Abstract—Although hyperaldosteronemia exerts detrimental impacts on vascular endothelium in addition to elevating blood pressure, the effects and molecular mechanisms of hyperaldosteronemia on early endothelial progenitor cell (EPC)–mediated endothelial repair after arterial damage are yet to be determined. The aim of this study was to investigate the endothelial repair capacity of early EPCs from hypertensive patients with primary hyperaldosteronemia (PHA). In vivo endothelial repair capacity of early EPCs from PHAs (n=20), age- and blood pressure–matched essential hypertension patients (n=20), and age-matched healthy subjects (n=20) was evaluated by transplantation into a nude mouse carotid endothelial denudation model. Endothelial function was evaluated by flow-mediated dilation of brachial artery in human subjects. In vivo endothelial repair capacity of early EPCs and flow-mediated dilation were impaired both in PHAs and in essential hypertension patients when compared with age-matched healthy subjects; however, the early EPC in vivo endothelial repair capacity and flow-mediated dilation of PHAs were impaired more severely than essential hypertension patients. Oral spironolactone improved early EPC in vivo endothelial repair capacity and flow-mediated dilation of PHAs. Increased oxidative stress, oxidative 5,6,7,8-tetrahydrobiopterin degradation, endothelial nitric oxide synthase uncoupling and decreased nitric oxide production were found in early EPCs from PHAs. Nicotinamide adenine dinucleotide phosphate oxidase subunit p47phox knockdown or 5,6,7,8-tetrahydrobiopterin supplementation attenuated endothelial nitric oxide synthase uncoupling and enhanced in vivo endothelial repair capacity of early EPCs from PHAs. In conclusion, PHAs exhibited more impaired endothelial repair capacity of early EPCs than did essential hypertension patients independent of blood pressure, which was associated with mineralocorticoid receptor–dependent oxidative stress and subsequently 5,6,7,8-tetrahydrobiopterin degradation and endothelial nitric oxide synthase uncoupling.

Key Words: blood pressure ■ hypertension ■ nitric oxide synthase type III ■ oxidative stress ■ sapropterin

Vascular endothelial injury contributes to the initiation and progression of atherosclerotic vascular disease. It has been demonstrated that early endothelial progenitor cells (EPCs) play a pivotal role in endothelial repair process. However, functional impairment of early EPCs observed in patients with several cardiovascular risk factors leads to decreased endothelium-reparative capacity and increased incidence of atherosclerotic vascular disease. Therefore, understanding novel mechanisms of early EPC dysfunction related to cardiovascular risk factors has important clinical relevance for the prevention and treatment of atherosclerotic vascular disease.

Primary hyperaldosteronemia patients (PHAs) displayed an increased rate of cardiovascular events when compared with essential hypertension patients (EHs) with similar blood pressure (BP) level, suggesting potential pathological effects of hyperaldosteronemia on cardiovascular system in addition to elevation of BP. Indeed, accumulating evidence indicates that hyperaldosteronemia has harmful effects on blood vessels via the direct action of aldosterone independent of rising
Recently, it has been reported that high level of aldosterone inhibited the early EPC formation from bone marrow precursor cells and vascularization capacity, suggesting that hyperaldosteronemia might also influence the early EPC-mediated endogenous repair mechanism apart from the direct detrimental effect on vascular wall. However, the in vivo effect of hyperaldosteronemia on early EPC function, especially the endothelial repair capacity, needed to be further determined. Therefore, in the present study, we compared the early EPC in vivo endothelial repair capacity of PHAs to age- and BP-matched EHs and age-matched healthy subjects (HSs) and analyzed the underlying molecular mechanism.

It has been demonstrated that aldosterone is a strong mediator stimulating oxidative stress in vascular cells. Oxidative stress–led 5,6,7,8-tetrahydrobiopterin (BH4) degradation is the main pathogenic cause of endothelial nitric oxide synthase (eNOS) uncoupling. The eNOS uncoupling has been proved as an important molecular mechanism underlying atherosclerotic vascular disease. On the basis of these data, we hypothesized that oxidative BH4 degradation and eNOS uncoupling may involve in the hyperaldosteronemia-caused alteration of early EPC function. Therefore, in the present study, we attempted to clarify the role of oxidative stress and BH4 bioavailability and eNOS/NO pathway in the regulation of early EPC-mediated endothelial repair in PHAs.

**Methods**

**Study Subjects**

Twenty patients identified with an aldosterone-producing adenoma, thus PHAs based on increased aldosterone/renin ratio (>50) and pathological saline infusion test and imaging test via high-resolution computed tomographic scan were recruited. All the patients were retrospectively defined to aldosterone-producing adenoma by histopathology after surgical treatment. All the antihypertensive drugs were stopped >2 weeks for the diagnostic tests before recruitment. After recruitment, all the PHAs were treated with spironolactone (60–120 mg/d) for 4 to 6 weeks before surgical treatment. Twenty newly diagnosed EHs without pharmacological therapy randomly matched for age and BP to enlisted PHAs were recruited from patients visited in the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). Twenty age-matched HSs were recruited as the controls. The PHAs and EHs were included without other known cardiovascular disease, and the included HSs had no cardiovascular risk factors. All the study subjects were excluded diabetes mellitus, malignant disease, infection, or inflammatory disorders. BP measurements were performed according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. All of the subjects underwent 3 BP measurements in 2 different visits, after 30 minutes of rest, and the measurements were spaced by 5- to 10-minute intervals, on both the left and right arm, in the sitting and lying positions. The clinical characteristics are summarized in Table S1 in the online-only Data Supplement. Our study was confirmed to the ethical principles outlined in the Declaration of Helsinki. The experimental protocol was approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), and written informed consent was obtained from every study participant.

**Statistical Analysis**

All results are expressed as mean±SEM. Comparisons between groups were analyzed by the Student t test. Statistical analysis was performed by a 1-way ANOVA, followed by multiple comparisons using either the Student–Newman–Keuls or Tukey–Kramer test. A value of P<0.05 was considered to denote statistical significance. All statistical analyses used SPSS statistical software (SPSS version 13.0).

Other detailed methods performed in the present study are provided in the online-only Data Supplement.

