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Original Article

Mineralocorticoid receptor antagonist eplerenone alleviates the reduction and dysfunction of endothelial progenitor cells in hypertensive patients

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Abstract: The aim of this study was to investigate the effects of antagonist of aldosterone/mineralocorticoid receptor (MR) on the amount, adhesion and migration capacity of EPCs of hypertensive patients. Human EPCs were isolated from peripheral blood by Ficoll density-gradient centrifugation and confirmed by uptake of acetylated LDL and binding of ulex-lectin. Adhesion assays and migration assays were used to determine the function of EPCs. The expression levels of MR, eNOS, and CYP11B2 were assessed by qRT-PCR and Western-blot. The serum SDF-1 level was determined by ELISA assay. Serum SDF-1 level was elevated in hypertensive patients and was suppressed by eplerenone. The migration and adhesion abilities of EPCs were reduced in patients but the reduction was partially reversed by eplerenone. The expression levels of MR and CYP11B2 were significantly increased but that of eNOS was markedly decreased in patients with hypertension, and the changes were suppressed after aldosterone/MR antagonist Eplerenone treatment. The present study indicated that elevated aldosterone level caused reduction and dysfunction of endothelial progenitor cells in hypertensive patients, aldosterone/MR antagonist Eplerenone can partially reverse effects caused by the increase of aldosterone.

Keywords: Hypertension, aldosterone, endothelial progenitor cells, cell migration, leukocyte adhesion

Introduction

Hypertension is a severe and progressive disease that has long been a major public health problem due to its increasingly high prevalence, poor control rates, and significant impact on the global burden of disease [1, 2]. Primary hyperaldosteronism has been identified as a frequent cause of hypertension and causes heart and kidney diseases [3-5]. Moreover, hyperaldosteronism is associated with decreased number and alteration of characteristics of endothelial progenitor cells (EPCs) [6, 7]. The detrimental effects of hyperaldosteronism are mediated through the activation of mineralocorticoid receptor (MR), which is widely expressed in many tissues and cell types including endothelial cells, myocardiocytes, and neutrophils [8, 9].

Endothelial progenitor cells are a heterogeneous population of cells that circulate in the blood and contribute to the formation of new blood vessels, endothelial repair, and vascular homeostasis [10-12]. Impairment of EPCs function is responsible for endothelial dysfunction [13, 14], cardiovascular diseases [15, 16], and worse prognosis [17, 18]. Endothelial progenitor cells express a variety of receptors including MR that mediate the effects of hormones and growth factors [19, 20]. Hypertension and hyperaldosteronism have been shown to cause impairment of endothelial repair capacity of EPCs [21, 22]. However, the effect of aldosterone receptor antagonists on EPC function and vascularization capacity in human is unclear. This study aimed to investigate the effects of aldosterone receptor antagonist Eplerenone on the amount and activity of EPCs in hypertension patients.

Material and methods

Patients and treatment

All protocols were reviewed and approval by the ethical committee of the Affiliated Hospital of Nanjing Medical University, aka Changzhou No.

Table 1. Characterization of controls and patients with hypertension

	Controls (n=15)	Patients (n=20)	*P*
Age (years)	58.7 ± 5.8	63.1 ± 4.9	0.558
Gender, M/F	9/6	13/7	0.762
Hypertension	0/15	20/20	< 0.0001
Diabetes	2/15	4/20	0.605

Values were expressed as mean ± SEM where appropriate. Differences between groups were examined by Chi-square tests. F, female; M, male.

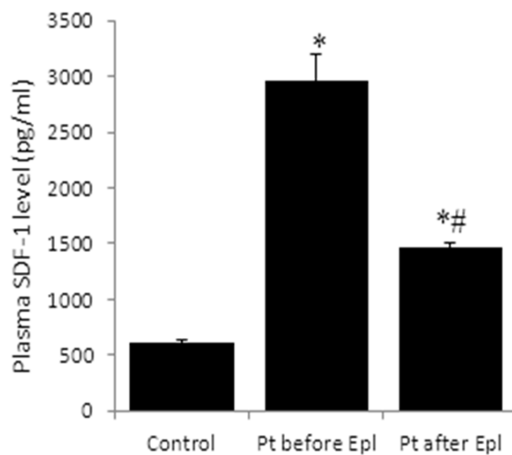

Figure 1. Serum SDF-1 levels of controls (n=15) and hypertensive patients (n=20) before and after Eplerenone treatment. Pt, Patients. $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to Pt before Eplerenone.

