Renovascular hypertension by two-kidney one-clip enhances endothelial progenitor cell mobilization in a p47phox-dependent manner
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\textbf{Background} Enhanced mechanical forces, e.g. in arterial hypertension, stimulate the formation of reactive oxygen species (ROS) by the NAD(P)H oxidase. Since bone marrow derived endothelial progenitor cells (EPCs) contribute to vascular remodeling and repair, we investigated whether renovascular hypertension stimulates EPC mobilization in a NAD(P)H oxidase-dependent manner.

\textbf{Methods} Renovascular hypertension was induced by two-kidney one-clip (2K1C) in C57BL/6 (WT) and in mice lacking the p47phox subunit of the NAD(P)H oxidase (p47phox\textsuperscript{−/−}).

\textbf{Results} In WT, 2K1C increased blood pressure levels by 32.4 \pm 4 mmHg, which was associated with a four-fold increase in circulating EPCs (Sca-1\textsuperscript{+};Flk-1\textsuperscript{+}). In p47phox\textsuperscript{−/−} mice, the increase in blood pressure was significantly reduced (15.1 \pm 1.8 mmHg, \(P<0.05\)) and not associated with increased EPCs. Inhibitors of the renin–angiotensin system (RAS) and nonspecific vasodilators normalized blood pressure and inhibited EPC mobilization in WT mice after 2K1C. In addition, p47phox deficiency and pharmacological ROS blockage abrogated 2K1C-induced blood pressure elevation and EPC mobilization. Stromal cell derived factor (SDF)-1 and matrix metalloproteinase (MMP)-9 activity in the bone marrow, required for EPC mobilization, were modulated in WT mice but not when stimulated with plasma from 2K1C p47phox\textsuperscript{−/−} mice.

\textbf{Conclusion} Enhanced mechanical stretch in renovascular hypertension induces EPC mobilization in a p47phox\textsuperscript{−/−}-dependent manner, involving bone marrow SDF-1 and MMP-9 which may contribute to compensatory vascular adaptation in renovascular hypertension. J Hypertens 26:257–268 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

\textbf{Keywords}: endothelial progenitor cell mobilization, mechanical stretch, NAD(P)H oxidase, p47phox, renovascular hypertension

\textbf{Abbreviations}: AngII, Angiotensin II; ACE, Angiotensin-converting Enzyme; BMMNC, Bone Marrow Mononuclear Cells; DHE, Dihydroethidium; EPC, Endothelial Progenitor Cells; EPO, Erythropoietin; ECM, Extracellular Matrix; MMP, Matrix Metalloproteinase; MCP-1, Monocyte Chemotaxis Protein; NO, Nitric Oxide; ROS, Radical Oxygen Species; RAS, Renin Angiotensin System; SMC, Smooth Muscle Cell; 2K1C, Two-Kidney One-Clip

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See editorial commentary on page 188

\textbf{Introduction} Under physiological conditions, the arterial wall is exposed to continuous mechanical forces induced by the rhythmical pulse wave of the bloodstream. A balanced yin–yan interplay of functional and structural adaptation mechanisms, e.g. extracellular matrix (ECM) turnover, nitric oxide (NO) production and smooth muscle cell (SMC) contraction, preserves the mature vessel function and morphology [1]. In contrast, in arterial hypertension excessive mechanical shear and longitudinal forces overcome this physiological balance and promote vascular remodeling, repair and maladaptive processes which subsequently contribute to the clinical manifestation of arterial hypertension [2–4].

Central to many of these processes is the activation of the renin–angiotensin system (RAS); regional synthesis and release of its effector peptide angiotensin II (Ang II), especially, contribute to the increase in systemic blood pressure [5]. Both Ang II and mechanical stretch have been shown to activate the vascular NAD(P)H oxidase [3,6] involved in vascular signal transduction and cell proliferation, as well as in the synthesis and secretion of cytokines, growth factors and ECM [7]. More recently, we reported that the vascular NAD(P)H oxidase is responsible for Ang II and mechanical stretch-induced expression and activity of matrix metalloproteinase (MMP)-2 [8,9].
In addition, enhanced mechanical forces of the vessel wall promote vascular maladaptation remodeling, leading to a diminished vessel wall compliance and elevated vessel stiffness [1]. Moreover, mechanical forces contribute to a reduction in the peripheral microvasculature [10], a vicious circle which may be compensated by neovascularization. Recent evidence indicates that bone marrow derived endothelial progenitor cells (EPCs) contribute to neovascularization and vascular repair under ischemic/hypoxic conditions [11,12]. We recently reported that enhanced mechanical stretch in vitro and in vivo induces the vascular expression of the pro-angiogenic factor CCN1 [13]. Potential cross-talk between the vessel wall and the bone marrow in hypertension remains to be elucidated.

