Endothelin 1 Activation of Endothelin A Receptor/NADPH Oxidase Pathway and Diminished Antioxidants Critically Contribute to Endothelial Progenitor Cell Reduction and Dysfunction in Salt-Sensitive Hypertension

Dan-Dan Chen, Yu-Gang Dong, Hong Yuan, Alex F. Chen

Abstract—Circulating endothelial progenitor cells (EPCs) are reduced in hypertension, which inversely correlates with its mortality. Deoxycorticosterone acetate (DOCA)-salt hypertension features elevated endothelin (ET) 1 and oxidative stress. We tested the hypothesis that ET-1 induces EPC dysfunction by elevating oxidative stress through the ETₐ/NADPH oxidase pathway in salt-sensitive hypertension. Both ETₐ and ETₐ receptors were expressed in EPCs, but only ETₐ receptors were significantly increased in EPCs of DOCA-salt rats. EPC number and function were reduced in DOCA-salt rats compared with sham controls, and both were reversed by in vivo blockade of ETₐ receptors or NADPH oxidase. The enzymatic activities of NAPDH oxidase and its subunits gp91phox, p22phox, and Rac1 were augmented in EPCs of DOCA-salt rats, with concomitantly decreased antioxidant enzymes manganese superoxide dismutase, copper-zinc superoxide dismutase, and glutathione peroxidase 1. Reactive oxygen species level was elevated in EPCs from DOCA-salt rats, accompanied by increased EPC telomerase inactivation, senescence, and apoptosis, which were rescued by ETₐ or NADPH oxidase blockade. Cell therapy of normal or treated DOCA EPCs, but not untreated DOCA EPCs, significantly increased capillary density and blood perfusion in ischemic hindlimbs of DOCA-salt rats. p53 and Bax/Bcl-2 ratios were increased in EPCs of DOCA-salt rats, which were reversed by ETₐ antagonist, NADPH oxidase inhibitor, or polyethylene glycol-superoxide dismutase. Finally, in ETₐ-deficient rats, plasma ET-1 was elevated, and EPC number and telomerase activity were diminished. These results demonstrate, for the first time, that both ET-1 activation of ETₐ/NADPH oxidase pathway and diminished antioxidants critically contribute to EPC reduction and dysfunction via increased oxidative stress in salt-sensitive hypertension. (Hypertension. 2012;59:1037-1043.) ● Online Data Supplement

Key Words: endothelin 1 ■ oxidative stress ■ NADPH oxidase ■ endothelial progenitor cell ■ hypertension

Endothelial dysfunction contributes to the pathogenesis and progression of hypertension and is an independent predictor of cardiovascular risk.1 The balance between endothelial injury and recovery is important to reduce cardiovascular incidences.1,2 Endothelial progenitor cells (EPCs) can differentiate into mature endothelial cells and regenerate the injured endothelium.2,3 There has been accumulating evidence for reduced availability and impaired function of EPCs in the presence of cardiovascular risks.3,5 Among them, hypertension is a strong predictor of EPC migratory impairment.5,6 Both clinical and animal studies have indicated that hypertension is inversely correlated with EPC reduction and/or dysfunction.7,8 However, investigations of in vivo EPC biology under hypertensive condition are limited, and the mechanisms underlying EPC dysfunction in hypertension remain poorly understood.

Deoxycorticosterone acetate (DOCA)–salt hypertension exhibits low renin, salt-sensitive but elevated arterial endothelin (ET) 1 and oxidative stress,10 due to vascular NADPH oxidase activation and superoxide formation via the ETₐ receptors,10 resulting in endothelial dysfunction.10 Elevated arterial ET-1 levels in DOCA-salt rats led to NADPH oxidase activation and superoxide formation via the ETₐ receptors,10 resulting in endothelial dysfunction. In contrast, ETₐ receptors may protect against vascular injuries in this setting.11 Circulating EPCs are important backups for endothelium integrity and function. In this study, we tested the hypothesis that ET-1 activation of ETₐ/NADPH oxidase pathway and diminished antioxidants both critically contribute to EPC dysfunction in DOCA-salt hypertension. Our findings may provide a mechanistic basis for restoring EPC number and function to combat endothelial dysfunction in hypertension.
Methods

All of the methods and data analysis are described in the online-only Data Supplement.

Results

Characterization of Bone Marrow–Derived EPCs
Bone marrow–derived EPCs used in the present study were characterized by flow cytometry, Dil-acLDL/lectin double staining and Western blot, which were presented in the online-only Data Supplement (Tables S1 and S2 and Figure S1).

ET<sub>A</sub> and ET<sub>B</sub> Receptor Expression in EPCs
The mRNA levels of ET<sub>A</sub> and ET<sub>B</sub> receptors were detected in normal EPCs (1.00±0.28 versus 1.27±0.68; P=0.732; n=4–6), which were further confirmed by immunohistochemistry (Figure S2). Furthermore, the expression of ET<sub>A</sub> receptors in EPCs of DOCA-salt rats was significantly increased compared with sham controls, whereas there were no significant differences in the expressions of ET<sub>B</sub> receptors in EPCs between DOCA-salt and sham rats (Figure 1).

Activation of NADPH Oxidase and Increased Reactive Oxygen Species in EPCs of DOCA-Salt Rats
Rac1, gp91<sup>phox</sup>, and p22<sup>phox</sup> proteins were significantly increased in EPCs of DOCA-salt rats compared with sham controls (Figure S3A). Consistently, NADPH oxidase activity was significantly increased in EPCs from DOCA-salt rats compared with sham rats. In vivo treatment with ETA antagonist (ABT-627) or NADPH oxidase inhibitor (Apocynin) blunted the activation of NADPH oxidase (Figure 2A). When EPCs of DOCA-salt rats were transfected with the dominant-negative Rac1 (DNRac1), which inhibits the key NADPH oxidase subunit Rac1, their NADPH activities were significantly decreased. The same effect was not observed in β-galactosidase–transfected EPCs (Figure 2A). Meanwhile, the intracellular reactive oxygen species (ROS) in DOCA-derived CD34<sup>+</sup>/Flk-1<sup>+</sup> progenitor cells was significantly elevated compared with that in sham rat

![Figure 1. Expressions of endothelin (ET) receptors in endothelial progenitor cells (EPCs). A, The expressions of ET<sub>A</sub> and ET<sub>B</sub> receptors in sham and deoxycorticosterone acetate (DOCA) rat cultured bone marrow–derived EPCs. B, Quantitative analysis of band density. Values were expressed as mean±SEM, which were normalized to sham rats (n=5 to 6). *P<0.05 vs sham rats (by a Student 2-tailed unpaired t test). The n values shown represented individual rats. □, sham; ■, DOCA.](http://hyper.ahajournals.org/lookup/figure/10.1161/HYPERTENSIONAHA.111.192830)

