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BASIC SCIENCE

Impairment of endothelial progenitor cell function and vascularization capacity by aldosterone in mice and humans

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Aims Hyperaldosteronism is associated with vascular injury and increased cardiovascular events. Bone marrow-derived endothelial progenitor cells (EPCs) play an important role in endothelial repair and vascular homeostasis. We hypothesized that hyperaldosteronism impairs EPC function and vascularization capacity in mice and humans.

Methods and results We characterized the effects of aldosterone and mineralocorticoid receptor (MR) blockade on EPC number and function as well as vascularization capacity and endothelial function. Treatment of human EPC with aldosterone induced translocation of the MR and impaired multiple cellular functions of EPC, such as differentiation, migration, and proliferation *in vitro*. Impaired EPC function was rescued by pharmacological blockade or genetic ablation of the MR. Aldosterone protein kinase A (PKA) dependently increased reactive oxygen species formation in EPC. Aldosterone infusion in mice impaired EPC function, EPC homing to vascular structures and vascularization capacity in a MR-dependent but blood pressure-independent manner. Endothelial progenitor cells from patients with primary hyperaldosteronism compared with controls of similar age displayed reduced migratory potential. Impaired EPC function was associated with endothelial dysfunction. MR blockade in patients with hyperaldosteronism improved EPC function and arterial stiffness.

Conclusion Endothelial progenitor cells express a MR that mediates functional impairment by PKA-dependent increase of reactive oxygen species. Normalization of EPC function may represent a novel mechanism contributing to the beneficial effects of MR blockade in cardiovascular disease prevention and treatment.

Keywords Aldosterone • Primary hyperaldosteronism • Endothelial progenitor cells • Endothelial function • Reactive oxygen species

Introduction

Endothelial progenitor cells (EPCs) are a heterogeneous cell population that circulate in the blood and contribute to the formation of new blood vessels, endothelial repair, and vascular homeostasis by direct and indirect mechanisms.[1–4] Impairment of EPCs is related to endothelial dysfunction,[5,6] coronary artery disease,[7,8] and adverse clinical outcome.[9,10] Endothelial progenitor cells express a variety of receptors that mediate functional effects of endogenous hormones and growth factors by changing the intracellular balance of reactive oxygen species and endothelial nitric oxide synthase-derived NO.[3,6,11–13]

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Activation of the renin–angiotensin–aldosterone system plays an important role in the pathogenesis of atherosclerosis and its complications, such as vascular dysfunction and coronary artery disease.[14,15] In contrast to the classical notion that mineralocorticoids were only involved in body electrolyte and water homeostasis mediated by the kidney, aldosterone exerts important (patho)physiological effects on the cardiovascular system.[14,16–19] Aldosterone targets cardiomyocytes,[17,20] cardiac fibroblasts,[21] and endothelial cells[18] leading to cardiac hypertrophy, fibrosis, and vascular injury. Upon binding to the mineralocorticoid receptor (MR), aldosterone increases vascular endothelial and vascular smooth muscle cell superoxide anion production.[15,18,22,23] High levels of aldosterone impair vascular function and predict mortality risk of patients with acute myocardial infarction or heart failure.[16,24] Patients with primary hyperaldosteronism (PHA) display a higher incidence of myocardial infarction and stroke.[25] Mineralocorticoid receptor antagonism in patients with heart failure ameliorates endothelial function[26–28] and large clinical trials have shown MR blockade to decrease mortality in cardiovascular disease.[29,30]

The effects of aldosterone on EPC function and vascularization capacity in mice and humans are unclear. We therefore studied MR expression in various subtypes of EPCs. *In vitro* and *in vivo* studies were performed to investigate the effects of aldosterone on EPC function, endothelial function, and overall vascularization capacity. Finally, we tested whether MR antagonism would improve EPCs and markers of endothelial function in mice infused with aldosterone and patients with PHA.

Methods

Isolation, characterization, and culture of human and mouse endothelial progenitor cell

This was done essentially as described.[11,31,32] A considerable number of different EPC subtypes exist and the nomenclature is rather heterogeneous (see[33] for review). We therefore here describe the different subtypes of cells we used in our studies and also mention this in the Results section; in brief, we used the following cells for our analyses, which have been extensively described and characterized in the literature: monocytic EPCs,[11,34] $CD34^+/KDR^+$ cells (human study),[35] sca1/flk1 cells (mouse study),[11] early outgrowth (colony-forming units, CFU assays or 'Hill' colonies)[31] and late-outgrowth EPCs.[32] Note, that alternatively to the term 'monocytic EPC' also 'early EPC', 'angiogenic progenitor cells', or 'circulating angiogenic cells'[33] are used in the literature. All of the employed EPC subtypes expressed the MR (see Supplementary material online, *Figure S1* and data not shown). For morphology and characteristics of late-outgrowth EPCs, see Supplementary material online, *Figure S2*. As cell culture media we used endothelial basal medium (EBM-2, Lonza, Germany) supplemented with EGM-2 BulletKit (for aldosterone experiments without addition of hydrocortisone to avoid cross-talk with the MR).

Mineralocorticoid receptor expression analysis and cellular distribution

Gene expression of the *MR* (*NR3C2*) was detected by RT-PCR as described[36] and using 5′-ATCACGATCGGCTAGAGACC-3′ and 5′-CCCATAATGGCATCCTGAAG-3′ as oligonucleotides. To determine protein expression, cell lysates (50 μg) from cultured EPCs or human umbilical vein endothelial cells (positive control[37]) were separated on 12% SDS-polyacrylamide gels. Primary antibodies included anti-MR (ab2774) and anti-GAPDH (ab8245) (both Abcam, Cambridge, UK). Mineralocorticoid receptor expression was additionally determined by immunohistochemistry after addition of an anti-MR-antibody (ab2774, Abcam) overnight. Thereafter cells were washed and a rhodamine-labelled mouse IgG antibody was added for 2 h. Cellular distribution of the MR was investigated before and after addition of aldosterone (1 μg) for 2 h.

Determination of endothelial progenitor cell number and function

A variety of assays were used to determine the effects of aldosterone and MR blockade on EPC number, differentiation, and function:

This included an EPC adhesion-related cell culture assay for the identification of monocytic (early) EPCs, the detection of $CD34^+$/VEGFR-2^+ cells (human study) or sca-1^+/flk-1^+ cells (mouse study), DiI-acLDL uptake, Ulex europeus-1 (UEA-1) binding, the determination of endothelial CFU, and analysis of the migratory capacity of EPCs towards an vascular endothelial growth factor (VEGF)/stromal-derived factor-1 (SDF-1) gradient. These assays were essentially done as described.[11,12,31]

Small interference RNA-mediated knockdown of the mineralocorticoid receptor

Endothelial progenitor cells were transfected with siRNA against *NR3C2* or scrambled controls using the Stealth™ Select RNAi Kit (Invitrogen, Germany; oligonucleotide concentration 150 nM, 48 h). FITC-labelled scrambled siRNA (control-FITC block-it fluorescent Oligo #2013, Invitrogen, Germany) was used as a transfection control. Transfection rate was >90% (data not shown). Forty-eight hours after transfection, *NR3C2* expression was monitored by RT-PCR and western blot analysis (see above-mentioned section).

Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) was determined using dihydroethidium (DHE) as described.[12] After 20 min of incubation with DHE (2.5 μM) at 37°C and 5% CO_2 in a humidified atmosphere, EPCs were evaluated using fluorescence microscopy. Signal intensity of cells and background was determined in at least four randomly chosen regions.

Aldosterone level

Plasma aldosterone levels were measured by commercially available radioimmunoassays (mice: DiaSorin GmbH, Dietzenbach, Germany; humans: DPC Biermann, Bad Nauheim, Germany).

Mouse *in vivo* studies

The study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). To test whether hyperaldosteronism would alter EPC biology, neovascularization capacity and endothelial function *in vivo*, we implanted osmotic mini-pumps (#1002, Alzet) to continuously infuse aldosterone (50 μg/kg/d) or vehicle into male mice (8 weeks of age, Harlan-Winkelmann, Germany). A subgroup of aldosterone-infused animals was co-treated with the MR antagonist eplerenone (100 mg/kg/d by oral gavage), the beta-blocker metoprolol (10 mg/kg/d by i.p. injection) or placebo. Matrigel plugs were implanted into a further subgroup of mice, which after 10 days received

aldosterone- or placebo infusion for 3 days followed by retroorbitally injection of 1×10^6 DiI-acLDL-labelled EPCs, which expressed the MR or in which the MR has been silenced by siRNA. Twenty-four hours later, the matrigel plugs were removed and numbers of EPCs within the matrigel plug were counted. Dosages for aldosterone infusion, eplerenone, and beta-blocker treatment were estimated based on previous studies.[18,38,39] Blood pressure of animals was determined using established tail-cuff measurements (Föhr Medical Instruments, Seeheim, Germany).

Determination of endothelial function in mice and humans

Fourteen days after continuous aldosterone or placebo infusion to mice, the descending aorta was dissected and cleaned of connective tissue. Three millimetres of aortic ring segments were studied in organ chambers (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurements as described.[40] Vessels were preconstricted with prostaglandine F2α to comparable constriction levels, and the relevant response to cumulative doses of acetylcholine and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NONOate) (both 1 pmol/l to 10 μmol/l) was assessed.

Endothelial function and arterial stiffness in patients with PHA and controls of the same age were determined by an established peripheral arterial tonometry method based on a plethysmographic device (EndoPAT2000, Itamar-Medical, Israel).[41]

Vascularization assays

Matrigel plug assay

Seven days after implantation of osmotic minipumps we injected Matrigel (400 μl, Becton Dickinson, #354248, supplemented with 600 ng/mL basic FGF and 300 ng/mL VEGF). After additional 7 days matrigel plugs were explanted, shock frozen in TissueTec, sliced, stained with a mouse CD31 antibody (PECAM-1, #MCA2388, Serotec, dilution 1:50) overnight and subsequently stained with a biotinylated antimouse antibody and Texas Red Avidin DCS (Vector #A2006) and investigated by fluorescence microscopy. Nuclei were counterstained by Hoechst 33342 (Molecular probes, dilution 1:500). Vascularization was calculated by determination of the amount of invading capillaries and measurement of haemoglobin content of the explanted Matrigel plug by standard ELISA (Mouse haemoglobin ELISA, E-90HM, Dunn Labortechnik, Asbach, Germany).

Aortic sprouting assay

In a subset of experiments, the transthoracic aorta was removed after infusion of aldosterone or placebo (PBS) for 14 days, sliced into 1 mm rings, placed in matrigel and cultured in EBM2 cell culture media (with supplements; LONZA, Switzerland) for 5 days. Then the amount of sprouting outgrowths per aortic ring was counted as a further marker of vascularization capacity as described.[42]

Clinical trial

Approval from the ethical committee of the University of Würzburg was obtained, as was informed written consent from patients and controls. We investigated EPC number and function, as well as endothelial function in patients admitted to the Division of Endocrinology (University of Wurzburg, Germany) because of suspected PHA. Based on an increase in the aldosterone/renin ratio (>50), pathological saline infusion test, imaging tests such as high-resolution computed tomography scan or magnetic resonance imaging and/or adrenal vein sampling tests, we identified 10 patients with an aldosterone-producing adenoma and thus PHA. Baseline characteristics of patients and controls are shown in *Table 1*. A subgroup of patients with PHA was treated with spironolactone (75–100 mg/day) for 4–6 weeks. Changes of EPC number and function, as well as endothelial function were determined before and after mineralocorticoid receptor blockade in this subgroup.

Table 1 Characterization of controls and patients with primary hyperaldosteronism

	Controls ($n = 13$)	Primary hyperaldosteronism ($n = 10$)	*P*
Age (years)	44.8 ± 3.0	50.6 ± 3.0	NS
Gender, M/F	9/4	8/2	NS[a]
Hypertension	3/13	10/10	<0.001[a]
CAD	3/13	3/10	NS[a]
Diabetes	2/13	2/10	NS[a]
Medication			
ACE/ARB	3/13	8/10	<0.01[a]
Beta blocker	3/13	8/10	<0.01[a]
Calcium antagonist	1/13	7/10	<0.01[a]
Statin	3/13	3/10	NS[a]
ASA	3/13	3/10	NS[a]
Diuretics	2/13	3/10	NS[a]
Metformin	1/13	2/10	NS[a]

Values were expressed as mean ± SEM where appropriate. ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid, CAD, coronary artery disease; F, female; M, male.
[a]χ^2 analysis.

Statistical analysis

Data are presented as mean and SEM unless otherwise stated. Statistical analysis was performed using the StatView (SAS Institute) package. For statistical comparison of two groups, we employed paired or unpaired, two-tailed Student's *t*-test as appropriate; for the comparison of three or more groups, we used ANOVA followed by post-tests. In the case of the clinical studies there was no normal distribution of tested parameters within the patients or controls and thus non-parametric tests were used. We used the Mann–Whitney test for comparison of parameters between two groups and the Wilcoxon signed rank test when paired samples were analysed. Differences were considered significant when $P \leq 0.05$.

