Original Article

Losartan Improves the Impaired Function of Endothelial Progenitor Cells in Hypertension *via* an Antioxidant Effect

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We evaluated the effects of the angiotensin II (Ang II) receptor blocker (ARB) losartan on the formation and number of endothelial progenitor cells (EPCs) in hypertensive rats. Wistar-Kyoto (WKY) rats and stroke-prone, spontaneously hypertensive rats (SHR-SP) were salt-loaded and then treated with losartan (10 mg/kg/day), trichlormethiazide (TCM; 1.6 mg/kg/day), or tempol (1 mmol/L) for 2 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 EPC migration. Oxidative stress in EPCs was evaluated by thiobarbituric acid reactive substance (TBARS) assay. The results showed that the number, colony formation, and migration of EPCs were markedly decreased in SHR-SP compared to those in WKY rats. The TBARS scores were significantly greater in SHR-SP than in WKY rats. Losartan and TCM decreased systolic blood pressure in SHR-SP to similar levels. Losartan and tempol increased the number of circulating EPCs and colony formation, and inhibited oxidation in SHR-SP. TCM did not affect the EPC number, colony formation, or oxidation. Both losartan and TCM stimulated EPC migration. Expression of gp91^{phox}, p22^{phox}, and p47^{phox} mRNA in tissues was significantly decreased by losartan but not by TCM. These results indicate that the formation and function of EPCs are impaired by oxidative stress in SHR-SP. This is the first report to show that losartan improves the proliferation and function of EPCs in hypertension, suggesting that ARBs are useful to repair hypertensive vascular injuries. (*Hypertens Res* 2007; 30: 1119–1128)

***Key Words*: angiotensin II receptor antagonist, endothelial progenitor cell, oxidative stress, hypertension**

Introduction

Hypertension is often accompanied by severe complications—*e.g.*, stroke, ischemic heart disease, and nephrosclerosis—that are associated with vascular damage. Angiotensin II (Ang II) receptor blockers (ARBs) effectively reduce high blood pressure in 90% of patients with essential hypertension

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This work was supported in part by a Grant-in-Aid for the High-Tech Research Center from the Japanese Ministry of Education, Culture, Sports, Science and Technology to Nihon University and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received March 9, 2007; Accepted in revised form June 6, 2007.

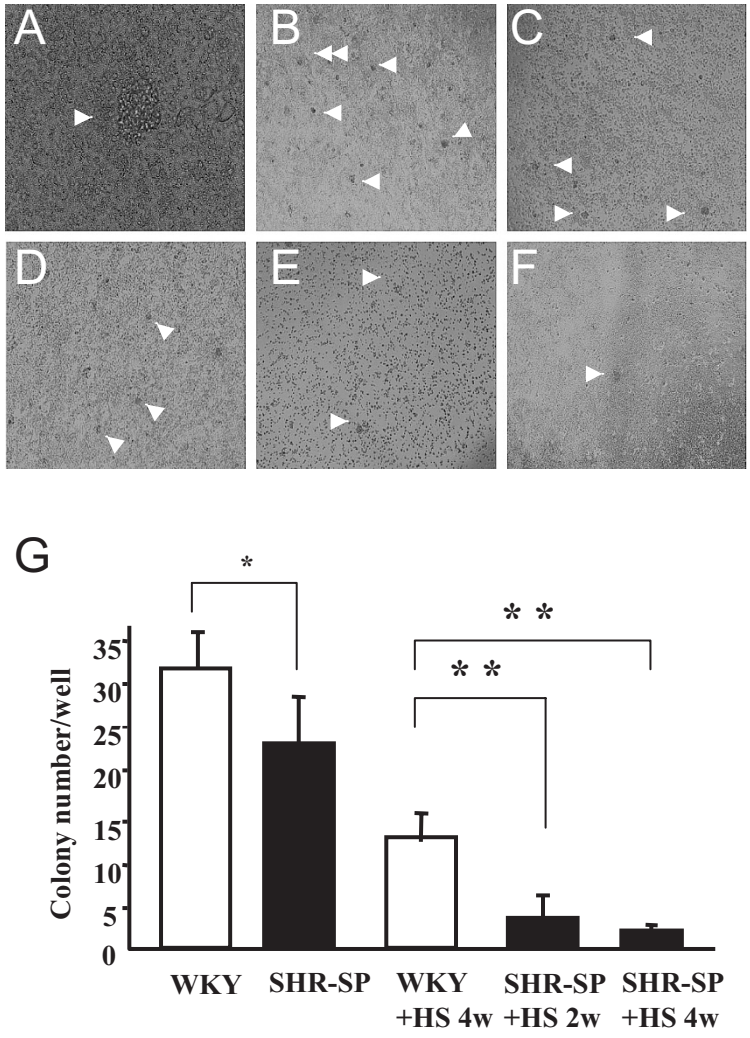

Fig. 1. *Endothelial progenitor cell (EPC) colony formation by cells from rats fed a normal or high-salt diet. Mononuclear cells isolated from rat peripheral blood were plated onto culture plates precoated with vitronectin. After 7 days of culture, nonadherent cells were removed, and attached cells formed distinct colonies (×200) (A). Representative micrographs of EPC colonies cultured from Wistar-Kyoto (WKY) rats fed a normal diet (B), stroke-prone spontaneously hypertensive rats (SHR-SP) fed a normal diet (C), WKY rats fed a high-salt diet for 4 weeks (D), and SHR-SP fed a high-salt diet for 2 weeks (E) or 4 weeks (F) are shown (×40). White arrowheads indicate EPC colonies. (G) Differences in EPC colony formation by cells from WKY rats and SHR-SP fed a normal or high-salt diet for 2 or 4 weeks. HS, high-salt diet. Data are shown as the means ± SEM (n = 6). *p < 0.05, **p < 0.01.*

