Effect of Eplerenone on Endothelial Progenitor Cells and Oxidative Stress in Ischemic Hindlimb

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BACKGROUND
We have demonstrated that angiotensin II receptor blocker (ARB) improved endothelial progenitor cells (EPCs) dysfunction through the antioxidative mechanism. Therefore, we investigate whether the selective mineralocorticoid receptor (MR) antagonist eplerenone improves EPCs function in rat hindlimb ischemia.

METHODS
Unilateral hindlimb ischemia was surgically induced in Wistar rats. After induced ischemia, rats received eplerenone (30 mg/kg/day), valsartan (3 mg/kg/day), or vehicle for 3 weeks. Peripheral blood mononuclear cells were isolated, subjected to flow cytometric analysis to determine the number of circulating EPCs, cultured to assay EPC colony formation, and subjected to a migration chamber assay to evaluate EPCs migration.

RESULTS
Blood perfusion by laser Doppler image was significantly higher in eplerenone than in vehicle. Capillary density by isoclin B4 stained of ischemic muscle was significantly increased in eplerenone compared with vehicle. Eplerenone significantly increased the number, colony formation, and migration of EPCs. Levels of endothelial nitric oxide synthase (eNOS) and angiogenic factor such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) protein expression by western blot were significantly higher in eplerenone than in vehicle. Eplerenone significantly decreased the NAD(P)H oxidase p22phox, p47phox, gp91phox and MR expression and expression of aldosterone effector kinase serum and glucocorticoid-induced protein kinase 1 (Sgk1). These effects of eplerenone are similar extent as valsartan.

CONCLUSIONS
This study showed that eplerenone improves the proliferation and function of EPCs in rat hindlimb ischemia, suggesting that eplerenone may provide a novel and effective therapeutic strategy for the repair of cardiovascular diseases.

Keywords: aldosterone; angiogenesis; blood pressure; endothelial progenitor cells; hypertension; nitric oxide synthase


Therapeutic availability of various angiogenic molecules has been reported in animal models or humans with ischemic heart disease. Endothelial progenitor cells (EPCs) are bone marrow-derived cells with the potential to differentiate into mature functional endothelial cells, and partly contribute to angiogenic mechanism.1 Numbers of atherosclerotic risk factors have been indicated to correlate with reduced numbers of circulating EPCs,2 and increases in numbers and colonies of EPCs have been reported to predict beneficial occurrence of cardiovascular events from cardiovascular causes.3,4 In addition, EPCs contribute to reendothelialization of injured vessels as well as neovascularization of ischemic lesions. Therefore, proliferation of circulating EPCs might be a beneficial novel preventive and therapeutic strategy for the treatment of ischemic event, and that EPCs play a pivotal role in the pathogenesis of atherosclerosis and cardiovascular diseases.5,6 Recently, we have demonstrated that the intracellular mechanism of angiotensin II receptor blocker (ARB)-induced EPCs mobilization may further result in the development of novel pharmacologic strategies to improve endothelial function, enhance angiogenesis, and protect oxidative stress.5,6 On the other hand, Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS),7 in which the efficacy of eplerenone, a novel mineralocorticoid receptor (MR) inhibitor with a higher selectivity, fewer and less severe adverse effects than spironolactone, was evaluated, revealed that administration of eplerenone to patients with left ventricular dysfunction and heart failure after myocardial infarction was effective in improving prognosis and reducing the incidence of cardiovascular events, confirming the potential usefulness of eplerenone.
for treating heart failure. However, the effects of eplerenone on mechanism are unclear. Therefore, the purpose of the present study was to investigate whether the eplerenone improves EPCs number and function and oxidative stress compared with ARB valsartan in rat hindlimb ischemia.