2 People's Hospital (Changzhou, China). Signed informed consent was obtained from patients and controls. The characteristics of controls and hypertension patients were listed in **Table 1**. The patients with hypertension were treated with Eplerenone (75-100 mg/day) for 3 weeks. Changes of EPCs number and function, as well as endothelial function were determined before and after aldosterone receptor blockade in this group.

Stromal cell-derived factor-1 level

Blood samples of 2 mL was transferred into clot tubes which were centrifuged for 10 minutes at 400 × g and the supernatant was used for ELISA. The ELISA assay was performed using a commercially available kit (DL-SDF1-Hu, Wuxi Donglin Sci & Tech Development Co, Wuxi, China) according to supplier's protocol. The optical density (OD) was read at a 450 nm wavelength using microplate reader.

Isolation, culture, and characterization of human endothelial progenitor cell

The isolation of endothelia progenitor cells was done essentially as previously described [23]. The peripheral blood mononuclear cells (PBMCs) were separated using Ficoll density-gradient centrifugation. After resuspension in endothelial basal medium (EBM-2) supplemented with EGM2-MV-SingleQuots (Lonaz, Guangzhou, China) containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and 10% FBS, one aliquot of PBMCs was stained with FITC-conjugated EGFR2 antibody and ran on a BD Accuri™ (BD Biosciences, Shanghai, China) to assess the amount of EPCs in peripheral blood mononuclear cells and the rest of the cells were plated on fibronectin-coated tissue culture flasks. After 4 days of culture, non-adherent cells were discarded by washing with PBS. All experiments were performed using cells harvested on day 7. To confirm the EPC phenotype, adherent cells were incubated with Dil-labeled acLDL at 37°C for 1 hour and then incubated with FITC-labeled Ulex europaeus agglutinin I for 1 hour after fixation with 4% paraformaldehyde. Cells were visualized with fluorescent microscope, and adherent cells positive for both FITC-ulex-lectin and Dil-acLDL were identified as EPCs [24].

Quantitative real-time PCR assay

Cultured EPCs were collected in Trizol, and RNA was extracted according to manufacturer's protocol. Gene expression of the CYP11B2, eNOS and MR was detected by RT-PCR. The primers used were as follows: CYP11B2, forward primer 5'-GGGAAAGGAATAAGGGGGCAA-3', reverse primer, 5'-GTGAGAGCCCCATAAACA-AGGA-3'; MR, forward primer 5'-CAACAGGTA-GACGGCGAGAG-3', reverse primer 5'-ACCTTC-AGGGAGACTGTGGT-3'; eNOS, forward primer 5'-TACAGGCTAAAACCTTAGAAGAGGA-3', reverse primer 5'-GATGCCAGCCTTCAGTCCAA-3'; GAPDH, forward primer 5'-AAAGTCCGCCATTTTG-CCACT-3', reverse primer 5'-CCAAATCGTTAG-CGCTCCTT-3'.

Western-blot assay

To determine protein levels, cell lysates (50 μg) from cultured EPCs were separated by 10% SDS-polyacrylamide gels and transferred onto PVDF memebranes. Primary antibodies ag-