independently of plasma renin activity, indicating that the pathogenesis of essential hypertension is related to the level of Ang II in tissue. In addition, unlike other antihypertensive drugs, ARBs are reported to have cardiovascular protective effects. The protective effects of ARBs with respect to vascular injury are associated with the inhibitions of Ang II–induced vascular growth (*1*), extracellular matrix formation (*2*), and oxidative stress (*3*). Oxidative stress has emerged as a common theme of many conditions associated with shortened lifespan, including hypertension. The cardiovascular damage caused by oxidative stress has been shown to be due to increased production of tissue Ang II (*4*).

Whereas conditions such as hypertension, dyslipidemia, insulin resistance, and diabetes mellitus cause vascular injury, including endothelial damage, abnormalities in the repair of endothelial damage enhance vascular injury. Endothelial progenitor cells (EPCs) are bone marrow–derived cells with the potential to differentiate into mature functional endothelial cells. In adults, repair of injured vessel walls and generation of new blood vessels require bone marrow–derived stem cells or EPCs (*5*, *6*). Postnatal neovascularization was initially thought to result exclusively from the migration and prolifer-

ation of pre-existing, fully differentiated endothelial cells (*7*). However, recent studies have shown that circulating EPCs localize to sites of neovascularization and differentiate into endothelial cells *in situ* (*5*, *8*, *9*). Impaired EPCs are reported to be a final determinant for vascular injury associated with endothelial dysfunction in disparate conditions such as hypertension, dyslipidemia, diabetes mellitus, and smoking-related diseases (*10*). EPCs in peripheral blood function in the search for and repair of endothelial damage, indicating that dysfunction of EPCs results in impaired vascular repair. The number of peripheral blood EPCs is decreased in patients with cardiovascular risk factors such as hypertension (*10*, *11*), and EPCs become dysfunctional in certain patients (*12–15*). In diseases of the vessel wall, such as atherosclerosis, the number of EPCs can be decreased by 40%, and the function of these cells is impaired (*11*). Multivariate analysis has revealed that hypertension is a major independent predictor of impaired EPC migration (*11*). These problems may limit endogenous repair of vascular lesions, leading to progression of atherosclerosis. Therefore, increasing the number and function of EPCs is necessary for the prevention and/or treatment of vascular diseases such as atherosclerosis.

The lifespan of stem cells and EPCs has been reported to be shortened by oxidative stress (*16*). It is therefore possible that oxidative stress induces endothelial damage by shortening the EPC lifespan and decreasing EPC function. However, the mechanisms underlying EPC reduction and dysfunction in hypertension remain to be determined.

Given the potential link between EPC and oxidative stress, we hypothesized that the decrease in circulating EPCs in hypertension may be due to oxidative stress induced by tissue Ang II. Thus, inhibition of oxidative stress in hypertension by ARBs may improve the number and function of circulating EPCs and improve the repair of the injured endothelium and ischemic organs, thereby preventing and/or attenuating the progression of atherosclerosis in hypertension. We investigated the effects of an ARB on oxidative stress and the number and function of EPCs in salt-loaded, stroke-prone, spontaneously hypertensive rats (SHR-SP) as a model of oxidative stress in hypertension.

Methods

Experimental Design

This study conformed to 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).

Experiments were performed on 12-week-old male Wistar-Kyoto (WKY) rats and SHR-SP (SHR Corp., Funabashi, Japan). Rats were housed 3 per cage under controlled conditions of temperature, humidity, and light, and had unrestricted access to water. WKY rats ($n=18$) and SHR-SP ($n=42$) received either a normal diet or a high-salt diet (8% NaCl) for 2 or 4 weeks, and SHR-SP fed a high-salt diet for 2 weeks