METHODS

All procedures were in accordance with our institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal models and experimental designs. Male Wistar rats (Oriental Bioservice Kanto, Ibaraki, Japan) weighing 290–330 g, were anesthetized with pentobarbital intraperitoneally and were always subjected to hindlimb ischemia by resection of the left femoral artery. The right foot served as a control. After resection of the left femoral artery, 30 mg/kg/day of eplerenone dissolved in water was administered orally in rats with an ischemic hindlimb for 3 weeks (n = 11). This dose of administration was determined to result in optimal pharmacokinetic characteristics for effective in vivo inhibition of MR in the rat.9 This amount of eplerenone was shown previously to ameliorate vascular remodeling without affecting systolic blood pressure.8,9 Another drugs were given at average doses of 3 mg/kg/day (valsartan; Novartis Pharma, East Hanover, NJ, n = 9) and 1 mmol/l (tempol, n = 8) by being weekly adjusted to the drinking habits of the animals for 3 weeks. Our preliminary data suggested that 3 mg/kg/day of valsartan would not influence blood pressure in rats,10 and Nagase et al.11 showed that 1 mmol/l of tempol did not affect systolic blood pressure by direct blood pressure measurement. Vehicle rats were administered only drinking water (n = 10).

Laser Doppler blood flow analysis. We measured hindlimb blood flow using a laser Doppler blood flowmetry (MoorLDI; Moor Instrument, Devon, UK), as described previously.12

Localization of eNOS, NAD(P)H oxidase p47phox, VEGF, and Ang-2 with dual immunofluorescence staining and analysis of capillary density. Immunohistochemistry for endothelial nitric oxide synthase (eNOS; Santa Cruz Biotechnology, Santa Cruz, CA), NAD(P)H oxidase p47phox (Santa Cruz Biotechnology), vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology), angiopoietin-2 (Ang-2; Alpha Diagnostic International, San Antonio, TX), analysis of capillary density (biotinylated isolecint B4; Vector Laboratories; Funakoshi, Tokyo, Japan) was described previously.13 Nuclei were stained with 4′,6-diamidino-2-phenylindole.

Western blot analysis. VEGF, angioptietin-1 (Ang-1), Ang-2, eNOS, NAD(P)H oxidase p22phox, p47phox, gp91phox, MR, and serum and glucocorticoid-induced protein kinase 1 (Sgk1) proteins were measured as described previously.8,9,14

Detection of superoxide anion in the ischemic hindlimb muscle. Histological detection of superoxide anion in the ischemic hindlimb muscle was performed using dihydroethidium as described previously.9,14,15

EPC colony formation assay. A modified EPC colony formation assay was performed as previously described.3,5,6,16,17 In brief, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and 10 ml of heparinized peripheral blood was immediately collected from the rat hepatic portal vein. Mononuclear cells (MNCs) were separated by centrifugation with Histopaque-1083 density gradient medium (Sigma-Aldrich, St Louis, MO). MNCs were suspended and mixed in 1 ml of endothelial growth medium-2 (Clonetics, San Diego, CA) containing 10% fetal bovine serum, 4 ml/l human basic fibroblast growth factor, 1 ml/l VEGF-A (R&D Systems, Minneapolis, MN), 1 ml/l recombinant 3 insulin-like growth factor-1 (Sigma-Aldrich), 1 ml/l human epidermal growth factor (Sigma-Aldrich), 1 ml/l ascorbic acid, and 1 ml/l GA-1000 (BioWhittaker, Walkersville, MD). Twenty-four-well plates (Falcon, San Jose, CA) were precoated with rat vitronectin (0.1 μg/cm²) plus 0.5% gelatin overnight at 37°C. MNCs were inoculated into 6-well plates (5 × 10⁴ cells/well) and cultured for 24 h. Nonadherent MNCs were reincubated into vitronectin-coated 24-well plates (2 × 10⁵ cells/well) and cultured in a CO₂ incubator at 37°C for 7 days. The average number of colonies was calculated manually under microscopy from a minimum of four wells by an observer who was unaware of the experimental design.

Flow cytometric analysis for EPCs. The number of circulating EPCs in peripheral blood was determined using a cell surface antigen as previously established.5,6,16,17 Circulating MNCs with CD34⁺ were quantified as tentative EPCs. Peripheral blood was drawn and MNCs were isolated by a density-gradient centrifuge method. MNCs were stained with a fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibody (Becton-Dickinson, San Jose, CA). Samples were subjected to a two-dimensional side scatter–fluorescence dot plot analysis (FACScan; Becton-Dickinson). After appropriate gating, the number of CD34⁺ cells with low cytoplasmic granularity (low sideward scatter) was quantified and expressed as the number of cells/10⁴ total events.