**Results**

**In Vivo Endothelial Repair Capacity of Early EPCs From PHAs, EHs, and HSs**

In vivo endothelial repair area was evaluated by Evans Blue staining, and the representative photographs are shown in Figure 1B. Transplantation of early EPCs accelerated in vivo endothelial repair of denuded injured carotid artery markedly compared with PBS injection (Figure 1A). In vivo endothelial repair capacity of early EPCs from PHAs and EHs was obviously impaired when compared with HSs (Figure 1A). The in vivo endothelial repair capacity of early EPCs from PHAs was impaired more severely than from EHs (Figure 1A). After oral spironolactone for 4 to 6 weeks, in vivo endothelial repair capacity of early EPCs from PHAs was significantly improved (Figure 1A).

Phycoerythrin-conjugated monoclonal mouse anti-human CD14 antibody-labeled early EPCs were detected homing to the sites of the injured carotid arteries but not in the contralateral uninjured carotid arteries via both the confocal laser scanning microscopy analysis (Figure S2A) and the fluorescence-activated cell sorter analysis (Figure S2B). The cultured human umbilical vein endothelial cells were injected into the tail vein of nude mice with carotid injury as the negative control. After human umbilical vein endothelial cells injection, little phycoerythrin-conjugated monoclonal mouse anti-human CD14 antibody-labeled cells were detected homing to the sites of the injured carotid arteries via the confocal laser scanning microscopy analysis and fluorescence-activated cell sorter analysis (data not shown). Moreover, human umbilical vein endothelial cells injection did not improve endothelial repair (Figure S2C).

In vitro early EPC function, such as migration to vascular endothelial growth factor/stromal cell–derived factor-1 and adhesion to fibronectin in flow, was also evaluated in the present study. As the results shown in Figure S4, in vitro migration and adhesion activity of early EPCs were impaired obviously in PHAs and EHs when compared with HSs, and a further impairment was shown in PHAs compared with EHs. The in vitro migration and adhesion activity of early EPCs in PHAs were obviously promoted after oral spironolactone treatment (Figure S4). The number of circulating EPCs (as assessed by CD34/kinase-insert domain receptor double-positive or CD133/kinase-insert domain receptor double-positive peripheral blood mononuclear cells via fluorescence-activated cell sorter) has no significant difference from PHAs, EHs, and HSs (Figure S5); similar to previous study, data reported here suggested that hyperaldosteronemia leads to qualitative rather than quantitative impairment on circulating EPCs.

**Endothelial Function in PHAs, EHs, and HSs**

Endothelial function expressed as the flow-mediated dilation (FMD) of brachial artery was obviously reduced in PHAs and EHs when compared with HSs (Figure 1C), and FMD...
was reduced more in PHAs than in EHs (Figure 1C). After oral spironolactone for 4 to 6 weeks, the FMD in PHAs was markedly improved (Figure 1C). In vivo endothelial repair capacity of early EPCs was positively related to FMD (Figure 1D).

Increased Oxidative Stress in Early EPCs From PHAs
Oxidative stress level of early EPCs was evaluated by intracellular reactive oxygen species (ROS) production via 2′,7′-dichlorodihydrofluorescein diacetate fluorescence and superoxide production via electron paramagnetic resonance spectroscopy analysis, respectively. Intracellular ROS production (Figure 2A) and superoxide production (Figure 2B and 2C) were obviously increased in early EPCs from PHAs when compared with HSs. After oral spironolactone for 4 to 6 weeks, both the intracellular ROS production (Figure 2A) and the superoxide production (Figure 2B and 2C) were reduced in early EPCs from PHAs.

Increased p47phox Transcription and Translocation to Plasma Membrane in Early EPCs From PHAs
The important nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p47phox transcription was increased compared with HSs (Figure 2D). After oral spironolactone for 4 to 6 weeks, p47phox transcription and translocation of early EPCs from PHAs were obviously inhibited (Figure 2D and 2E). The transcription and expression of NADPH oxidase isoforms (NOX1, NOX2, and NOX4) in early EPCs showed no apparent difference between the HSs and the PHAs (Figure S6); the result was consistent with the in vitro effect of aldosterone on endothelial cells reported by Nagata et al.21 previously.

Decreased NO Generation in Early EPCs From PHAs
NO generation was evaluated both as intracellular cGMP concentration via ELISA system and l-arginine/l-citrulline conversion via high-performance liquid chromatography system analysis. Our data showed that NO generation was obviously decreased in early EPCs from PHAs when compared with HSs (Figure 3A and 3B). After oral spironolactone for 4 to 6 weeks, NO generation was restored in early EPCs from PHAs (Figure 3A and 3B). The representative chromatograms of high-performance liquid chromatography for l-arginine/l-citrulline conversion are shown in Figure 3C.

Oxidative BH₄ Degradation and eNOS Uncoupling in Early EPCs From PHAs
BH₄ plays a crucial role as a cofactor of eNOS for generating NO. In its absence, eNOS produces superoxide rather than
which was reported to mechanistically correlate with EPC dysfunction. Therefore, we detected the intracellular biopterin content via high-performance liquid chromatography system and analyzed the amount of BH$_4$ in early EPCs. There was no obvious difference of the total biopterin content in early EPCs from PHAs and HSs (Figure 4A and 4B). In the early EPCs from PHAs compared with HSs, the BH$_4$ content was substantially reduced, and the oxidized forms of BH$_4$ (BH$_2$ and biopterin) were correspondingly increased (Figure 4A and 4B). After oral spironolactone for 4 to 6 weeks, oxidation of BH$_4$ in early EPCs from PHAs was obviously attenuated and BH$_4$ content was significantly increased (Figure 4A and 4B).

The eNOS uncoupling-dependent impairment of NO production was mechanistically involved in EPC dysfunction, and the decreased eNOS dimer/monomer ratio can be served as a marker of eNOS uncoupling. Therefore, we performed low-temperature SDS-PAGE and immunoblotting to investigate eNOS dimerization. There was no difference in total eNOS expression in early EPCs from PHAs and HSs using conventional SDS-PAGE (Figure 4C), but low-temperature SDS-PAGE showed that the eNOS dimer/monomer ratio was decreased in early EPCs from PHAs compared with HSs (Figure 4C). After oral spironolactone for 4 to 6 weeks, the eNOS dimer/monomer ratio in early EPCs from PHAs was markedly increased (Figure 4C).