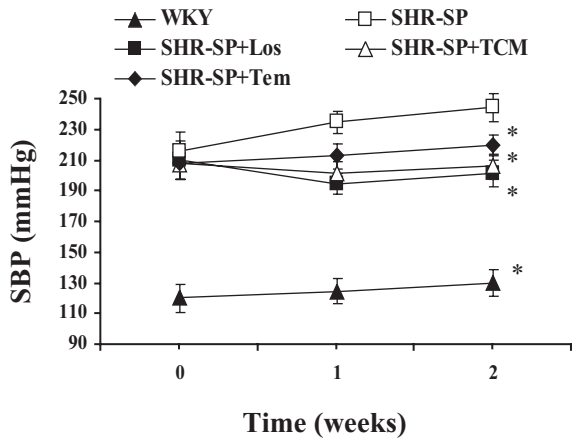

Fig. 2. *Changes in systolic blood pressure (SBP) in salt-loaded WKY rats and SHR-SP treated without (SHR-SP, n = 6) or with losartan (10 mg/kg/day) (SHR-SP+Los, n = 6), trichlormethiazide (1.6 mg/kg/day) (SHR-SP+TCM, n = 6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n = 6) for 2 weeks. Data are shown as the means ± SEM. *p < 0.01 vs. untreated SHR-SP.*

were randomly assigned to 1 of 4 groups: 1) untreated SHR-SP ($n=6$) received drinking water; 2) SHR-SP treated with trichlormethiazide (TCM; Sigma, St. Louis, USA) ($n=6$) received the diuretic TCM (1.6 mg/kg/day) dissolved in drinking water and administered orally for 2 weeks; 3) SHR-SP treated with losartan (Merck Pharmaceutical Co., Ltd., Wilmington, USA) ($n=6$) received losartan (10 mg/kg/day) dissolved in drinking water and administered orally for 2 weeks; and 4) SHR-SP treated with 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (tempol; Sigma) ($n=6$) received an antioxidant tempol (1 mmol/L) dissolved in drinking water and administered orally for 2 weeks.

Systolic blood pressure (SBP) was measured weekly in the morning throughout the 2-week period by means of the tail-cuff method and a blood pressure monitor (model BO-98A; Softron Co., Tokyo, Japan). The average of three successive readings was recorded. The rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) after 2 or 4 weeks, and the kidneys, aorta, and heart were flushed with ice-cold phosphate-buffered saline (PBS) and removed immediately. The kidney cortex, aorta, and heart were stored at −80°C until use in RNA extraction.

EPC Colony Formation Assay

A modified EPC colony formation assay was performed as previously described (*10*, *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 Histopaque-1083 density gradient

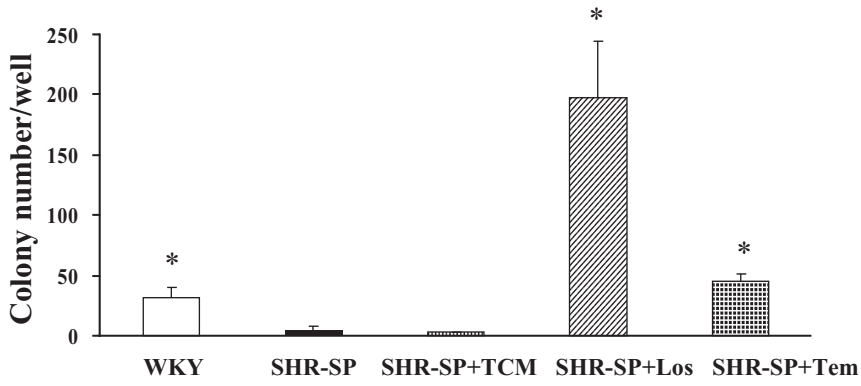

Fig. 3. *Effect of losartan, TCM, or tempol on EPC colony formation by cells from SHR-SP. WKY rats and SHR-SP were fed a high-salt diet for 2 weeks. SHR-SP were then treated without (SHR-SP, n = 6) or with TCM (1.6 mg/kg/day) (SHR-SP+TCM, n = 6), losartan (10 mg/kg/day) (SHR-SP+Los, n = 6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n = 6) for 2 weeks. Data are the means ± SEM. *p < 0.01 vs. untreated SHR-SP.*

medium (Sigma). MNCs were suspended and mixed in 1 mL of EGM-2 (endothelial growth medium-2) medium (Clonetics, San Diego, USA) containing 10% fetal bovine serum (FBS), human basic fibroblast growth factor, vascular endothelial growth factor (VEGF), recombinant 3 insulin-like growth factor-1, human epidermal growth factor, ascorbic acid, and GA-1000. Twenty-four-well plates (Falcon) were precoated with rat vitronectin (0.1 μg/cm^2) plus 0.5% gelatin overnight at 37°C. MNCs were inoculated onto 6-well plates (5×10^6 cells/well) and cultured for 24 h. Nonadherent MNCs were reinoculated onto vitronectin-coated plates (2×10^6 cells/well) and cultured in a CO_2 incubator at 37°C for 7 days. The average number of colonies was calculated manually under microscopy from a minimum of 4 wells by an observer who was unaware of the experimental design.

Flow Cytometric Analysis for EPCs

We determined the number of circulating EPCs in peripheral blood using a cell surface antigen as previously described (*5*, *18*). 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 (FITC)–conjugated anti-CD34 monoclonal antibody (Becton-Dickinson, San Jose, USA). Samples were subjected to a two dimensional side scatter-fluorescence dot plot analysis (FACScan; Becton-Dickinson) (*19*). After appropriate gating, the number of CD34$^+$ cells with low cytoplasmic granularity (low sideward scatter) was quantified and expressed as the number of cells per 10^4 total events.

