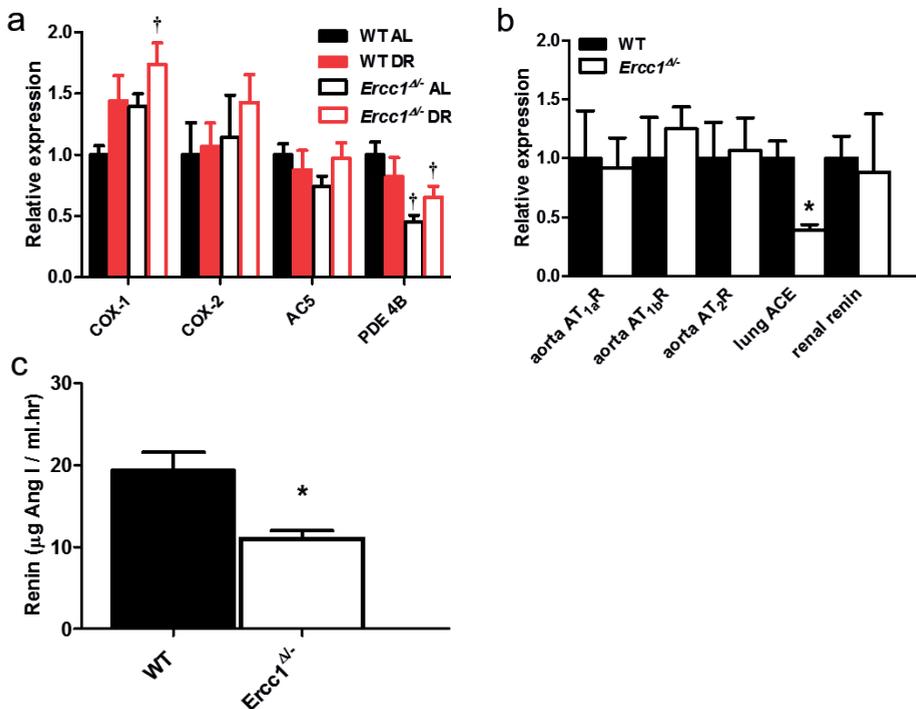


Figure 3. (A) Relative mRNA expression levels in lung tissue of COX-1, COX-2, AC 5/6, and PDE4B in 16-wk-old *Ercc1*^{Δ/Δ} mice and 20-wk-old wild-type (WT) littermates from the diet intervention study. (B) Relative mRNA expression levels of AT_{1a}-, AT_{1b}- and AT₂-receptors in abdominal aortic tissue, of ACE in lung tissue and of renin in renal tissue of 12-wk-old *Ercc1*^{Δ/Δ} mice and WT littermates from the losartan treatment study. All values are corrected for β -actin and normalized to WT expression levels. Results were similar when corrected for HPRT-1 (data not shown). (C) Plasma renin concentration in *Ercc1*^{Δ/Δ} mice and WT littermates from the losartan treatment study. Error bars: S.E.M. †= $P < 0.05$ vs. WT-AL (one way- ANOVA followed by Dunnett's post-hoc test vs. WT-AL); *= $p < 0.05$ vs. WT, t-test.

Effects of genomic instability and DR on Ang II responses

To explore a possible role of the renin-angiotensin system we first investigated vasoconstriction to Ang II in a subset of the diet intervention mice. Ang II responses were in general highly variable within each strain, and tended to be higher in AL-fed *Ercc1^{dl/-}* vs. AL-fed WT mice (Fig. 2D), although this did not reach significance over the entire CRC (GLM-RM). DR-fed *Ercc1^{dl/-}* animals showed a trend for a decreased response to Ang II as compared to AL-fed *Ercc1^{dl/-}* mutants (GLM-RM, $p=0.059$). The results suggest a genomic instability-induced upregulation of the Ang II response, which is normalized by DR.

The losartan intervention study

In a separate cohort of *Ercc1^{dl/-}* and WT mice we evaluated the effect of chronic AT₁ receptor blockade on blood pressure and vascular function. In agreement with our previous study⁴ blood pressure tended to be slightly higher in *Ercc1^{dl/-}* mice, mainly reflected by systolic blood pressure (SBP), and to a lesser extent by diastolic blood pressure (DBP) (Fig. 4). The difference reached borderline significance for SBP ($p = 0.076$, t-test). Chronic AT₁ receptor blockade by losartan significantly lowered SBP and DBP in both mouse strains.