**P47$^{phox}$-siRNA Improved In Vivo Endothelial Repair Capacity of Early EPCs From PHAs**

Treatment with p47$^{phox}$-siRNA decreased the transcription and expression of p47$^{phox}$ in early EPCs from PHAs obviously (Figure S7). Knockdown of p47$^{phox}$ via siRNA attenuated...
BH4 Promoted In Vivo Endothelial Repair Capacity of Early EPCs From PHAs

Although BH4 treatment (1 mmol/L) did not change ROS production (data not shown) and oxidative BH4 degradation (Figure 6A) of early EPCs from PHAs, it increased the total biopterin and BH4 content (Figure 6A) of early EPCs from PHAs. Correspondingly, BH4 treatment was able to attenuate eNOS uncoupling (Figure 6B) and increased NO generation (Figure 6C and 6D), as well as promoted in vivo endothelial repair capacity (Figure 6E and 6F) of early EPCs from PHAs. Moreover, the in vitro migration and adhesion activity of early EPCs from PHAs were also enhanced by BH4 treatment (Figure S8).

Discussion

The present study demonstrated first that in vivo endothelial repair capacity of early EPCs was severely impaired in PHAs accompanied with endothelial dysfunction. Increased oxidative stress, oxidative BH4 oxidation, and reduced NO generation were observed to mechanistically correlate with impaired in vivo endothelial repair capacity of early EPCs from PHAs. Oral mineralocorticoid receptor (MR) antagonist spironolactone improved the
early EPC in vivo endothelial repair capacity and endothelial function of PHAs. Knockdown of the NADPH oxidase subunit p47^phox^ via siNRA or BH_4 treatment was able to restore early EPC in vivo endothelial repair capacity of PHAs.

Abnormalities in endothelial structure and function thought as important processes underlying the development of atherosclerotic vascular disease are likely the result of imbalance between endothelial damage and repair. The early EPCs, also termed as monocytic EPCs or circulating angiogenic cells, have been proved homing to injured vascular wall and promoting endothelial repair by direct and indirect mechanism in our and other previous studies. To investigate the homing and endothelial repair capacity of early EPCs derived from clinical subjects in vivo, the athymic nude mice with carotid endothelial denuded injury were introduced to accept human early EPC transplantation in the present study. This nude mouse model can nearly avoid species rejection and satisfy the EPC transplantation research. In the present study, the confocal laser scanning microscopy analysis showed a sub-endothelial homing of intravenously injected early EPCs on the endothelial repair zone of the injured carotid artery, what the result in line with the recent study by Giannotti et al suggested that early EPCs promote the endothelial repair process likely, in particular, by paracrine mechanisms. However, the beneficial effect of early EPCs for endothelial repair was limited when the functional impairment occurred in the presence of some cardiovascular risk factors. Raising BP regardless of whether the cause is essential or secondary to endocrine or renal processes is positively correlated with the incidence of cardiovascular events and leads to endothelial injury. Impaired endothelial repair capacity of early EPCs accompanied by endothelial dysfunction in EHs has been reported previously. The present study provided the first evidence that in vivo endothelial repair capacity of early EPCs was also impaired in secondary hypertensive patients with PHA, suggesting a novel pathological mechanism for

Figure 4. 5,6,7,8-tetrahydrobiopterin (BH_4) level and endothelial nitric oxide synthase (eNOS) dimerization. A. The representative chromatograms of high-performance liquid chromatography (HPLC) for intracellular biopterin measurement in early endothelial progenitor cells (EPCs). B. Intracellular biopterin content was detected by HPLC system in early EPCs from healthy subjects (HSs; n=12) and primary hyperaldosteronemia patients (PHAs; n=10) before or after treatment with spironolactone (Spi). *P<0.01 vs BH_4 content of early EPCs from HSs, **P<0.01 vs oxidized biopterin content of early EPCs from HSs, #P<0.01 vs BH_4 content of early EPCs from PHAs before Spi treatment, ##P<0.01 vs oxidized biopterin content of early EPCs from PHAs before Spi treatment. C. The eNOS dimerization of early EPCs from HSs (n=12) and PHAs (n=10) before or after treatment with Spi was analyzed by low-temperature SDS-PAGE and immunoblotting; quantification expressed as eNOS dimer/monomer rate.
vascular complications caused by hyperaldosteronemia. To further investigate the possible difference between the EHs and the PHAs on the early EPC-based endogenous endothelial repair capacity, we compared the in vivo endothelial repair capacity of early EPCs from PHAs to the age- and BP-matched EHs in the present study. We found that in vivo endothelial repair capacity of early EPCs from EHs was impaired when compared with HSs similar with previous study,7 but the early EPCs from PHAs showed a more severely impaired in vivo endothelial repair capacity than from EHs. Correspondingly, a further reduced FMD was showed in PHAs when compared with age- and BP-matched EHs. These results provided further evidence to prove that impaired early EPC-mediated endogenous endothelial repair capacity contributes to endothelial dysfunction and proposed a novel mechanism to explain the increased rate of cardiovascular events in PHAs when compared with EHs independent of BP.

Increasing evidence indicates that acute and chronic oxidative stress in cardiovascular system is an essential molecular mechanism underlying the development of atherosclerotic vascular disease.26 Previous studies reported that aldosterone treatment in vitro induced ROS production of early EPCs.15,16 In the present study, we observed that the intracellular ROS and superoxide production were obviously increased in early EPCs from PHAs, which further proved that the high level of aldosterone induced oxidative stress of early EPCs in vivo. In endothelial cells, aldosterone-induced oxidative stress increases oxidative degradation of BH₄ that leads to eNOS uncoupling and subsequently decreased NO generation.21 The eNOS uncoupling has also been reported to contribute to EPC functional impairment.22 The oxidative BH₄ degradation and eNOS uncoupling and decreased NO generation were showed in early EPCs from PHAs in the present study as well, suggesting that oxidative BH₄ degradation and derangement of eNOS/NO pathway may be also involved in the functional impairment of early EPCs in PHAs.