EPC Migration Assay

To investigate EPC migration activity, a modified Boyden chamber assay was performed with a 96-well microchemotaxis chamber (Neuroprobe, Gaithersburg, USA) as previously described (*20*, *21*). In brief, after inoculation onto 6-well plates for 24 h, nonadherent MNCs were resuspended in EBM-2 (endothelial basic medium-2) medium (Clonetics). Chemotaxis assays were performed in 96-microwell chemotaxis chambers (Neuroprobe) with 5-μm–pore-size, vitrogen (Cohesion Technologies Inc., Palo Alto, USA)-coated polycarbonate filters (Neuroprobe). EBM-2 medium containing 10% FBS, 50 ng/mL stromal-derived factor 1 (SDF-1) (R&D Systems, Minneapolis, USA), 50 ng/mL VEGF-A (R&D Systems), or 0.1% bovine serum albumin (negative control) was applied to the lower compartment, and cells were adjusted to 5×10^4 cells/mL and applied to the upper compartment. After incubation in a CO_2 incubator at 37°C for 24 h, the filter was removed, and migrated cells on the filter were washed once with PBS, fixed, stained with Diff-Quick (Sysmex International Reagents Co., Ltd., Hyogo, Japan), and counted by microscopy at 40× magnification. The migration index was calculated by dividing the number of migrated cells in the presence of chemoattractants by the number of cells migrated in negative controls. All assays were performed at least in triplicate.

Thiobarbituric Acid Reactive Substance Assay

Thiobarbituric acid (TBA) reactive substances (TBARS) in MNCs were measured with a commercial kit (Oxi-Tek TBARS assay kit; Zeptometrix Corp., Buffalo, USA) (*22*). In brief, MNCs (10^6/100 μL) were mixed with 100 μL sodium dodecyl sulfonate (SDS) solution. TBA/buffer reagent was prepared by mixing 0.5 g TBA with 50 mL acetic acid and 50 mL NaOH. TBA/buffer reagent (2.5 mL) was added to 200 μL sample/SDS mixture and incubated at 95°C in capped tubes for 60 min. Samples were cooled to room temperature in an ice bath for 10 min and centrifuged at 3,000 rpm for 15 min. The supernatants were removed, and the fluorescence

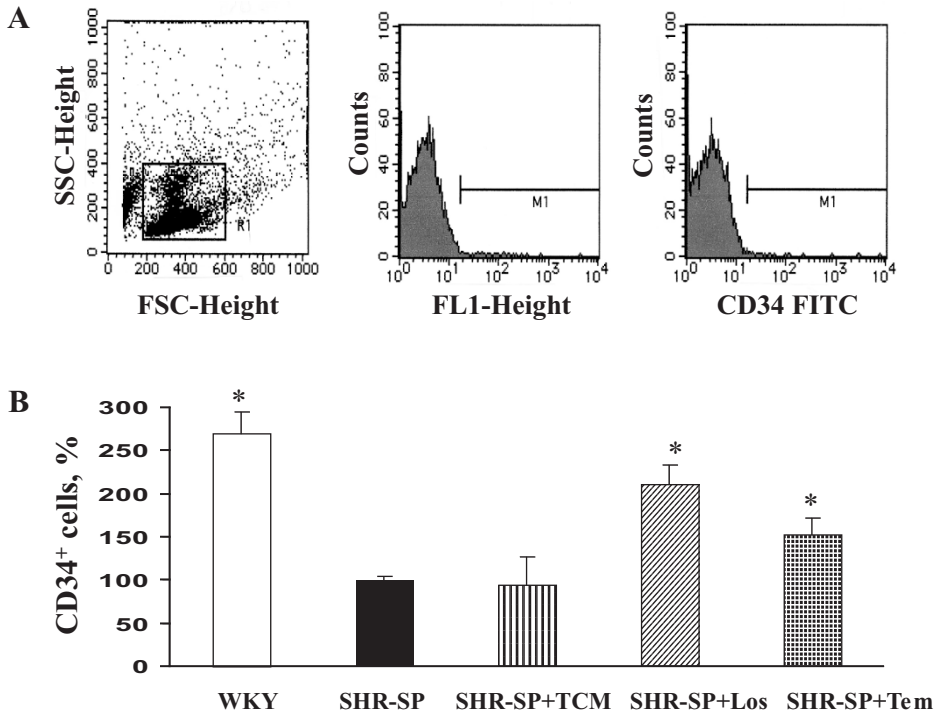

Fig. 4. *A: Quantification of EPC by flow cytometric analysis. Circulating EPCs were identified by flow cytometry according to the expression of cell surface antigen CD34. Representative results of a flow cytometry analysis are shown. B: The number of circulating EPCs (CD34-expressing cells) in peripheral blood. WKY rats and SHR-SP were fed a high-salt diet for 2 weeks. SHR-SP were then treated without (SHR-SP, n = 6) or with TCM (1.6 mg/kg/day) (SHR-SP+TCM, n = 6), losartan (10 mg/kg/day) (SHR-SP+Los, n = 6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n = 6) for 2 weeks. Data are the means ± SEM. *p < 0.01 vs. untreated SHR-SP.*

intensity was measured in semimicrocuvettes in a fluorometer (Bio-Rad, Hercules, USA). The concentration of TBARS was expressed in pmol/10^6 cells by interpolation from a standard curve of malondialdehyde (MDA) at concentrations of 0–200 pmol/L.