![Figure 2. Activation of NADPH oxidase and increased reactive oxygen species (ROS) in endothelial progenitor cells (EPCs) of deoxycorticosterone acetate (DOCA)-salt rats. A, NADPH oxidase activity in EPCs of DOCA-salt rats was significantly increased, which was inhibited by 4-week treatment with an endothelin (ET)<sub>A</sub> antagonist (ABT-627, 5 mg · kg<sup>-1</sup> · d<sup>-1</sup>) or NADPH oxidase inhibitor (Apocynin [APO] 1.5 mmol/L). Dominant-negative Rac1 (DNRAc1) blunted NADPH oxidase activity in EPCs of DOCA-salt rats. EPCs were transfected with adenoviral (Ad) DNRAc1 or Adβ-galactosidase (gal) at a titer of 500 multiplicity of infection (MOI) for 24 hours. NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay. The enzyme activity was expressed as relative light units (RLUs) per microgram of protein. Values were expressed as mean±SEM, which was normalized to sham rats (n=5 to 6). *P<0.05 vs sham rats; #P<0.05 vs DOCA rats or the β-gal+DOCA group. The n values shown represented individual rats. β-Gal indicates β-gal as a reporter gene; DNRAc1, DNRAc1 inhibiting the endogenous NADPH oxidase subunit Rac1. B, Mean fluorescence of dichlorofluorescein (DCF) in freshly isolated circulating CD34<sup>+</sup>/Flk-1<sup>+</sup> progenitor cells by flow cytometry. CM-H<sub>2</sub>DCFDA, a membrane-permeable probe, enters the cells and produces a green fluorescent signal (DCF fluorescence) after intracellular oxidation by ROS. Values were normalized to sham rats and expressed as mean±SEM (n=6 to 8). *P<0.05 vs sham rats; #P<0.05 vs DOCA rats. All of the data were analyzed by 1-way ANOVA with Bonferroni posttest.)
cells, which was blunted after 4 weeks of treatment with ABT-627 or Apocynin (Figure 2B).

**Antioxidant Enzyme in the EPCs of DOCA-Salt Rats**
The expressions of major antioxidant enzymes were measured in EPCs from sham and DOCA-salt rats. Except for catalase, the expressions of manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), and glutathione peroxidase 1 (GPx-1) in EPCs from DOCA-salt rats were significantly decreased when compared with those from sham rats. In vivo blockade of ABT-627 or Apocynin for 4 weeks significantly reversed the expressions of the decreased antioxidant (Figure 3).

**EPC Dysfunctions in DOCA-Salt Rats**
The tube formation capacity of EPCs was significantly impaired in EPCs from DOCA-salt rats compared with sham controls. In vivo treatment with ABT-627 or Apocynin significantly preserved EPC tube formation capacity (Figure 4). In addition, the adhesion activity of EPCs from DOCA-salt rats was decreased by 50% to 60% compared with sham controls, which was reversed by in vivo blockade of ETA receptors (n=4–8; P<0.05) but not by Apocynin (n=3 in Apocynin group and n=5 in DOCA group; P=0.7234).

**Increased EPC Apoptosis and Senescence in DOCA-Salt Rats**
Both apoptosis and senescence were significantly increased in EPCs from DOCA-salt rats, which were prevented in rats treated with ABT-627 or Apocynin (Figure 5A and 5B). The expressions of apoptosis-related proteins were detected in EPCs. p53 expression in EPCs from DOCA-salt rats was significantly increased and accompanied by upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2 as compared with sham controls. The ratio of Bax/Bcl-2 was significantly increased by ~2-fold in the EPCs of DOCA-salt rats. The above effects were reversed by chronic blockades of ETA receptors or NADPH oxidase (Figure 5C and Figure S3C and S3D). The ROS scavenger, polyethylene glycol-superoxide dismutase (PEG-SOD, 100 U/mL, 24 hours) can reverse these effects (Figure S3B). Consistently, the telomerase activity in the EPCs of DOCA-salt rats was decreased by ~70% compared with sham rats, which was rescued after in vivo treatment with ABT-627 or Apocynin for 4 weeks (Figure 5D).

**EPC Therapy Restored Impaired Angiogenesis in DOCA-Salt Hypertensive Rats**
Serial blood flow measurements by laser Doppler showed that limb perfusion recovery was severely delayed and impaired in DOCA-salt rats compared with sham rats (Figure 6A). The ratio of perfusion in ischemia relative to that in nonischemic hindlimb was 0.18±0.04 for DOCA rats versus 0.29±0.02 for sham rats at day 7 and 0.27±0.06% versus 0.62±0.05% at day 14, respectively (Figure 6B). To determine the effects of EPCs on blood flow repercussions after the DOCA-salt regiment, 1×10⁷ EPCs were injected intramus-
intramuscularly into the anterior tibial muscle. Some BrdU–5-bromodeoxyuridine (BrdU)–labeled EPCs were injected into the vessels to participate in angiogenesis in hindlimb ischemia, to investigate whether implanted EPCs incorporate into existing vessels to improve blood flow perfusion in DOCA-salt ischemia limbs from day 7 until day 14 (0.51 ± 0.03 versus 0.27 ± 0.06). Implantation with EPCs from ABT-627-treated DOCA rats or EPCs treated with DNRac1 significantly accelerated delayed reperfusion in ischemic hind limbs from day 7 to day 14. In contrast, implantation of DOCA EPCs improved the ischemic limb reperfusion at day 7, but they failed to improve the reperfusion in the DOCA-salt ischemia limb at day 14 (0.33 ± 0.04 versus 0.27 ± 0.06; \( P = 0.849 \); Figure 6B and 6C).

The capillary density of ischemia hindlimb was significantly decreased in DOCA-salt rats compared with sham rats at day 14. EPCs from sham rats significantly increased the capillary number in ischemia hindlimb compared with the untreated DOCA rats (Figure 6D and 6E). Meanwhile, EPCs from ABT-627-treated DOCA rat or EPCs treated with DNRac1 restored capillary density in DOCA rats as well. To investigate whether implanted EPCs incorporate into existing vessels to participate in angiogenesis in hindlimb ischemia, 5-bromodeoxyuridine (BrdU)–labeled EPCs were injected intramuscularly into the anterior tibial muscle. Some BrdU–positive cells (red fluorescence) integrated into the CD31-positive vessels (green fluorescence), whereas the remaining 5-bromodeoxyuridine–positive cells were found in perivascular spaces or the matrix between muscle fibers surrounding the vessels (Figure 4S).

