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Original Paper

Phosphodiesterase 1 regulation is a key mechanism in vascular aging

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Abstract

Reduced nitric oxide (NO)/cGMP signalling is observed in age-related vascular disease. We hypothesize that this disturbed signalling involves effects of genomic instability, a primary causal factor in aging, on vascular smooth muscle cells (VSMCs) and that the underlying mechanism plays a role in human age-related vascular disease. To test our hypothesis, we combined experiments in mice with genomic instability resulting from the defective nucleotide excision repair gene *ERCC1* (*Ercc1*$^{d/-}$ mice), human VSMC cultures and population genome-wide association studies (GWAS). Aortic rings of *Ercc1*$^{d/-}$ mice showed 43% reduced responses to the soluble guanylate cyclase (sGC) stimulator sodium nitroprusside (SNP). Inhibition of phosphodiesterase (PDE) 1 and 5 normalized SNP-relaxing effects in *Ercc1*$^{d/-}$ to wild-type (WT) levels. PDE1C levels were increased in lung and aorta. cGMP hydrolysis by PDE in lungs was higher in *Ercc1*$^{d/-}$ mice. No differences in activity or levels of cGMP-dependent protein kinase 1 or sGC were observed in *Ercc1*$^{d/-}$ mice compared with WT. Senescent human VSMC showed elevated *PDE1A* and *PDE1C* and *PDE5* mRNA levels (11.6-, 9- and 2.3-fold respectively), which associated with markers of cellular senescence. Conversely, PDE1 inhibition lowered expression of these markers. Human genetic studies revealed significant associations of *PDE1A* single nucleotide polymorphisms with diastolic blood pressure (DBP; $\beta = 0.28$, $P = 2.47 \times 10^{-5}$) and carotid intima–media thickness (cIMT; $\beta = -0.0061$, $P = 2.89 \times 10^{-5}$). In summary, these results show that genomic instability and cellular senescence in VSMCs increase PDE1 expression. This might play a role in aging-related loss of vasodilator function, VSMC senescence, increased blood pressure and vascular hypertrophy.

Key words: aging, blood pressure, genetic association, phosphodiesterases, vascular disease.

INTRODUCTION

Since the identification of endothelial nitric oxide (NO) as an important vasodilator signal in blood vessels acting through the second messenger cGMP in vascular smooth muscle cells (VSMCs), many studies have shown that this vascular signalling pathway is disturbed in cardiovascular diseases [1,2]. It is now widely assumed that disturbed NO/cGMP signalling is

Abbreviations: ACTB, β–actin; BP, blood pressure; BCA, bicinchoninic acid; 8-Br-PET-cGMP, 8-bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphate; CAD, coronary artery disease; CFPWV, carotid-femoral pulse wave velocity; CHF, chronic heart failure; cIMT, carotid intima–media thickness; CRC, concentration–response curve; DBP, diastolic blood pressure; DEA NONOate, diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate; *Ercc1*$^{d/-}$ mice, mice with defective nucleotide excision repair gene *ERCC1*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLM–RM, general linear model–repeated measures; GWAS, genome-wide association studies; HPRT-1, hypoxanthine phosphoribosyltransferase 1; IBMX, 3-isobutyl-1-methyl xanthine; ICBP-GWAS, International Consortium of Blood Pressure Genome-Wide Association Studies; L-NAME, N^{ω}-nitro-L-arginine-methyl ester; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; PP, pulse pressure; qPCR, quantitative PCR; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; TBS-T, Tris Buffered Saline with Tween; VSMC, vascular smooth muscle cell; WT, wild-type.
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importantly involved in the pathogenesis of age-related vascular dysfunction and cardiovascular disease resulting thereof [3,4]. However, actual evidence in humans is not abundant. A recent combined human genetic study with mouse experiments shows that mutations in the human cGMP-producing machinery, consisting of soluble guanylate cyclase (sGC) and chaperonin containing t-complex polypeptide subunit η (CCTη), a protein that stabilizes sGC, increases the risk of myocardial infarction due to increased thrombogenesis [5]. Since this is a rare variant leading to increased familial risk for myocardial infarction, it is unclear whether this potential pathogenic mechanism acts in the general population. Even more recently, it was found that genetic variants in phosphodiesterase (PDE) type 1A, an enzyme that degrades cGMP, might contribute to dysregulation of blood pressure [6]. Notably, dysregulation of PDE1 sub-types has been suggested to play a role in atherosclerosis and a potential target for treatment of VSMC and cardiomyocyte dysfunction [7–12]. However, it is unclear which function of cGMP would be involved. Also, it is unclear what circumstances related to vascular aging would lead to decreased levels of this cyclic nucleotide.

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

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

MATERIALS AND METHODS

To accomplish our aims, we followed an integrative approach combining experiments in animal models, human VSMC cultures and population genome-wide association studies (GWAS).

Animals

A thorough description of the generation and of the overall phenotype of the mice with defective nucleotide excision repair gene *ERCC1* (*Ercc1*$^{d/-}$ mice) can be found in previous publications [14,15]. *Ercc1*$^{d/-}$ mice of 16 weeks in an F1 hybrid Fvb/C57Bl/6 background and their wild-type (WT) littermates of the same age were bred at the Erasmus MC animal facility. The animals were housed in individually ventilated cages with access to normal chow and water *ad libitum*. As required by Dutch law, all animal studies were approved by an independent Animal Ethical Committee.

Experiment in human VSMCs

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

Reciprocally, the effect of PDE1 and PDE5 inhibition on p16 and p21 was tested in n=6 samples from three independent duplicate experiments. Coronary artery smooth muscle cells passage 6 (CC-2583, Lonza) cultured in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS were treated with vinpocetine (10 μmol/l) or sildenafil (100 nmol/l) and incubated for 24 h, after which RNA isolation was performed and cDNA was prepared from 500 ng of total RNA.

Organ bath experiments

Ercc1$^{d/-}$ and WT mice were asphyxiated in a CO_2 chamber. The thoracic aortas were isolated and stored overnight in ice-cold oxygenated Krebs–Henseleit buffer (in mmol/l: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3, pH 7.4) solution. The following day, vessel segments were mounted in 6 ml organ baths (Danish Myograph Technology) containing Krebs–Henseleit buffer at 37 °C and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure. Maximum contractile responses were determined using 100 mmol/l KCl. After washout of KCl, pre-constriction was elicited with 30 nmol/l U46619 resulting in 50–100% of the previously obtained 100 mmol/l KCl pre-contraction. During pre-constriction, relaxation concentration–response curves (CRCs) were constructed to sodium nitroprusside (SNP) and diethylammonium (*Z*)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA NONOate). To detect a possible NO/sGC/cGMP-independent vasorelaxant activity of SNP, we performed SNP CRCs after inactivation of sGC with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 μmol/l). To investigate the involvement of increased activity of PDE enzyme in VSMC dysfunction of *Ercc1*$^{d/-}$ mice, segments were pre-incubated for 20 min with 100 nmol/l sildenafil or 10 μmol/l vinpocetine. Sildenafil and vinpocetine were used together with 100 μmol/l N^{ω}-nitro-L-arginine-methyl ester (L-NAME) to allow the measurement of the response to the exogenous NO donor SNP in the absence of endogenous NO. To test for differences in cGMP-dependent protein kinase 1α (PKGI) sensitivity, CRCs to 8-bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphate (8-Br-PET-cGMP) were constructed during NOS inhibition with L-NAME (100 μmol/l).