EPC migration assay. To investigate the EPC migration activity, a modified Boyden chamber assay was performed with a 96-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) as previously described.5,6,17–19

Statistical analysis. All of the values are expressed as mean ± s.e.m. Mean values were compared between the three or four groups by analysis of variance and the Bonferroni post hoc test for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

Effect of eplerenone and valsartan on blood flow and neovascularization in ischemic hindlimb

First, we examined whether eplerenone improves hindlimb blood flow perfusion using a laser Doppler blood flowmetry compared
with valsartan treatment. The ischemic (left side)/normal (right side) blood perfusion ratios were significantly greater in eplerenone to a similar extent as valsartan treatment than in vehicle at 14 days ($P < 0.05$) and 21 days ($P < 0.01$) (Figure 1a,b). Next, to investigate the extent of angiogenesis at the microcirculation level, we measured capillary density in a histological section from the ischemic tissues. Figure 1d shows representative photomicrographs stained with endothelium-specific isolectin B4 at 21 days after surgery. Quantitative analysis revealed that the capillary density was significantly ($P < 0.01$) increased in eplerenone to a similar extent as valsartan treatment compared with vehicle (Figure 1c,d). These results suggest that eplerenone actually induced neovascularization in ischemic hindlimbs.

**Effect of eplerenone, valsartan, and tempol on expression of angiogenic factors, eNOS, MR, Sgk1, NAD(P)H oxidase subunits, and superoxide anion production**

Next, we examined the effect of eplerenone and valsartan on angiogenic factors such as VEGF, Ang-1, and Ang-2 expression, and eNOS, MR, Sgk1: an effector kinase of MR activation, and NAD(P)H oxidase p22phox, p47phox, gp91phox expression, and superoxide anion production. In addition, the inhibitory effect of eplerenone on NAD(P)H oxidase subunits expression in the ischemic tissue suggests the possibility of the crosstalk between MR and oxidative stress. Leong et al. reported that oxidative stress induced Sgk1 expression in mammalian epithelial cells. Therefore, we also examined whether the treatment with the superoxide dismutase mimetic tempol reduced MR and Sgk1 expression, NAD(P)H oxidase expression, and superoxide production, and upregulated angiogenic factors and eNOS expression in the ischemic tissue. Expression of VEGF, Ang-1, Ang-2, and eNOS protein was significantly increased in eplerenone to a similar extent as valsartan and tempol treatment compared with vehicle (Figure 2a,b).

Level of MR and Sgk1 protein expression was significantly decreased in eplerenone to a similar extent as valsartan and tempol treatment compared with vehicle (Figure 2c). Level of NAD(P)H oxidase p22phox, p47phox, and gp91phox expression was significantly inhibited in eplerenone to a similar extent as valsartan treatment compared with vehicle. In addition, treatment of tempol significantly decreased p22phox and p47phox expression compared with vehicle (Figure 2d). Moreover, superoxide anion production was significantly reduced in eplerenone to a similar extent as valsartan and tempol treatment compared with vehicle (Figure 2e).

**Effect of eplerenone and valsartan on EPC colony formation, circulating EPC numbers, and EPC migration**

Next, we examined the effect of eplerenone and valsartan on EPC colony formation, circulating EPC number (CD34+ cell ratio), and EPC migration index such as VEGF and stromal cell-derived factor-1. Treatment with eplerenone and valsartan markedly increased the number of EPC colonies in comparison with vehicle group (Figure 3a). The number of circulating EPCs in peripheral blood was significantly higher in eplerenone and valsartan than in vehicle (Figure 3b). EPC migration in response to VEGF or stromal cell-derived factor-1 was significantly higher in rat hindlimb ischemia treated with eplerenone and valsartan than in treated with vehicle (Figure 3c).