### Effects of ET\(_A\) Receptor Antagonist, NADPH Oxidase Inhibitor, or Diuretic on Blood Pressure and Circulating EPCs in DOCA-Salt Rats

Average systolic blood pressure in DOCA-salt rats began to increase on day 5 after the DOCA regimen compared with sham controls (Figure S5A) accompanied by a significantly elevated level of circulating CD34\(^+\)/Flk-1\(^+\) progenitor cells (Figure S5B). On day 28, average systolic blood pressure was further increased in DOCA-salt rats compared with sham controls (Figure S5A). However, the level of circulating CD34\(^+\)/Flk-1\(^+\) progenitor cells in DOCA-salt rats was reduced by 50% (Figure S5B). In vivo blockade of ET\(_A\) receptors, inhibition of NADPH oxidase, or diuretic (all in drinking water) for 4 weeks significantly lowered blood pressure and reserved circulating EPCs in DOCA-salt rats (Figure SSC and SSD).

### EPC Number and Telomerase Activity in ET\(_B\) Receptor-Deficient Rats

The circulating CD34\(^+\)/Flk-1\(^+\) progenitor cell level was significantly decreased in ET\(_B\) receptor-deficient (ET\(_B^{-/-}\)) rats compared with ET\(_B^{++}\) rats (Figure S6A). The telomerase activity in EPCs was also significantly reduced in ET\(_B^{-/-}\) rats (Figure S6B), paralleled with elevated systolic blood pressure (129±0.9 versus 151±1.2 mmHg; \( n = 6 \); \( P < 0.05 \)) and plasma ET-1 levels (3.61±0.14 versus 5.17±0.26 pg/mL; \( n = 6 \); \( P < 0.05 \)).

### Direct Effects of ET\(_A\) Receptor Antagonist and NADPH Oxidase Inhibitor on EPCs

ET-1–induced EPC reduction and NADPH oxidase activation were reversed by pretreatment with the ABT-627 or Apocynin. Transfection of EPCs with DNRac1, which inhibits the key NADPH oxidase subunit Rac1, blunted ET-1–induced EPC reduction and NADPH oxidase activity (Figure S7A through S7C). Consistently, ROS level and apoptosis in normal EPCs were significantly increased after ET-1 treatment in vitro, which was abolished by pretreatment with ABT-627 or Apocynin (Figure S7D and S7E). Telomerase activity was markedly reduced in ET-1–treated normal EPCs, which were rescued by ABT-627 or Apocynin pretreatment (Figure S7F).

### Discussion

The present study demonstrates for the first time that in DOCA-salt hypertension: (1) both ET\(_A\) and ET\(_B\) receptors are expressed in rat EPCs, and the expression of ET\(_A\) receptors is significantly increased; (2) in vivo EPC angiogenesis is significantly impaired; (3) ET\(_A\)-mediated NADPH oxidase activation leads to decreased EPC number and function; and (4) blockade of the ET\(_A\)/NADPH oxidase pathway rescues EPC number and function.

Seven-day cultured EPCs used in the present study are heterogeneous populations containing progenitor cells with the potency differentiating into endothelial cells, as we have...
shown recently. A number of clinical and animal studies have shown that the number and function of EPCs are decreased in hypertension. We showed that EPCs from DOCA-salt rats failed to promote capillary formation and blood flow recovery, whereas EPCs from DOCA-salt rats with ETA receptors or NADPH oxidase blockade significantly restored peripheral perfusion. Of note, while the capillary density in DOCA-EPC–implanted hindlimbs was enhanced, blood reperfusion was not proportionally improved after DOCA-EPC implantation on day 14, suggesting that the newly formed capillaries on DOCA-EPC implantation are not functional, leading to impaired angiogenesis and reduced reperfusion. We demonstrated previously that EPCs from DOCA-salt mice secrete thrombospondin 1 (a well-documented antiangiogenic factor) inhibiting Matrigel tube formations, which can be reversed by antioxidant treatments. Thus, it is possible that altered EPC paracrine functions impair their ability in angiogenesis in DOCA-salt hypertension.

An important finding in the present study is the presence of ET receptors on rat early cultured EPCs. ET-1, as a major humoral contributor to the development of DOCA-salt hypertension, exerts its biological effects through binding to 2 G protein–coupled membrane receptors in a mature vascular system, namely, ETA and ETB subtypes. Decreased circulating EPC number and telomerase activity were found in ETB receptor deficiency (ETB−/−) rats that possess a phenotype of increased plasma ET-1 levels (because of the lack of ET-1 clearance), increased ROS level, and hypertension. These data indicate that elevated ET-1 in ETB−/− rats may inactivate EPC telomerase activity and reduce EPC number through the unmasked effect on ETA receptors. Our study provides first evidence that both ETA and ETB receptors are expressed in rat EPCs. More importantly, the upregulated expression of ETA receptors, but not ETB receptors, suggests that ETA receptors may be the therapeutic target for EPC dysfunction in salt-sensitive hypertension. It has been reported that protein kinase C induces ETA receptors at a transcriptional level in rat fibroblasts or in cardiac cells from DOCA-salt hypertensive rats. However, whether these possibilities exist in EPCs remain unknown.

NADPH oxidase is a complex enzyme with multiple membrane and cytosolic subunits, and pharmacological interventions are rather limited and often difficult for specific inhibition of the enzyme subunits. To this end, we transfected EPCs with the DNARac1 by adenoviral vectors to abrogate endogenous Rac1 expression, a key GTPase component of the NADPH oxidase complex. Our results show that excessive intracellular ROS in EPCs were dominantly generated by activated NADPH oxidase in DOCA-salt rats. Furthermore, blockades of ETα receptors with ABT-627...
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Figure 6. The effects of endothelial progenitor cell (EPC) therapy on hindlimb ischemia in deoxycorticosterone acetate (DOCA)-salt rats. A, Representative images of hindlimb blood flow analyzed by laser Doppler perfusion imaging 14 days after transplantation. Color-coded images represent blood flow distribution. Low or no perfusion is displayed as dark blue, whereas highest perfusion is displayed as yellow or red. Seven-day cultured bone marrow–derived EPCs were used for cell therapy. Preoperation shows an intact hindlimb blood flow before femoral ligation artery surgery; postoperation, the hindlimb blood flow right after surgery; sham, sham rats; DOCA, DOCA rats; DOCA + Sham-EPCs, DOCA rats treated with EPCs from sham rats; DOCA + DOCA-EPCs, DOCA rats treated with EPCs from DOCA rats; DOCA + DOCA-EPCs; DOCA rats treated with EPCs from DOCA rats; DOCA + ABT-EPCs; DOCA rats treated with EPCs from ABT-627 rats; DOCA + DNRac1-EPCs, DOCA rats treated with DNRac1 EPCs. B and C, Quantitative analysis of blood flow in hindlimb expressed as perfusion ratio of ischemic hindlimb:nonischemic limb. A 2-way repeated-measures ANOVA with Bonferroni posttest was used to analyze blood flow perfusion in hindlimb ischemia models, and a significant overall difference was detected (P < 0.05); n = 5 for each group. The perfusion ratios of ischemic:nonischemic limb of sham and DOCA-salt rats representing the baseline were the same for B and C (P = 0.849). DOCA rats that received sham EPCs showed significant improvement of reperfusion in ischemic limbs compared with that of DOCA rats receiving DOCA EPCs (#P < 0.05 vs DOCA + DOCA-EPCs). D, Representative images of CD31-positive capillaries 14 days after EPC implantation. CD31-positive stainings (red) are identified as capillaries. Sham indicates sham rats; DOCA, DOCA rats; DOCA + Sham-EPCs, DOCA rats treated with EPCs from sham rats; DOCA + DOCA-EPCs, DOCA rats treated with EPCs from DOCA rats; DOCA + ABT-EPCs; DOCA rats treated with EPCs from ABT-627 rats; DOCA + DNRac1-EPCs, DOCA rats treated with DNRac1 EPCs. E, Quantitative analysis of capillary. *P < 0.05 vs sham; #P < 0.05 vs DOCA or DOCA + DOCA-EPCs, by a 1-way ANOVA with Bonferroni posttest, n = 5 each group.