Results

Expression and functional importance of mineralocorticoid receptor activation for endothelial progenitor cells

Monocytic (early) EPCs expressed the MR (NR3C2) both at the gene (*Figure 1A*) and protein levels (*Figure 1B*). In addition, the MR was expressed in all employed types of EPC (Supplementary material, *Figures S1* and *S2*). In untreated EPC the MR was detectable throughout the cell, but upon stimulation with aldosterone mainly translocated to the peri- and intranuclear region suggesting activation of the receptor (*Figure 1C*). The functional effects of MR

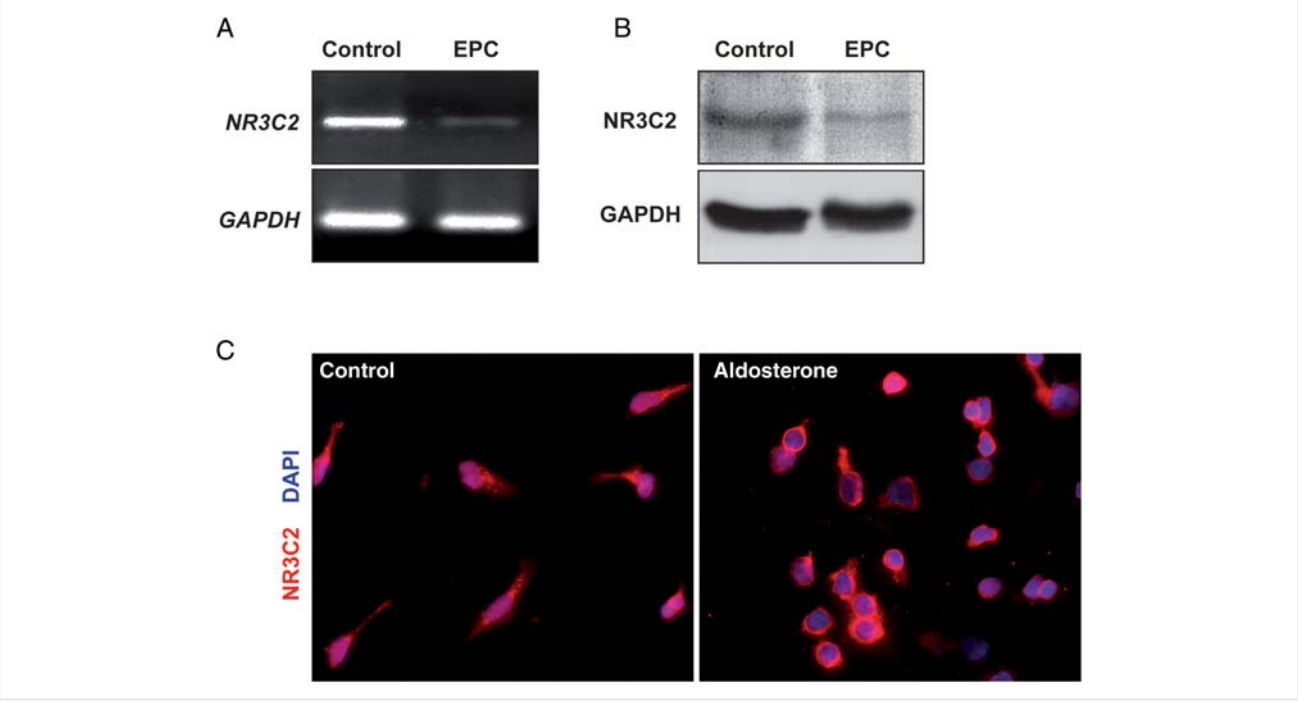

Figure 1 Expression of mineralocorticoid receptor (NR3C2) in endothelial progenitor cells (EPCs). (*A*) Gene expression, (*B*) protein expression of NR3C2 (mineralocorticoid receptor) and GAPDH in human monocytic EPCs. Human umbilical vein endothelial cells (control) served as positive controls for NR3C2 expression.[37] (*C*) Fluorescence microscopic detection of NR3C2 expression in untreated (left) and aldosterone-treated (1 μg, 2 h) human EPCs. 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. Exemplary pictures/blots from at least three independent experiments are shown.

stimulation on EPC biology were then tested in a series of *in vitro* assays. Aldosterone treatment impaired formation of endothelial CFU and of UEA-1$^+$/dil-acLDL$^+$ cells from cultured peripheral blood mononuclear cells, indicating inhibition of EPC differentiation and functional impairment (*Figure 2A* and *B*). Migratory capacity of monocytic (early) EPCs towards a SDF-1/VEGF gradient was dose dependently attenuated by aldosterone but not hydrocortisone (see *Figure 2C* and Supplementary material online, *Figure S3*). Likewise, aldosterone inhibited migration of human late-outgrowth EPCs (see Supplementary material online, *Figure S2*). Expression of the SDF-1 receptors CXCR4 and CXCR7 was unaffected by aldosterone treatment (data not shown). Selective pharmacological inhibition of the MR by eplerenone (*Figure 2A–D*) or genetic ablation of the MR by siRNA abolished aldosterone-mediated functional impairment of EPCs (see *Figure 2D* and Supplementary material online, *Figure S2*). As EPCs may additionally contribute to angiogenesis by a paracrine mode of action,[43] we screened for altered production of different angiogenic cytokines and growth factors (VEGF), stem cell factor, hepatocyte growth factor, platelet-derived growth factor bb, fibroblast growth factor 2, granulocyte macrophage colony-stimulating factor, IL-2, IL-4, IL-6, monocyte chemotactic protein-1, chemokine (C-C motif) ligand 5 (CCF5 or RANTES) and tumour necrosis factor alpha (TNF-alpha), but did not detect significant differences (data not shown). We thus conclude that aldosterone directly impairs EPC function without major effects on paracrine activity.

Aldosterone increases oxidative stress in monocytic (early) endothelial progenitor cells in a protein kinase A-dependent manner

Endothelial progenitor cell function is impaired by ROS.[6,12,31] We tested whether aldosterone would alter ROS formation in monocytic (early) and late-outgrowth EPC. Treatment of EPC with aldosterone led to an up to four-fold increase in intracellular ROS production, which was blocked by pre-treatment of EPC with eplerenone or siRNA-mediated silencing of the MR (see *Figure 3A* and *B* and Supplementary material online, *Figure S2*). The protein kinases (PK) A and C are involved in endothelial ROS production. Inhibition of aldosterone PKA alleviated aldosterone-mediated ROS production of EPC, whereas PKC inhibition had only minor effects (*Figure 3B*). Aldosterone-stimulated ROS formation impaired the migratory potential of EPC, which could be completely normalized by concomitant PKA, but not PKC inhibition (*Figure 3C*).