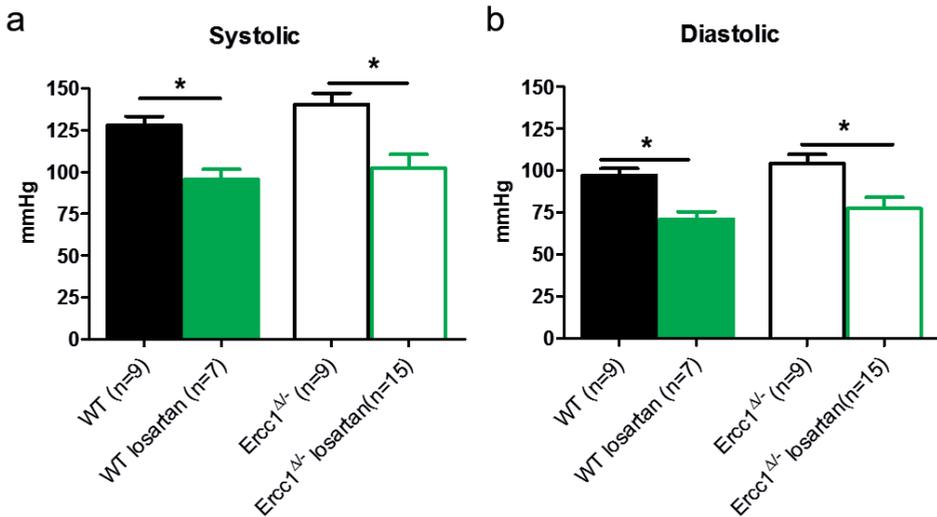


Figure 4. (A) Systolic and (B) diastolic blood pressure in conscious *Ercc1^{dl/-}* and wild-type (WT) mice of the losartan intervention study as measured by the tail cuff method. *: $p < 0.05$, t-test

Vasomotor responses to Ang II in the losartan intervention study

In the losartan intervention cohort, *Ercc1^{dl/-}* mice displayed an exaggerated response to Ang II as compared to WT mice (Fig. 5A). Vasoconstrictions are mediated by AT₁ receptors and we therefore explored other indicators of increased AT₁ receptor activity such as negative feedback on renin activity and ACE expression. Plasma renin activity was reduced (Fig.

3C). This was not due to a change in mRNA level in the kidney (Fig. 3B). ACE mRNA in the lung was reduced (Fig. 3B). Both findings are in agreement with increased AT₁ receptor activity.

To further explore mechanisms leading to increased Ang II vasoconstrictions we studied vascular AT₁ and AT₂ receptor expression and function. We and others previously reported that AT₂ receptor stimulation counteracts AT₁ receptor-mediated vasoconstriction.¹⁶ To explore the effect of genomic instability on AT₂ receptor activity, Ang II responses in *Ercc1*^{dl/-} and WT animals in the presence of AT₂ receptor antagonist PD123319 were compared to those in the absence of this antagonist. PD123319 did not change in *Ercc1*^{dl/-} mice, but tended (*p*=NS) to increase the Ang II response in WT (Fig. 5A). Chronic treatment with losartan, starting from week 5 after birth until the end of week 12, normalized this exaggerated response in the *Ercc1*^{dl/-} mice (Fig. 5B), but had no effect on the Ang II response in WT mice (data not shown). Therefore, genomic instability leads to loss of counterregulation of AT₁ receptor-mediated vasoconstriction by AT₂ receptor, and not due changes in receptor expression.

To explore the possible involvement of counterregulation of Ang II-induced constriction by NO-cGMP signaling, which can be the result of endothelial AT₂ receptor stimulation, Ang II responses were studied in the presence of the guanylyl cyclase inhibitor ODQ. This approach, rather than adding an eNOS inhibitor, was chosen because *Ercc1*^{dl/-} mice show both changes in endothelial NO production as well as in cGMP responses of VSMC. Although the presence of ODQ tended to increase Ang II responses, the increase was very modest and did not reach significance in *Ercc1*^{dl/-} animals (Fig. 5B), nor in WT (not shown). Apparently, the loss of NO-cGMP signaling cannot entirely explain the increase Ang II vasoconstrictions.

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

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

In the 12-wk-old *Ercc1*^{dl/-} mutants vasodilator responses to ACh were significantly decreased as compared to WT animals (Fig. 5C). The dilation response to a SNP concentration-response curve was also decreased in *Ercc1*^{dl/-} mice (Fig. 5D). Chronic AT₁ receptor blockade with losartan *in vivo* did not significantly change any of the responses. Our findings indicate that the observed vasodilator dysfunction (persistent after losartan) was not blood pressure-dependent and that the detrimental effect of genomic instability cannot be opposed by chronic AT₁ receptor blockade with Losartan.