DOCA-salt rats. This evidence from the present study suggests that ET_{a}/NADPH oxidase is a potential pathway involved in EPC dysfunction in DOCA-salt hypertension.

Increased apoptotic and senescence EPCs were observed in DOCA-salt rats, suggesting that some molecules controlling cell survival and cell cycle may be the downstream targets in the ET_{a}/NADPH oxidase pathway. Telomerase protects EPCs from senescence and improves their survival and regenerative properties against excessive ROS, whereas the activity of telomerase in EPCs from DOCA-salt rats was markedly decreased, which partially contributes to impaired EPC angiogenic functions. In addition, the apoptosis-related molecules (p53 and Bax) were upregulated in DOCA EPCs,
which promote EPC apoptosis in this hypertensive setting. Blockades of the ET<sub>A</sub> receptors or NADPH oxidase significantly blunted telomerase activity and inhibited p53 expression and Bax/Bcl-2 ratio. Moreover, PEG-SOD, a membrane-permeable superoxide scavenger, reversed the above changes. Together, oxidative stress induced by ET<sub>A</sub>/NADPH oxidase activation leads to EPC apoptosis and senescence via blunting telomerase activity, downregulated p53 expression, and Bax/Bcl-2 ratio in DOCA-salt hypertension.

Although ET<sub>A</sub> receptor antagonism or NADPH oxidase inhibition could preserve the EPC number and its resistance to oxidative stress in DOCA-salt rats, the question remained regarding their direct antihypertensive effect on EPCs. To address this issue, we treated the DOCA-salt rats with the diuretic trichlormethiazide, a nonselective blood pressure–lowering agent, for 4 weeks and found that trichlormethiazide failed to rescue the circulating EPC number in DOCA-salt rats despite its blood pressure–lowering effect. A recent study also showed that diuretics did not reverse the EPC number in spontaneously hypertensive rats when the blood pressure is reduced. In addition, it is important to note that, although the systolic blood pressure was markedly reduced in DOCA-salt rats after in vivo ET<sub>A</sub> receptor blockade or NADPH oxidase inhibition, it was significantly higher (i.e., ~30 mm Hg) compared with sham rats. In contrast, the number of circulating EPCs in the 2 groups of pharmacological intervention maintained the similar level to that of sham rats. Moreover, inhibition of ET<sub>A</sub> receptors or NADPH oxidase also ameliorated ET-1–induced EPC reduction in vitro, without the compounding effect of the blood pressure. Finally, we found that the circulating EPCs were increased at the beginning of the blood pressure elevation, which suggests that high blood pressure itself may not decrease circulating EPCs in DOCA-salt rats and the elevation in the number of EPCs in the beginning of the DOCA-salt regimen may be attributed to increased compensation of EPC mobilization from the bone marrow. Thus, inhibition of ET-1/ET<sub>A</sub> NADPH oxidase–induced oxidative stress, more than blood pressure reduction, may account for the preservation of circulating EPC number in DOCA-salt rats.

Perspectives

The present study demonstrates, for the first time, that ET-1 activation of ET<sub>A</sub>/NADPH oxidase pathway and diminished antioxidants both critically contribute to EPC reduction and dysfunction via increased oxidative stress in salt-sensitive hypertension. Oxidative stress–induced telomerase inactivation, senescence, and apoptosis may represent important cellular mechanisms underlying ET-1–induced EPC reduction and dysfunction in low-renin, salt-sensitive hypertension. Hypertension with exacerbated systemic oxidative stress impairs EPC function, resulting in loss of its regenerative capacity, with obvious implications for endothelial dysfunction. Our finding on how the ET<sub>A</sub>/NADPH oxidase pathway and diminished antioxidants affect EPC function may provide a mechanistic basis for EPC genetic modification and therapeutic rejuvenation in hypertension.

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Disclosures

None.

References

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Endothelin 1 Activation of Endothelin A Receptor/NADPH Oxidase Pathway and Diminished Antioxidants Critically Contribute to Endothelial Progenitor Cell Reduction and Dysfunction in Salt-Sensitive Hypertension

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Supplemental Methods

All the procedures in this study were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals

All male Sprague-Dawley (SD) rats (250-285 g) were obtained from Charles River Laboratories. Breeding pairs of ETB receptor deficiency (DβH; ETB<sup>al</sup>/al, ETB<sup>−−</sup>) rats and ETB wild-type (DβH; ETB<sup>++</sup>, ETB<sup>++</sup>) rats were a generous gift from Dr. Cheryl E. Gariepy (University of Michigan, Ann Arbor, MI) 5. In the present study, male ETB<sup>−−</sup> and ETB<sup>++</sup> rats (240-280 g) were used. ETB<sup>−−</sup> rats are a novel single-locus genetic model of severe salt-sensitive hypertension, which express ETB receptors in adrenal glands and other adrenergic neurons but not in other tissues, such as the kidney, vascular endothelium, and vascular smooth muscle 5.