Aldosterone impairs monocytic (early) endothelial progenitor cell function, vascularization capacity, and endothelial function *in vivo*

To determine whether the *in vitro* findings of aldosterone-mediated EPC dysfunction were operative *in vivo*, we implanted osmotic

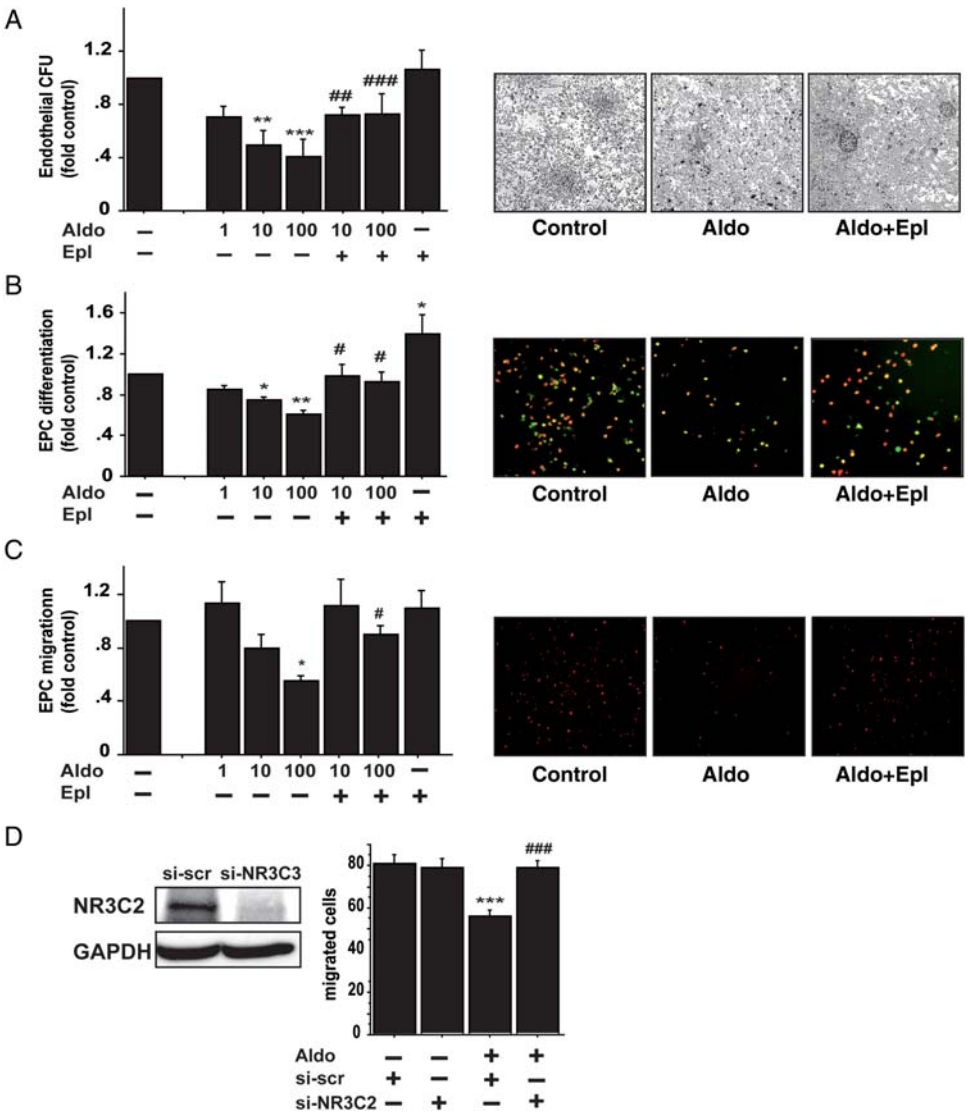

Figure 2 Mineralocorticoid receptor-dependent effects of aldosterone on endothelial progenitor cell (EPC) function. Effects of aldosterone (Aldo, 1–100 ng/mL) and selective blockade by concomitant eplerenone treatment (Epl, 10 μM) on EPC-colony forming unit capacity (*A*), monocytic (early) EPC differentiation measured by dil-acLDL uptake and UEA-1 staining (*B*) and EPC migratory capacity using modified Boyden chambers (*C*). (*D*) SiRNA-mediated knockdown of mineralocorticoid receptor (NR3C2; left) alleviated the detrimental effects of aldosterone on EPC migration in a modified Boyden chamber assay (right). Data are mean and SEM. *Significance against control; #Significance against respective Aldo dose without Epl; $^{*,\#}P \leq 0.05$, $^{**,\#\#}P \leq 0.01$, $^{***,\#\#\#}P \leq 0.005$. $n = 3–8$ per group.

minipumps in the wild-type mice to infuse either aldosterone (50 μg/kg/day) or vehicle for 14 days. Aldosterone infusion increased blood pressure, which was attenuated by concomitant eplerenone treatment (*Figure 4A* and *B*). We found no changes in the number of circulating sca-1^+/flk-1^+ EPC (control: 0.45 ± 0.06% sca-1^+/flk-1^+ cells; aldosterone infusion: 0.40 ± 0.06% sca-1^+/flk-1^+ cells; P = n.s.), but an increase of intracellular ROS levels in response to aldosterone infusion (*Figure 4C*). This resulted in impaired monocytic (early) EPC differentiation and function based on migratory activity studies and could be completely prevented by co-treatment with the selective MR blocker eplerenone (*Figure 4D* and *E*). In control animals, eplerenone had no significant effects on cellular ROS formation or migratory capacity, but improved EPC differentiation (*Figure 4C–E*). Hyperaldosteronism prevented homing of monocytic (early) EPCs to implanted matrigel plugs, which was completely normalized if the MR had been silenced by a siRNA approach (*Figure 4F*). Note that infused EPC both integrated and/or firmly adhered to preformed capillaries in matrigel plugs (*Figure 4G*). In a further study we wanted to exclude significant effects of aldosterone-induced blood pressure increase *per se* on number and function of circulating sca-1^+/flk-1^+ EPC. Here, we co-treated the aldosterone-infused mice additionally with the beta-blocker metoprolol, which prevented blood pressure increase (see Supplementary material