Here we investigate whether renovascular hypertension in the two-kidney one-clip (2K1C) high renin hypertension model may stimulate bone marrow cell mobilization as a potential compensatory vascular adaptive mechanism [14]. To determine the role of NAD(P)H oxidase in the process of EPC mobilization from the bone marrow, we used mice lacking the cytosolic NAD(P)H oxidase subunit p47phox, known to be critically involved in the activation of the oxidase and subsequently in the production of superoxide anions in vitro and in vivo [15–17].

**Materials and methods**

**Mice and reagents**

Male C57BL/6 wild-type mice (WT) were obtained from Jackson Laboratories (Bar Harbor, Massachusetts, USA). Mice lacking the p47phox subunit of the NAD(P)H oxidase were a generous gift from Steven Holland [18] and were backcrossed for 10 generations with C57BL/6 mice. Animals were housed under Specific Pathogen Free conditions, and controlled temperature and light exposure, and fed with a normal rodent diet and water *ad libitum*. Angiotensin II (Ang II, Sigma, Taufkirchen, Germany) in increasing dosages of 10⁻², 10⁻⁵ and 3 x 10⁻⁴ mol/l [19–21] was administered daily by intraperitoneal injection in WT and p47phox−/− mice for 5 days. In further experiments, Ang II-injected WT mice were simultaneously treated with the vasodilator hydralazine, with a daily dose of 50 mg/kg [22] by gavage. Subcutaneous injection of lenogastrim (hrG-CSF) at 300 μg/kg/day (Chugai Pharmaceutical, Tokyo, Japan) was used as a positive control for progenitor cell mobilization [23].

**Blood pressure measurement**

Systolic blood pressure was measured by tail-cuff plethysmography (BP-2000 system, VisiTech Systems, Apex, NC, USA). Mice were adapted to this procedure over a 7-day period prior to the 2K1C experiments and systolic blood pressure was further recorded over up to 21 days after 2K1C.

**Two-kidney one-clip hypertension (2K1C)**

Renovascular hypertension was induced in male WT and p47phox−/− mice (10 weeks old, weight 20–22 g) [24]. First, mice were anesthetized with ketamine (400 mg/kg) and xylazine (5 mg/kg) intraperitoneally (i.p.). In rapid succession, the abdominal wall was opened by an incision along the mid line and the left kidney was exposed. A short segment of the left renal artery was isolated by blunt dissection and a standard silver clip (120 μm gap) was placed around the renal artery [14]. The kidney was returned to the retroperitoneal cavity and muscle and skin layers of the abdominal wall were sutured. Sham procedures were performed as described above except clipping of the renal artery. In additional experiments, the NAD(P)H oxidase inhibitor apocynin (4-hydroxy-3-ethoxyacetophenone; Sigma) and the radical scavenger tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, Fluka/Sigma, Taufkirchen, Germany) were administered daily by i.p. injection at dosages of 10 mg/kg [25] and 250 mg/kg [26], respectively, starting 1 day before the induction of renovascular hypertension by 2K1C. In another set of experiments, the angiotensin-converting enzyme (ACE) inhibitor quinapril (Pfizer, Karlsruhe, Germany), the angiotensin (AT₁)-receptor antagonist telmisartan (Boehringer Ingelheim, Germany) and hydralazine were administered daily by gavage at dosages of 10 mg/kg [27], 10 mg/kg [27] and 50 mg/kg [22], respectively, starting the day of the induction of renovascular hypertension by 2K1C. All procedures were approved by the local animal care committee (02/568).

**Plasma renin activity**

Plasma renin activities from WT and p47phox−/− mice were measured by radioimmunoassay for the end product angiotensin I using the commercially available Gamma-Coat Plasma Renin Activity iodine-125 radioimmunoassay kit (Diasorin, Dietzenbach, Germany). In brief, the test is based on the estimation of the renin-dependent rate of angiotensin I formation under optimal conditions, which is subsequently measured by a radioimmunoassay.