One main source of superoxide in vasculature stimulated by aldosterone is the NADPH oxidase.27 Nagata et al21 have recently proved that p47phox subunit of the NADPH oxidase plays an important role in aldosterone-stimulated ROS production in endothelial cells.21 The present study showed an obviously increased p47phox transcription and translocation to the plasma membrane fraction in early EPCs from PHAs, indicating the NADPH oxidase activation. To further investigate the connection between the increased ROS production and the NADPH oxidase activation, we treated early EPCs of PHAs with p47phox-siRNA or NADPH oxidase inhibitor apocynin. It showed clearly that either p47phox-siRNA or apocynin markedly inhibited the oxidative stress of early EPCs from PHAs. In parallel,
BH₄ oxidation and eNOS uncoupling were attenuated, and NO generation was increased in early EPCs from PHAs by p47phox-siRNA. Importantly, p47 phox-siRNA restored in vivo endothelial repair capacity of early EPCs from PHAs. The eNOS was also recognized to be a source of superoxide with the BH₄ deficiency. To identify the source of superoxide production in early EPCs from PHAs, the early EPCs of PHAs were treated with eNOS inhibitor L-nitro-arginine methyl ester. The data showed that L-nitro-arginine methyl ester treatment had little effect to inhibit superoxide production of early EPCs from PHAs when compared with apocynin (Figure S9). These data indicated that hyperaldosteronemia impaired in vivo endothelial repair capacity of early EPCs from PHAs. The BH₄ treatment group: n=5; solution medium used as the control: n=5.

BH₄ has been recognized gradually as a potential therapeutic target in atherosclerotic vascular disease for regulating eNOS activity. BH₄ supplementation with BH₄ was able to slow the progression of atherosclerosis. Recently, Nagata et al reported that BH₄ treatment prevented aldosterone-induced eNOS uncoupling of endothelial cells. In the present study, the high-performance liquid chromatography analysis showed that BH₄ treatment increased intracellular BH₄ content of early EPCs from PHAs, indicating that intracellular BH₄ can be supplemented exogenously. We then demonstrated that the exogenous supplementation of BH₄ markedly increased the eNOS dimerization and the NO generation, as well as improved in vivo endothelial repair capacity of early EPCs from PHAs, indicating that BH₄ treatment has beneficial effect on modifying hyperaldosteronemia-caused functional impairment of early EPCs.

BH₄, 5,6,7,8-tetrahydrobiopterin (BH₄). The MR that mediated the response to aldosterone expressing in early EPCs has been proved previously and was identified in the present study again (Figure S10). Spironolactone, a MR antagonist, has been generally applied in clinic for treatment with PHAs or the condition of secondary activation of renin–angiotensin–aldosterone system such as heart failure and myocardial infarction. The present study showed that oral spironolactone was able to improve in vivo endothelial repair capacity of early EPCs and endothelial function in PHAs. Oral spironolactone also decreased oxidative stress level, reduced BH₄ oxidation, attenuated eNOS uncoupling, and increased NO generation in early EPCs of PHAs. These results demonstrated that hyperaldosteronemia-induced functional impairment of early EPCs was MR dependent and put forward a novel...
mechanism contributing to beneficial effects of MR blockade for vascular protection.

Hyperaldosteronemia whether occurred in PHAs or secondary cases such as patients with acute myocardial infarction or chronic heart failure has been proved to be adverse to cardiovascular outcome.32,29,30 The present study demonstrated the negative effect of hyperaldosteronemia on early EPC-mediated endogenous endothelial repair process via the well-established simple PHAs avoiding underlying heterogeneous interference of other cardiovascular risk, providing novel evidence to support the hyperaldosteronemia as an independent risk factor for the pathogenesis of atherosclerotic vascular disease. The present study also demonstrated that suppressing oxidative stress or exogenous supplement with BH4 was beneficial for early EPC function, suggesting that antioxidant therapy and BH4 supplementation may be novel supplementary treatment means for hyperaldosteronemia particularly in the situation where the high dose of MR antagonist should be avoided because of its side effect in long-term treatment.

PERSPECTIVES
The present study demonstrated that the hyperaldosteronemia leads to diminished early EPC-mediated endothelial repair capacity, and the effect is MR dependent. NADPH oxidase–derived oxidative stress activation and subsequently oxidative capacity, and the effect is MR dependent. NADPH oxidase–BH4 degradation, as well as eNOS uncoupling, were responsible for hyperaldosteronemia–caused early EPC dysfunction. MR antagonist, suppressing oxidative stress or exogenous BH4 supplementation, has a beneficial effect to improve early EPC-mediated endothelial repair capacity, which was impaired by hyperaldosteronemia. Discrimination and intervention of hyperaldosteronemia should draw more attention in clinic practice to prevent and retard the pathogenesis of atherosclerotic vascular disease.

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DISCLOSURES
None.

REFERENCES
What Is New?

• Endothelial repair capacity of early endothelial progenitor cells and endothelial function are substantially impaired in hypertensive patients with primary hyperaldosteronemia, and the impairment is more severe than essential hypertension patients with similar blood pressure level.
• Nicotinamide adenine dinucleotide phosphate oxidase–derived oxidative stress activation and subsequently oxidative 5,6,7,8-tetrahydrobiopterin degradation, as well as endothelial nitric oxide synthase uncoupling, are mechanistically related to declined early endothelial progenitor cell endothelial repair capacity of primary hyperaldosteronemias.
• Mineralocorticoid receptor antagonist, suppressing oxidative stress or exogenous 5,6,7,8-tetrahydrobiopterin supplementation, has beneficial effect to improve early endothelial progenitor cell–mediated endothelial repair of PHAs.

What Is Relevant?

• The findings in this study demonstrate a novel mechanism for hyperaldosteronemia contributing to the development of endothelial injury and provide a new evidence to support the hyperaldosteronemia as an independent risk factor for the pathogenesis of atherosclerotic vascular disease.