**Effect of eplerenone and valsartan on VEGF and Ang-2 expression in hindlimb ischemia**

Next, we examined the effect of eplerenone and valsartan on the cellular localization of VEGF and Ang-2 expression in the ischemic hindlimb skeletal muscle tissues at 21 days after surgery, we performed the immunofluorescence staining with double labeling for isolectin B4 and VEGF and Ang-2. Figure 4 shows that VEGF and Ang-2 and isolectin B4 were colocalized...
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We investigated the mechanisms of eplerenone on EPCs function in the ischemic hindlimb skeletal muscle tissue. The major findings of the present study are that (i) angiogenesis by laser Doppler blood flowmetry and capillary density was increased in eplerenone compared with vehicle; (ii) EPC colony formation and EPC migration index were upregulated in eplerenone compared with vehicle; (iii) eNOS and angiogenic factors, including VEGF, Ang-1, and Ang-2 were higher in eplerenone than vehicle; and (iv) NAD(P)H oxidase p22phox, p47phox, gp91phox expression and superoxide, and MR expression and expression of aldosterone effector kinase Sgk1 were decreased in eplerenone compared with vehicle. Moreover, these effects

Effect of eplerenone and valsartan on eNOS and NAD(P)H oxidase p47phox expression in hindlimb ischemia

Next, we examined the effect of eplerenone and valsartan on the cellular localization of eNOS and p47phox expression in the ischemic hindlimb skeletal muscle tissues at 21 days after surgery, we performed the immunofluorescence staining with double labeling for isolectin B4, myosin and eNOS, and p47phox. Figure 3 shows that eNOS and isolectin B4, and p47phox and myosin were colocalized in the same cells, and that eNOS expression was significantly increased, and p47phox was decreased in eplerenone and valsartan group compared with vehicle group.

DISCUSSION

in the same cells, and that VEGF and Ang-2 expression was significantly increased in eplerenone and valsartan group compared with vehicle group.
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Figure 5 | Effect of eplerenone and valsartan on immunofluorescence staining with double labeling for eNOS and p47phox. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Bar = 25 μm. eNOS, endothelial nitric oxide; EPL, eplerenone; VAL, valsartan.

were also indicated in valsartan treatment. These findings suggest that the effect of eplerenone on the protein levels of the angiogenic factors and NAD(P)H oxidase subunits may occur via MR and Sgk1. Thus, these findings provide a novel mechanism for the pleiotropic effects of eplerenone underlying EPCs mobilization.

EPCs represent a bone marrow-derived cell population implicated in vascular healing and endothelial cell regeneration whose number is closely correlated to the risk factor profile in both healthy subjects and patients affected by coronary artery disease. Reduced EPC count is associated with coronary artery disease, diabetes, smoking, and ageing. In addition, augmentation of circulating EPCs or other cells with proangiogenic properties results in improved coronary collateral development in coronary artery disease. Previous study indicated that incubation with aldosterone determined a dose-dependent inhibition on the formation of rat bone marrow-derived EPCs due to a decrease in the expression of VEGF receptor 2; this effect was reversed by spironolactone and N-acetyl-cysteine, indicating specificity of action through the MR and involvement of oxidative pathways. In addition, Suzuki et al. demonstrated that chronic monotherapy with eplerenone prevents progressive left ventricular systolic and diastolic dysfunction and attenuates left ventricular chamber remodeling in dogs with moderate heart failure. They showed that eplerenone appeared to cause an increase in capillary density as a result of attenuating the development of fibrosis. A critical review of the literature on these subjects has been shown by Stier, and it is concluded that eplerenone should prove to be of great therapeutic value in hypertension control and prevention of cardiovascular disease and associated end-organ damage. Moreover, an important role for nitric oxide in regulation of progenitor cell mobilization and function has been described previously. Landmesser et al. demonstrated that the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor atorvastatin increased EPC mobilization in eNOS+/+ but not eNOS−/− mice, which underlines the pivotal role of endothelial-derived nitric oxide in regulation of the transit of EPCs from bone marrow to circulation. In a recent report on endothelial functions, Schäfer et al. demonstrated that downregulated expression of eNOS protein in the aorta of failing rats with myocardial ischemia could be restored with eplerenone administration. Also, we showed that reduced eNOS expression in the left ventricular of Dahl rats with cardiac dysfunctions and myocardial remodeling was restored with eplerenone administration. Based on these findings together, it is considered that eplerenone may improve the proliferation and function of EPCs by enhancing eNOS production. Furthermore, increased oxidative stress may play a further role for the development of EPC dysfunction in cardiovascular diseases. Imanishi et al. showed that exposure of EPCs to oxidized low-density lipoprotein significantly induces EPC senescence and cellular dysfunction. Dernbach et al. investigated antioxidative systems in EPCs, and showed that the inhibition of antioxidant enzymes increased reactive oxygen species (ROS) levels in EPCs and impaired EPC survival and migration. Likewise, impaired angiogenesis in glutathione peroxidase-1-deficient mice with enhanced oxidative stress is associated with EPC dysfunction. With regard to the ROS and aldosterone, Sun et al. demonstrated that inhibition of NAD(P)H oxidase ameliorates the adverse myocardial effects of aldosterone, suggesting that NAD(P)H oxidase may be a source of ROS in response to MR activation. In addition, eplerenone treatment in hyperlipidemic rabbits reduced their aortas superoxide generation and NAD(P)H oxidase activity. These findings suggest that the biochemical basis of EPC dysfunction is excessive oxidative stress and that EPCs subjected to the unfavorable vascular environment of oxidative stress may undergo senescence.

