Combination of the Angiotensin-Converting Enzyme Inhibitor Perindopril and the Diuretic Indapamide Activate Postnatal Vasculogenesis in Spontaneously Hypertensive Rats

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ABSTRACT

Cardiovascular risk factors are associated with reduction in both the number and function of vascular progenitor cells. We hypothesized that 1) hypertension abrogates postnatal vasculogenesis, and 2) antihypertensive treatment based on the combination of perindopril (angiotensin-converting enzyme inhibitor) and indapamide (diuretic) may counteract hypertension-induced alteration in progenitor cell-related effects. Postischemic neovascularization was significantly lower in untreated spontaneously hypertensive rats (SHRs) compared with Wistar Kyoto (WKY) rats (p < 0.05). Treatment of SHRs with perindopril and the combination of perindopril/indapamide reduced the blood pressure levels and normalized vessel growth in ischemic area. Cotreatment with perindopril and indapamide increased vascular endothelial growth factor and endothelial nitric-oxide synthase protein contents, two key proangiogenic factors. It is interesting to note that 14 days after bone marrow mononuclear cell (BM-MNC) transplantation, revascularization was significantly lower in ischemic SHRs receiving BM-MNCs isolated from SHRs compared with those receiving BM-MNCs isolated from WKY rats (p < 0.05). Alteration in proangiogenic potential of SHR BM-MNCs was probably related to the reduction in their ability to differentiate into endothelial progenitor cells in vitro. Furthermore, the number of circulating endothelial progenitor cells (EPCs) was reduced by 3.1-fold in SHRs compared with WKY rats (p < 0.001). Treatments with perindopril or perindopril/indapamide restored the ability of BM-MNCs to differentiate in vitro into EPCs, increased the number of circulating EPCs, and re-established BM-MNC proangiogenic effects. Therefore, hypertension is associated with a decrease in the number of circulating progenitor cells and in the BM-MNC proangiogenic potential, probably leading to vascular complications in this setting. The combination of perindopril and indapamide counteracts hypertension-induced alterations in progenitor cell-related effects and restores blood vessel growth.

The ability of organisms to spontaneously develop collateral vessels represents an important response to vascular occlusions and operates to improve perfusion of ischemic tissues (Carmeliet, 2003). However, in most clinical settings these natural adaptive responses to a compromised perfusion are insufficient to block the progression of ischemic diseases. Hence, diabetes, aging, and hypercholesterolemia have been shown to inhibit vessel growth (Couffinhal et al., 1999; Takeshita et al., 2001; Hill et al., 2003; Ebrahimian et al., 2006). Likewise, hypertension, another major cardiovascular risk factor, hampers posts ischemic vessel growth. This effect is probably mediated by a reduction in the protein levels of key proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor in hypertensive animals (Nakano et al., 1997; Emanueli et al., 2001). In addition, endothelial dysfunction may also contribute to impaired angiogenesis in spontaneously hypertensive rats (SHRs) (Konishi and Su, 1983; Lüscher et al., 1987; Panza et al., 1990). Alternatively, the participation of pro-
Progenitor Cells and Hypertension

Genitor cell dysfunction to the pathogenesis of hypertension can be speculated. The bone marrow and nonbone-marrow derived endothelial progenitor cells have been shown to promote vessel growth through their ability to incorporate into vascular structures and their paracrine activity (Fazel et al., 2006; Jin et al., 2006; You et al., 2006; Aicher et al., 2007). Specific risk factors, such as, for example, type I and II diabetes, are associated with reduced EPC numbers and angiogenicity and, subsequently, reduction in postschismic revascularization (Tepper et al., 2002; Loomans et al., 2004; Tamarat et al., 2004; Ebrahimian et al., 2006). In adult subjects without a history of cardiovascular diseases, the number of circulating endothelial progenitor cells (EPCs) was inversely correlated with the Framingham risk score, which includes systolic blood pressure as a major component (Hill et al., 2003). In a recent study, accelerated senescence of EPCs was demonstrated in hypertensive animals and humans (Imanishi et al., 2005). We can then speculate that hypertension abrogates postnatal vasculogenesis. Perindopril and indapamide are both well established effective agents that are used as first line antihypertensive treatment. Perindopril is a long-acting angiotensin-converting enzyme (ACE) inhibitor, and indapamide is an indoline derivative of chlorsulfonamide that has both diuretic and antihypertensive properties. Combination of perindopril and indapamide displays proangiogenic properties. In particular, perindopril/indapamide combination increased the capillary density in cardiac tissue of SHRs and of stroke-prone hypertensive rats (Rakusan et al., 2000; Levy et al., 2001). In normotensive rats, combination of perindopril and indapamide also induces an early and sustained effect on the revascularization process (Silvestre et al., 2002). We therefore hypothesized that hypertension hampers progenitor cell proangiogenic function and that antihypertensive treatments with perindopril, indapamide, or the combination of perindopril/indapamide may restore postschismic vasculogenesis and subsequently revascularization.