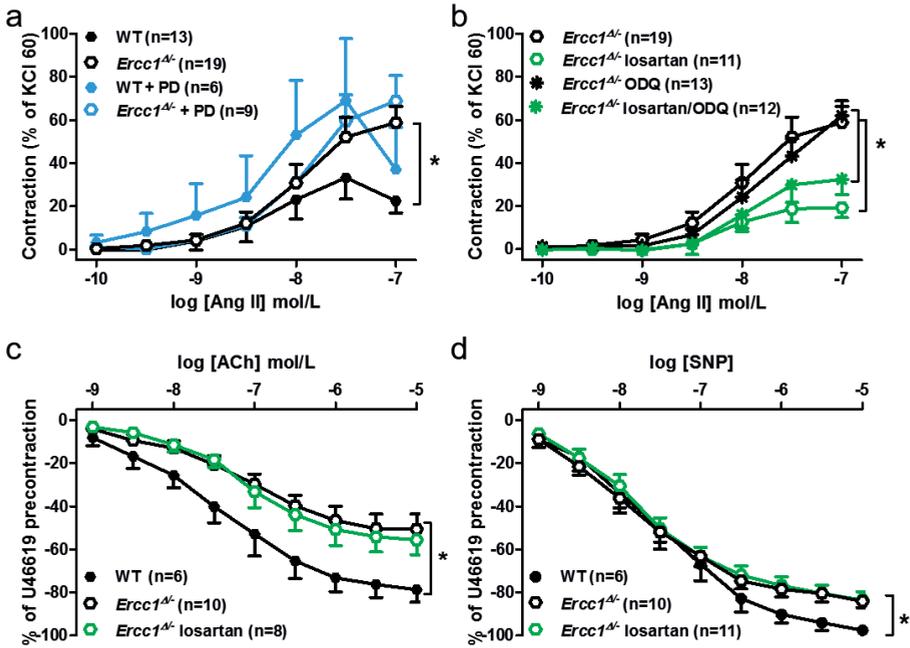


Figure 5. Vasoconstriction to angiotensin II (Ang II) of isolated iliac arteries from mice of the (A) diet intervention study, and (B) the losartan intervention study. (C, D) Vasodilation in isolated aortic tissue of the mice from the losartan intervention study to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator, NO-donor sodium nitroprusside (SNP). All responses were measured *ex vivo* in small wire organ baths. Aortic tissue used to measure vasodilator responses was precontracted with thromboxane analogue U46619. Error bars: S.E.M. *: $p < 0.05$, general linear model for repeated measures. #: $p < 0.05$, t-test on individual concentrations of Ang II of *ad libitum* (AL) fed *Ercc1^{d/r}* mice vs. wild-type (WT) littermates.

DISCUSSION

In the present study we explored potential mechanisms that lead to DR-mediated improvement of vasodilator dysfunction caused by DNA damage. Loss of vasodilation was entirely due to loss of NO-cGMP signaling, both as a result of decreased endothelial NO release and decreased VSMC responsiveness to NO. In previous publications we showed that the decreased NO function was due to decreased eNOS expression and activation, increased PDE1 and possibly also PDE5 activity, and for a small part due to increased ROS production.^{4,5} The present results indicate that DR improves vascular dilations up to an age of at least 30 weeks. This is because DR enables aortic tissue to recruit endothelium-derived vasodilatory prostaglandins, which are normally absent. In addition, the responsiveness of VSMC to NO is improved. A possible explanation for the emerging prostaglandin response is the increase in COX-1 combined with a decreased in PDE4B, which together should lead

to improved vasodilator cAMP signaling. We have also explored the possible involvement of Ang II. Although defective DNA repair increased vasoconstrictive responses to Ang II, chronic blockade of AT₁ receptors with losartan did not rescue vasodilator responses. Also, blood pressure is not a driving mechanism in the observed vasodilator dysfunction, since blood pressure-lowering did not affect this dysfunction. Ang II-induced vasoconstriction most likely increased due to the loss of counterregulatory action of AT₁ receptor by AT₂ receptor when *Ercc1*^{dl/-} mutants age.