**Flow cytometry analysis**

Peripheral blood and bone marrow cells were analyzed in WT and p47phox−/− mice as described previously [23,28]. Peripheral blood samples were obtained from heart puncture directly before mice were sacrificed; 500 μl of blood was subjected to erythrocyte lysis and kept on ice-cold phosphate-buffered saline (PBS, pH 7.4) containing 2% fetal calf serum (FCS) for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated Sca-1 and phycoerythrin (PE)-conjugated Flk-1 antibodies or their corresponding Ig-specific isotype control antibodies (BD Biosciences, San Jose, California, USA). Bone marrow cells were extracted from femurs and tibias. Briefly, cells were harvested by flushing bones with ice-cold PBS containing 5% FCS. After dispersion, cells were passed through a 60 μm nylon mesh, washed twice with ice-cold
5% FCS–PBS and immediately double stained with FITC-conjugated Sca-1 and allophycocyanin (APC)-conjugated c-Kit antibodies or matched isotype control (BD Biosciences). Cell samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software (BD Biosciences). Sca-1+/FlK-1+ cells and Sca-1+, c-Kit+ cells were evaluated in the lymphocyte/monocyte population and expressed as a percentage of gated cells.

**Measurement of vascular superoxide production**

Superoxide production was measured in aortic samples using two approaches, i.e. dihydroethidium (DHE) fluorescence staining and lucigenin-enhanced chemiluminescence. DHE fluorescence staining was described previously [29]. Briefly, 30 μm frozen sections were obtained from cryo-embedded thoracic aortas from WT and p47phox−/− mice, and 6–8 sectioned aortic rings per mouse were placed on glass slides with 2 μmol/l DHE in Krebs/HEPES buffer and incubated at 37°C for at least 30 min in the dark. Tissue sections were visualized with a confocal microscope (Leica TCS SP2) using identical acquisition parameters and processed with image processing software (ImageJ, National Institutes of Health, Bethesda, Maryland, USA). Vascular superoxide production is presented as the percentage of DHE positive vessel area per total vessel area. In an additional approach, vascular superoxide was determined by lucigenin-enhanced chemiluminescence as described previously [30]. Briefly, intact aortic tissues (2–4 mm segments) were suspended in Krebs/HEPES buffer and transferred to a scintillation counter (LS 6500, Beckman Instruments Inc., Fullerton, California, USA) for measurement of lucigenin (5 μmol/l)-enhanced chemiluminescence. Vascular superoxide production is expressed as recorded photon counts per μg of protein.

**Gelatine zymography**

Gelatinolytic activity of bone marrow samples was evaluated by zymography 7 days after 2K1C, as reported recently [8]. Therefore, 5 μg of protein extracts were mixed with nonreducing loading buffer (Roti-Load2, Roth, Karlsruhe, Germany) in a ratio of 4:1 and loaded on to a 10% SDS-PAGE gel containing 1 mg/ml of gelatine (Sigma). Gels were rePRECATED. Unauthorized reproduction of this article is prohibited.
Germany) or medium without serum were used as positive and negative controls, respectively. After 6 h the number of migrated cells was determined using a hemocytometer.

**Statistical analysis**

Data are given as mean ± SEM. All analyses were performed using Student's t-test and analysis of variance (ANOVA). P values <0.05 were considered statistically significant.

**Results**

**Blood pressure elevation and plasma renin activity are reduced in p47phox−/− mice after 2K1C hypertension**

Renovascular hypertension in WT and p47phox−/− mice was induced by the 2K1C model [14]. We observed a strong increase in absolute blood pressure in WT mice, starting at day 3 after 2K1C and persisting up to day 21, compared to sham (131.1 ± 2.5 versus 104 ± 2.5 mmHg, P < 0.01) (supplementary Fig. 1). As reported previously, p47phox−/− mice exhibited higher basal blood pressure levels [27]; however, the increase in absolute blood pressure following 2K1C was less pronounced in these animals compared to sham (141.7 ± 2.0 mmHg versus 129.0 ± 2.9 mmHg, P < 0.05) (supplementary Fig. 1). Accordingly, blood pressure elevation in p47phox−/− mice after 2K1C hypertension was significantly reduced compared to WT mice, as presented in delta values (15.1 ± 1.8 mmHg versus 32.4 ± 4, P < 0.05) (Fig. 1a). Since 2K1C is a high renin model of hypertension we evaluated the plasma renin activity. In accordance with our recent findings [27] p47phox−/− mice exhibited somewhat higher plasma renin activity compared to WT mice after sham operation (4.3 ± 0.6 ng/ml per h versus 3.0 ± 0.3 ng/ml per h, P < 0.05); however, 2K1C hypertension clearly enhanced plasma renin activity in WT mice compared to sham operation (6.9 ± 0.7 ng/ml per h versus 3.0 ± 0.3 ng/ml per h, P < 0.01) which was completely suppressed in p47phox−/− mice (Fig. 1b).