Summary

The present study demonstrates for the first time that hyperaldosteronemia impairs early endothelial progenitor cell–mediated endothelial repair capacity in addition to elevating blood pressure and provides a novel therapeutic means for increased endothelial repair capacity in hypertensive patients with primary hyperaldosteronemia.
Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Hypertensive Patients With Primary Hyperaldosteronemia: Role of 5,6,7,8-Tetrahydrobiopterin Oxidation and Endothelial Nitric Oxide Synthase Uncoupling
Long Chen, Mei-Lin Ding, Fang Wu, Wen He, Jin Li, Xiao-Yu Zhang, Wen-Li Xie, Sheng-Zhong Duan, Wen-Hao Xia and Jun Tao

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Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Hypertensive Patients with Primary Hyperaldosteronemia: Role of BH₄ oxidation and eNOS uncoupling

**Authors:** Long Chen,* Mei-Lin Ding,* Fang Wu,* Wen He, Jin Li, Xiao-Yu Zhang, Wen-Li Xie, Sheng-Zhong Duan, Wen-Hao Xia, Jun Tao.

From the Department of Hypertension and Vascular Disease (L. C., F. W., X.-Y. Z., W.-L. X., W.-H. X., J. T.), the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China; Department of Geriatrics (L. C., M.-L. D., F. W., W. H., J. L., J. T.), the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China; Key Laboratory of Nutrition and Metabolism (S.-Z.D), Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of the Chinese Academy of Sciences, Shanghai, China.

*These authors contributed equally to this work.

**Short title:** Chen et al Early EPCs in Hyperaldosteronemia

Correspondence to Jun Tao and Wen-Hao Xia, Department of Hypertension and Vascular Disease, The First Affiliated Hospital, Sun Yat-sen University, NO. 58, Zhongshan Road 2, Guangzhou 510080, China. Tele/Fax: 0086-20-87335700. Email: taojungz@yahoo.com.
Supplemental Materials and Methods

Early Endothelial Progenitor Cells (EPCs) Cultivation and Identify
Early EPCs were cultured and identified as described previously.\(^1\) In brief, peripheral blood mononuclear cells (PBMCs) from subjects were isolated by Ficoll density gradient centrifugation and cultured on fibronectin-coated 6-well plates in endothelial cell basal medium-2 (EBM-2) supplemented with endothelial growth medium-SingleQuots exactly as indicated by the manufacturer (Clonetics, San Diego, CA, USA). After 4 days culture, nonadherent cells were removed by washing plates with phosphate buffered solution (PBS), and new medium was applied. Adherent cells were maintained for 7 days and then used for early EPC experiments.

Early EPCs were defined as cells dually positive for DiI-acLDL (0.02 mg/mL; Invitrogen, Carlsbad, CA, USA) uptake and FITC-labelled BS-1 lectin (0.01 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) binding as described previously.\(^1\) Furthermore, early EPCs were characterized by endothelial marker (>80% cells expressing endothelial markers, such as CD31 and kinase-insert domain receptor [KDR] and von Willebrand factor [vWF]) and monocytic lineage marker (70~80% of the cells expressing monocytic lineage marker CD14) expression via fluorescence-activated cell sorter (FACS) (Beckman-Coulter, Fullerton, CA, USA) analysis as described previously.\(^1\)

In Vitro Migration Assay
Migration assays were performed as described previously.\(^1\) In brief, a total of 2 x 10\(^4\) early EPCs were isolated, resuspended in 250 μL EBM-2 and pipetted at the 7th day in the upper chamber of a modified Boyden chamber (Costar Transwell ® assay, 8 μm pore size, Corning, NY, USA). The chamber was placed in a 24-well culture dish containing 500 μL EBM-2 supplemented with 50ng ng/mL vascular endothelial growth factor (VEGF) and 100 ng/mL stromal cell-derived factor-1 (SDF-1). After 24 hours incubation at 37°C, transmigrated cells were counted by at least 3 independent investigators blinded to treatment randomly.

In Vitro Assay of Adhesion on fibronection in flow
Laminar flow assays were performed as described previously.\(^1\) In brief, dishes were coated with fibronectin (10 mg/mL), and early EPCs (5x10\(^5\)/mL) were resuspended in assay buffer (HEPES-buffered Hank’s balanced salt solution, 1 mmol/L Mg\(^{2+}\)/Ca\(^{2+}\), and 0.5% BSA) and perfused into the flow chamber (proprietary item; commercially obtained from RWTH Aachen University) at a shear rate of 1.5 dyn/cm\(^2\) for 4 minutes at 37°C. The number of adherent cells after 4 minutes was quantified in multiple fields by at least 3 independent investigators blinded to treatment randomly.

Circulating EPCs Sorted by FACS Analysis
Circulating EPCs were evaluated as the number of CD34\(^+\)/KDR\(^+\) or CD133\(^+\)/KDR\(^+\) cells per 10\(^5\) PBMCs of subjects by FACS (Beckman-Coulter) analysis as previously described.\(^2\)

Animal Model and in Vivo Endothelial Repair Assay
Male NRMI\(\text{nu/nu}\) athymic nude mice (SLAC laboratory animal center, Shanghai, China),
aged 8 to 10 weeks, were used to allow injection of human early EPCs. Animals were anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Carotid artery denudated injury was performed as described previously.\textsuperscript{1-4} Surgery was carried out using a dissecting microscope. The left carotid artery was exposed via a midline incision on the ventral side of the neck. The bifurcation of the carotid artery was located, and two ligatures were placed around the external carotid artery, which was then tied off with the distal ligature. An incision hole was made between the two ligatures to introduce the denudation device. The curved flexible wire (0.35-mm diameter) was introduced into the common carotid artery, and passed three times in order to denude endothelium. The wire was then removed, and the external carotid artery was tied off proximal to the incision hole with the proximal ligature. The endothelial denudation of carotid artery was identified by Evans Blue staining (Online Figure S1A) and electron microscope scanning (Online Figure S1B) and FITC-labelled BS-1 lectin staining (Online Figure S1C).

Early EPCs (5×10\(^5\) cells) were resuspended in 100 μL of prewarmed PBS (37°C) and transplanted 3 hours after carotid artery injury via tail vein injection with a 27-gauge needle and the same volume of PBS was injected into placebo mice. 3 days after carotid injury, endothelial repair was evaluated as reendothelialization area of the denudated endothelial zone by staining with 100 μL of solution containing 3% Evans Blue dye via tail vein injection as described in detail previously.\textsuperscript{1-4} Early EPCs from each subject were transfused into 2 nude mice with carotid endothelial injury, the mean values of the reendothelialization area were used for analysis. All experimental protocols complied with 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) and the Animal Care and Use Committees of Sun Yat-sen University (Guangzhou, China).