Preservation of endothelium-dependent responses by DR has been previously reported in aging WT rodents, involving nuclear factor erythroid-2-related factor-2 (Nrf2)-mediated upregulation of antioxidants.¹⁷ In various tissues of AL-fed *Ercc1*^{dl/-} mice Nrf2-related antioxidants are already increased as a protective mechanism (² and unpublished observations), but *ex vivo* aortic vasodilator responses are still improved by the oxygen radical scavenger N-acetylcysteine due to an interaction in VSMC.^{2, 4} After DR, Nrf2 is further activated² and SNP responses are totally normalized (Fig. 1), establishing the potential role of Nrf2. The present study now demonstrates that DR recruits yet another system to enhance endothelial function, namely vasodilatory prostaglandin signaling, acting through cAMP. To achieve this, COX-1 expression increased in DR-fed *Ercc1*^{dl/-} mice, on top of already decreased PDE4B expression in those mice. Potentially, this points at the genoprotective effect of DR improving transcriptional output in *Ercc1*^{dl/-}, as was demonstrated in our previous publication.² Although the fate of COX products in the aging vasculature is certainly not uniform in diverse studies in rodents and humans¹⁸, our results confirm the observation that DR prevents the decline of plasma and renal prostacyclin levels in aging rats.^{19, 20} Another study shows rather diverse changes, claiming lower levels of both vasoconstrictive and vasodilatory COX products after DR in the aging rat aorta.²¹ Although both findings indicate the participation of COX products in DR-induced changes, this topic has attracted little attention until now. Nevertheless, the combined improvement of VSMC responses to NO-cGMP and increased prostaglandin-cAMP by the endothelium explains the effect of DR.

Ang II responses increased in AL-fed *Ercc1*^{dl/-} mice compared to WT, whereas plasma renin activity decreased. The latter result is in agreement with the observation that renin levels decrease with age.^{22, 23} Although renin levels start decreasing already when approaching middle age, our present findings indicate that the aging process might contribute to this decrease. With respect to the effect of aging on AT₁ receptor-mediated vasoconstrictions many contrasting findings have been described, depending on the species or the vessels that have been used.²⁴⁻²⁸ Nevertheless, our observation is in agreement with findings showing that in older persons blood pressure and blood flow responses to Ang II are elevated, especially in the presence of diabetes or the absence of counterregulation by AT₂ receptors.^{26, 29-31} Counterbalancing of AT₁-mediated pathogenesis is the basis for development of AT₂ receptor agonists as clinical drugs against cardiovascular diseases.^{32, 33} Conversely,

increased Ang II activity via AT₁ receptors due to a loss of counterregulation by AT₂ receptor is a mechanism observed in various disease models and in aging wild-type rats.^{32, 34} The counterbalancing effect by AT₂ receptors is often ascribed to endothelial NO release, or might relate to a change in dimerization of the two Ang II receptor subtypes.³⁵ Loss of NO signaling clearly does not play a role in *Ercc1*^{d/-} mutants nor their WT littermates given the absence of an effect of ODQ, leaving receptor interaction as the alternative mechanism.

The endothelial dysfunction observed in the present study was clearly not related to Ang II and increased blood pressure. In patients, the effect of chronic AT₁ receptor blockade on preservation of vasodilator function is variable, having either a protective effect or not.³⁶ It was assumed that this might depend on the underlying disease or the vessel type that is investigated. However, our study suggests that the aging process might explain such variation. Unfortunately, most of the human studies exploring the effect of chronic AT₁ receptor blockade focus on patients around 60 and younger, and not on the oldest old. Nevertheless, there are clues that aging affects the effectiveness of AT₁ receptor blockade. It has been shown that losartan becomes gradually less effective in aging rats, especially in the presence of hypertension and after loss of endothelium-independent NO function.³⁷ Clinical observations show that losartan/antihypertensive treatment can lead to adverse cognitive effects in elderly, which is ascribed to perfusion problems.³⁸ However, the same study suggests that this perfusion problem is a result of both blood pressure lowering and a persisting vascular dysfunction, at least in the brain. This implicates that vasodilator function is not improved. More dedicated studies in the oldest patients are necessary to resolve this paradox. There are mechanistic explanations available for this paradigm. In patients or in models of heart failure, hypertension, diabetes etc. Ang II blockade largely improves endothelial function due to an acute reduction of ROS formation by NAPH oxidase, increasing NO bioavailability.⁹ DNA damage largely lowers NO independently from ROS⁴, and apparently this undermines the treatment efficacy of AT₁ antagonists in our mouse model. It is therefore relevant to find tools to investigate this possibility in humans. Suboptimal effectiveness of and treatment response variability to RAS inhibition are well-known phenomena, but remain largely unexplained, and therefore drive studies that attempt to find prediction markers for therapy effectiveness.^{39, 40}

CLINICAL PERSPECTIVES

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

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

AUTHORS CONTRIBUTION

Anton Roks, Jan Danser and Jan Hoeijmakers designed the research. Bibi van Thiel and Paula Bautista-Niño conducted the research, collected data, provided important scientific input and participated in writing of the paper. Haiyan Wu, Erwin Reiling, Matej Durik, Frank Leijten, Yanto Ridwan and Renata Brandt helped in conducting the research and collecting data. Harry van Steeg, Martijn Dolle, Wilbert Vermeij, Jeroen Essers and Ingrid van der Pluijm provided important scientific input. All authors read and approved the final paper. There are no conflicts of interest to disclose.

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