Determination of mRNA Expression

Total RNA was isolated and reverse-transcribed as described previously (*23*). Real-time quantitative polymerase chain reaction (PCR) was performed with cDNA diluted four times, TaqMan Universal Master Mix, and an ABI 7500 sequence detector (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Assay-on-Demand primers and probes ($p22^{phox}$: Rn00577357_m1; $p47^{phox}$: Rn00586945_m1; $gp91^{phox}$: Rn00576710_m1; and TaqMan Rodent GAPDH control reagents) were purchased from Applied Biosystems. The comparative ΔCT method was used for relative quantification and statistical analysis (*24*), and fold changes are presented graphically. A unit increase in cycle value represents a 2-fold change in the amount of mRNA.

Statistical Analysis

Values are reported as the means ± SEM. Statistical analysis was performed with Student's *t*-test for unpaired data, two-way analysis of variance (ANOVA), or Duncan's multiple range test. Values of $p < 0.05$ were considered statistically significant.

Results

EPC Colony Formation by Cells from Rats Fed a Normal or High-Salt Diet

EPC colony formation of cells from WKY rats and SHR-SP fed a normal or high-salt diet for 2 or 4 weeks is shown in Fig. 1. Without salt-loading, the number of EPC colonies formed by cells from SHR-SP was significantly smaller than that formed by cells from WKY rats ($p < 0.05$). With salt-loading for 2 or 4 weeks, the number of EPC colonies formed by cells from SHR-SP was significantly decreased compared to that formed by cells from WKY rats with salt-loading ($p < 0.01$).

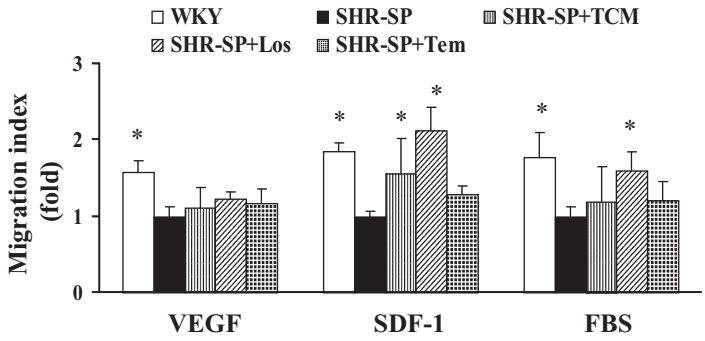

Fig. 5. *Effect of losartan, TCM, or tempol on EPC migration in SHR-SP. WKY rats and SHR-SP were fed a high-salt diet for 2 weeks. SHR-SP were then treated without (SHR-SP, n=6) or with TCM (1.6 mg/kg/day) (SHR-SP+TCM, n=6), losartan (10 mg/kg/day) (SHR-SP+Los, n=6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n=6) for 2 weeks. Data are shown as the means±SEM. *p<0.05 vs. untreated SHR-SP.*

Effect of Losartan, TCM, or Tempol on SBP

Changes in SBP in salt-loaded WKY rats and SHR-SP treated without or with losartan, TCM, or tempol for 2 weeks are shown in Fig. 2. SBP was significantly increased in salt-loaded SHR-SP compared to that in salt-loaded WKY rats. Treatment with TCM or losartan significantly decreased SBP in salt-loaded SHR-SP to similar levels. Treatment with tempol also decreased SBP in salt-loaded SHR-SP.

Effect of Losartan, TCM, or Tempol on EPC Colony Formation

The number of EPC colonies was significantly smaller in SHR-SP than in WKY rats ($p<0.01$; Fig. 3). Treatment with TCM did not increase the number of EPC colonies in SHR-SP. However, treatment with losartan or tempol significantly increased the number of EPC colonies in SHR-SP compared to that in untreated SHR-SP ($p<0.01$).

Effect of Losartan, TCM, or Tempol on Circulating EPC Number

The number of circulating EPCs in peripheral blood was significantly smaller in SHR-SP than that in WKY rats ($p<0.01$; Fig. 4). Treatment with TCM did not increase the number of circulating EPCs in SHR-SP. However, treatment with losartan or tempol significantly increased the number of EPCs in SHR-SP compared to that in untreated SHR-SP ($p<0.01$).

Effects of Losartan, TCM, or Tempol on EPC Migration

The effects of losartan, TCM, or tempol on EPC migration in response to VEGF-A, SDF-1, or FBS are shown in Fig. 5. EPC migration was significantly impaired in SHR-SP compared to that in WKY rats ($p<0.05$). Treatment with TCM increased EPC migration in response to SDF-1 compared to that in untreated SHR-SP, and treatment with losartan significantly increased EPC migration in response to SDF-1 or FBS ($p<0.05$). However, treatment with tempol did not increase EPC migration.