**Detection of Homing of Early EPCs to Carotid Artery by Confocal Laser Scanning Microscopy Analysis**

Early EPCs (5×10\(^5\) cells) cultured from health subjects (HSs; n=5) were were injected into the tail vein of nude mice with carotid injury. After 24 hours the animals were sacrificed, the injured section of the carotid arteries and the corresponding uninjured section of the contralateral carotid arteries were collected and dissected. After fixed with 4% (v/v) paraformaldehyde, the dissected carotid arteries were respectively incubated with phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD14 antibody (1:100; abCAM, Cambridge, MA, USA) overnight at 4°C to label the injected human early EPCs (red signal) and FITC-labelled BS-1 lectin (0.01 mg/mL) for 1 hour at room temperature to stain the endothelium layer (green signal), finally the DAPI staining was used for nuclei staining (blue signal). Then the confocal laser scanning microscopy (Carl Zeiss LSM 710, German) analysis was used to observe the homing early EPCs.

**Detection of Homing of Early EPCs to Carotid Artery by FACS Analysis**

Early EPCs (5×10\(^5\) cells) cultured from HSs (n=5) were transduced and injected into the tail vein of nude mice with carotid injury. After 24 hours, the animals were sacrificed, blood was immediately removed, injured section of carotid arteries and the corresponding uninjured section of contralateral carotid arteries were dissected. These carotid arteries were then opened and incubated with 0.2% collagenase for 30 minutes at 37 °C, and then flushed with pre-cooled washing buffer (10 mM HEPES, 0.1% BSA in HBSS). Then the cell suspension
was filtered through a 100 μm mesh and centrifuged at 1200 rpm for 5 minutes. The cells were resuspended and fixed with 4% (v/v) paraformaldehyde, the cells then incubated with PE-conjugated monoclonal mouse anti-human CD14 antibody (abCAM) for 1 hour at room temperature and analyzed by the BD FACSCanto II system (BD Biosciences, San Jose, California, USA).

**Endothelial Function Evaluation**
Flow-mediated dilation (FMD) of the brachial artery was performed in subjects by high-resolution ultrasound (Acuson 128XP/10, Mountain View, CA, USA, with a 7.0 MHz linear-array transducer) to evaluate the endothelial function as previously described.

**Measurement of Reactive Oxygen Species (ROS) via Fluorescence Microscope**
Intracellular production of ROS in early EPCs was measured semi-quantitatively by 2',7'-dichlorodihydrofluorescein diacetate (DCF; Alexis, Plymouth Meeting, PA, USA) according to the manufacturer’s instructions. Early EPCs were loaded with DCF (10 μmol/L) for 20 minutes at 37°C, washed 3 times in PBS, and then observed with a fluorescence microscope equipped with a high-resolution digital CCD camera (Olympus, Tokyo, Japan). Images were collected by single rapid scans with identical parameters being used for all of the samples. Fluorescence intensity data were quantified in 4 independent experiments.

**Electron Paramagnetic Resonance (EPR) Spectroscopy Analysis of Early EPC Superoxide Production**
Early EPC superoxide (O$_2^-$) production was measured by EPR spectroscopy analysis using the paramagnetic trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CM-H; Noxygen, Denzlingen, Germany) as following the protocol described by other research lab.$^{5,6}$ In brief, 2.5x10$^5$ early EPCs were resuspended in 46 μL Krebs-Hepes-buffer containing diethyldithiocarbamic acid sodium salt (DETC) and deferoxamine. EPR measurements were performed after CM-H addition in 50 μL glass capillaries. EPR spectra were recorded using an EPR spectrometer (Bruickner; Leonberg, Germany). Early EPC O$_2^-$ production was determined by following the oxidation of CM-H to paramagnetic CM*. EPR instrumental settings were as follows: field sweep 100G, sweep time 60 seconds, microwave frequency 9.86GHz, microwave power 20mW, modulation amplitude 1G.

**cGMP Assay**
To evaluate NO output in early EPCs, intracellular cGMP concentration was measured using a enzyme immunoassay system (R&D Systems, Minneapolis, MN, USA). The cells were washed with serum-free medium followed by stimulated with 50 ng/mL VEGF, and then detected with the enzyme immunoassay kit as indicated by the manufacturer instruction.

**L-arginine/L-citrulline Conversion Assay**
The enzymatic reaction catalyzed by eNOS converts the L-arginine into L-citrulline while the equimolar amounts of NO generates. Therefore, L-citrulline production serves as a marker of NO generation in this enzymatic reaction. Early EPCs were washed with PBS and equilibrated in HEPES buffer (pH 7.4) for 1 hour at 37°C. Then 0.5 μmol/L L-arginine
(Sigma-Aldrich) and 10 minutes later 1 μmol/L of the calcium ionophor A23187 (Sigma-Aldrich) were added. After incubation with another 15 minutes at 37°C the reaction was stopped by lysing cells with 96% ethanol on ice. Cell extract was pooled and dried under vacuum. The derivatization was performed by the reagent solution consisted of methanol/ethanol/triethylamine/ultra-pure water/phenylisothiocyanate (PITC) = (6/1/1/1/1, v/v/v/v/v), which was made freshly. The mobile phase A consisted of 0.12 mmol/L sodium acetate and 2.5 μmol/L EDTA buffer with 2.5% (v/v) acetonitrile (pH 6.5). The mobile phase B consisted of 15% methanol, 45% acetonitrile, and 40% ultra-pure water. Chromatography separation was performed on a high performance liquid chromatography (HPLC) system (Shimadzu LC-20AD, Kyoto, Japan).

Measurements of Intracellular Biopterin Content in Early EPCs
The intracellular biopterin content was detected by HPLC system following the protocol described by other research lab.7 Biopterin content was determined in early EPCs lysate after iodine oxidation with KI/I₂ under acidic and basic conditions using HPLC system (Shimadzu) with a fluorescence detector. The amount of BH₄ was determined from the difference between total (BH₄ plus BH₂ plus biopterin) and alkaline-stable oxidized (BH₂ plus biopterin) biopterin.