DOCA-salt hypertensive rats and in vivo pharmacologic intervention

DOCA-salt hypertension was created in adult male SD rats as we previously described 6-8. From the beginning of DOCA administration, some DOCA-salt rats received 4 weeks of ABT-627 (5 mg·kg<sup>−1</sup>·d<sup>−1</sup> in drinking water, Abbott Laboratories), a selective ET<sub>A</sub> receptor antagonist 9. Some DOCA-salt rats received diuretic trichlormethiazide (TCM, 10 mg·kg<sup>−1</sup>·d<sup>−1</sup> in drinking water, Sigma) 10. Some DOCA-salt rats received Apocynin (1.5 mmol/L in drinking water, Sigma), a NADPH oxidase inhibitor that impedes the assembly of the p47<sup>phox</sup> and p67<sup>phox</sup> within the membrane NADPH oxidase complex 7, 11, 12. Our previous studies have shown that these doses of ABT-627 and Apocynin are effective in blocking the ET<sub>A</sub> receptors and the NADPH oxidase activity in vivo, respectively 8, 9, 11. Average systolic blood pressure (SBP) of Sham and DOCA-salt rats was measured by the non-invasive tail-cuff method in conscious rats6-8. The peripheral blood of some rats was collected when the blood pressure was significantly elevated on Day 5 after DOCA-salt regimen. The peripheral blood and bone marrow of other rats were collected at Day 28 (4 weeks) following DOCA-salt regimen. The blood pressure of ETB<sup>−−</sup> rats was monitored by radiotelemetry as we described previously 13. Briefly, arterial blood pressure was monitored remotely using a commercially available radiotelemetry data acquisition program (Dataquest ART 3.1, Data Sciences International). Data were reported as 24-hour averages.

EPC isolation and characterization

Bone marrow-derived EPCs (BM-EPCs) were isolated from the femur and tibia of rats according to our recent publications 1-4. All assays were preformed after day 7. BM-EPCs were used in EPC function assays, Western blot analysis, NADPH oxidase activity and cell implantation. Freshly isolated MNCs and cultured BM-EPCs were characterized by flow cytometry according to our recent publications 1, 3, 4, 14. To detect CD133, Flk-1, and VE-Cadherin, cells were incubated with rabbit anti-rat CD133 antibodies (Abcam), or rabbit anti-rat Flk-1 antibodies (Abcam), or mouse anti-rat VE-Cadherin antibodies (Santa Cruz) on ice for one hour right after pre-permeabilization with fresh-prepared methanol for 15 minutes. Then, cells were incubated with FITC-conjugated goat anti-rabbit secondary antibodies (Abcam) or PE-conjugated goat anti-mouse secondary antibodies (Santa Cruz) on ice for another one hour. 0.5% rabbit serum or 0.5% mouse serum was used for isotype staining. To detect CD34 or CD45, cells were incubated with mouse anti-rat CD34-PE antibodies (Santa Cruz) or mouse anti-rat CD45-PECy5 antibodies (BD Bioscience) on ice for one hour. Isotype specific conjugated anti-IgG was used as negative control. Quantifications of CD34<sup>+</sup>/Flk-1<sup>+</sup> cells, CD34<sup>+</sup>/CD133<sup>+</sup> cells, CD34<sup>+</sup> cells, CD133<sup>+</sup> cells, Flk-1<sup>+</sup> cells, VE-Cadherin<sup>+</sup> cells, and
CD45+ cells were performed with a BD Vantage Flow Cytometer. Each analysis included at least 10,000 events.

Expressions of ET\textsubscript{A} and ET\textsubscript{B} receptors in EPCs by real-time PCR, immunocytochemistry, and Western blot analysis

ET\textsubscript{A} and ET\textsubscript{B} receptors on EPC were assessed by real-time RT-PCR, immunocytochemistry, and Western blot as described previously\textsuperscript{15}. For all experiments, 18\textsubscript{s} was used as an internal control. The specific primer sequences were as follows: ET\textsubscript{A} receptor, forward: CCTGGCAACCATGAACTCTTGCAT; reverse: TGGACTGGTGACACAACAGCAACAGA; ET\textsubscript{B} receptor, forward: ATTCTGAAGCTCACCCTTTATGAC; reverse: AGAAGCCCATGTGATATCCATGTGA; and 18\textsubscript{s}, forward: GGGCCCGAAGCGTTTACTTTGAA; reverse: ACCCGGGTCCTATTCCATTATCC. Amplification was performed on a 7500 Real-Time PCR system (Applied Biosystems) according to the manufacturer’s instructions. Relative mRNA expressions of the ET\textsubscript{A} and ET\textsubscript{B} receptors were calculated by the comparative C\textsubscript{T} method, normalized to the endogenous 18\textsubscript{s} control, and calculated as a relative expression\textasciitilde2^{-\Delta C_{T}}\textsuperscript{1}.

For immunocytochemistry, cells were fixed with 4\% PFA in PBS for 20 minutes at room temperature\textsuperscript{15}. Antibodies against the ET\textsubscript{A} and ET\textsubscript{B} receptors were from Alomone (Jerusalem, Israel). To verify the specificity of antibody binding, the primary antibody was incubated with the control peptide prior to application to cells. The absence of immunoreactivity confirmed the specificity of antibodies for ET receptors. The images were taken with a Zeiss Pascal confocal microscope at a resolution of 1,024\times1,024 pixels.

Western blots were performed by using primary antibodies directed against ET\textsubscript{A} receptors (1:200 dilution; Almone Labs) and ET\textsubscript{B} receptors (1:200 dilution; Almone Labs). To verify equal protein loading and transfer, the \textbeta-actin (1:10,000 dilution; Sigma) was used as the internal control. Bands were visualized with an Odyssey Imager and quantified with Quantity One software (Bio-Rad)\textsuperscript{15}.

EPC angiogenic function assays and pharmacologic treatments

EPC angiogenic function assays including tube formation capacity by Matrigel assay and adhesion function assay were described in detail in our previous studies\textsuperscript{1,3,4}.

In vitro gene transfer

The propagation, purification, and titration of replication-incompetent adenoviral vectors were prepared as we described\textsuperscript{7,16}. After 7-days in culture, EPCs were transfected with the adenoviral vector encoding dominant-negative Rac1 (DNRac1) that inhibits endogenous NADPH oxidase subunit Rac1\textsuperscript{16} or reporter gene \textbeta-galactosidase (\textbeta-gal) at a titer of 500 multiplicity of infection (MOI) in EGM-2 supplemented with 2\% fetal bovine serum for 24 hours, followed by change of fresh 5\% FBS EGM-2\textsuperscript{1}. After 48 more hours of cultivation, transfected EPCs were subjected to ET-1 treatment as described above\textsuperscript{16}.

Circulating EPC flow cytometry

Circulating EPCs were isolated according to our published methods\textsuperscript{1,3}. Briefly, freshly isolated peripheral blood mononuclear cells (PB-MNCs, 1\times10\textsuperscript{6} cells, Abcam) for one hour on ice, then incubated with a biotin-labeled goat anti-mouse IgG antibody (2.6\mu g/10\textsuperscript{6} cells, Jackson Immuno Research) for 30 minutes, followed by one hour incubation with APC-conjugated streptavidin (20\mu l/10\textsuperscript{6} cells, BD Biosciences) and PE-conjugated CD34 (1\mu g/10\textsuperscript{6} cells, Santa Cruz) on ice\textsuperscript{17}. After washing and centrifugation, the cell pellets were suspended in 500\mu l 5\% BSA-PBS, and co-expressions of CD34 and Flk-1 were determined by flow cytometry.
Intracellular ROS measurement with dichlorofluorescein fluorescence (DCF) by flow cytometry

The intracellular ROS level in circulating EPCs was evaluated by DCF fluorescence using flow cytometry according to the manufacturer’s recommended protocol and previous publications 18, 19. 5-(6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) enters the cells and produces a green fluorescent signal (DCF fluorescence) after intracellular oxidation by ROS. The DCF/CD34+/Flk-1 triple-positive cells were determined by flow cytometry (FACScan, Becton Dickenson) gating 30,000 events. The mean fluorescence of DCF was taken as an indicator of intracellular ROS level in circulating CD34+/Flk-1 progenitor cells.