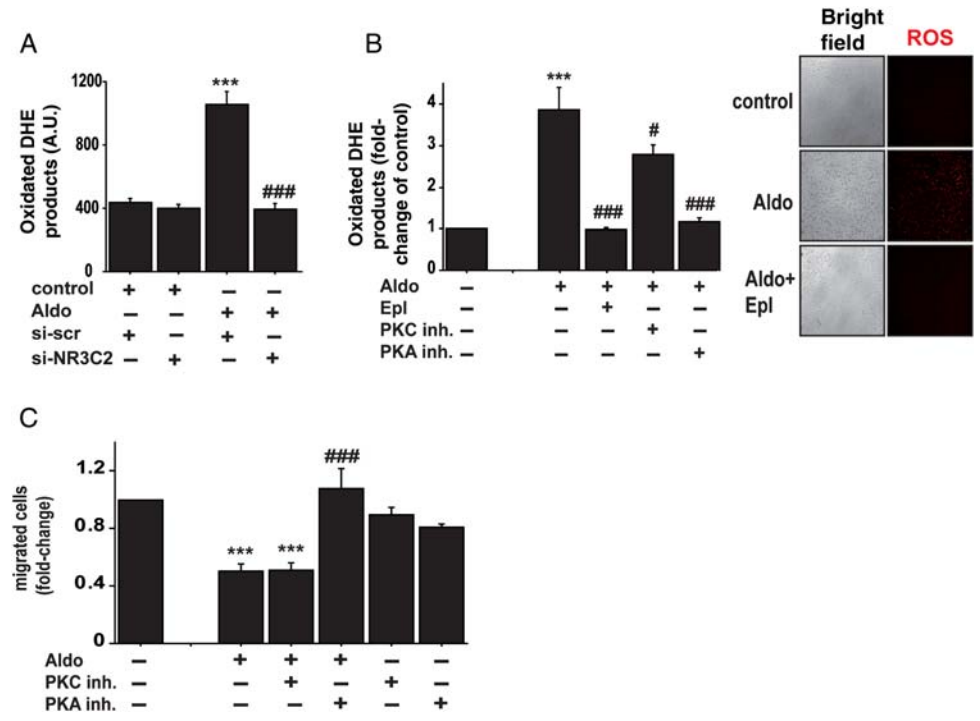

Figure 3 Protein kinase-dependent effects of aldosterone on superoxide production in endothelial progenitor cells (EPCs). (*A*) Fluorescence-microscopic detection of oxidated dihydroethidium (DHE) in human monocytic (early) EPC treated with aldosterone or vehicle control after transfection with siRNA against NR3C2 or a scrambled control. (*B*) Measurement of oxidative stress (oxidated DHE) in EPCs co-treated with aldosterone and a protein kinase A inhibitor (H-89, 24 h), a protein kinase C inhibitor (chelerythrine, 24 h) or eplerenone (Epl, 10 μM, 24 h). (*C*) Monocytic EPC migratory capacity after aldosterone treatment with and without inhibition of PKA or PKC. Data are mean and SEM. *Significance against control; #significance against respective Aldo dose without Epl; $^{*,\#}P \leq 0.05$, $^{***,\#\#\#}P \leq 0.005$. $n = 3–8$ per group.

online, *Figure S4A*). Importantly, this treatment did not prevent the aldosterone-mediated impairment on monocytic EPC function (see Supplementary material online, *Figure S4B*), demonstrating that the effects are independent from blood-pressure alterations.

We then evaluated the consequences of hyperaldosteronism on endothelial function. Compared with controls, aldosterone-infused mice demonstrated a significant impairment of endothelium-dependent vasodilatation, whereas treatment with eplerenone alleviated this effect (*Figure 5A*). There were also minor effects on endothelium-independent vasodilatation suggesting lower sensitivity to nitric oxide of aldosterone-infused mice without changes in the maximal response to DEA-NONOate.

In addition to effects of aldosterone on large vessels, we determined alterations in the capacity for vascularization in two further models. Aldosterone infusion markedly reduced endogenous vascularization of implanted matrigel plugs (see *Figure 4F*) based on measurements of the number of invading capillaries and total haemoglobin content (*Figure 5B*). Next, sprouting capacity of explanted aortae was significantly impaired after aldosterone infusion independent from blood-pressure alterations (*Figure 5C* and see Supplementary material online, *Figure S4C*). Importantly, co-treatment with eplerenone normalized vascularization capacity in both models of vascularization (*Figure 5B* and *C*).

Endothelial progenitor cell and endothelial dysfunction in patients with primary hyperaldosteronism: improvement by mineralocorticoid receptor blockade

To determine the clinical impact of the detrimental effects of aldosterone seen *in vitro* and in mice experiments, we conducted a *clinical trial* in patients with PHA. For comparison we recruited controls of the same age with normal aldosterone plasma levels. Beside a higher rate of antihypertensive medications there was no difference in drug treatment, coronary artery disease, or diabetes in PHA patients compared with controls (*Table 1*). Patients with established PHA had significantly increased aldosterone levels and aldosterone/renin ratios (*Figure 6A*). There was no statistical significant difference between the number of circulating EPCs ($CD34^+/KDR^+$ cells) in PHA patients when compared with controls (21.9 ± 6.1 $CD34^+/KDR^+$/100 000 cells vs. 24.9 ± 8.9 $CD34^+/KDR^+$/100 000 cells; $P = 0.81$). However, monocytic (early) EPCs from patients with PHA showed an impaired migratory potential compared with controls (*Figure 6B*). Treatment of PHA patients with the MR blocker spironolactone for 4–6 weeks significantly improved EPC function (*Figure 6C*).