Real Time-PCR Analysis
The primers of p47phox used for quantitative real-time RT-PCR were purchased from Invitrogen (Carlsbad, Calif, USA). Total RNA was isolated with the mRNA abstraction kit (Tiangen Biotech, Beijing, China). The experiments were performed using SYBR One-Step quantitative RT-PCR kits (Invitrogen) according to the manufacturer’s instructions. GAPDH was used as the internal control.

Western Blot Analysis
Early EPC protein was harvested by cell lysis buffer (Cell Signaling Technology, Boston, MA, USA). For immune-blotting analysis of the dimeric form of eNOS, samples were subjected to 6% to 9% gradient polyacrylamide gels without preheating, and the temperature of the gels was maintained at 4°C during electrophoresis (low-temperature SDS-PAGE). To separate membrane and cytosolic protein fraction for evaluating p47phox translocation, we used the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, Darmstadt, Germany) according to the manufacturer’s instructions. The following primary antibodies were used: rabbit anti-human antibodies for eNOS (1:1000; Cell Signaling Technology), NADPH oxidase subunits p47phox (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NOX1 (1:1000; Santa Cruz Biotechnology), NOX2 (1:1000; Santa Cruz Biotechnology), NOX4 (1:1000; Santa Cruz Biotechnology), actin (1:2000; Cell Signaling Technology). Proteins were visualized with HRP-conjugated anti-rabbit IgG (1:2000; Cell Signaling Technology) then followed by use of the ECL chemiluminescence system (Cell Signaling Technology). The density of the bands was quantified using the NIH Image program. Each experiment was repeated at least 3 times.

Small-interfering RNA Transfection
To knock down the expression of NADPH oxidase subunit p47\textsuperscript{phox} of early EPCs, the p47\textsuperscript{phox}-siRNA duplex (sc-29422) was purchased from Santa Cruz Biotechnology. After determining optimal transfection conditions, cultured human early EPCs were transfected with siRNA according to the manufacturer’s protocol (data not shown). A scrambled-siRNA (sc-36869, Santa Cruz Biotechnology) was used as a negative control. After 24 hours of transfection, the early EPCs were used for subsequent measurement.


## Supplemental Table

### Supplemental Table S1. Characterization of subjects

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>PHAs (n=20)</th>
<th>EHs (n=20)</th>
<th>HSs (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>56.8 ± 3.9</td>
<td>57.5 ± 3.4</td>
<td>54.7 ± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (Men/women)</td>
<td>11/9</td>
<td>12/8</td>
<td>10/10</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>23.5 ± 1.5</td>
<td>22.9 ± 2.1</td>
<td>23.8 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>176 ± 22</td>
<td>170 ± 23</td>
<td>116 ± 16*</td>
<td>*P &lt;0.01 vs PHAs or EHs</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>108 ± 12</td>
<td>99 ± 14</td>
<td>73 ± 8*</td>
<td>*P &lt;0.01 vs PHAs or EHs</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.38 ± 1.05</td>
<td>5.42 ± 1.02</td>
<td>5.32 ± 1.10</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-c (mmol/L)</td>
<td>3.46 ± 0.98</td>
<td>3.49 ± 1.02</td>
<td>3.45 ± 1.03</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>92 ± 20</td>
<td>95 ± 25</td>
<td>86 ± 23</td>
<td>NS</td>
</tr>
<tr>
<td>FBG (mmol/L)</td>
<td>5.2 ± 0.83</td>
<td>5.5 ± 0.81</td>
<td>5.3 ± 0.92</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma aldosterone (pg/mL)</td>
<td>302 ± 66*</td>
<td>86 ± 37</td>
<td>82 ± 33</td>
<td>*P &lt;0.01 vs EHs or HSs</td>
</tr>
<tr>
<td>Aldosterone/renin ratio</td>
<td>169 ± 45*</td>
<td>12 ± 7</td>
<td>15 ± 9</td>
<td>*P &lt;0.01 vs EHs or HSs</td>
</tr>
</tbody>
</table>

Value expressed as mean ± SEM. PHAs = primary hyperaldosteronemia patients; EHs = essential hypertension patients; HSs = healthy subjects; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; LDL-c = low-density lipoprotein cholesterol; FBG = fast blood glucose. Medication: In the present study, there was not any included subject who was being treated with the medication (such as statins, insulin-sensitizing agents, ACEIs or ARBs) which has possible effect on early endothelial progenitor cell bioactivity according to the excluded criterion and drug withdrawal protocol.
**Supplemental Figures and Figure Legends**

**Supplemental Figure S1**

**Figure S1.** Identification of endothelial denudated injury in carotid artery of nude mice. **A,** Endothelium injury was confirmed by Evans Blue dye staining immediately after wire-mediated carotid artery injury in nude mice. Representative photographs: injured artery and contralateral uninjured artery (arteries without discission). **B,** Electron microscope pictures (×1000 magnification) showed that endothelial layer was intact on contralateral uninjured carotid artery (upper panel) and endothelial layer was denudated on the injured carotid artery (low panel). **C,** Confocal laser scanning microscopy analysis (×400 magnification): the endothelial layer was visualized by FITC-lectin-stained (green signal), and DAPI staining was used for nuclei staining (blue signal). The uninjured zone of the artery covered by intact endothelial layer (upper panel), the endothelial layer was denudated and the sub-endothelial layer was exposed on the injured zone of the artery (low panel).
Supplemental Figure S2

A

B

C

Endothelial Repair Capacity [%]

PBS  HUVECs  early EPCs

5mm  PBS  HUVECs  early EPCs

Number of Labelled Early EPCs

Homing of the Carotid Artery

Uninjured Carotid Artery  Injured Carotid Artery

$P < 0.01$
Figure S2. Detection of homing of early endothelial progenitor cells (EPCs) to the injured carotid arteries. **A**, Confocal laser scanning microscopy analysis (×400 magnification): the endothelial layer was visualized by FITC-lectin-stained (green signal), DAPI staining was used for nuclei staining (blue signal). Phycoerythrin (PE)-conjugated monoclonal mouse anti-human CD14 antibody-labeled early EPCs (red signal) were detected beneath the endothelial layer by repeated serial imaging with increasing depth of the endothelial repair zone of the injured carotid artery (**A** a-c) and the sub-endothelial layer where the denudated endothelium was not repaired yet (**A** d-f). **B**, Early EPCs from healthy subject (HSs; n=5) were injected into the tail vein of nude mice with carotid injury. After 24 hours, the injured and the corresponding section of the contralateral uninjured carotid artery were homogenized respectively, the number of PE-conjugated monoclonal mouse anti-human CD14 antibody-labeled early EPCs was quantified by using fluorescence-activated cell sorter analysis. **C**, HUVECs were injected into the tail vein of nude mice with carotid injury as the negative control. After 3 days, the effect of HUVECs injection for endothelial repair was not different compared with PBS injection; Early EPCs injection showed a distinct effect to improve endothelial repair compared with PBS injection or HUVECs injection (n=5 per group). * P<0.01 versus reendothelialization area of PBS injection group or HUVECs injection group.
Supplemental Figure S3