Materials and Methods

Animals. All the experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (70430). Twelve-week-old male normotensive Wistar Kyoto (WKY) rats and SHRs were separated into 10 groups (n = 6–8) receiving the following treatment for 3 weeks in drinking water: PBS, ACE inhibitor (perindopril, 0.76 mg/kg/day; Servier, Courbevoie, France), diuretic (indapamide, 0.24 mg/kg/day; Servier), and combination of perindopril and indapamide. Untreated rats served as control groups. In an additional set of experiments, WKY rats were treated with or without hypertensive dose of angiotensin II (Ang II; 120 ng/kg/min; Sigma-Aldrich, St. Quentin Fallavier, France; osmotic minipump).

Measurement of Arterial Pressure. In conscious rats, systolic blood pressure was measured by the tail-cuff method (BP2000, VisiTech system; VisiTech Systems, Inc., Apex, NC). Blood pressure was measured weekly for 10 consecutive cycles.

Model of Ischemic Hindlimb. After 1 week of treatment, WKY rats and treated and untreated SHRs were anesthetized, and the right femoral artery was occluded (6-0 silk suture) by ligation. The ligature was performed on the right femoral artery, 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries, as described previously (Silvestre et al., 2005). After 2 weeks of ligation (3 weeks of treatment), vessel density was determined by high-definition microangiography and capillary density analysis. Laser-Doppler perfusion imaging was also performed to assess in vivo tissue perfusion in the paw (Silvestre et al., 2005).

Determination of VEGF and eNOS Protein Expression. Tissue samples were thawed and homogenized in 500 μl of buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, pH 7.4) containing protease inhibitors. Protein content was measured by the method of the Dc Protein Assay (Bio-Rad, Hercules, CA). Proteins were separated in denaturing 9% SDS-polyacrylamide gels and then blotted onto a nitrocellulose sheet (Hybond enhanced chemiluminescence; GE Healthcare, Chalfont St. Giles, UK). Antibodies directed against VEGF (Tebu Bio, Le Perray en Yvelines, France), eNOS (Cell Signaling, Saint Quentin Yvelines, France), and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at a dilution of 1:1000.

Isolation of BM-MNCs. Bone marrow was obtained by flushing tibia and femur, and low-density mononuclear cells were then isolated by centrifugation on a Ficoll gradient, as described previously (Ebrahimian et al., 2006; You et al., 2006). Alternatively, 5 ml of blood was collected in EDTA-containing tubes. After centrifugation on a Ficoll gradient and red cell lysis, circulating blood mononuclear cells were then analyzed in a flow analysis cytometry system.

Flow Analysis Cytometry System. BM-MNCs (10⁶) or circulating MNCs were incubated for 15 min at 4°C with 5% fetal bovine serum buffer and then with 20 μl of fluorescein-conjugated anti-CD34 antibody (Tebu Bio) for 30 min at 4°C with PBS buffer. MNCs were also incubated with 20 μl of phycoerythrin/Cy5-conjugated anti-CD117 antibody (BD Pharmingen, San Diego, CA) for 30 min at 4°C with PBS buffer. After washing, the samples were centrifuged, and the pellets were suspended in 500 μl of PBS cell. The percentage of CD34+ and CD117+ positive cells was analyzed using CellQuest software (Beckman Coulter EPICS-XL, Roissy-Charles de Gaulle, France).