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

cGMP measurement

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

Measurement of PDE5 activity

To measure the cGMP-hydrolysing activity of PDE5, lung tissue of *Ercc1*$^{d/-}$ and WT mice was isolated, frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were crushed in liquid nitrogen and then homogenized at 0 °C in a buffer solution containing 50 mmol/l Tris/HCl, pH 7.5, 1.5 mmol/l EDTA, 1 mmol/l DTT, Roche complete protease inhibitor cocktail, phosphatase inhibitor cocktail 1 and 1 mmol/l sodium orthovanadate. In order to assure appropriate linear kinetics of cGMP hydrolysis, the protein concentration of all samples was determined and subsequently they were diluted to yield 15–30 % of total hydrolysis. Diluted sample, containing 0.9 mg/ml protein was added to an assay buffer (40 mmol/l Tris/HCl, pH 7.5, 10 mmol/l $MgCl_2$, 0.2 mg/ml BSA, 1 mmol/l EGTA and 1 μmol/l cGMP) containing 10^5 counts per min [^{3}H]cGMP per sample. Homogenization buffer without protein was used as a negative control and undiluted protein solution (8 mg/ml) was used to determine full hydrolysis. Assay mixture was incubated at 30 °C for 30 min. After this, the reaction was stopped by heating the mixture to 100 °C. Subsequently, the mixture was cooled by placing it on ice and [^{3}H]5′-GMP was further hydrolysed by incubation for 30 min at 37 °C with 0.25 mg/ml *Ophiophagus hannah* snake venom to [^{3}H]guanosine. Following incubation, 1 ml of Dowex ion-exchange slurry was added (1:0.9:0.1 mixture of Dowex AG 1-X8/water/methanol) and shaken for 2 min on an Eppendorf shaker. The mixture was centrifuged for 4 min at 18 000 ***g*** and 700 μl of supernatant was added to a scintillation cocktail and the number of counts per min was determined.

Quantitative real-time PCR analyses

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

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

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

Western blot analyses

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

Protein content of the murine aortic and human VSMC lysates was determined using the BCA method (Thermo Scientific). Lysates were subsequently run on a criterion Bis-Tris gradient gel and blotted to a nitrocellulose membrane.

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

For PDE1A, membranes were blocked with 5% BSA in Tris Buffered Saline with Tween (TBS-T), whereas for PDE1C and PDE5, 5% dried skimmed milk powder in TBS-T was used. Subsequently incubation with the primary antibodies was as follows.

In mice lung tissue: PDE1A (Santa Cruz Biotechnology, 1:500 dilution in 5% BSA in TBS-T), PDE1C (Santa Cruz Biotechnology, 1:1000 dilution in 5% dried skimmed milk powder in TBS-T), PDE5 (Alexis Biochemicals, 1:1000 dilution in 5% dried skimmed milk powder in TBS-T) and p-PDE5 (FabGennix, 1:1000 dilution in 5% dried skimmed milk powder in TBS-T).

In human VSMCs: PDE1A (Santa Cruz Biotechnology, 1:500 dilution in 5% BSA in TBS-T) and (Santa Cruz Biotechnology, 1:100 dilution in 5% dried skimmed milk powder in TBS-T), PDE1C (Santa Cruz Biotechnology, 1:100 dilution in 3% dried skimmed milk powder in TBS-T), PDE5 (Alexis Biochemicals, 1:1000 dilution in 5% dried skimmed milk powder in TBS-T) and p21 (Cell Signaling Technology, 1:250 dilution in 2% BSA in TBS-T).

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

Genetic association studies

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

For CFPWV, we used data from The AortaGen Consortium which comprises 20 634 individuals of European ancestry from nine cohort studies [19]. As CFPWV increases non-linearly and exhibits marked variance inflation with advancing age, resulting in a strongly right-skewed distribution and varies depending on the method used to ascertain transit distance, genetic association analyses were performed using a sex-specific standardized residual that was based on the inverse of CFPWV, which normalizes the distribution and that was further adjusted for age, age squared, height and weight.

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

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

Statistical methods

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

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

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

RESULTS

Studies in mice

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

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

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

Key enzymes in cGMP signalling

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

Phosphodiesterase activity. Both the PDE5 inhibitor sildenafil (100 nmol/l) and the PDE1 inhibitor vinpocetine (10 μmol/l) significantly increased responses to SNP as compared over the entire concentration-effect range in *Ercc1*$^{d/-}$ and WT mice ($P < 0.05$, GLM–RM test). In *Ercc1*$^{d/-}$ mice, the increase in

Table 1 Pharmacodynamic parameters of SNP CRCs

pEC_{50}: Log_{10}-transformed SNP concentrations at which the half-maximal response occurred. E_{max}: maximal effect of SNP (percentage of U46619 pre-contraction). #$P < 0.05$, Student's t test compared with control.

	pEC_{50}			E_{max}		
	Control	**Sildenafil**	**Vinpocetine**	**Control**	**Sildenafil**	**Vinpocetine**
WT	-7.8 ± 0.2	$-8.8 \pm 0.1^{\#}$	-8.4 ± 0.1	-85.5 ± 4.3	-93.4 ± 3.1	-96.4 ± 1.2
Ercc1$^{d/-}$	-7.7 ± 0.4	$-8.4 \pm 0.1^{\#}$	$-8.2 \pm 0.1^{\#}$	-48.6 ± 7.6	$-74.9 \pm 5.2^{\#}$	$-87.4 \pm 3.1^{\#}$