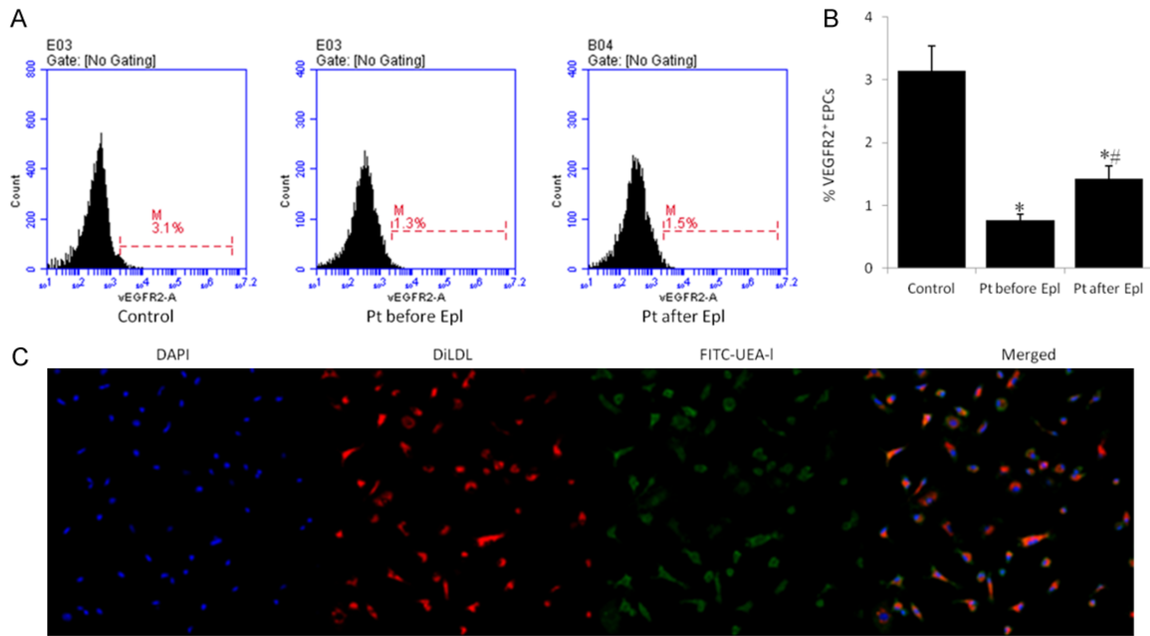

Figure 2. Eplerenone reversed hypertension-caused reduction of EPCs. A. The amount of circulating VEGFR2-positive EPCs was detected by flow cytometry. B. The results of quantitative analysis. C. The identity of EPCs was confirmed by uptake of Dil-labeled acetylated LDL and binding of FITC-ulex-lectin binding. Pt, Patients. **P* < 0.05 compared to control. #*P* < 0.05 compared to Pt before Eplerenone.