**2K1C hypertension enhances endothelial progenitor cell mobilization in wild-type but not in p47phox−/− mice**

EPCs positive for Sca-1;Flk-1 in the peripheral blood were determined by flow cytometry in WT and p47phox−/− mice following 2K1C hypertension and sham operation. In WT mice, 2K1C hypertension significantly enhanced circulating Sca-1-;Flk-1- cells from 0.4 ± 0.1% at baseline to 1.2 ± 0.3% at day 3, with a peak of 2.1 ± 0.7% at day 7 (P < 0.05 versus sham). Circulating Sca-1-;Flk-1- cells returned to baseline levels after 21 days. In contrast, the increase in circulating Sca-1-;Flk-1- cells was blunted in p47phox−/− mice following 2K1C hypertension (Fig. 2a). Sham operation procedure did not affect Sca-1-;Flk-1- cells in either, WT or p47phox−/− mice. Since EPCs derive predominantly from bone marrow stem cells, we investigated the Sca-1-;c-Kit+ cell population in the bone marrow of WT and p47phox−/− mice 7 days after the induction of 2K1C hypertension. In WT mice, we observed a significant increase in bone marrow Sca-1-;c-Kit+ cells compared to sham controls (3.6 ± 0.2% versus 1.6 ± 0.1%; P < 0.05) which was completely blocked in p47phox−/− mice (1.7 ± 0.2%...
versus 1.8 ± 0.1%) (Fig. 2b). Renovascular hypertension induced by 2K1C operation is a high renin model due to an activated RAS [31], therefore we next investigated whether exogenous Ang II enhances circulating Sca-1+/Flk-1+ cells in a dose-dependent manner. Increasing Ang II concentrations of 10⁻⁷, 10⁻⁵ and 3 × 10⁻⁴ mol/l [19–21] were applied intraperitoneally for 5 days. The highest dose of Ang II caused a blood pressure elevation in WT mice which was completely blunted in p47phox⁻/⁻ mice (27.0 ± 4.2 mmHg versus 4.1 ± 6.7 mmHg, P < 0.05) (supplementary Fig. 2, Fig. 2c). Importantly, Ang II at this dosage increased Sca-1+/Flk-1+ cells in the peripheral blood of WT mice, which was again blunted in p47phox⁻/⁻ mice (1.06 ± 0.17% versus 0.4 ± 0.08%, P < 0.05) (Fig. 2d). Of interest, G-CSF used as a positive control mobilized EPCs in WT and in p47phox⁻/⁻ mice. To further clarify the role of the RAS in enhanced EPC mobilization in renovascular hypertension, WT mice were treated with the angiotensin-converting enzyme (ACE) inhibitor quinapril and the Ang II type 1 (AT₁) receptor antagonist telmisartan for 7 days after 2K1C. Renovascular hypertension was normalized by both
Increased blood pressure and progenitor cell mobilization after two-kidney one-clip (2K1C) hypertension and angiotensin II (Ang II) administration is suppressed by quinapril, telmisartan and hydralazine. (a) Systolic blood pressure was assessed by tail-cuff measurements in wild-type (WT) mice with and without quinapril, telmisartan and hydralazine administration 7 days after 2K1C and sham operation. Changes in blood pressure are presented as delta values and expressed as mean ± SEM (n = 5). (b) Endothelial progenitor cells (Sca-1⁺;Flk-1⁺) in peripheral blood were determined by FACS analysis from WT mice with and without quinapril, telmisartan and hydralazine administration 7 days after 2K1C and sham operation. Results are presented as percentage of gated cells and are expressed as mean ± SEM (n = 5). (c) Systolic blood pressure was assessed by tail-cuff measurements in WT mice with and without hydralazine administration 7 days after application of Ang II. Changes in blood pressure are presented as delta values and expressed as mean ± SEM (n = 5). (d) Endothelial progenitor cells (Sca-1⁺;Flk-1⁺) in peripheral blood were determined by FACS analysis from WT mice with and without hydralazine administration 7 days after application of Ang II. Results are presented as percentage of gated cells and are expressed as mean ± SEM (n = 5).