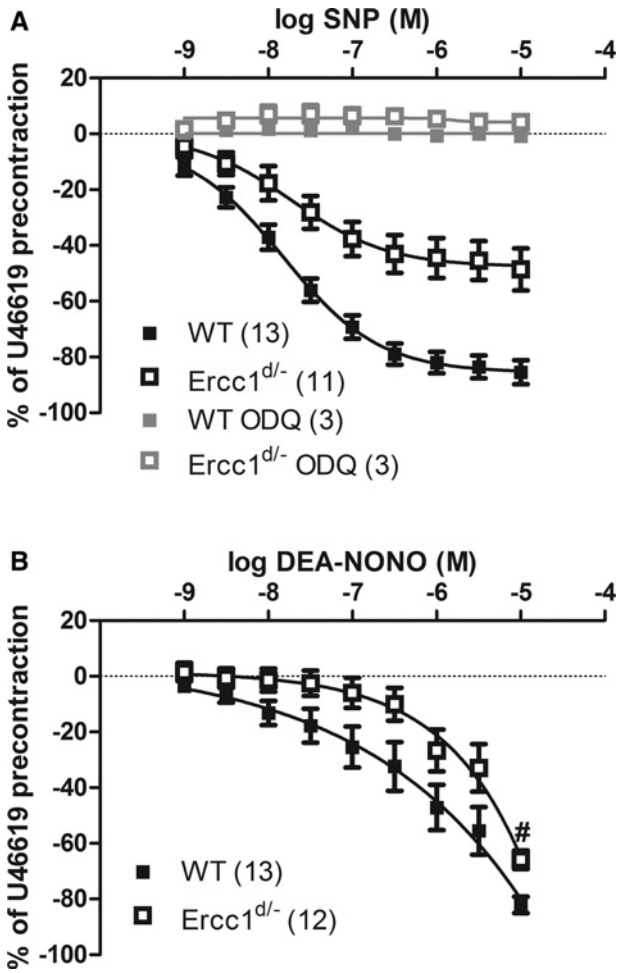

Figure 1 VSMC relaxing responses in progeroid *Ercc1*$^{d/-}$ mice

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

SNP responses induced by vinpocetin was significantly higher than by sildenafil ($P < 0.05$, GLM–RM on entire concentration effect range for SNP, sildenafil compared with vinpocetin). Looking further into qualitative aspects of the SNP effects, potency of SNP increased significantly ~10-fold in *Ercc1*$^{d/-}$ mice for both PDE inhibitors and also for sildenafil in WT mice, whereas in WT mice potentiation by vinpocetine did not reach significance (Table 1). In addition, the two drugs increased the maximal effect (E_{max}) of SNP (percentage of U46619 pre-contraction) in *Ercc1*$^{d/-}$ mice by 53% ($P = 0.03$) and 80% ($P = 0.0023$) respectively, whereas no statistically significant increase was observed in WT animals (Figures 3A–3D).

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

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

Studies in human VSMCs

To study the relationship between PDE1 expression and cellular senescence, passage-dependent expression of PDE1, p16 and p21, a cyclin-dependent kinase and an accepted senescence marker [13,23,24], was studied in five VSMC cultures; typical results from one of the cultures are presented in Figures 6(A)–6(D). There was a passage-dependent increase in *PDE1A* and *PDE1C* mRNA levels and this corresponded with an increase in *p16* and *p21*. Summary results are presented in Figure 6(E), senescent human aortic VSMCs showed a significant 11.6- and 9-fold increase in *PDE1A* and *PDE1C* mRNA

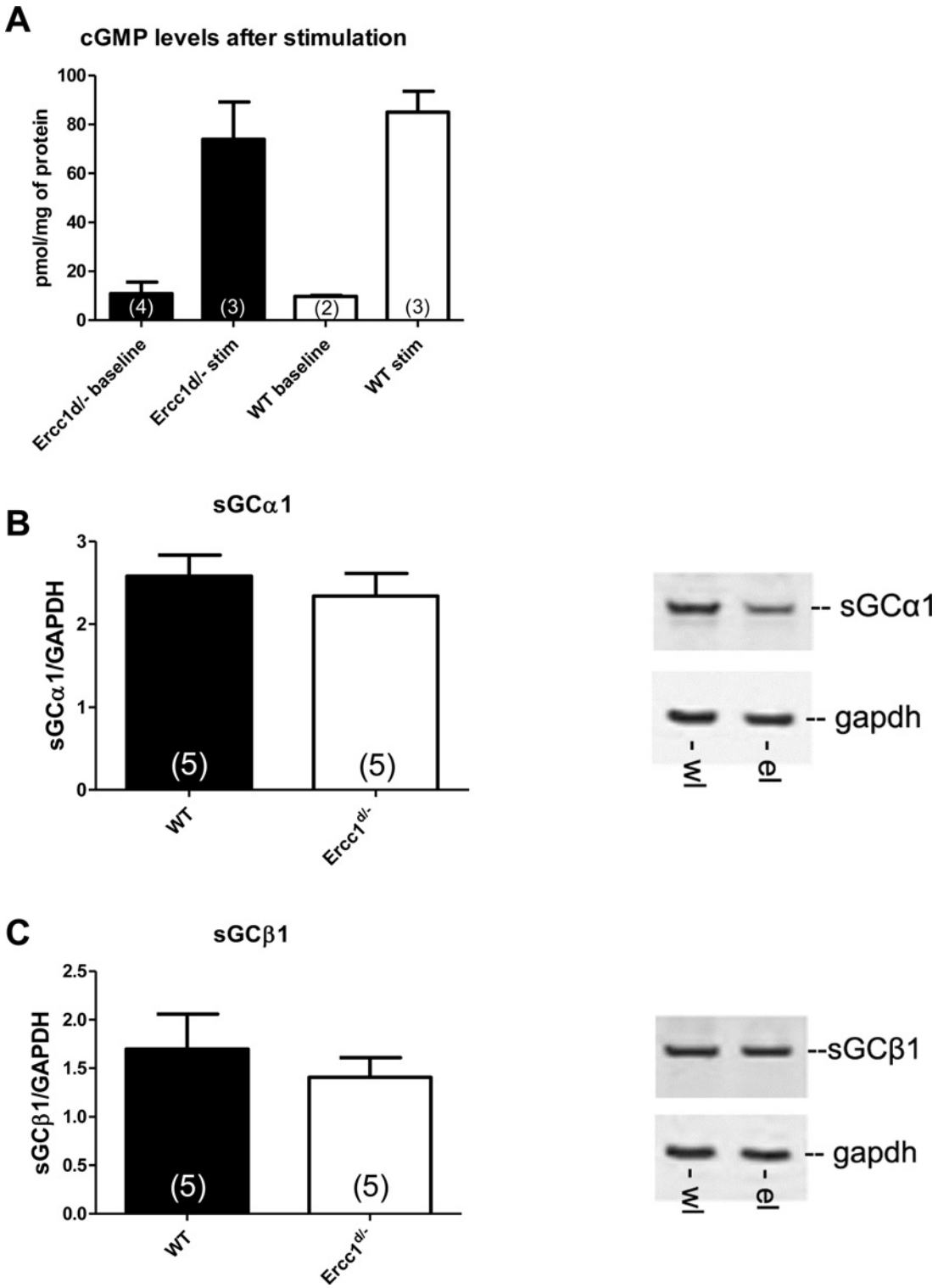

Figure 2 **cGMP production and sGC subunit protein levels in thoracic aortas of *Ercc1*$^{d/-}$ mice**

Production of cGMP in aortic tissue of *Ercc1*$^{d/-}$ and WT mice with and without stimulation with SNP (10^{-5} mol/l) for 5 min (**A**). sGC α1 and β1 sub-types (**B** and **C**) protein levels were measured by Western blotting and corrected for GAPDH levels. Numbers of observations are given in parentheses.