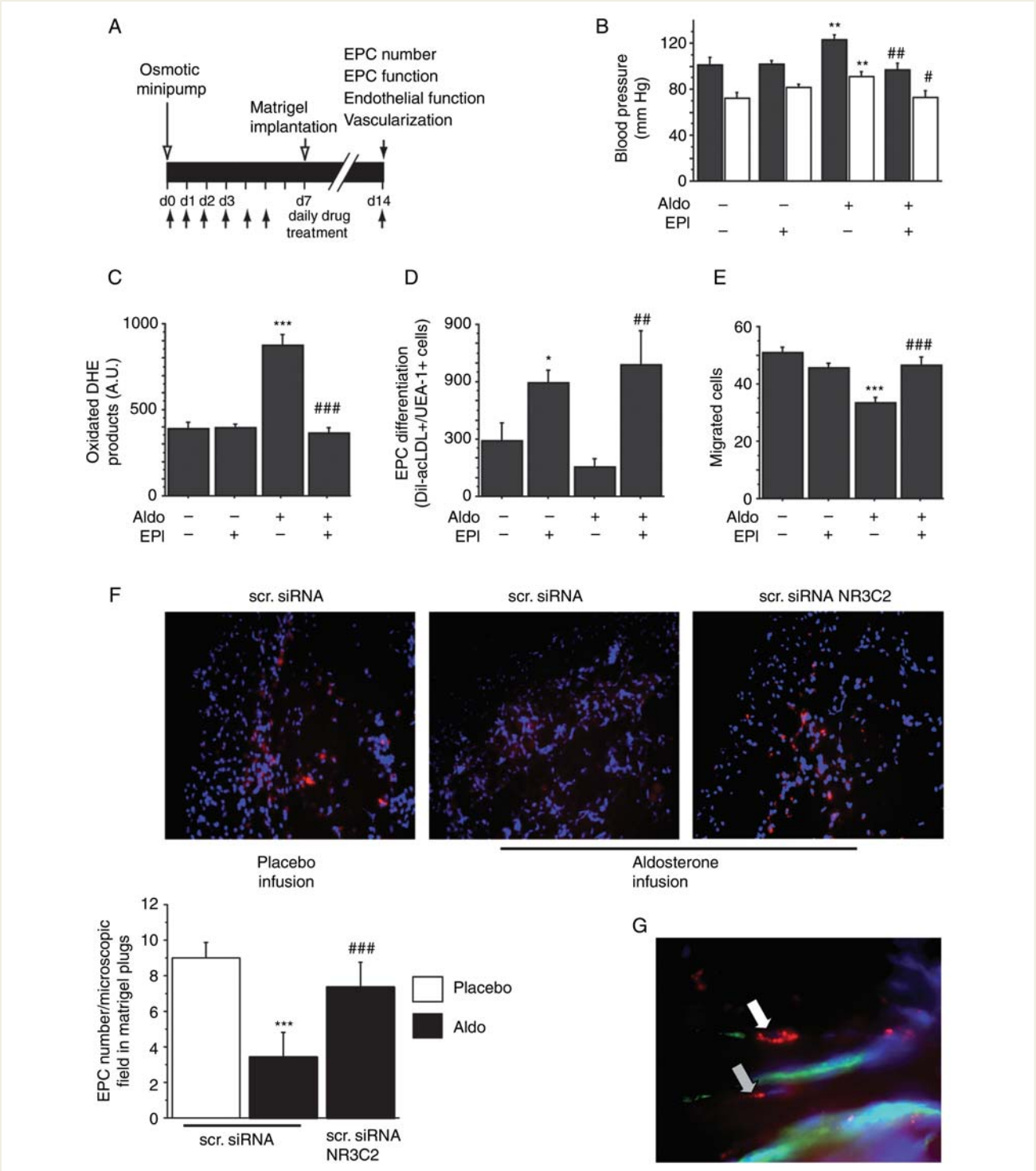

Figure 4 Effects of aldosterone treatment *in vivo* on intracellular oxidative stress and function of endothelial progenitor cells (EPCs). (*A*) Scheme of *in vivo* experiments: aldosterone or vehicle was delivered by implanted osmotic minipumps continuously for 2 weeks at a dose rate of 50 μg/kg/day. After 7 days matrigel plugs were implanted. After 14 days EPC function and number were determined, as well as vascularization of the implanted matrigel plug and sprouting capacity of explanted aortic rings. (*B*) Systolic and diastolic blood pressure of mice after 14 days of aldosterone- or vehicle infusion with and without concomitant oral treatment with the mineralocorticoid receptor-antagonist eplerenone (Epl, 100 mg/kg of body weight). Effects of aldosterone treatment and mineralocorticoid receptor antagonism *in vivo* on oxidative stress (oxidated dihydroethidium) in monocytic (early) EPCs (*C*), EPC differentiation (*D*) and EPC migratory capacity (*E*). (*F*) Number of Dil-acLDL-labelled monocytic (early) EPCs in implanted matrigel plugs after infusion of 1×10^6 mineralocorticoid receptor-positive or -negative EPCs to hyperaldosteronemic or control mice. (*G*) Integration (white arrow) or adhesion (gray arrow) of infused EPCs into CD31-positive vascular structures in explanted matrigel plugs. Data are mean and SEM.; *Significance against control; $^{\#}$significance against respective Aldo dose without Epl; $^{*,\#}P \leq 0.05$, $^{**,\#\#}P \leq 0.01$, $^{***,\#\#\#}P \leq 0.005$. $n = 4-6$ individual experiments or animals per group.