- *P < 0.05 WT 2K1C versus WT sham
- #P < 0.05 WT 2K1C + quinapril versus WT 2K1C
- §P < 0.05 WT 2K1C + telmisartan versus WT 2K1C
- &P < 0.05 WT 2K1C + hydralazine versus WT 2K1C
- %P < 0.05 WT Ang II versus WT control
- yP < 0.05 WT Ang II + hydralazine versus WT Ang II

Quinapril (6.8 ± 1.2 mmHg versus 35.0 ± 7.2 mmHg, P < 0.05) and telmisartan (−9.2 ± 3.0 mmHg versus 35.0 ± 7.2 mmHg, P < 0.05) (supplementary Fig. 3, Fig. 3a) significantly reduced Sca-1⁺;Flk-1⁺ cells after 7 days of 2K1C as compared to 2K1C hypertension alone (0.5 ± 0.1% and 0.6 ± 0.1% versus 1.5 ± 0.4%, P < 0.05) (Fig. 3b). In order to evaluate the impact of vascular mechanical stretch on 2K1C-induced EPC mobilization, we additionally treated WT mice with the RAS-independent nonspecific vasodilator hydralazine for 7 days after 2K1C. Notably, hydralazine not only normalized blood pressure (3.4 ± 3.5 mmHg versus 35.0 ± 7.2 mmHg, P < 0.05) (supplementary Fig. 3, Fig. 3a) but also reduced the mobilization of EPC into the peripheral blood at day 7 of 2K1C (0.49 ± 0.06% versus 1.5 ± 0.4%, P < 0.05) (Fig. 3b). Moreover, administration of hydralazine in WT mice normalized Ang II-induced blood pressure (−8.2 ± 4.3 versus 27 ± 4.1 mmHg, P < 0.05) (Fig. 3c) and significantly reduced Ang II-induced mobilization of Sca-1⁺;Flk-1⁺ cells into the peripheral blood (0.6 ± 0.2% versus 1.1 ± 0.2%, P < 0.05) (Fig. 3d).
Endothelial progenitor cell mobilization after 2K1C hypertension is reactive oxygen species dependent

To verify the role of oxidative stress in the mobilization of EPC after 2K1C hypertension, we first measured superoxide levels in aortic rings from WT and p47phox−/− mice 7 days after the induction of 2K1C hypertension by DHE staining. Two-kidney one-clip hypertension significantly enhanced superoxide production in WT aorta as compared to sham controls (38.5 ± 4.6% versus 20.7 ± 6.7%, P < 0.05). In contrast, 2K1C hypertension failed to increase aortic superoxide levels in p47phox−/− mice (22.8 ± 5.1% versus 18.6 ± 5.0%, P < 0.05) (Fig. 4a), which could be confirmed by lucigenin-enhanced chemiluminescence (supplementary Fig. 4a).

To further analyze the role of oxidative stress in 2K1C-induced EPC mobilization, we treated WT mice with the general radical scavenger tempol and the specific NAD(P)H oxidase inhibitor apocynin for 7 days after 2K1C hypertension. Like p47phox−/− deficiency, tempol and apocynin administration completely inhibited 2K1C-induced aortic superoxide production (17.7 ± 5.4% and 22.0 ± 4.3% versus 38.5 ± 4.6%, P < 0.05) (Fig. 4a). 2K1C-induced blood pressure elevation (12.3 ± 3.0 mmHg and 1.9 ± 0.7 mmHg versus 27.3 ± 5.8 mmHg, P < 0.05) (supplementary Fig. 4b, Fig. 4b) 2K1C-induced mobilization of Sca-1+/c-Kit+ cells into the peripheral blood (0.6 ± 0.1% and 0.5 ± 0.1% versus 1.5 ± 0.3%, P < 0.05) (Fig. 4c) and the increase of Sca-1+/c-Kit+ cells in the bone marrow (1.5 ± 0.4% and 1.4 ± 0.3% versus 2.8 ± 0.4%, P < 0.05) (Fig. 4d).