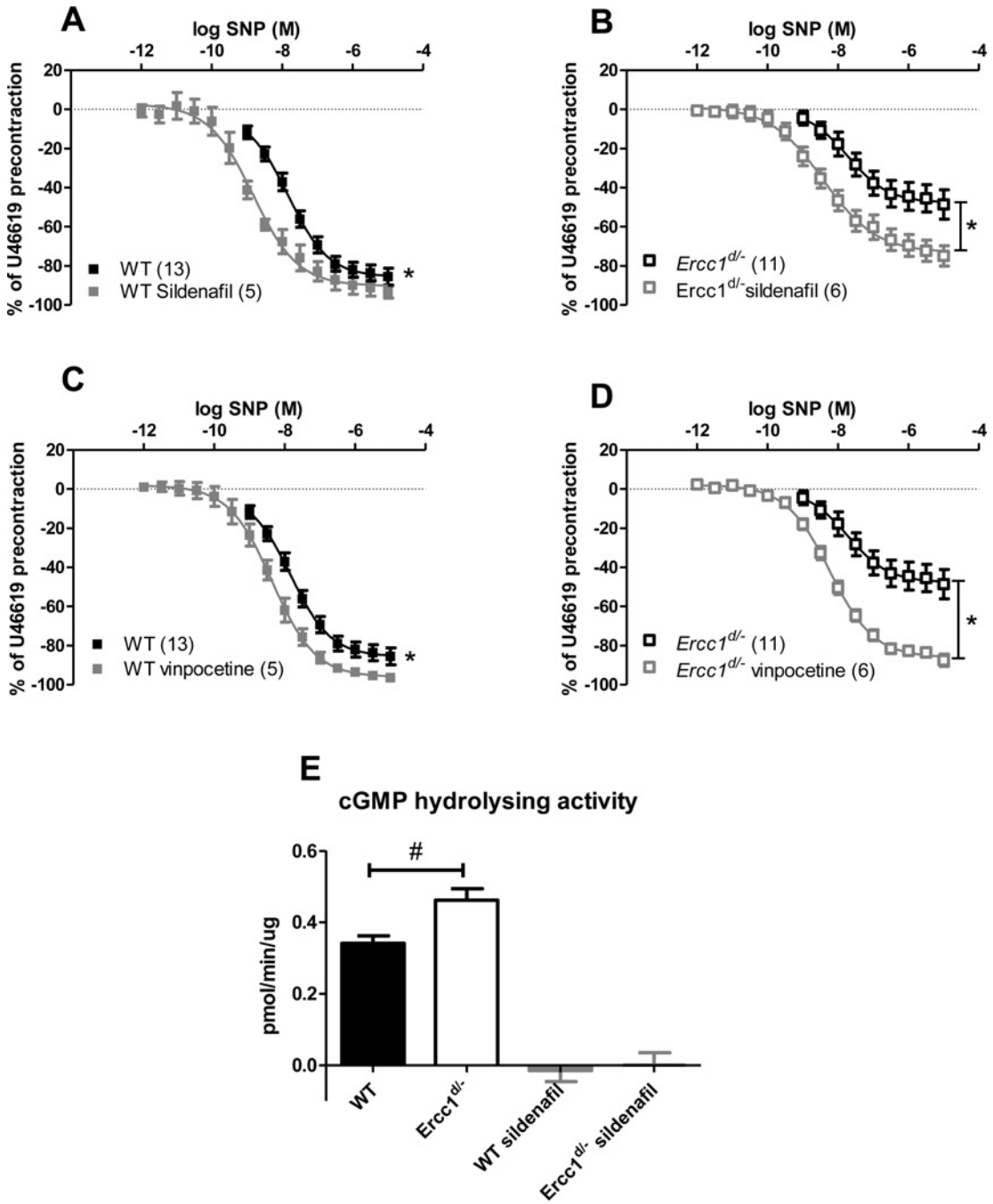

Figure 3 Effects of PDE inhibitors on SNP-induced relaxation and PDE activity in *Ercc1*$^{d/-}$ lungs

Relaxation of isolated thoracic aorta segments from *Ercc1*$^{d/-}$ and WT animals to SNP studied in organ bath set-ups during contraction induced by U46619 in the absence and presence of sildenafil and vinpocetine (**A**–**D**). cGMP hydrolysis in lung of *Ercc1*$^{d/-}$ mice as compared with WT mice, in the presence or absence of sildenafil (**E**). $^{\#}*P < 0.05$ (Student's *t* test and GLM–RM respectively). Numbers of observations are given in parentheses.

levels respectively (Figures 6A and 6B), whereas the *PDE5* level was only modestly increased (Figure 6E; $P = 0.06$). The pattern of PDE1C protein expression (Figure 7A) corresponded with qPCR data and p21 protein expression (Figure 7B) but we could not specifically detect PDE1A or PDE5 protein in human samples despite trying several different antibodies. When we treated VSMCs with the PDE1 and PDE5 inhibitors vinpocetin and sildenafil to accomplish loss-of-function of the PDEs we found that vinpocetin (predominant PDE1 inhibition) lowered both p16 and p21 expression, whereas sildenafil (predominant PDE5 inhibition) only appeared to affect p21 (Figures 7C and 7D).