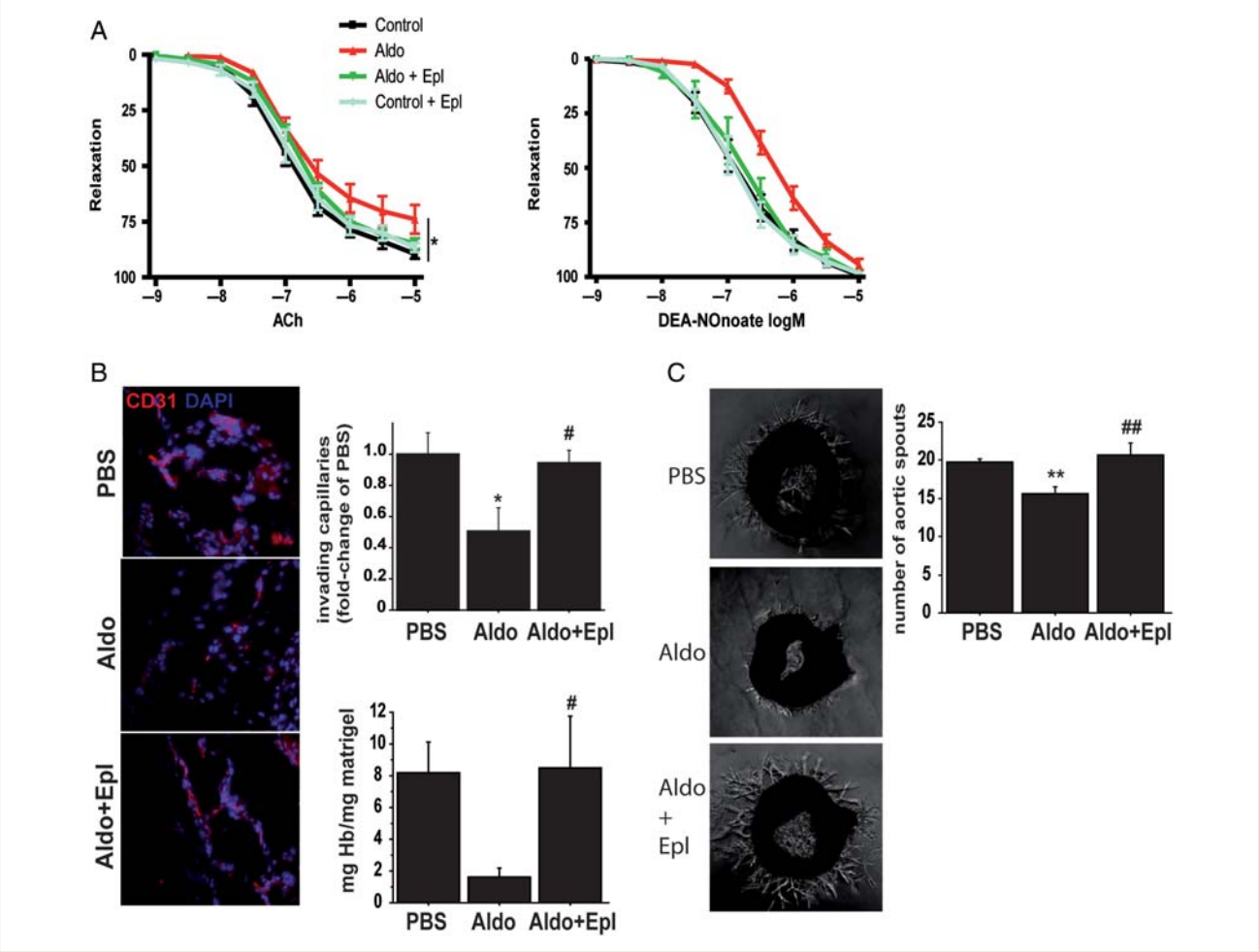

Figure 5 Effects of aldosterone-infusion and mineralocorticoid receptor antagonism on endothelial function and vascularization capacity *in vivo*. (*A*) Endothelium-dependent relaxation induced by acetylcholine (left) and endothelium-independent relaxation induced by 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (right) function measured in aortic ring preparations from mice treated with aldosterone (aldo) or control and/or concomitant eplerenone (Epl) treatment (see Scheme *Figure 4A*). (*B*) *Left* CD31 staining of invading capillaries in matrigel plugs explanted 2 weeks after continuous aldosterone- or placebo infusion to mice. (*right*) Statistical summary of the amount of invading capillaries into matrigel plugs per section (*upper*) and haemoglobin content of matrigel plugs as a further marker of vascularization (*bottom*). (*C*) Number of sproutings from aortic ring preparations explanted from mice treated for 2 weeks with aldosterone or placebo and/or mineralocorticoid receptor antagonism. Data are mean and SEM. *Significance against control; $^{\#}$significance against respective Aldo dose without Epl; $^{*,\#}P \leq 0.05$, $^{**,\#\#}P \leq 0.01$. $n = 5-6$ individual experiments or animals per group.

Primary hyperaldosteronism (PHA) patients additionally displayed signs of endothelial dysfunction, such as reduced reactive hyperaemia index and increased arterial stiffness based on PAT analysis (*Figure 6D–F*). Pharmacological MR blockade of PHA patients did not significantly alter reactive hyperaemia index but normalized increased arterial stiffness thus indicating improvement of endothelial biology (*Figure 6G* and *H*).

Discussion

The current study identified aldosterone to induce oxidative stress and dysfunction of EPCs in a PKA-dependent manner. Mice infused with aldosterone and patients with PHA displayed EPC functional impairment, vascularization defects, and endothelial dysfunction. Pharmacological inhibition or genetic ablation of the MR but not blood pressure normalization by beta-blocker treatment prevented EPC dysfunction in mice and patients with PHA.

Endothelial progenitor cells emerged as a surrogate biologic marker for vascular function and cumulative cardiovascular risk.[5,9,10] Mechanistically, various types of EPC contribute to neovascularization and overall endothelial homeostasis. Indeed, neoangiogenesis is significantly suppressed when transplanted EPC are depleted by activation of an inducible suicide gene,[44] whereas high numbers of circulating EPC seem to protect cardiovascular diseased patients from future cardiovascular events.[9] However, next to EPC number, their functional capacity may be of additional and even greater importance for the biological outcome. For instance, selective impairment of progenitor cell function contributes to an