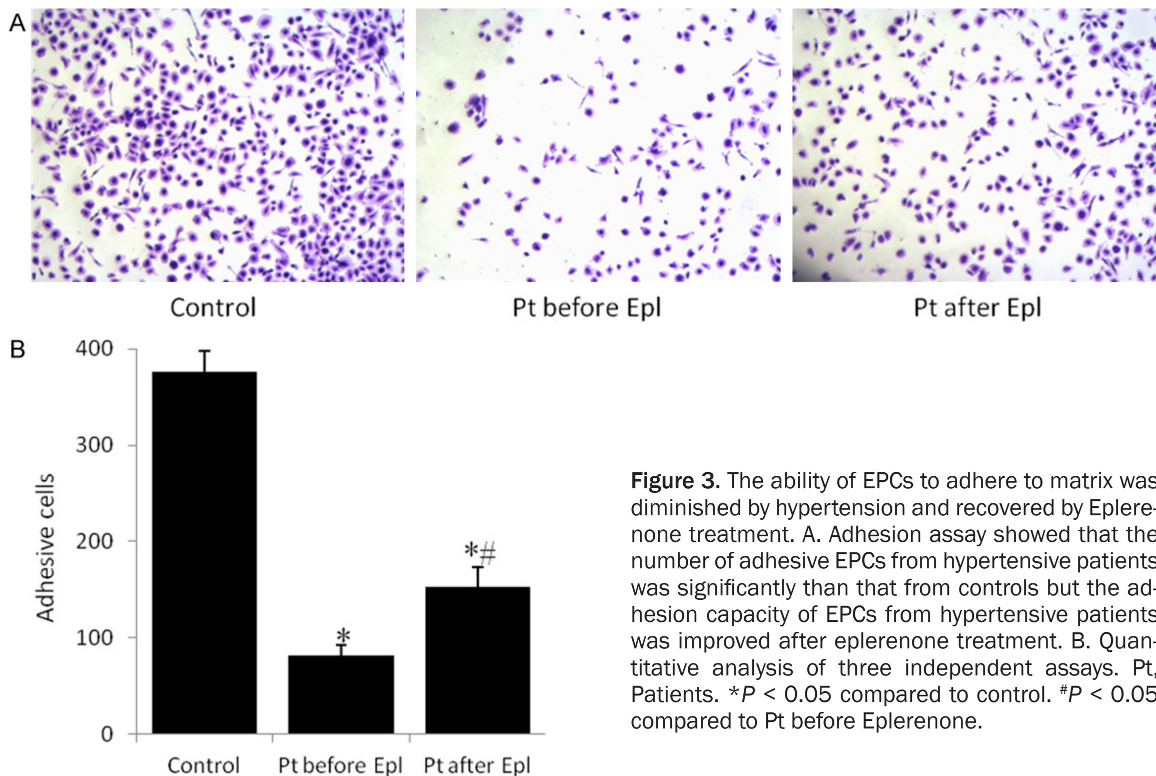

Figure 3. The ability of EPCs to adhere to matrix was diminished by hypertension and recovered by Eplerenone treatment. A. Adhesion assay showed that the number of adhesive EPCs from hypertensive patients was significantly than that from controls but the adhesion capacity of EPCs from hypertensive patients was improved after eplerenone treatment. B. Quantitative analysis of three independent assays. Pt, Patients. **P* < 0.05 compared to control. #*P* < 0.05 compared to Pt before Eplerenone.

ainst CYBP11B2, eNOS, MR and β-actin were incubated separately with membranes at 4°C overnight. Thereafter membranes were washed and a HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody was added and incubated for 2 h at room temperature.

Then the membrane was developed with ECL methods.

Adhesion assays

Matrigel basement membrane matrix purchased from BD Biosciences was used as an adhesion sheet at 5 μg per well. Cells (45000 per well) were added. After 60 minutes incubation, the wells were vigorously washed to remove unbound cells. Adherent cells were then fixed and stained with crystal violet. Stained cells were later counted in 10 random fields under a 20 × objective.

Migration assays

The EPCs (5×10^4) were resuspended in serum-free medium and seeded into the top chambers of Matrigel-coated Transwell inserts (BD Biosciences). The bottom compartment of the chamber contained complete medium as a chemo attractant. After 48 hrs incubation, cells on the top surface of the membrane were removed by dry cotton swabs and the cells on the bottom surface of the membrane were washed with PBS, fixed with formaldehyde, and stained with crystal violet and counted in five representative 200 × fields.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using SPSS software. For statistical comparison of two groups, we used two-tailed Student's t-test, for the comparison of three groups; we used ANOVA followed by post-tests. Differences were considered significant when $P < 0.05$.

Results

MR antagonist eplerenone reduced plasma SDF-1 level in hypertension patients

To determine the effects of mineralocorticoid receptor antagonist Eplerenone on endothelial progenitor cells of hypertension patients, we first checked the change of plasma levels of chemokine stromal cell-derived factor-1 (SDF-1) after 3-week Eplerenone treatment (75-100 mg/day). The plasma SDF-1 level was significantly higher in hypertension patients compared to controls, which was drastically reduced by Eplerenone treatment (**Figure 1**).

The amount of circulating EPCs in hypertension patients was regulated by aldosterone-MR system

Circulating EGFR2-positive EPCs were significantly depleted in hypertension patients and were partially recovered after 3-week Eplerenone treatment (**Figure 2A**, **2B**). Similarly, the number of circulating mononuclear cells adhered to matrigel after 60 min incubation was substantially lower for hypertension patients compared to non-hypertensive controls, which was markedly elevated by Eplerenone treatment (**Figure 3A**, **3B**). The identity of EPCs was verified by DiLDL and UEA-I labeling of adherent cells after culturing mononuclear cells for 4 days (**Figure 3C**).

Eplerenone increased migrating ability of EPCs in hypertension patients

EPCs from hypertension patients showed much lower migrating ability than those of non-hypertensive controls whereas Eplerenone treatment greatly restored migrating capability of EPCs in hypertension patients (**Figure 4A**, **4B**).

Eplerenone modulated the expression of aldosterone/MR genes

The mRNA (**Figure 5A-C**) and protein (**Figure 5D**, **5E**) levels of CYP11B2 (**Figure 5A**, **5D**, **5E**) and MR (**Figure 5B**, **5D**, **5E**) were significantly elevated while that of eNOS (**Figure 5C-E**) was markedly reduced in EPCs of hypertension patients compared to that of controls. These changes were substantially reversed by Eplerenone treatment (**Figure 5**).