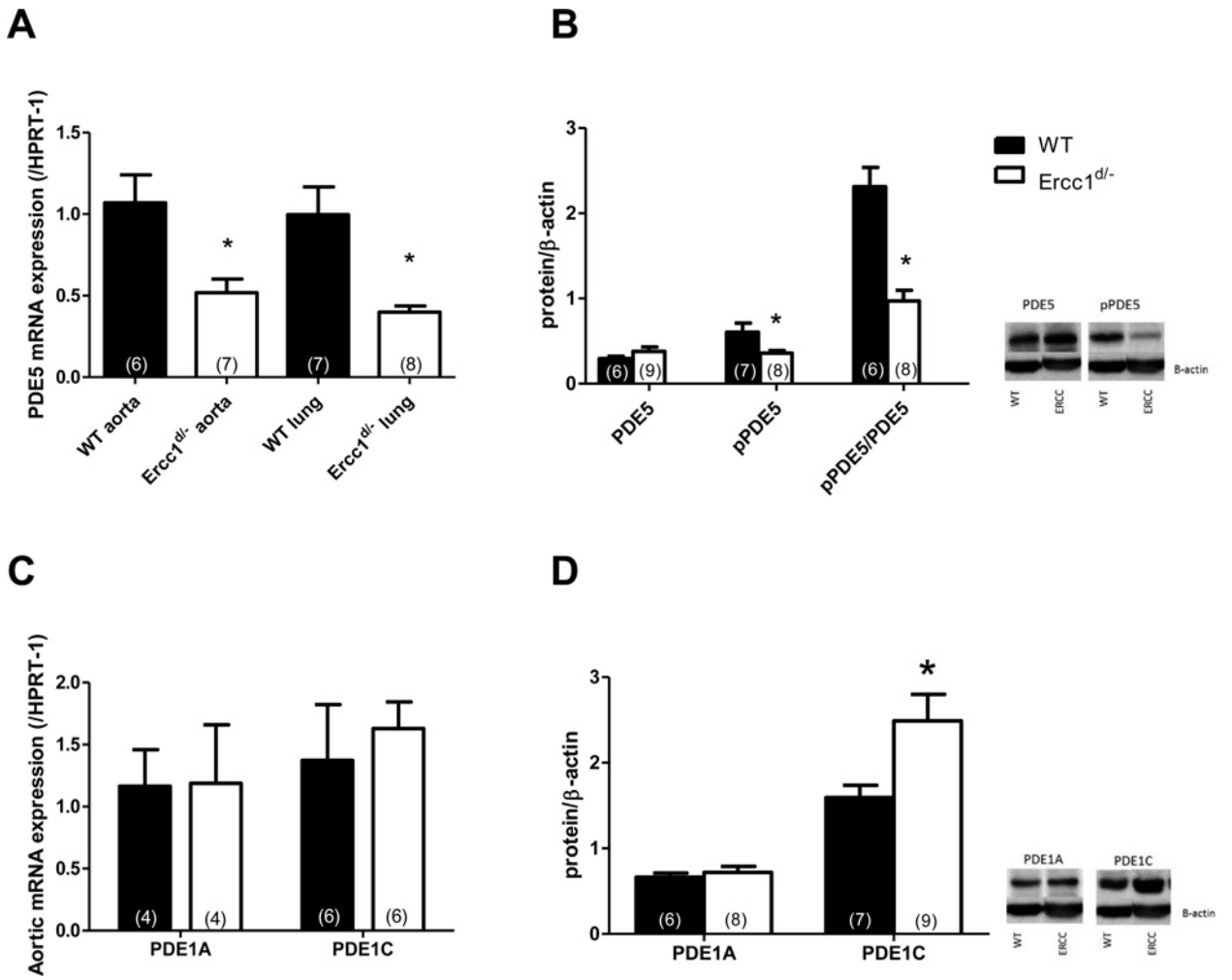

Figure 4 Expression of PDE1 and PDE5 sub-types in mouse tissues
Aortic and lung *PDE5* mRNA (**A**) and lung PDE5 protein expression (**B**), aortic *PDE1A* and *PDE1C* mRNA expression (**C**) and lung PDE1A and PDE1C protein expression (**D**) were measured by qPCR and Western blot (WB), correspondingly. $*P < 0.05$ (Student's *t* test). Numbers of observations are given in parentheses.

Genetic association studies

Since *in vitro* cellular senescence is not synonymous with vascular aging, we tested the involvement of PDE1 and PDE5 by means of genetic association studies. After Bonferroni correction for multiple testing, 14 single nucleotide polymorphisms in the *PDE1A* gene remained significantly associated with DBP at the threshold of $P \leqslant 2.87 \times 10^{-4}$ (Table 2; Figure 8A). The biggest effect size was found for the single nucleotide polymorphism rs1430158 ($\beta = 0.281$, $P = 2.47 \times 10^{-5}$).

We found six single nucleotide polymorphisms in the *PDE1A* gene significantly associated with cIMT (Table 2; Figure 8B). Among all, rs2887202 showed the lowest *P*-value ($\beta = -0.0061$, $P = 2.89 \times 10^{-5}$)

The results for the association of the single nucleotide polymorphisms in the *PDE1A*, *PDE1C* and *PDE5* genes with SBP, DBP, PP, CFPWV, cIMT and CAD at $2.87 \times 10^{-4} < P < 0.05$ are presented in Supplementary Table S1. There are some suggestive associations of *PDE5A* with SBP (rs6844263, $\beta = 0.312$, $P = 1.60 \times 10^{-3}$) and DBP (rs1155577, $\beta = 0.214$, $P = 5.16 \times 10^{-4}$) and of *PDE1A* with PP (rs17343395, $\beta = -0.354$, $P = 5.05 \times 10^{-3}$) and cIMT (rs12989198, $\beta = -0.0053$, $P = 3.28 \times 10^{-4}$). One single nucleotide polymorphism in the *PDE1C* gene showed a suggestive association with CAD (rs10226190, $\beta = 0.080$, $P = 5.75 \times 10^{-3}$). CFPWV did not show any association that might be relevant.

DISCUSSION

In our studies in mice with accelerated vascular aging due to genomic instability, we found decreased vasodilator function and increased cGMP metabolism in lung tissue that can both be restored by PDE1 and PDE5 inhibition, whereas the highly vascularized lung shows increased PDE1C expression. In humans, VSMC senescence, which can be caused by genomic instability, also leads to increased PDE1 and possibly also PDE5 activity, whereas in the general population single nucleotide polymorphisms in the *PDE1A* gene are associated with increased DBP and

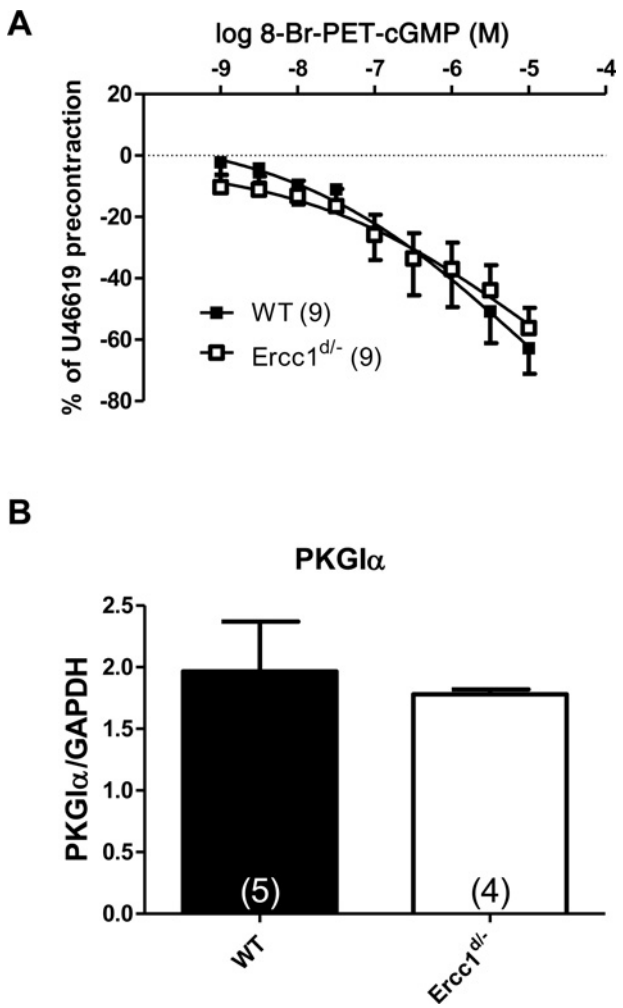

Figure 5 Responses to PKGI activator and PKGIα protein levels in aortic tissue of *Ercc1*$^{d/-}$ mice

Relaxation of thoracic aortas from 16-week-old *Ercc1*$^{d/-}$ and WT mice to cell membrane-permeant PKGI activator 8-Br-PET-cGMP during U46619-induced contraction, as measured in organ bath set-ups (**A**). PKGIα protein levels were measured by Western blotting and corrected for GAPDH levels (**B**). Numbers of observations are given in parentheses.

cIMT, two hallmarks of human age-related vascular dysfunction. Therefore, in general, our data suggest that increase in PDE1A and PDE1C in VSMCs might play a pivotal role in aging-related vasodilator dysfunction and vascular hypertrophy and that this increase is linked to cellular senescence, a consequence of genomic instability. A similar observation, but with more reservations, can be made for PDE5.