NADPH oxidase enzymatic activity

NADPH oxidase activity in EPCs was measured by a lucigenin-enhanced chemiluminescence assay, as we described with minor modifications 6, 20, 21. Briefly, the enzyme activity was measured by lucigenin (5×10^-6 mol/L, Sigma) and indicated as the amount of O_2^- levels in the presence of their relative substrate NADPH (1×10^-4 mol/L, Sigma) 6. No enzymatic activity could be detected in the absence of NADPH.

Western blot analyses

Western blots were performed by using primary antibodies directed against Rac1 (1:1,000 dilution; Abcam), gp91^phox (1:1,000 dilution; BD Biosciences), p22^phox (1:500 dilution; Santa Cruz), MnSOD (1:1,000 dilution; BD Biosciences), CuZnSOD (1:1,000 dilution; Abcam), catalase (1:1,000 dilution; BD Biosciences), GPx-1 (1:500 dilution; Santa Cruz), p53 (1:2,500 dilution; Cell Signals), Bcl-2 (1:1,000 dilution; BD Biosciences), and Bax (1:1,000 dilution; BD Biosciences). To verify equal protein loading and transfer, β-actin (1:1,000 dilution; Sigma) was used as the internal control. Bands were visualized with an Odyssey Imager and quantified with Quantity One software (Bio-Rad) 1, 3, 4, 14.

Senescence assay

EPC senescence was determined by acidic β-galactosidase staining kit (Sigma), according to the manufacturer’s recommended protocol and our recent publication 4.

Apoptosis assay

EPC apoptosis was determined by DeadEnd Fluorometric TUNEL System staining (Promega) according to the manufacturer’s instructions 22. Cell nuclei were co-stained with the fluorescent dye Hoechst33528 1. The green-staining nuclei (TUNEL-positive cells) were detected under a fluorescence microscope equipped with a digital camera and the MetaMorph 6.1 image analysis software (Universal Imaging Corporation) 22. TUNEL-positive cells were counted under ten random high power fields (magnifications 200×) of each sample.

Telomerase activity

Telomeric Repeat Amplification Protocol Assay (TRAP Assay) was employed, through which the telomerase reaction product is amplified by PCR 23. Telomerase activity was measured with 2 µg proteins by the Telo TAGGG Telomerase PCR ELISA® plus Kit (Roche, USA) according to the manufacturer’s instructions 23. The absorbance values were determined by using a Micro-plate reader; the absorbance of the samples was measured at 450 nm wavelengths.
Hindlimb ischemia and in vivo EPC therapy

Hindlimb ischemia was carried out in Sham or DOCA-salt rats on Day 14 after DOCA or Sham surgery. Briefly, the right femoral artery and its branches were exposed proximal to the origin of the arterial popliteal, ligated, and the incision was closed in layers. The left femoral artery and its branches were only exposed without dissection, which served as non-ischemia control. DOCA-salt rats were randomly divided into five groups, which received PBS (vehicle) or BrdU-labeled EPC implantation: 1) DOCA rats + PBS, 2) DOCA rats + sham rat-derived EPCs (Sham-EPCs), 3) DOCA rats + DOCA rat-derived EPCs (DOCA-EPCs), 4) DOCA rats + ABT treated DOCA rat-derived EPCs (ABT-EPCs), and 5) DOCA rats + DNRac-1 treated DOCA rat-derived EPCs (DNRac1-EPCs). In the vehicle or EPC implantation groups, 100 μl of PBS or 1×10^7 EPCs in 100μl, was randomly injected intramuscularly into anterior tibial muscle at the six points with a 27-gauge needle 24 hours after femoral artery dissection. Hindlimb blood flow was measured before, right after, 7 days after, and 14 days after right femoral artery ligations. The rats were pre-warmed for 10 minutes, and then placed on 37°C heated pads through the measurement. The hindlimb blood flow was measured by a Laser Doppler Perfusion Imager (LDPI) as described previously. Low or no perfusion is displayed as dark blue, whereas the high perfusion is displayed as yellow or red. The blood flow images were measured with Image-Pro Plug 5.0 and expressed as perfusion ratio of ischemic hindlimb to non-ischemic limb.

Immunohistochemistry and measurement of capillary density

The effect of EPC implantation (or PBS) on neovascularization was assessed under light microscopy by measuring the number of capillaries in sections taken from the ischemic muscles. Tissue specimens were obtained from anterior tibial muscles on day 14. This muscle was chosen because it is one of the principal muscles of the lower limb and is commonly used to evaluate the distal reperfusion after the femoral arteries are excised. The muscle was fixed in 10% Formalin and embedded in paraffin. Sections (5 μm) were de-paraffinized and incubated with a goat anti-human CD31 antibody (1:100 dilution, Santa Cruz). Antibody distribution was visualized with the use of the avidin-biotin-complex technique and Vector Red chromogenic substrate (Vector Laboratories), followed by counterstaining with hematoxylin. Negative controls were performed by avoiding the primary antibodies. Capillaries were identified by positive staining for CD31. Five fields from each sample were randomly selected and photographed with a digital camera (Olympus), and visible capillaries (positive for CD31) were counted. Capillary density was expressed as the number of capillaries per square millimeter. To investigate whether implanted EPCs were incorporated the capillaries, BrdU labeled EPCs were injected intramuscularly into anterior tibial muscle. On Day 14, the anterior tibial muscle was collected and fixed with 10% Formalin. The sections were incubated with BrdU antibody (1:50 dilution, Amersham Biosciences) and CD31 antibody (1:50 dilution, Santa Cruz), followed by secondary antibodies anti-mouse PE and anti-goat FITC, respectively. Implanted EPCs were identified as BrdU positive cells.

Data analysis

All obtained values were expressed as mean ± SEM. A GraphPad Prism (Version 5) was used for data analysis. Statistical analysis between two groups was performed using the Student’s two-tailed unpaired t test. A one-way ANOVA was used in data analysis when more than two groups were compared, followed by the Bonferroni’s procedure to control the Type I error. A two-way Repeated-measures ANOVA followed by the Bonferroni’s post-test was used to analyze blood flow perfusion in hindlimb ischemia models, and a significant overall difference was detected. Specifically, the group of DOCA-salt rats was compared with the Sham group, to determine the effect...
of DOCA-salt treatment on EPC functions and subsequent molecular changes. The groups of ABT-627 + DOCA and Apocynin + DOCA were compared with DOCA-salt rats to determine the effects of the blockade of ET$_A$/NADPH oxidase pathway on EPC functions and subsequent molecular changes. A value of $P<0.05$ was considered as a statistically significant finding.