Discussion

Endothelial progenitor cells are derived from bone marrow, which are circulating cells with ability to differentiate into mature endothelial cells. It is suggested that EPCs may involve in maintaining and restoring the endothelial function, and contribute to *in vivo* vasculogenesis and/or vascular homeostasis [14, 25]. It has been known that EPCs play a key role in the maintenance of endothelial homoeostasis [26]. The function of the endothelium and endothelial integrity are impaired in hypertension [25, 27]. Both the number and function of EPCs are affected by several cardiovascular risk factors

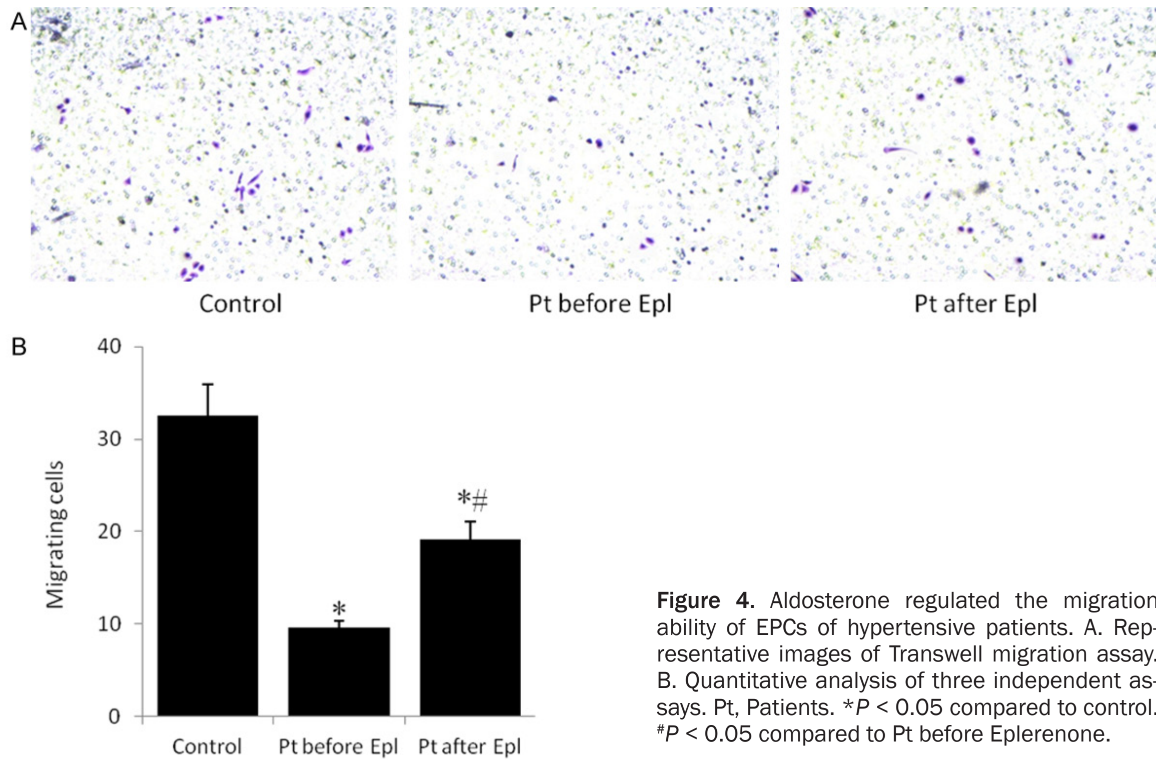

Figure 4. Aldosterone regulated the migration ability of EPCs of hypertensive patients. A. Representative images of Transwell migration assay. B. Quantitative analysis of three independent assays. Pt, Patients. **P* < 0.05 compared to control. #*P* < 0.05 compared to Pt before Eplerenone.