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

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

Relevant to advanced vascular aging is the occurrence of senescence in vascular cells. When we measured the gene expression of three PDE sub-types in human VSMCs, we found that predominantly *PDE1A* and *PDE1C* mRNA levels increased with increasing passages and p16 and p21 expression. The association between VSMC senescence and PDE1 sub-types was confirmed when we treated VSMCs with vinpocetin, which caused a reduced *p16* and *p21* mRNA expression. PDE5 inhibition only reduced *p21* mRNA expression, again pointing to a stronger association of senescence with PDE1. Overall, our results show that PDE1 and cell senescence are reciprocally associated and suggest that PDE1

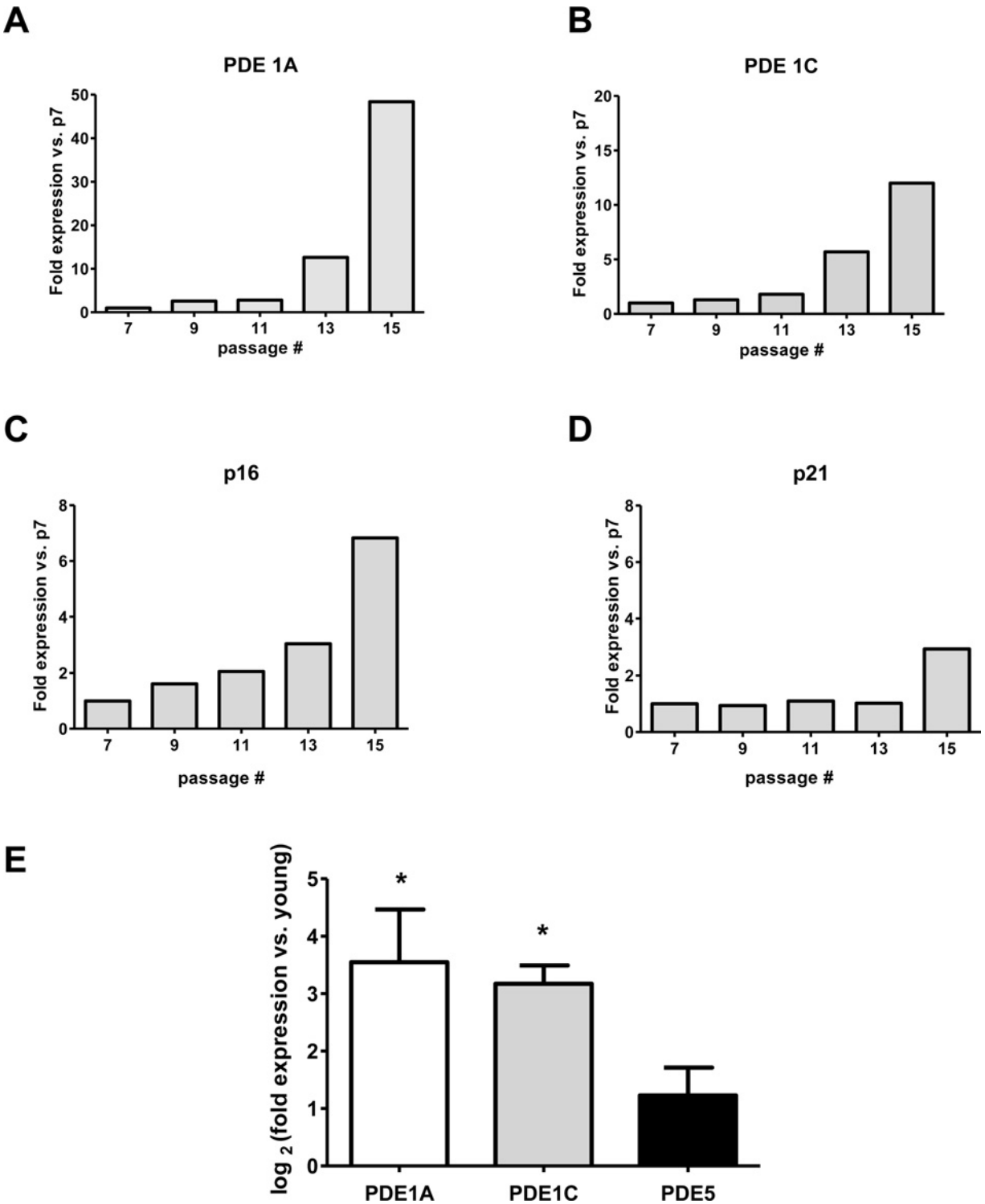

Figure 6 mRNA expression of PDE1, p16 and p21 in human VSMCs

Pattern of *PDE1A* (**A**), *PDE1C* (**B**), *p16* (**C**) and *p21* (**D**) mRNA expression at different passages measured by qPCR. *PDE1A*, *PDE1C* and *PDE5* mRNA expression in young compared with senescent human VSMCs was measured by qPCR. Fold expression values were $\log_2$-transformed, $^*P < 0.05$ (**E**). All C_T values of mRNA expression were corrected for *TBP* gene expression.