Effect of Losartan, TCM, or Tempol on TBARS Score

The TBARS score was significantly greater in SHR-SP than in WKY rats ($p<0.05$; Fig. 6). Treatment with TCM did not affect the increased TBARS score in SHR-SP. However, treatment with losartan or tempol significantly decreased the TBARS score in SHR-SP ($p<0.05$).

Effect of Losartan, TCM, or Tempol on mRNA Expression of Nicotinamide Adenine Dinucleotide Phosphate Oxidase Subunits

The effects of losartan, TCM, or tempol on mRNA expression of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase subunits in the aorta, heart, and renal cortex are shown in Fig. 7. The amounts of $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ mRNA in SHR-SP were significantly greater than those in WKY rats ($p<0.01$). Treatment with TCM did not affect the mRNA expression of NAD(P)H oxidase subunits in SHR-SP. However, treatment with losartan significantly decreased $gp91^{phox}$ and $p22^{phox}$ mRNA levels in the aorta, heart, and renal cortex in SHR-SP and also significantly decreased $p47^{phox}$ mRNA expression in the aorta ($p<0.01$). Treatment with tempol significantly decreased $p22^{phox}$ mRNA levels in the heart and renal cortex in SHR-SP.

Discussion

In the present study, EPC number and colony formation were significantly inhibited in SHR-SP compared to those in WKY

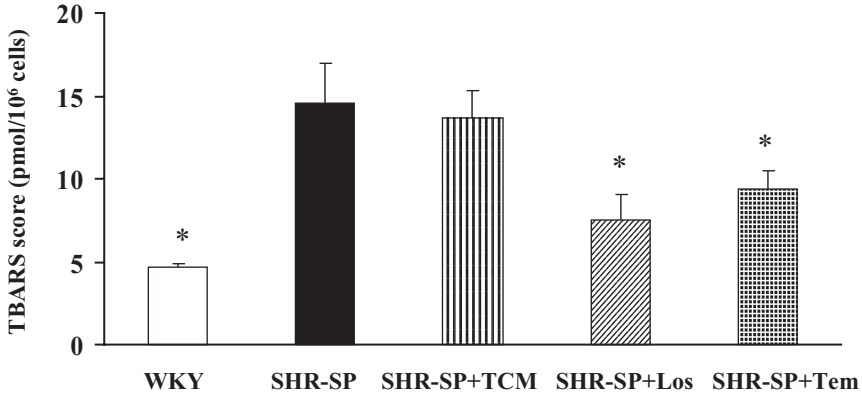

Fig. 6. *Effect of losartan, TCM, or tempol on thiobarbituric acid reactive substance (TBARS) scores in mononuclear cells (MNCs). WKY rats and SHR-SP were fed a high-salt diet for 2 weeks. SHR-SP were then treated without (SHR-SP, n = 6) or with TCM (1.6 mg/kg/day) (SHR-SP+TCM, n = 6), losartan (10 mg/kg/day) (SHR-SP+Los, n = 6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n = 6) for 2 weeks. Data are shown as the means ± SEM. *p < 0.05 vs. untreated SHR-SP.*