References


Results

Characterization of bone marrow-derived EPCs

To characterize bone marrow derived EPCs, the stem cell markers (CD34, CD133), endothelial cell markers (Flk-1, VE-cadherin) and a hematopoietic cell marker (CD45) were examined by flow cytometry according to the procedure described in our recent publications 1-4. The cell populations were showed in Supplemental Table 1 and Table 2. These data suggest that EPCs cultured for 7 days (termed as early-cultured EPCs) are heterogeneous in nature, and they contain significantly higher percentage of progenitor cells with endothelial differentiating potential compared with the freshly isolated mononuclear cells. The early-cultured EPCs were also identified by Dil-acLDL/lectin double staining (Supplemental Fig. S3A) and the expressions of endothelial lineage markers between HUVECs and EPCs cultured for 7 days. Similar to HUVECs, early-cultured EPCs expressed endothelial markers including Flk-1, VE-cadherin, and eNOS (Supplemental Fig. S3B).
Supplemental Tables

Table S1. The cell population of freshly isolated mononuclear cells.

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>Sham (n=7)</th>
<th>DOCA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>17.65±4.98</td>
<td>13.84±2.40</td>
</tr>
<tr>
<td>CD133</td>
<td>39.51±7.38</td>
<td>25.71±5.30</td>
</tr>
<tr>
<td>Flk-1</td>
<td>8.98±2.23</td>
<td>5.51±1.72</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>2.84±0.87</td>
<td>2.92±1.60</td>
</tr>
<tr>
<td>CD34/CD133</td>
<td>3.36±0.46</td>
<td>2.60±0.35</td>
</tr>
<tr>
<td>CD34/Flk-1</td>
<td>3.64±0.64</td>
<td>4.03±1.79</td>
</tr>
<tr>
<td>CD45</td>
<td>53.37±3.91</td>
<td>41.97±5.89</td>
</tr>
</tbody>
</table>

MNCs (Day 0): freshly isolated mononuclear cells; EPCs (Day 7): 7-day cultured endothelial progenitor cells. The data were expressed as mean ± SEM, n=7 each group.

Table S2. The cell population of 7-day cultured EPCs.

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>Sham (n=7)</th>
<th>DOCA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>31.91±2.50</td>
<td>24.90±3.04</td>
</tr>
<tr>
<td>CD133</td>
<td>20.50±1.20</td>
<td>14.99±3.52</td>
</tr>
<tr>
<td>Flk-1</td>
<td>19.32±2.91</td>
<td>17.8±3.96</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>10.72±0.35</td>
<td>7.74±1.54</td>
</tr>
<tr>
<td>CD34/CD133</td>
<td>20.02±1.12</td>
<td>13.13±6.80</td>
</tr>
<tr>
<td>CD34/Flk-1</td>
<td>15.9±1.39</td>
<td>12.76±2.43</td>
</tr>
<tr>
<td>CD45</td>
<td>9.98±3.45</td>
<td>13.60±4.15</td>
</tr>
</tbody>
</table>

The data were expressed as mean ± SEM, n=7 each group.
Fig. S1. A. Representative images of Dil-ac-LDL up-taking (red) and lectin binding (green) from circulating EPCs (yellow). The nuclei were stained with Hoechst33258 (blue). After 7 days of cultivation, cells were stained for Dil-ac-LDL uptaking and lectin binding. Dil-ac-LDL/lectin dual positive cells (yellow) were identified as EPCs. Bar=100 μm. B. The expressions of endothelial cell specific genes (VE-cadherin, eNOS, and VEGFR-2) in 7-day cultured EPCs. Human umbilical vein endothelial cells (HUVECs) were used as positive controls.
Fig. S2. Representative immunocytochemical images of ET$_A$ and ET$_B$ receptor staining in normal EPCs. The green fluorescence demonstrated expressions of ET$_A$ and ET$_B$ receptors on normal EPCs, respectively. Bar=10 µm. The second FITC-labeled IgG antibodies were used. To verify the specificity of antibody binding, the primary antibody was incubated with the control peptide prior to application to the cells. The absence of immunoreactivity confirmed the specificity of antibodies for ET receptors.
Fig. S3

A. Rac1
gp91\(^{phox}\)
p22\(^{phox}\)
actin

B. p53
Bax
Bcl-2
actin

Arbitrary unit/actin

Rac1, gp91\(^{phox}\) (Nox2), p22\(^{phox}\)

Sham DOCA DOCA+PEG-SOD

Arbitrary unit/actin

p53, Bax, Bcl-2, Bax/Bcl-2

Sham DOCA DOCA+PEG-SOD
**Fig. S3**  
**A. Expressions of NADPH oxidase subunits Rac1, gp91\(^{\text{phox}}\), and p22\(^{\text{phox}}\).** Western blot analysis was performed in cultured EPCs from Sham and DOCA-salt rats. Values were expressed as mean±SEM, which were normalized to Sham rats, n=5-6, *P<0.05 vs. Sham rats (By a Student’s 2-tailed unpaired t test). The n values shown representative individual rat. **B. The expressions of p53, Bax, and Bcl-2 EPCs of DOCA-salt rats in the presence or absence of PEG-SOD (100 U/ml, 24 hours).** Values were expressed as mean±SEM, which were normalized to Sham controls, n=4-6, *P<0.05 vs. Sham rats (By a Student’s 2-tailed unpaired t test). The n values shown representative individual rat. PEG-SOD: polyethylene glycol-superoxide dismutase, an established membrane-permeable O\(_2^\cdot\) scavenger. Bax/Bcl-2: the ratio of Bax to Bcl-2. **C and D.** The expressions Bax and Bcl-2 in EPCs of DOCA-salt rats with or without chronic treatments of ET\(_A\) receptor blockade (ABT-627, 5 mg·kg\(^{-1}\)·d\(^{-1}\)) or NADPH oxidase inhibitor (Apocynin, APO, 1.5 mmol/L). The n values shown represented individual rat. Values were expressed as mean±SEM,
Fig. S4

Fig. S4 Representative images of EPC incorporation with vascular culture by confocal microscopy. CD31 indicated endothelial cell (green fluorescence), BrdU indicated implanted EPCs (red fluorescence). The arrows point to the positive staining. DAPI indicated nuclear staining (blue fluorescence).
Fig. S5

A. SBP (mmHg)

- Sham (open bars)
- DOCA (filled bars)