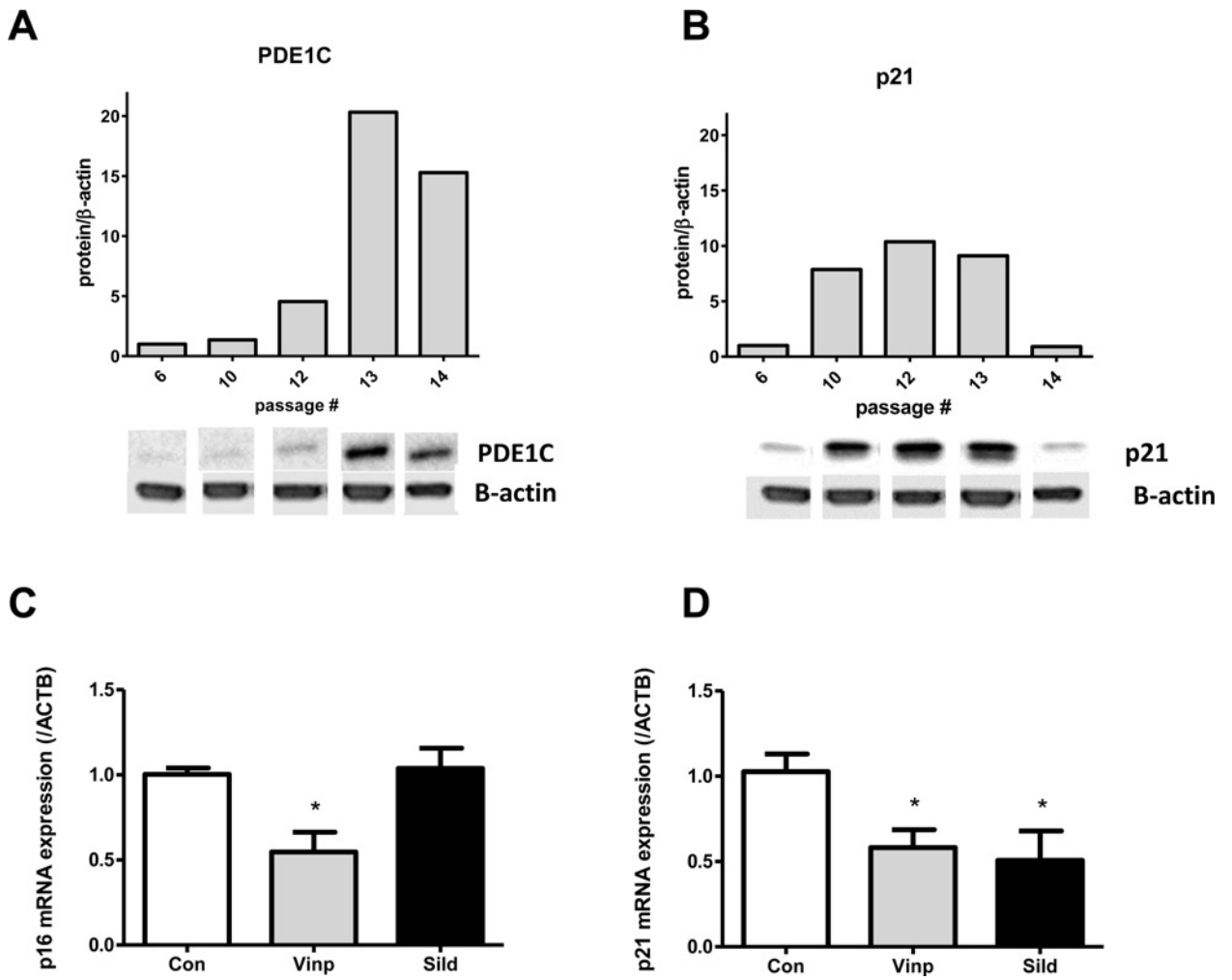

Figure 7 Protein expression of PDE1C and p21 in human VSMCs and mRNA expression of p16 and p21 in human VSMCs treated with PDE1 and PDE5 inhibitors

Pattern of PDE1C (**A**) and p21 (**B**) protein expression at different passages measured by Western blotting (WB). Protein levels were corrected for β-actin levels. *p16* (**C**) and *p21* (**D**) mRNA expression in control, vinpocetine- and sildenafil-treated VSMCs. $*P < 0.05$. All C_T values of mRNA expression were corrected for *ACTB* gene expression.

might even be involved in regulation of VSMC senescence. Other roles for PDE1 in the aging vasculature have been proposed previously. As VSMCs age, their phenotype changes from a contractile to a synthetic state in which they exhibit increased proliferation, migration and production of extracellular matrix proteins, thus contributing to the neointima formation in aged vessels. It has been shown that PDE1A localization is different in contractile compared with synthetic VSMCs; in contractile VSMCs in the media, PDE1A is predominantly cytoplasmic, whereas in synthetic VMSCs in the neointima, it is nuclear and associated with VSMC proliferation [39]. The impact of cytoplasmic PDE1 subtypes on decreased vasodilator capacity is most probably reflected in our organ bath studies in mice and the genetic association with DBP in humans. The role of *PDE1A* gene variants in cIMT may be related to nuclear localization of the enzyme.

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

The variability in associations with respect to the clinical variable that is explored suggests that PDE1 and PDE5 would be involved in very specific functions in the vascular system. For instance, DBP might be relatively more dependent on basal tone of VSMCs and medial thickness and less dependent on cardiac contractility and vascular compliance as determined by changes in extracellular matrix than SBP and PP. CAD is an even more complex phenomenon, also involving inflammation, dyslipidaemia and thrombogenesis. In other words, our genetic association study might hint at the most relevant biological role of PDE1A and perhaps also of PDE5, which would be in regulation of vasomotor function and vascular remodelling by increasing