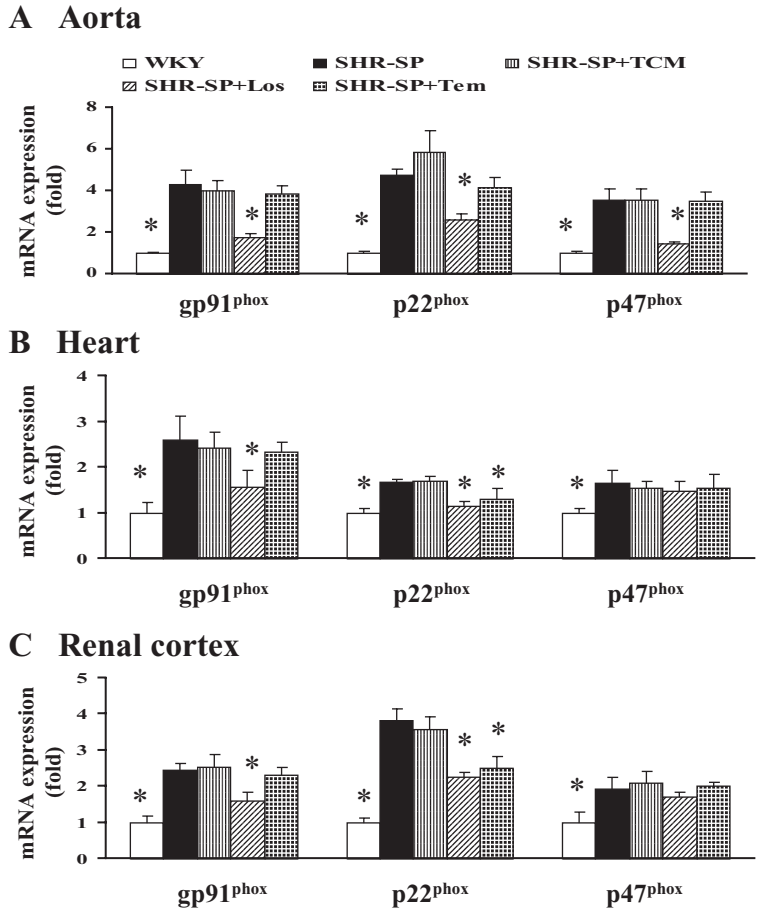

Fig. 7. *Real-time PCR analysis of mRNA expression of NAD(P)H oxidase subunits gp91^{phox}, p22^{phox}, and p47^{phox} in the aorta (A), heart (B), and renal cortex (C) without or with TCM, losartan, or tempol treatment for 2 weeks. WKY rats and SHR-SP were fed a high-salt diet for 2 weeks. SHR-SP were then treated without (SHR-SP, n = 6) or with TCM (1.6 mg/kg/day) (SHR-SP+TCM, n = 6), losartan (10 mg/kg/day) (SHR-SP+Los, n = 6), or tempol (1 mmol/L in drinking water) (SHR-SP+Tem, n = 6) for 2 weeks. Ratios of target mRNA to GAPDH mRNA were evaluated. Data are shown as the means ± SEM. *p < 0.01 vs. untreated SHR-SP.*

rats. SHR-SP have been reported to show severe cardiovascular damage associated with tissue Ang II (*25*) and oxidative stress (*26*, *27*). In hypertension, cardiovascular damage is caused by oxidative stress due to the increased production of tissue Ang II. Ang II promotes oxidative stress, and superoxide is an important transduction signal for this effect (*28*). Superoxide is the most prominent reactive oxygen species (ROS), and the primary enzyme in superoxide generation is NAD(P)H oxidase. Vascular NAD(P)H oxidase is upregulated by vasoactive factors including Ang II, cytokines, growth factors, stretch, shear stress, and pulsatile strain (*29*). At low concentrations, ROS act as signaling molecules in endothelial function and vascular contractility (*4*). NAD(P)H oxidase is a major source of ROS in the cardiovascular system (*30*). Pathologic increases in ROS levels result in a plethora of effects that damage the vessel wall (*31*). Inhibition of NAD(P)H oxidase–mediated generation of superoxide by pharmacologic or gene-targeting approaches improves endothelial function and causes regression of vascular remodeling (*32*). In the present study, the expression of NAD(P)H oxidase component mRNAs such as $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ was increased in the heart, aorta, and renal cortex in salt-loaded SHR-SP. Losartan treatment significantly inhibited the increased expression of these NAD(P)H oxidase components. Thus, tissue Ang II increases NAD(P)H oxidase components and induces oxidative stress in salt-loaded SHR-SP. TBARS scores in MNCs from SHR-SP were also significantly greater than those from WKY rats, and these increased scores were significantly decreased by losartan treatment, indicating that cellular oxidative stress increases with tissue or cellular Ang II levels. Thus, the oxidative stress in hypertension induces the impairment of EPC function.

Imanishi *et al*. (*33*) reported that EPC senescence is accelerated in SHR and deoxycorticosterone acetate (DOCA)/salt-hypertensive rats. They also recently reported that Ang II–induced EPC senescence is significantly inhibited by treatment with valsartan *in vitro* (*34*). However, their methods for the detection of EPCs differed from ours. They isolated MNCs from rats, cultured them in endothelium-basement medium, and identified acetyl low-density lipoprotein (LDL) incorporation as an indicator of EPCs. Acetyl LDL–incorporating cells do not differ from endothelial cells. We seeded MNCs isolated from rats and then reseeded floating cells to eliminate contamination by monocytes and macrophages, and cultured cells for 7 days for colony formation and migration assays. We treated SHR-SP with losartan for 2 weeks *in vivo*, whereas Imanishi *et al*. (*34*) incubated cultured cells with valsartan *in vitro*. Thus, we were able to detect pure EPCs and to evaluate the effects of losartan on hypertension *in vivo*.

In the present study, the number of circulating EPCs, number of EPC colonies formed, and EPC migration were all significantly decreased in SHR-SP compared to those in WKY rats. Losartan treatment markedly increased the EPC number and colony formation, whereas TCM treatment did not increase the EPC number or colony formation but did increase migration, even though TCM and losartan induced similar decreases in blood pressure. Treatment of the SHR-SP with tempol, a cell-permeable antioxidant, significantly increased the number of circulating EPCs and number of EPC colonies formed, and significantly decreased the TBARS score in circulating MNCs. These results indicate that the effect of losartan on the EPC number and function in SHR-SP was independent of its effect on blood pressure, and that cellular Ang II suppresses EPC function *via* the induction of oxidative stress in SHR-SP. Whereas only losartan treatment increased the EPC number and colony formation in SHR-SP, TCM or losartan enhanced EPC migration in SHR-SP. The mechanisms underlying EPC colony formation and migration are different. The migration of EPCs in response to VEGF or SDF-1 reflects an angiogenic ability, similar to that of endothelial cells (*35*), whereas the EPC number and colony formation may reflect the vascular protective effects (*10*). In addition, treatment with the antioxidant tempol did not increase the migration of EPCs in the present study. Thus, it may be that only the EPC number and colony formation are regulated by cellular Ang II and oxidative stress. The blood pressure reduction may be one of the possible mechanisms by which TCM enhanced EPC migration. Further *in vivo* studies are required to determine the precise mechanisms responsible for TCM-induced alterations in the EPC migration.