BM-MNC Differentiation into EPCs. BM-MNCs (2 × 10⁶/ml), isolated from treated or nontreated rats, were plated on 11-mm cell culture dishes coated with gelatin (0.1%) and rat plasma vitronectin (Sigma-Aldrich). BM-MNCs were maintained in endothelial basal medium for 7 days (EGM2; Lonza Verviers SPRL, Verviers, Belgium) with or without ACE inhibitor (10 μM perindopril) and/or diuretic (10 μM indapamide). Nonadherent cells were then removed, and adherent cells were analyzed by immunocytochemical assay with 1,1-diiodoacetetyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (AcDiI-LLD) (Tebu Bio) and fluorescein isothiocyanate-labeled BS-1 lectin (Sigma-Aldrich). Cells were incubated in EGM2 containing AcDiI-LLD at 37°C for 1 h. Cells were then fixed in 2% paraformaldehyde and incubated with fluorescein isothiocyanate-labeled BS-1 lectin (Sigma-Aldrich). Cells were characterized as cells with double-positive staining for both AcDiI-LLD and BS-1 lectin. EPCs were counted and expressed in cells per well by using epifluorescence microscopy, as described previously (Ebrahimian et al., 2006; You et al., 2006). Five replicates were counted for each treated or nontreated rat. Results are expressed as percentages of total cell numbers.

Progenitor Cell Proangiogenic Potential. Five hours after hindlimb ischemia, SHRs received i.v. injections of 1.10⁸ BM-MNCs isolated from treated or nontreated rats. Animals were sacrificed 14 days after cell transplantation, and vascular density was determined by three different methods: high-definition microangiography, capillary density analysis, and laser-Doppler perfusion imaging, as described above.

Statistical Analysis. Results are expressed as mean ± S.E.M. One-way analysis of variance was used to compare variables. Comparisons between two groups were performed using nonparametric Mann-Whitney test. A value of p < 0.05 was considered significant.
Results

Hemodynamical Parameters

Systolic blood pressure was markedly increased in SHRs compared with WKY rats throughout the experiment (see Table 1). Hindlimb ischemia did not affect systolic blood pressure compared with groups of rats without right femoral artery ligation. Treatment with perindopril or the combination of perindopril/indapamide reduced systolic blood pressure compared with the untreated SHRs. A low dose of indapamide alone did not affect systolic blood pressure in SHRs compared with untreated SHRs.

Expression of VEGF and eNOS Proteins

We next analyzed VEGF protein content, a key angiogenic factor. Ischemia increased by 1.7-fold VEGF protein levels in ischemic muscle of WKY rats in reference to nonischemic muscle of WKY rats ($p < 0.05$). However, VEGF protein content was decreased by 2.4-fold in the ischemic hindlimb of SHRs compared with that of WKY rats ($p < 0.001$). Treatment with perindopril or indapamide alone did not significantly change VEGF protein contents. It is interesting to note that coadministration of perindopril and indapamide markedly increased VEGF protein expression in ischemic SHRs in reference to untreated ischemic SHRs (Fig. 3a). We next determined eNOS protein levels. eNOS content was unaffected in the nonischemic leg, whatever the treatments. Ischemia increased eNOS protein content by 1.7-fold in WKY rats compared with nonischemic WKY rats but not in ischemic SHRs in reference to nonischemic SHRs ($p < 0.05$). Treatments with perindopril or perindopril/indapamide restored eNOS protein expression in ischemic SHRs ($p < 0.05$ versus untreated ischemic SHRs) (Fig. 3b).

Postischemic Neovascularization

Microangiography. The angiographic score was significantly reduced by 1.5-fold in untreated SHRs in reference to control WKY rats ($p < 0.05$). It is interesting to note that treatments with perindopril, indapamide, and perindopril/indapamide combination increased vessel density by 1.4-, 1.5-, and 1.5-fold, respectively, compared with untreated SHRs (Fig. 1).

Capillary Density. Microangiographic data were confirmed by capillary density analysis. The capillary number was decreased by 1.6-fold in ischemic SHRs compared with ischemic WKY rats ($p < 0.001$). Perindopril, indapamide, and the combination of perindopril/indapamide increased the ischemic/nonischemic capillary number ratio by 1.6-, 1.7-, and 1.6-fold, respectively, compared with control SHRs (Fig. 1).

Foot Perfusion. Microangiographic and capillary density measurements were associated with changes in paw perfusion. The ischemic/nonischemic foot ratio was reduced by 1.6-fold in ischemic SHRs compared with ischemic WKY rats ($p < 0.05$). Administration of perindopril alone, indapamide alone, and the combination of perindopril/indapamide enhanced the ischemic/nonischemic foot perfusion ratio of treated ischemic SHRs versus control ischemic SHRs (Fig. 2). In the absence of hindlimb ischemia, administration of perindopril alone, indapamide alone, and the combination of perindopril/indapamide did not affect the right/left leg ratio of angiographic score, capillary density, and foot perfusion.