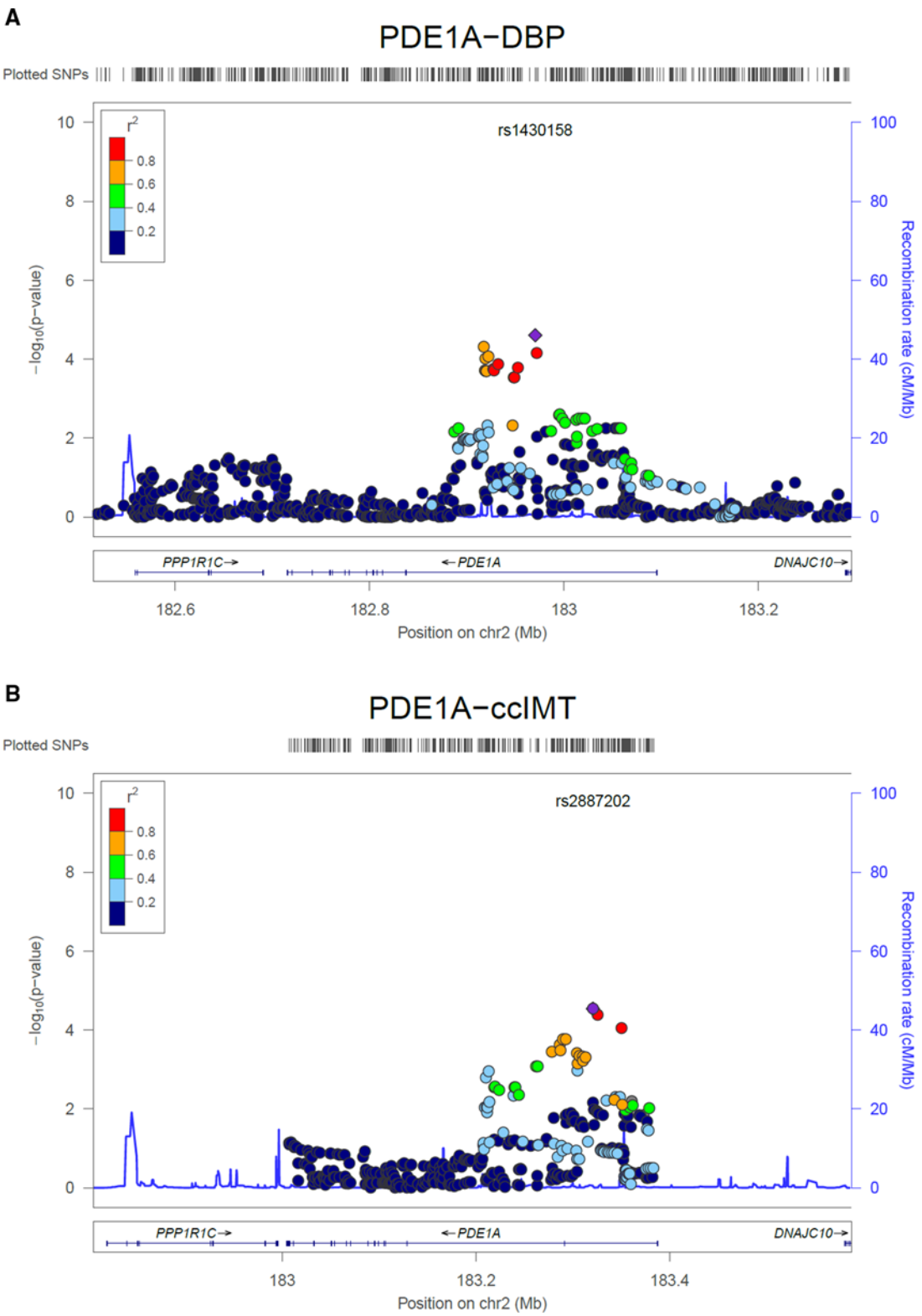

Figure 8 **Regional plots for the association between *PDE1A* single nucleotide polymorphisms and DBP and intima–media thickness**

Regional association plot showing $-\log_{10}$ (*P*-values) for all *PDE1A* single nucleotide polymorphisms ordered by their chromosomal position and DBP (**A**) and IMT (**B**). Each single nucleotide polymorphism is coloured according to its correlation with the single nucleotide polymorphism showing the lowest *P*-value (index single nucleotide polymorphism) within the region as specified in the colour scheme.

Table 2 Significant associations at the threshold of $P \leqslant 2.87 \times 10^{-4}$ of the single nucleotide polymorphisms in the *PDE1A* gene with DBP and cIMT

Chromosome				Allele					
Gene	**single nucleotide polymorphism**	***n***	**Position**	**Coded**	**Frequency**	***β***	**S.E.M.**	***P***	**Outcome**
PDE1A	rs1430158	2	182970373	T	0.77	0.282	0.067	2.47×10^{-5}	DBP
PDE1A	rs833166	2	182917392	T	0.78	0.278	0.068	4.85×10^{-5}	DBP
PDE1A	rs10497597	2	182971998	T	0.23	0.269	0.068	6.84×10^{-5}	DBP
PDE1A	rs2623431	2	182922232	T	0.78	0.266	0.068	8.61×10^{-5}	DBP
PDE1A	rs833168	2	182919236	T	0.78	0.264	0.068	9.56×10^{-5}	DBP
PDE1A	rs16823124	2	182932372	G	0.78	0.254	0.066	1.35×10^{-4}	DBP
PDE1A	rs16823150	2	182952598	T	0.81	0.254	0.067	1.65×10^{-4}	DBP
PDE1A	rs1438065	2	182927238	G	0.78	0.249	0.067	1.79×10^{-4}	DBP
PDE1A	rs11682598	2	182927925	G	0.78	0.248	0.067	1.90×10^{-4}	DBP
PDE1A	rs864417	2	182918692	T	0.75	0.250	0.067	1.94×10^{-4}	DBP
PDE1A	rs12693302	2	182919688	G	0.26	0.248	0.067	1.96×10^{-4}	DBP
PDE1A	rs12996836	2	182920273	T	0.25	0.248	0.067	1.97×10^{-4}	DBP
PDE1A	rs7558737	2	182948186	T	0.22	0.243	0.067	2.84×10^{-4}	DBP
PDE1A	rs10931009	2	182949102	T	0.22	0.243	0.067	2.87×10^{-4}	DBP
PDE1A	rs2887202	2	183029113	A	0.58	−0.006	0.002	2.89×10^{-5}	cIMT
PDE1A	rs934264	2	183033988	T	0.58	−0.006	0.002	4.14×10^{-5}	cIMT
PDE1A	rs1897104	2	183058624	A	0.42	0.006	0.002	8.91×10^{-5}	cIMT
PDE1A	rs10931012	2	182998273	A	0.40	0.006	0.002	1.70×10^{-4}	cIMT
PDE1A	rs11684406	2	183001113	T	0.60	−0.006	0.002	1.70×10^{-4}	cIMT
PDE1A	rs17343416	2	182994934	T	0.59	−0.005	0.002	2.38×10^{-4}	cIMT

VSMC proliferation and migration [39], remodelling processes that alter the vessel tone and therefore blood pressure [40].

It might seem remarkable that *PDE1C* single nucleotide polymorphisms did not show associations, with the exception of perhaps a weak relationship with PP and CAD. However, this observation does not necessarily mean that PDE1C is not important for human vascular disease. It might simply be explained by the absence of a relevant genetic variant. Our mouse data and previous studies in human VSMCs and atherosclerotic tissue are very suggestive of a relevant role of PDE1C [10,41]. In addition, the fact that the mRNA and protein levels of PDE1A and PDE1C are increasing more than those of PDE5 does not mean that both type 1 sub-types are more important. Our data show relative increases of gene product and this is not indicative for the absolute contribution of each PDE sub-type. In fact, previous studies show that PDE1 sub-type expression might be low in cultured VSMCs, hence leading to high increases in gene expression relative to baseline even in the case that absolute increases are small [42].

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

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

CLINICAL PERSPECTIVES

- After the failure of several clinical trials to show effectiveness of PDE5 inhibitors in chronic cardiovascular disease, our results underpin the previously suggested importance to further evaluate the potential of PDE1 as a treatment target.
- Our present study suggests that vascular aging related to genomic instability might be a major focus in this endeavour. Inhibition of specific PDE sub-types is an attractive option because of their strict localization–function relationship, which was explained above for PDE1A, theoretically allows very specific pharmacological interventions.
- Unfortunately, the PDE1-specific inhibitor IC86340 has been discontinued and a search for new inhibitors is required.

Stimulators or activators of sGC might be an alternative drug group, although it might not provide the same specificity.

AUTHOR CONTRIBUTION

Abbas Dehghan, Anton Roks, Jan Danser and Jan Hoeijmakers designed the research. Paula Bautista Niño, Matej Durik, René de Vries, Usha Musterd-Bhaggoe, Marcel Meima, Maryam Kavousi, Mohsen Ghanbari, Christopher O'Donnell, Nora Franceschini, Ger Janssen, Jo De Mey, Yiwen Liu, Catherine Shanahan and Oscar Franco conducted the research, collected data, provided important scientific input and participated in writing of the paper. All authors read and approved the final paper.

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