Day 1: 100
Day 3: 130
Day 5: 160
Day 28: 190

B. CD34+ Flk-1+ Cells (fold)
in circulation

- Sham (open bars)
- DOCA (filled bars)

Day 5: 2
Day 28: 1

C. SBP (mmHg) Day 28

- Sham
- DOCA
- ABT-627 + DOCA
- APO + DOCA
- TCM + DOCA

D. CD34+ Flk-1+ cells (fold) in circulation

- Sham
- DOCA
- ABT-627 + DOCA
- APO + DOCA
- TCM + DOCA
Fig. S5  A. Time course of systolic blood pressure (SBP) in DOCA-salt rats. Values were expressed as mean±SEM, n=7-10, *P<0.05 vs. Sham rats (By a Student’s 2-tailed unpaired t test).  B. Quantitative analysis of CD34+/Flk-1+ progenitor cell in circulation on Day 5 and on Day 28 after DOCA-salt regimen. Freshly isolated peripheral blood mononuclear cells from DOCA-salt and Sham rats were analyzed for the co-expressions of CD34 and Flk-1 by flow cytometry. Values were normalized to Sham rats and expressed as mean±SEM, n=6-8, *P<0.05 vs. Sham rats (By a Student’s 2-tailed unpaired t test).  C. Effects of ABT-627 (5 mg·kg⁻¹·d⁻¹), apocynin (APO, 1.5 mmol/L) or trichlormethiazide (TCM, 10 mg·kg⁻¹·d⁻¹) on average systolic SBP in DOCA-salt rats. Values were expressed as mean±SEM, n=8-15, *P<0.05 vs. Sham rats, #P<0.05 vs. DOCA rats (By a one-way ANOVA with Bonferroni post-test). ABT-627, a selective ETA antagonist; APO, an inhibitor of NADPH oxidase; TCM (trichlormethiazide), a diuretic.  D. Effects of ABT-627 (5 mg·kg⁻¹·d⁻¹), Apocynin (APO, 1.5 mmol/L) or trichlormethiazide (TCM, 10 mg·kg⁻¹·d⁻¹) on CD34+/Flk-1+ progenitor cell levels after DOCA-salt regimen. Values were normalized to Sham rats and expressed as mean±SEM, n=6-8, *P<0.05 vs. Sham rats, #P<0.05 vs. DOCA rats (By a one-way ANOVA with Bonferroni post-test).
Fig. S6

A. The level of CD34+/Flk-1+ progenitor cells in ETB-/- rats. Freshly isolated peripheral blood mononuclear cells from ETB+/+ and ETB-/- rats were analyzed for the co-expressions of CD34 and Flk-1 by flow cytometry. The CD34+/Flk-1+ progenitor cells were considered as one subpopulation of circulating EPC. Values were normalized to ETB+/+ rats and expressed as mean±SEM, n=5-6, *P<0.05 vs. ETB+/+ rats, (By a Student’s 2-tailed unpaired t test).

B. EPC telomerase activity was measured by the TRAP assay in ETB-/- rats. Seven-day cultured EPCs were used. Values were expressed as mean±SEM, n=6. *P<0.05 vs. ETB+/+ rats, (By a Student’s 2-tailed unpaired t test).

Fig. S6 The circulating progenitor cell number and telomerase activity in ETB receptor-deficient (ETB-/-) rats.
Fig. S7

A. Dil-ac-LDL/lectin cells per hpf (X 400)

B. Relative DCF fluorescence

C. Relative NADPH oxidase activity (RLU/μg)

D. Relative DCF fluorescence

E. Apoptotic cells (fold)

F. Telomerase activity (arbitrary unit)
Fig S7. The effects of ET-1 on EPC number, NADPH oxidase activity, ROS level, telomerase activity and apoptosis in normal cultured EPCs. A. The effect of ET-1 receptor blockade on ET-1 induced EPC reduction. After cultivation, normal EPCs were pretreated with the selective ETA receptor antagonist ABT-627 for 1 hour, and then incubated with ET-1 (1×10^{-8} mol/L) for 48 hours. Values were expressed as mean±SEM, n=4-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only (By a one-way ANOVA with Bonferroni post-test). B. The effect of NADPH oxidase inhibition on ET-1 induced EPC reduction. After cultivation, normal EPCs were transfected with AdDNRac1 or Adβ-gal at a titer of 500 multiplicity of infection (MOI) for 24 hours, or pretreated with NADPH oxidase inhibitor Apocynin for 1 hour, followed by a 48-hour treatment with ET-1. Adherent Dil-ac-LDL/lectin dual positive cells were counted as EPCs. Values were expressed as mean±SEM, n=4-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only or ET-1+ β-gal treatment (By a one-way ANOVA with Bonferroni post-test). C. The effect of ET-1 on NADPH oxidase activity. NADPH oxidase activity of EPCs was measured by a lucigenin-enhanced chemiluminescence assay. The enzyme activity was expressed as relative light units (RLU)/µg protein. Values were expressed as mean±SEM, n=5-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only or ET-1+ β-gal treatment (By a one-way ANOVA with Bonferroni post-test). D. The effect of ET-1 on EPC intracellular ROS. ROS level in EPCs was estimated by DCF microscopy. Values were normalized to control groups and expressed as mean±SEM, n=4-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only (By a one-way ANOVA with Bonferroni post-test). E. The effect of ET-1 on EPC apoptosis. Apoptotic EPCs were determined by TUNEL assay. ET-1 (1×10^{-8} mol/L) treated normal EPCs for 48 hours with or without pre-incubation of ETA receptor antagonist ABT-627 or NADPH oxidase inhibitor Apocynin. Values were normalized to control groups and expressed as mean±SEM, n=4-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only (By a one-way ANOVA with Bonferroni post-test). F. The effect of ET-1 on EPC telomerase activity. Telomerase activity in EPCs was measured by TRAP assay. Values were normalized to control groups and expressed as mean±SEM, n=4-6. *P<0.05 vs. Control, #P<0.05 vs. ET-1 treatment only (By a one-way ANOVA with Bonferroni post-test). β-gal: β-galactosidase; DNRAc1: dominant-negative Rac1; APO: apocynin.
Fig. S8. Schematic illustration of possible mechanisms underlying EPC reduction and dysfunction in DOCA-salt hypertension. Endothelin-1 (ET-1) interacts with increased ET_A receptors on endothelial progenitor cells (EPCs), resulting in NADPH oxidase activation and decreased expressions of antioxidant enzymes including MnSOD, CuZnSOD, and GPx-1, which together contribute to ROS accumulation in EPCs of DOCA-salt rats. The elevated ROS may diminish EPC survival by two mechanisms: 1) increased EPC apoptosis via up-regulations of p53 and Bax/Bcl-2 ratio and 2) increased EPC senescence via decreased telomerase activity. Both mechanisms could consequently lead to EPC reduction and dysfunction.