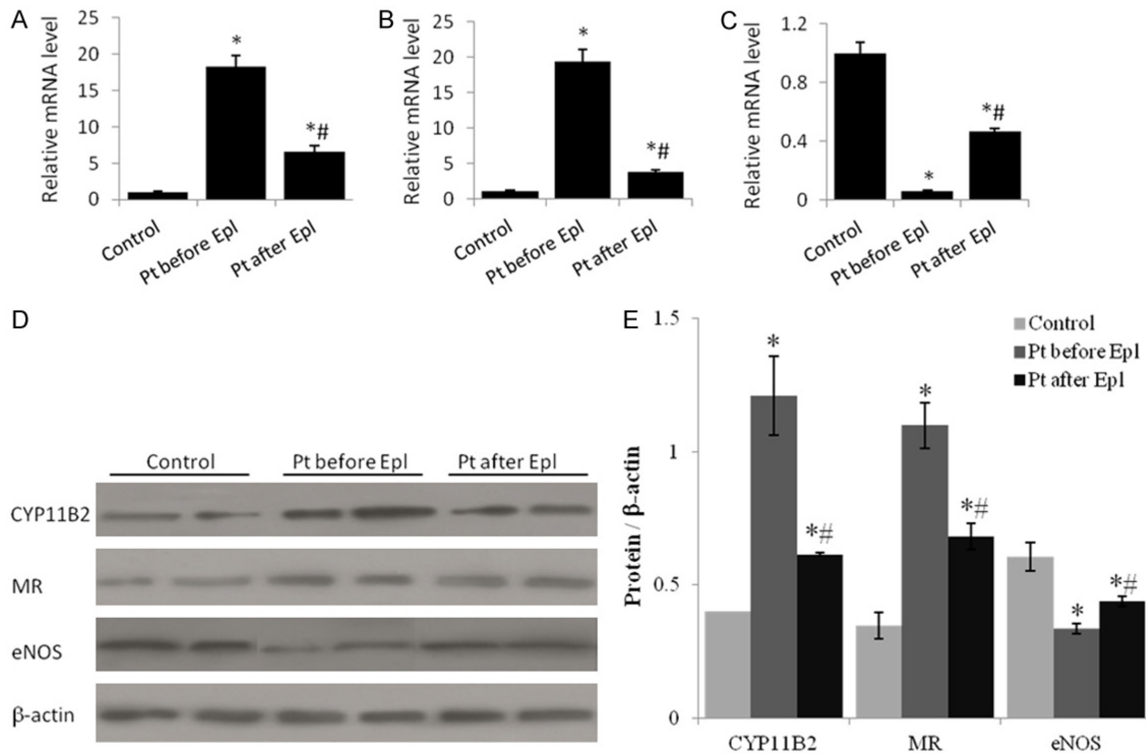

Figure 5. The genes involved in aldosterone production and signaling were dysregulated in hypertensive patients. The mRNA levels (A-C) and protein levels (D, E) of CYP11B2 (A, D, E), MR (B, D, E), and eNOS (C-E) were assessed by quantitative real-time PCR and western blot. Pt, Patients. **P* < 0.05 compared to control. #*P* < 0.05 compared to Pt before Eplerenone.

as well as various cardiovascular disorders, such as hypertension, hypercholesterolemia and coronary heart disease [28]. In consistence with the findings of previous studies [29, 30], the current study showed that the number of EPCs was decreased in hypertensive patients. In addition, the migration and adhesion abilities of hypertensive EPCs were also diminished.

It has been known that the mobilization and migration of EPCs from the bone marrow to sites of endothelial injuries or ischemia are primarily regulated by the SDF-1 gradient *in vivo* [31]. We found that the level of SDF-1 decreased in patients with hypertension along with the reduction of EPCs number, which increased after treating with Eplerenone. These data indicated that inhibiting the activation of aldosterone receptor might improve the number and function of EPCs. Aldosterone has been shown to cause cardiac hypertrophy, fibrosis, and vascular injury through targeting cardiomyocytes [32], cardiac fibroblasts [33], and endothelial cells [34]. Upon binding to the mineralocorticoid receptor (MR), aldosterone increases vascular endothelial and vascular smooth muscle cell superoxide anion production [34-36]. As shown in present study, the mRNA and protein level of MR and CYP11B2 decreased in patients treated with aldosterone receptor antagonist (Epleronone). It had been demonstrated that eNOS was also a crucial regulator of putative EPC release from the bone marrow through activation of matrix metalloproteinase-9 (MMP-9) and increase of VEGF expression [20]. Aldosterone synthetase CYP11B2 was found to be reduced in hypertensive patients treated with spironolactone, which largely regulated the secretion of aldosterone [37]. As shown by adhesion and migration assays in our study, the function of EPCs was significantly influenced by aldosterone.

Taken together, the results in the present study indicated that elevated aldosterone level caused the reduction and dysfunction of endothelial progenitor cells in hypertensive patients, which could be partially reversed by aldosterone receptor antagonist Epleronone.

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Disclosure of conflict of interest

None.

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