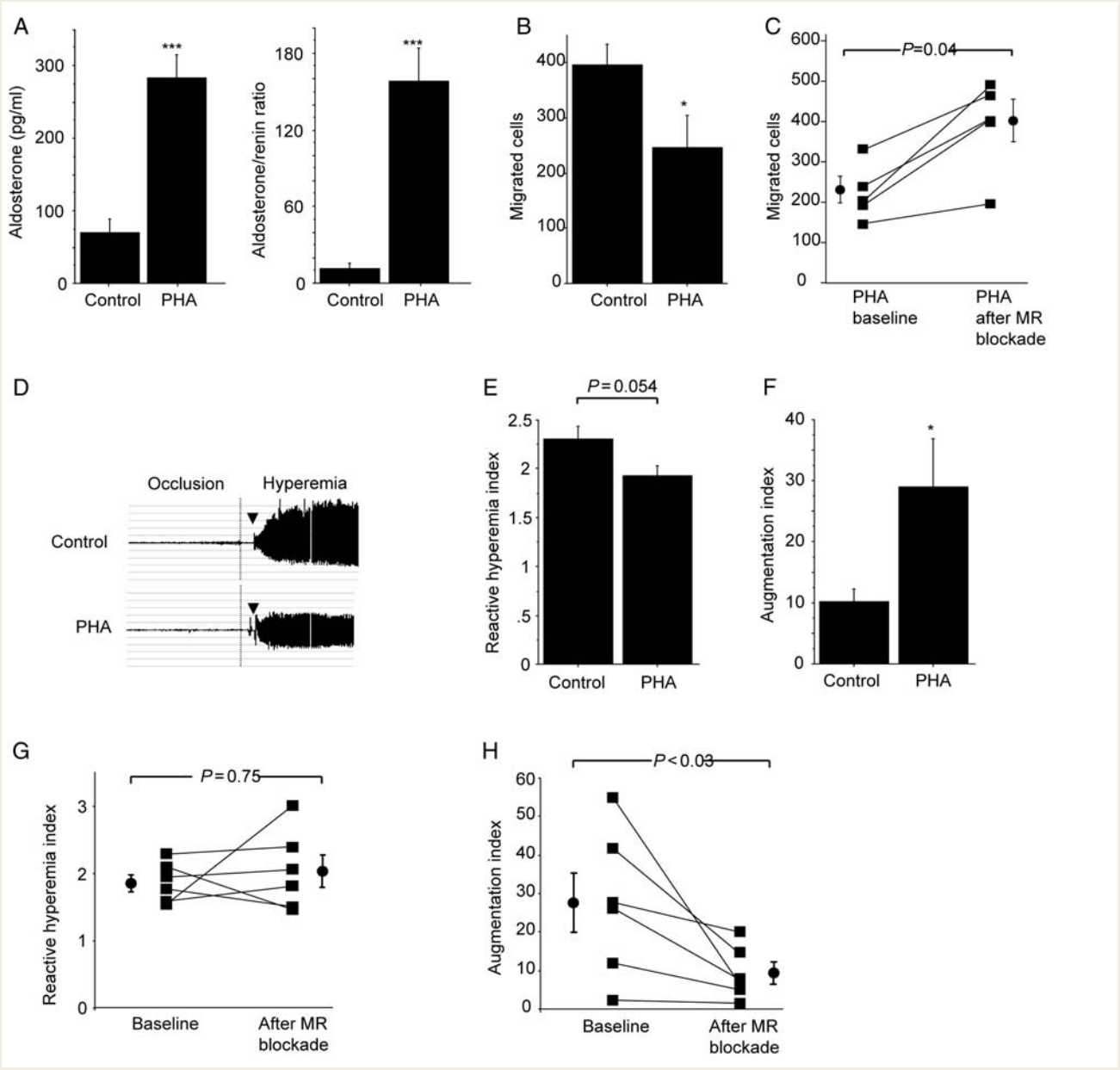

Figure 6 Endothelial progenitor cells (EPCs) and endothelial function in patients with primary hyperaldosteronism (PHA) and controls. (*A*) Aldosterone plasma levels (left) and aldosterone/renin ratio (right) in patients with PHA and controls. Migratory capacity of monocytic (early) EPCs isolated from controls and patients with PHA (*B*) before and after pharmacologic mineralocorticoid receptor (MR) antagonism (*C*). Determination of endothelial function by reactive hyperaemia ratio (*D*, *E*) and the augmentation index (*F*) by a peripheral arterial tonometry method based on a plethysmographic device (EndoPAT2000) in patients with PHA and controls. For patient characterization and *n*-values see *Table 1*. Note, that only a subgroup of five to six patients with primary hyperaldosteronism was treated with spironolactone (C, G, H). Effects of MR blockade with spironolactone on the reactive hyperaemia (G) and augmentation index (H) in patients with PHA. Data are mean and SEM. $^{*}P \leq 0.05$, $^{***}P \leq 0.005$.

unfavourable left ventricular (LV) remodelling process[45] and fosters vascular remodelling due to chronic impairment of endothelial maintenance.[46] A previous study did not find differences of EPC proliferation and cell-cycle changes in patients with PHA.[47] However, other functional end points were not investigated and control subjects had about three-fold higher plasma aldosterone levels than the controls in our study. Here, we did not identify changes of circulating EPC number in an animal model of hyperaldosteronism or in a clinical trial with patients suffering from PHA compared with controls of the same age. In strong contrast, high aldosterone levels severely impaired multiple functions of EPC such as differentiation, proliferation, migration, and subsequently vascularization capacity. Thus, aldosterone selectively led to *qualitative* rather than *quantitative* impairment of circulating EPC.

Endothelial progenitor cells express a variety of receptors, which makes them responsive to changes of many growth factors and hormones, such as oestrogen[3] or insulin-like growth factor-1.[11] Here we show that various subtypes of EPC express a functional MR that translocated to the nucleus upon aldosterone challenge leading to production of oxidative stress. Increased intracellular ROS results in EPC dysfunction and impaired neovascularization capacity.[6,12] This explains at least in part the functional impairment of monocytic (early) EPC in conditions with high aldosterone concentrations. We show that pharmacologic blockade and genetic ablation of the MR in EPCs prevents aldosterone-mediated oxidative stress end cellular dysfunction. Mechanistically, aldosterone inhibits the formation of bone marrow-derived progenitor cells by attenuating VEGFR-2, but effects on circulating EPCs were not investigated.[48] Aldosterone also impairs vascular reactivity by decreasing glucose-6-phosphate dehydrogenase (G6PD) expression and activity in mature endothelial cells.[18] We did not find changes in G6PD expression in monocytic EPCs by aldosterone treatment, suggesting that this mechanism is not involved in aldosterone-mediated EPC dysfunction (data not shown). Protein kinase C has been described to be involved in vascular oxidative stress.[49] We found that in EPCs aldosterone-induced oxidative stress by PKA rather than PKC-dependent mechanisms, which is line with findings that PKA inhibition reduces ischaemia/reperfusion injury of myocardial tissue.[50] The PKA inhibitor H-89 prevented both aldosterone-mediated ROS formation as well as EPC functional defects. Interestingly, paracrine activity appeared unaffected by aldosterone challenge.

Impairment of EPC function was also observed in mice infused with aldosterone for 14 days, which resulted in an overall reduced vascularization capacity in different models, e.g. shown by impaired endothelial invasion of implanted matrigel plugs and attenuated endothelial cell sprouting capacity from vascular segments. The inhibitory effects of aldosterone on monocytic (early) EPC function and vascularization capacity were independent of the increase in blood pressure, as treatment with beta-blocker normalized blood pressure but did not rescue the aldosterone-mediated pathological effects. Our findings fit well with a report that aldosterone infusion exerts a detrimental vascular inflammatory phenotype with destruction of endothelial cells in myocardial tissue.[51] However, it should be noted that under certain conditions such as oxygen-induced retinopathy aldosterone may also exert proangiogenic effects and stimulates pathological angiogenesis in the retina.[52] We show that pharmacological blockade of the MR normalized attenuated vascularization capacity in a mouse model of hyperaldosteronism. Moreover, genetic knockdown of the MR prevented aldosterone-mediated impaired homing of monocytic EPC to vascular structures in an *in vivo* matrigel plug assay. Our findings thus provide at least in part an explanation for the findings that MR blockade improves neovascularization and cardiac function after experimental myocardial infarction[38] and leads to an improved clinical outcome in patients with cardiac ischaemia and/or heart failure.[29,30,53,54]

The observations of the present study are also of clinical importance for patients with PHA, which suffer from higher indices of cardiovascular damage and face an increased risk of cardiovascular events than controls of the same age, sex, and blood pressure status.[25,55] Those patients display an increased media-to-lumen ratio and administration of MR antagonists is associated with amelioration of vascular remodelling.[27,56] We selected well-characterized patients with PHA for functional analysis of EPC and endothelial function, although patients with secondary hyperaldosteronism, e.g. after myocardial infarction or heart failure are more common. However, the latter groups and the underlying causes of hyperaldosteronism are rather heterogeneous and therefore those patients were not within the scope of the present investigation. We observed impaired cellular function of circulating monocytic (early) EPC and endothelial dysfunction in patients with PHA. Treatment with MR antagonists improved EPC and normalized increased vascular stiffness, a major factor determining increased systolic blood pressure and an independent marker of cardiovascular risk.[57] Long-term effects of MR blockade (selective vs. unselective MR blockers) on EPC biology and endothelial function in patients with PHA remain to be investigated.

The current study identifies aldosterone as an important negative regulator of EPC function. Correction of aldosterone-mediated impairment in EPC function by MR blockade may favour endothelial regeneration at sites of tissue damage and thus vascular homeostasis and endothelial function. Further prospective studies are needed that determine the effects of MR blockers as a novel therapeutic strategy against vascular disorders with impaired function of EPC.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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