EPCs circulate in the peripheral blood and are directly exposed to systemic oxidative stressors. The pro-oxidant conditions of hypertension may affect EPCs and cells that interact with EPCs to determine their function and fate. For example, Imanishi *et al*. (*36*) reported that exposure of EPCs to oxidized LDL significantly induces EPC senescence and cellular dysfunction. Dernbach *et al*. (*37*) reported impaired EPC proliferation and migration in the presence of high levels of oxidative stress. 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 or apoptosis. Increased resistance to stress, beyond protection against apoptosis, may be a general characteristic of stem and progenitor cells. The expression of stress response genes involved in redox balance is a characteristic feature of stem and progenitor cells (*38*, *39*). Studies of neural progenitor cells have revealed that factors promoting self-renewal cause these cells to enter a decreased redox state, whereas exposure to molecules that promote differentiation leads to excess oxidation in these cells (*40*). Thus, ROS may be involved in the balance between self-renewal and differentiation. The redox balance changes as cells mature, suggesting that antioxidant enzymes may play major roles in the preservation of characteristics of stem and progenitor cells. It is possible that increased expression of antioxidant enzymes such as manganese superoxide dismutase (MnSOD) in EPCs compared to that in mature endothelial cells maintains EPCs in an undifferentiated, self-renewing state to allow for progenitor cell expansion at sites of repair. Other functions of EPCs, such as migration, which

affect patient improvement after cell transplantation (*35*), may be critically influenced by the redox equilibrium state (*37*).

While changes in NAD(P)H oxidase subunit expression in EPCs were not assessed in this study, Imanishi *et al*. (*34*) recently showed that expression of the NAD(P)H oxidase subunit $gp91^{phox}$ increases in EPCs *via* Ang II, which leads to an increased rate of EPC senescence. When ROS-scavenging enzymes, such as SOD, were administered, Ang II–induced senescence was ameliorated. Dernbach *et al*. (*37*) investigated antioxidative systems in EPCs, and showed that the inhibition of antioxidant enzymes increased ROS levels in EPCs and impaired EPC survival and migration. In the present study, the antioxidant tempol significantly increased the number of EPCs and the formation of EPC colonies, suggesting that oxidation decreases EPC formation and function in hypertension. The ability of Ang II to induce cell senescence also involves suppression of telomerase activity (*34*). Kobayashi *et al*. (*41*) reported that telomerase activity was significantly lower in EPCs from Ang II–treated rats, and that Ang II accelerated the senescence of EPCs *via* Ang II type 1 receptor (AT_1R). Telomerase maintains the telomere number at the ends of chromosomes. Telomere loss is emerging as an important factor in the pathogenesis of various conditions, including hypertension, atherosclerosis, and heart failure (*42*). The above-mentioned results show that EPCs are sensitive to Ang II signaling, and that this may affect the number and function of EPCs, as well as EPC senescence. The results of the present study show that levels of TBARS, a marker of lipid peroxidation and oxidative stress, are increased in circulating MNCs from SHR-SP. EPCs are believed to comprise a subset of circulating MNCs. Thus, our results, together with those of previous studies, indicate that oxidative stress can decrease the number and function of EPCs in hypertension. Bahlmann *et al*. (*43*) recently showed that the number of EPCs in patients with type II diabetes mellitus, which is decreased, can be increased by treatment with olmesartan or irbesartan, suggesting that ARBs may have novel beneficial effects on cardiovascular disease. In this study, we also showed that an ARB had beneficial effects on EPC function in hypertension *in vivo*.

Aging is generally associated with vascular damage. The number of patients with essential hypertension in Japan is approximately 20 million, most of whom would certainly die of cardiovascular diseases such as stroke, hypertensive heart disease, or renal sclerosis without treatment. Cardiovascular damage is caused by oxidative stress due to increased production of tissue Ang II. Oxidative stress also shortens the lifespan of stem and progenitor cells, leading to decreased repair of damaged tissue and organs. Thus, the lifespans of stem and progenitor cells, which are regulated by oxidative mechanisms, influence the human lifespan. ARBs may be beneficial antihypertensive agents with cardiovascular protective effects.

In conclusion, the results of the present study showed that the number and function of EPCs were impaired in hypertensive rats showing oxidative stress. Such impairment may reduce the vascular regeneration potential and contribute to the pathogenesis of vascular complications in hypertension. Treatment with losartan ameliorated the loss in the number and function of EPCs in hypertensive rats. Thus, therapy with ARBs may provide a novel and effective therapeutic strategy for the repair of vascular injury.

Perspectives

The number and function of EPCs were found to be impaired in hypertensive rats, and these impairments were ameliorated in response to treatment with losartan. Losartan treatment may prove to be a novel strategy for the treatment of vascular injury involving endothelial damage in patients with hypertension.

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