Expression of VEGF and eNOS Proteins

We next endeavored to define the molecular and cellular mechanisms associated with these proangiogenic effects. We first analyzed VEGF protein content, a key angiogenic factor.
percentage of double positive cells was lower by 1.3-fold in nonischemic SHRs compared with nonischemic WKY rats ($p < 0.05$). Administration of perindopril, indapamide, or combination of perindopril/indapamide did not affect the percentage of double-positive cells in this setting. After 2 weeks of ischemia, the percentage of double positive cells was increased by 1.6-fold in WKY rats ($p < 0.01$). However, ischemia was not able to increase the number of BM-MNC-derived EPCs in SHRs. It is noteworthy that treatment with perindopril, indapamide, or both increased the number of cells double positive for both AcDil-LDL and BS-1 lectin by 1.7-, 1.4-, and 2.0-fold ($p < 0.01$), respectively, in ischemic SHRs in reference to untreated ischemic SHRs (Fig. 5).

Proangiogenic Potential of BM-MNCs

Finally, we sought to investigate the effect of antihypertensive agents on the proangiogenic potential of BM-MNCs. Angiography score, capillary density, and foot perfusion were decreased by 1.4-, 1.5-, and 1.2-fold, respectively, in ischemic SHRs receiving BM-MNCs isolated from ischemic SHRs compared with those treated with BM-MNCs isolated from ischemic WKY rats. Treatment with perindopril, indapamide, or combination of perindopril/indapamide restored the proangiogenic potential of BM-MNCs isolated from ischemic SHRs compared with untreated ischemic SHRs (Fig. 6).

Model of Ang II-Induced Hypertension

Ang II infusion (120 ng/kg/min) increased blood pressure levels by 1.3-fold (138 ± 12 versus 186 ± 19 mm Hg in control and Ang-II-treated rats, $p < 0.01$, n = 6/group). Angiographic score, capillary number and foot perfusion were reduced by 1.4-, 1.5-, and 1.4-fold, respectively, in Ang-II-treated rats compared with normotensive animals. Likewise, the ability of BM-MNCs to differentiate into EPCs and their proangiogenic potential were markedly reduced in Ang-II treated rats compared with untreated rats (Fig. 7). Altogether, these results highlight the concept that hypertension hampers the proangiogenic potential of progenitor cells and subsequently postischemic neovascularization.

Discussion

The present study showed that hypertension-induced impairment in postischemic neovascularization is mediated, at least in part, by a reduction in the number and proangiogenic potential of progenitor cells. Treatment with a combination of perindopril and indapamide counteracted the hypertension-induced abrogation of progenitor cell-related effects and restored postischemic revascularization.

Hypertension, a major risk factor for cardiovascular diseases, is characterized by endothelial dysfunction and an altered control of vascular cell growth and death (Tea et al.,...
Reparative neovascularization is also impaired in SHRs as a function of progression of the hypertensive disease. We confirm and extend these previous studies because we demonstrated that alteration of vessel growth is related, at least in part, to a reduction in the ischemia-induced up-regulation of two key angiogenic factors, VEGF-A and eNOS (Murohara et al., 1998; Couffinhal et al., 1999). VEGF-A receptor ligation triggers a cascade of intracellular signaling pathways that initiate neovascularization. VEGF can activate the survival and cell proliferation promoting phosphatidylinositol 3'-kinase/Akt pathway (Gerber et al., 1998; Dimmelmer and Zeiher, 2000; Kureishi et al., 2000) or eNOS signaling, eNOS plays an essential role in postnatal neovascularization. Endothelial NOS knockout mice are characterized by impaired neovascularization in response to ischemia (Murohara et al., 1998). In mice ischemic hindlimb, the angiogenic response to VEGF involves the activation of the eNOS gene (Murohara et al., 1998). Likewise, in ischemic heart, the induction of angiogenesis by VEGF requires the production of NO (Matsunaga et al., 2000). Finally, human eNOS gene delivery promotes neovascularization in the rat ischemic leg, supporting the idea that eNOS works in conjunction with VEGF to promote vessel growth (Smith et al., 2002).