2K1C hypertension alters bone marrow microenvironment in a p47phox−/−-dependent manner

Previous observations indicated that mobilization of bone marrow derived cells into the peripheral blood depends on the generation of local cytokine gradients, such as SDF-1, and migration of cells along this gradient [32]. To analyze the role of 2K1C hypertension in the modulation of cytokines involved in bone marrow cell mobilization, we investigated SDF-1 levels in the bone marrow and in the plasma of WT and p47phox−/−/− mice. Two-kidney one-clip hypertension significantly decreased SDF-1 levels in bone marrow protein extracts in WT mice after 7 days compared to the sham group (0.55 ± 0.1 pg/μg versus 1.27 ± 0.09 pg/μg; P < 0.05). This decrease was not present in p47phox−/−/− mice. In contrast, we observed reduced SDF-1 levels in the sham group which were rather induced after 2K1C (0.4 ± 0.09 pg/μg versus 0.2 ± 0.03 pg/μg; P < 0.05), whereas circulating SDF-1 levels in the plasma of WT and p47phox−/−/− mice were not significantly different (Fig. 5a).

In this context of interest, bone marrow SDF-1 is degraded by MMP-9 which, in turn, allows the release of progenitor cells from the bone marrow [33]. In addition, MMP-9 is known to be involved in the cleavage of progenitor cells from stromal cells required for their mobilization [34]. In order to evaluate this mechanism, MMP-9 in the bone marrow of WT and p47phox−/−/− mice after 2K1C was investigated by zymography. In WT mice, gelatinolytic MMP-9 activity was significantly enhanced at day 7 as compared to sham controls (1.95 ± 0.1-fold, P < 0.05), which was completely blunted in p47phox−/−/− mice (0.86 ± 0.4-fold, P < 0.05) (Fig. 5b). Furthermore, we investigated the relative concentration of cytokines known to be involved in progenitor cell mobilization. Two-kidney one-clip hypertension significantly induced the secretion of M-CSF, IL-1, IL-3, IL-2, thrompoietin, TNF-α and MCP-1 into the plasma in WT mice after 1 week of 2K1C, which was reduced in p47phox−/−/− mice (Table 1). Finally, to analyze the impact of 2K1C hypertension on the migratory capacity of bone marrow cells, Lin− BMNMC cells were isolated from bone marrow of WT mice and subjected to plasma isolated from WT and p47phox−/−/− mice after 7 days of 2K1C hypertension. We observed a significant increase in the number of migrated Lin− BMNMC with the plasma of WT mice following 2K1C hypertension as compared to plasma of WT sham mice (30 857 ± 4152 versus 18 000 ± 2274; P < 0.05) (Fig. 5c). In contrast, plasma of p47phox−/−/− mice after 2K1C hypertension was not able to induce migration of Lin− BMNMC compared to plasma isolated from sham-operated p47phox−/−/− mice (10 500 ± 2727 versus 10 000 ± 9591, P > 0.05). SDF-1 was used as a positive control for migration of bone marrow derived cells.

Discussion

In summary, we demonstrate here that 2K1C-induced blood pressure elevation is associated with the mobilization of EPCs from the bone marrow to the peripheral blood and depends on the NAD(P)H oxidase subunit p47phox. Impaired EPC mobilization in p47phox−/−/− mice is correlated with altered bone marrow SDF-1 and MMP-9 levels, suggesting a critical role for the NAD(P)H oxidase in renovascular hypertension-induced EPC mobilization. Moreover, these results imply cross-talk between the vessel wall and the bone marrow in renovascular hypertension.