It has been demonstrated that some ARB increase the number of EPC in patients with type 2 diabetic mellitus and coronary artery diseases via probably antioxidative mechanism. These effects are consistent with the evidences that angiotensin II induced the cellular senescence of EPC through oxidative stress and endothelial dysfunction by producing of ROS, activating apoptotic signaling pathways and secretion of inflammatory cytokines of endothelial cells. Moreover, the effects of ARB on EPC function and cardiovascular oxidation were recently investigated in stroke-prone spontaneously hypertensive rats. EPC colony formation was markedly suppressed with increases in oxidation, and treatment with ARB markedly increased EPC colony formation with decreases in NAD(P)H oxidase. In the present study, the colony number of EPCs from rat hindlimb ischemia in eplerenone and valsartan treatment was markedly increased. In addition, eplerenone, valsartan, and superoxide dismutase mimetic tempol significantly decreased the expression of NAD(P)H oxidase p22phox and p47phox protein and superoxide anion production. Therefore, eplerenone and valsartan may improve EPC function through antioxidative mechanism independent...
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of its blood pressure-lowering action. Moreover, eplerenone, valsartan, and tempos significantly decreased the expression of MR and an effector kinase of MR activation Sgk1. The inhibitory effect of eplerenone on NAD(P)H oxidase subunits expression in the ischemic tissue suggests the possibility of the crosstalk between MR and oxidative stress. These findings suggest that EPC function is impaired in hindlimb ischemia by ROS induced by tissue MR, and that eplerenone improves EPC function by suppressing MR-induced oxidative stress in rat hindlimb ischemia.

Aldosterone breakthrough is a state of sustained aldosterone synthesis in the adrenal during relative long-term treatment with angiotensin-converting enzyme inhibitors or ARB. Based on literature reports, the incidence of the aldosterone breakthrough phenomenon ranges from 10% over 6 months to 53% over 1 year. The mechanism of aldosterone breakthrough is still unclear, but subanalysis of the Valsartan Heart Failure Trial (Val-HeFT) revealed that the plasma aldosterone concentration did not differ significantly whether an angiotensin-converting enzyme inhibitor was administered or not. It is concluded that valsartan added to background therapy for heart failure produces sustained reduction in plasma aldosterone, consistent with the observed significant reduction in the combined mortality and morbidity end point. In the present study, valsartan inhibited the expression of MR and a downstream effector of aldosterone Sgk1 with a similar degree to the treatment with eplerenone. The mechanism of this observation is not known. However, future studies are necessary to determine this mechanism.

In conclusion, the findings of the present study demonstrated that the number and function of EPCs were impaired in rat hindlimb ischemia showing reduced eNOS production and increased oxidative stress. Such impairment may reduce the vascular regeneration potential and contribute to the pathogenesis of vascular complications in rat hindlimb ischemia. Treatment with eplerenone ameliorated the loss in the number and function of EPCs in hindlimb ischemia. The intracellular mechanisms of eplerenone-induced EPC mobilization may further result in the development of novel pharmacologic strategies to improve endothelial dysfunction and oxidative stress, enhance angiogenesis, and protect cardiac function.

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