**Figure S3.** The early EPCs-mediated endothelial repair after carotid injury at different time points: 3, 7, 14 days. A, Reendothelialization area at 3 days, 7 days, and 14 days after carotid injury in nude mice with transplantation of early EPCs from healthy subjects (HSs; n=5), essential hypertension patients (EHs; n=5), and primary hyperaldosteronemia patients (PHAs; n=5). Representative photographs showed in B. * P<0.05 versus early EPC in vivo endothelial repair capacity of HSs, # P<0.05 versus early EPC in vivo endothelial repair capacity of EHs.
Supplemental Figure S4

**Figure S4.** In vitro early endothelial progenitor cells (EPCs) function assays. A, Quantification analysis of migration to vascular endothelial growth factor/stromal cell-derived factor-1 of early EPCs from healthy subjects (HSs; n=12), essential hypertension patients (EHs; n=10) or primary hyperaldosteronemia patients (PHAs; n=10) before and after treatment with spironolactone (Spi). The representative photographs showed in (B; ×200 magnification, scale bar = 100μm). C, Quantification analysis of early EPC adhesion on fibronectin in flow of early EPCs from HSs (n=12), EHs (n=10) or PHAs (n=10) before and after treatment of Spi. The representative photographs showed in (D; ×200 magnification, scale bar = 100 μm).
**Supplemental Figure S5**

**Figure S5.** Fluorescence-activated cell sorter analysis of circulating endothelial progenitor cells (EPCs). **A,** Percentage of circulating CD34⁺/kinase-insert domain receptor [KDR]⁺ peripheral blood mononuclear cells (PBMNCs) in healthy subjects (HSs; n=12), essential hypertension patients (EHs; n=10) and primary hyperaldosteronemia patients (PHAs; n=10) before or after treatment of spironolactone (Spi). **B,** Percentage of circulating CD133⁺/KDR⁺ PBMNCs in HSs (n=12), EHs (n=10) and PHAs (n=10) before or after treatment of Spi.
Supplemental Figure S6

Figure S6. Transcription and expression of NADPH oxidase isoforms. A, Transcription of NOX1, NOX2 or NOX4 in early endothelial progenitor cells (EPCs) from healthy subjects (HSs) and primary hyperaldosteronemia patients (PHAs) by real time-PCR analysis; n=5 per group. B, Expression of NOX1, NOX2 or NOX4 in early EPCs from HSs and PHAs were detected by western blot analysis; n=5 per group.
Supplemental Figure S7

**Figure S7.** The knockdown effect of p47\textsuperscript{phox}-siRNA. The transcription (A) and expression (B) of p47\textsuperscript{phox} in early EPCs from PHAs were obviously down-regulated by p47\textsuperscript{phox}-siRNA.
Supplemental Figure S8

Figure S8. In vitro early endothelial progenitor cell (EPC) function of PHAs enhanced by p47phox-siRNA or 5,6,7,8-tetrahydrobiopterin (BH₄). A, The effect of p47phox-siRNA or BH₄ treatment on in vitro migration activity of early EPCs from primary hyperaldosteronemia patients (PHAs). n=5 per each bar. B, The effect of p47phox-siRNA or BH₄ treatment on in vitro adhesion activity of early EPCs from PHAs. n=5 per each bar. The scrambled-siRNA was used as the negative control for p47phox-siRNA and the solution medium was used as the negative control for BH₄.
Supplemental Figure S9

**Figure S9.** The effect of L-NAME or apocynin on oxidative stress of early endothelial progenitor cells (EPCs) from primary hyperaldosteronemia patients (PHAs). **A and B,** Electron paramagnetic resonance spectroscopy analysis showed that the apocynin rather than L-NAME inhibited superoxide production of early EPCs from PHAs; n=5 per group. **C,** Intracellular reactive oxygen species (ROS) production of early EPCs from PHAs evaluated via 2′,7′-dichlorodihydrofluorescein diacetate (DCF)-fluorescence analysis was suppressed obviously by apocynin rather than L-NAME; n=5 per group.
Figure S10. Expression of mineralocorticoid receptor (MR) in early endothelial progenitor cells (EPCs). A, The mRNA level of MR in human early EPCs was detected by real time RT-PCR. The GAPDH was used as the internal control; the MR mRNA level of human umbilical vein endothelial cells (HUVECs) as the positive control. n=5 per each bar. B, MR protein expression in human early EPCs was detected by immune-blotting. The actin was used as the internal control; the MR protein expression of HUVECs as the positive control. n=5 per each bar.
Figure S11. The effect of in vitro treatment with aldosterone (Ald) on early endothelial progenitor cells (EPCs) activity. A, In vitro migration to VEGF/SDF-1 of early EPCs from healthy subjects (HSs) was inhibited by in vitro of Ald treatment as a concentration-dependent manner; Co-treatment with spironolactone (Spi; 1 μmol/L), NADPH oxidase inhibition by p47phox-siRNA, or BH4 (10 μmol/L) addition reversed significantly the Ald-inhibited migration function of early EPCs. n=5 per each bar; * P<0.05 versus Ald, # P<0.05 versus Ald (100 nmol/L). B, In vitro adhesion to fibronectin in flow of early EPCs from HSs was suppressed by in vitro Ald treatment as a concentration-dependent manner; Co-treatment with Spi (1 μmol/L), NADPH oxidase inhibition by p47phox-siRNA, or BH4 (10 μmol/L) addition reversed significantly the Ald-suppressed adhesion function of early EPCs. n=5 per each bar; * P<0.05 versus Ald, # P<0.05 versus Ald (100 nmol/L).