Chronically altered mechanical forces in arterial hypertension lead to marked alterations in vascular structure and function by promoting ECM production, cell proliferation of endothelial cells and smooth muscle cells [35]. Increased ROS production via vascular NAD(P)H oxidase plays a central role in the modulation of this pathological process [36]. Vascular cells express components of this multisubunit enzyme, including its cell membrane-associated subunits p2p91phox (Nox2) or Nox2 homologues Nox1 and Nox4, and cytosolic subunits, p47phox, p67phox and p40phox [37,38]. The functional significance and individual role varies in a cell type-specific manner and has not yet been fully elucidated.
Studies from several groups have demonstrated an essential role for p47\textsuperscript{phox} in the activation of the vascular NAD(P)H oxidase [15,16,39]. In this regard, we were just able to demonstrate the pivotal role of p47\textsuperscript{phox} for NAD(D)P oxidase activation for left ventricle remodeling/dysfunction and survival after myocardial infarction [40]. Of interest, we recently observed that p47 phox\textsuperscript{-/-} mice exhibited elevated blood pressure levels at baseline,
Bone marrow levels of stromal cell derived factor (SDF-1) and matrix metalloproteinase-9 (MMP-9) and migration of bone marrow cells are altered in \( p47^{phox-/-} \) mice after two-kidney one-clip (2K1C) hypertension. (a) SDF-1 levels are downregulated in the bone marrow after 2K1C hypertension in wild-type (WT) but not in \( p47^{phox-/-} \) mice. Bone marrow and plasma levels of SDF-1 were determined by enzyme-linked immunosorbent assay (ELISA) in WT and \( p47^{phox-/-} \) mice 7 days after 2K1C and sham operation. Results are presented as pg/mg protein and ng/ml, respectively, and expressed as mean ± SEM (\( n=6 \)). (b) The increase in gelatinolytic MMP-9 activity after 2K1C hypertension is blocked in \( p47^{phox-/-} \) mice. MMP-9 activity was analyzed by gelatine zymography in WT and \( p47^{phox-/-} \) mice 7 days after 2K1C and sham operation. Results are expressed as x-fold increase ± SEM (\( n=5 \)). (c) Migration of Lin- bone marrow mononuclear cells (BMMNC) is enhanced by plasma from WT but not by plasma from \( p47^{phox-/-} \) mice after 2K1C hypertension. Migration capacity of BMMNC in response to the plasma from WT and \( p47^{phox-/-} \) mice 7 days after 2K1C and sham operation was determined using transwell cell culture inserts. SDF-1 was used as the positive control. Results are presented as migrated cells per well and expressed as mean ± SEM (\( n=3 \)). *\( P<0.05 \) WT 2K1C versus WT sham; #\( P<0.05 \) \( p47^{phox-/-} \) 2K1C versus WT 2K1C; §\( P<0.05 \) WT SDF-1 versus WT control.
which could not be normalized by antioxidant treatment, pointing to a ROS-independent mechanism [27]. We and others reported that Ang II exerts its effects on SMCs via intracellular signal transduction events critically involving ROS generated by a p47phox-containing NAD(P)H oxidase [9,41,42]. Interestingly, Ang II has been shown to trigger ROS production via vascular NAD(P)H oxidase, which may contribute to functional alterations in hypertension [36] and markedly attenuated blood pressure response to Ang II in p47phox−/− mice [30]. In this regard, the 2K1C model is an established high renin model of renovascular hypertension with increased RAS activity and consequently enhanced levels of circulating Ang II [31] which, in turn, increases total peripheral resistance and raises mean arterial blood pressure. Here we demonstrate that WT mice subjected to 2K1C showed a robust increase in blood pressure of approximately 35 mmHg. Interestingly, in the present study blood pressure elevation was significantly lower in p47phox−/− mice, probably by an impaired responsiveness to Ang II. This observation was further supported by the findings that high-dosed Ang II administration failed to enhance blood pressure levels in p47phox−/− mice. Our observations suggest that blood pressure elevation after 2K1C is—at least in part—under the control of the NAD(P)H oxidase.

Moreover, hypertension is accompanied by enhanced mechanical forces of the vessel wall, not only promoting vascular remodeling but also increasing vascular resistance which, in turn, leads to hypoxia in most of the organ systems [10,35]. Recent observations indicated that bone marrow-derived EPCs play a crucial role in tissue regeneration after vascular injury and hypoxia [12,43–45]. Here we hypothesized that 2K1C hypertension enhances the mobilization of EPCs from the bone marrow to the peripheral blood in a NAD(P)H oxidase-dependent manner in order to initiate vascular adaptation and remodeling. In fact, we observed an early induction of circulating Sca-1+;Flk-1+ EPCs and, in parallel, an increase in the Sca-1−;c-Kit+ progenitor cell population in the bone marrow after 2K1C in WT mice. In contrast, EPC levels were not enhanced in p47phox−/− mice following 2K1C. Importantly, p47phox deficiency abolished not only 2K1C-induced aortic superoxide production but also blood pressure elevation and EPC mobilization. In addition, and to confirm the observations found in the genetic p47phox−/− deficiency mouse model, we used the general radical scavenger tempol and the NAD(P)H oxidase specific inhibitor apocynin in WT mice subjected to 2K1C. Both inhibitors were able to block increased blood pressure and EPC mobilization, further demonstrating the importance of NADP(H) oxidase-derived ROS in renovascular hypertension-induced EPC mobilization.