We also demonstrated for the first time that the number of circulating progenitor cells is reduced in the setting of hypertension. In addition, the ability of BM-MNCs to differentiate into EPCs and promote therapeutic revascularization was strongly impaired. Finally, BM-MNC-related effects were abrogated in Ang II-treated rats, another model of hypertension. Altogether, these results suggest that hypertension hampers vasculogenesis and subsequently postischemic revascularization. In support of this view, patients with coronary artery diseases show reduced levels and functional impairment of EPCs, which correlated with risk factors for coronary artery diseases (Vasa et al., 2001). Likewise, hyperlipidemia is associated with EPC senescence and reduced EPC number (Chen et al., 2004; Imanishi et al., 2004). Chronic smokers also exhibit reduced EPC levels that can be restored after smoking cessation within 4 weeks (Kondo et al., 2004). The rate of transplanted homologous bone marrow cells incorporated into capillaries in ischemic tissues is lower in precocious-aging klotho mouse than in wild-type mice, in combination with a decrease in the number of e-CRT + CD31 + EPC-like mononuclear cells in bone marrow and peripheral blood (Shimada et al., 2004). Although there are no differences in the number of circulating EPCs in peripheral blood of old subjects, their migration and proliferation capacity are hampered (Heiss et al., 2005). The reduction in EPC proangiogenic effect associated with age and cardiovascular diseases, such as hypertension, may limit their therapeutic usefulness in the population of these patients.

It is interesting to note that antihypertensive treatment based on perindopril-indapamide combination restored VEGF-A and eNOS protein contents. In addition, treatment with antihypertensive agents also increased the number of circulating progenitor cells, re-established the ability of BM-MNCs to differentiate into EPCs, and activated BM-MNC proangiogenic potential. Hence, it is possible that blood pressure reduction is the principal mechanism leading to activation of vasculogenesis and blood vessel growth in this setting.
In line with these results, intensive blood pressure lowering after combination therapy has been shown to confer larger reduction risks compared with single-drug therapy in patients with cardiovascular diseases or with type 2 diabetes (Turnbull, 2003; Patel et al., 2007). Nevertheless, pressure-dependent and -independent effects of such therapy are difficult to dissociate. Pressure-independent effects are suggested by some data. ACE inhibition, through activation of bradykinin signaling, promotes neovascularization in normotensive and hypertensive rats (Fabre et al., 1999; Rakusan et al., 2000; Levy et al., 2001; Silvestre et al., 2001, 2002). Although indapamide alone (0.24 mg/kg/day) could not affect blood pressure levels in SHRs, it restored postischemic neovascularization. The mechanisms involved in the nonblood pressure-dependent effects of indapamide remain unclear. The development of endothelial dysfunction related to the duration of the hypertensive disease has been shown to contribute to the impaired response to tissue ischemia (Emmanuelli et al., 2001). Indapamide normalized impaired endothelium-dependent relaxations in aortic rings in vitro and in vivo (Schini et al., 1990; Hayakawa et al., 1997; Pickkers et al., 1998) and may have subsequently restored vessel growth in the setting of ischemia. Indapamide also potentiates bradykinin-related actions and raises prostaglandin generation, which has been reported to affect VEGF gene expression and angiogenesis (Junquero et al., 1991; Tsujii et al., 1998). VEGF and eNOS are involved in progenitor cell mobilization from the bone marrow, BM-MNC differentiation into EPCs, and progenitor cell proangiogenic function (Aicher et al., 2003; You et al., 2006). Up-regulation of VEGF and eNOS protein levels may also promote perindopril- and/or indapamide-related effects on vasculogenesis. Finally, indapamide may affect the vascular smooth cell growth defect associated with hypertension (Hadrafi et al., 1991). Altogether, one can speculate that both blood pressure reduction and blood pressure-independent effects may be involved in the beneficial effects of combination therapy observed in our experimental conditions but also in patients with stroke or type 2 diabetes. In addition, activation of vasculogenesis after combination therapy may participate in the reduction in major macrovascular and microvascular events observed in these patients (Turnbull, 2003; Patel et al., 2007).

In conclusion, we show for the first time that hypertension is associated with a reduction in the number and proangiogenic potential of progenitor cells. The antihypertensive agents, indapamide and perindopril, restored progenitor cell function and blood vessel growth. However, pressure-dependent and -independent effects of such therapy are difficult to dissociate. In addition, the driving mechanisms that lie behind the increase in vasculogenesis induced by each component of this combination remain to be clarified. Nevertheless, the use of perindopril/indapamide may provide a promising
strategy to improve the efficiency of stem cell therapy in hypertensive patients with vascular diseases.

References


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