Mobilization of EPCs requires the migration, maturation, and redistribution of progenitor cells from the bone marrow into the peripheral blood, and constitutes a critical step in the initiation of potential regenerative processes after vascular injury. Several factors such as G-CSF, VEGF, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) or erythropoietin (EPO) have been shown to strongly act on EPC mobilization and are correlated with improved neovascularization and reendothelialization after vascular injury [11,23,28]. We report here that 2K1C hypertension induced EPC mobilization under the control of the NAD(P)H oxidase. Blood pressure elevation and EPC mobilization could be blocked by ACE inhibition or AT1-receptor inhibition, confirming the impact of the RAS. Of interest, the RAS-independent nonspecific vasodilator hydralazine likewise inhibited both 2K1C- and Ang II-induced blood pressure elevation and EPC mobilization, suggesting that enhanced mechanical stretch of the vessel wall is crucially involved in EPC mobilization after 2K1C. Of interest, hydralazine is also known to inhibit vascular superoxide production by the NAD(P)H oxidase [46]. Thus, EPC mobilization in renovascular hypertension seems to be regulated in a tied interplay of mechanical stretch, the RAS and NAD(P)H oxidase (Fig. 6).

At the cellular level, EPC mobilization is mainly directed by a dynamic process taking place inside the bone marrow microenvironment involving disruption of cell-to-cell and cell-to-matrix interactions by proteolytic enzymes (e.g. MMPs), as well as the establishment of chemokine gradients which attract progenitor cells into the peripheral circulation [33,34]. In this regard, SDF-1 plays a critical role in progenitor cell mobilization under vascular stress and injury [44,45]. Here we observe a strong decrease in bone marrow SDF-1 levels in WT mice correlated with the maximum peak of EPC mobilization at day 7 of 2K1C hypertension. The fact that we only observed an increase in SDF-1 in bone marrow samples of WT mice may be explained by the short half-life of SDF-1 and a dilution effect in the peripheral

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Induction of cytokines after 7 days of two-kidney one-clip (2K1C) determined in the plasma from wild-type (WT) and p47phox−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine</td>
<td>x-fold induction versus sham</td>
</tr>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>IL-1</td>
<td>2.0</td>
</tr>
<tr>
<td>IL-2</td>
<td>2.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.6</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.4</td>
</tr>
<tr>
<td>M-CSF</td>
<td>2.7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.8</td>
</tr>
<tr>
<td>Thrombopoietin</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Plasma samples (n = 5) from WT and p47phox−/− mice 7 days after 2K1C and sham operation were pooled and subjected to a cytokine protein array. The relative plasma concentration was determined densitometrically and given as x-fold induction versus sham. IL, interleukin; MCP-1, monocyte chemotaxis protein; M-CSF, macrophage colony stimulating factor; TNF-α, tumor necrosis factor-α.
circulation. In addition, we observed markedly lower SDF-1 levels in the bone marrow of sham-operated p47<sup>phox</sup>−/− mice as compared to WT mice. These results point to p47<sup>phox</sup> as a crucial regulator of bone marrow SDF-1 synthesis and suggest that p47<sup>phox</sup> deficiency is responsible for the inability to create a SDF-1 gradient required for the mobilization of EPC in 2K1C hypertension.

In addition to the required chemokine gradient, proteolytic cleavage of precursor cells is critically involved in the migration and mobilization of EPC from the bone marrow [47]. MMP activity is known to be important for the cleavage of progenitor cells, especially MMP-9 [34]. In this regard, we recently demonstrated that MMP-2 expression and activity in vascular cells depend on NAD(P)H oxidase-derived superoxide formation [8]. Here we demonstrate that the gelatinolytic activity of MMP-9 was significantly increased in bone marrow samples isolated from WT mice after 7 days of 2K1C hypertension, whereas this increase was completely blunted in bone marrow samples from 2K1C p47<sup>phox</sup>−/− mice. Together these observations point to the NAD(P)H oxidase system as an essential regulator for EPC mobilization in renovascular hypertension. This hypothesis is supported by the observation that plasma from 2K1C WT mice but not plasma from 2K1C p47<sup>phox</sup>−/− mice enhanced migration of WT bone marrow Lin<sup>+</sup> cells.

In summary, we here demonstrate that enhanced mechanical stretch in renovascular hypertension enhances EPC mobilization, which involves bone marrow SDF-1 synthesis and MMP-9 activation in a p47<sup>phox</sup>-dependent manner. Moreover, our observations suggest a cross-talk between the vessel wall and the bone marrow under the control of the NAD(P)H oxidase, which may contribute to vessel regeneration and adaptation in renovascular hypertension.

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References

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