Hypertension Impairs Postnatal Vasculogenesis
Role of Antihypertensive Agents

Dong You, Clément Cochain, Céline Loinard, José Vilar, Barend Mees, Micheline Duriez, Bernard I. Lévy, Jean-Sébastien Silvestre

Abstract—We analyzed the effect of hypertension on postischemic vasculogenesis. Ischemia was induced by right femoral artery ligation in Wistar Kyoto rats (WKY) or spontaneously hypertensive rats (SHR) treated with or without angiotensin-converting enzyme inhibitor (Perindopril, 0.76 mg/kg/d) and angiotensin type 1 receptor blocker (losartan, 30 mg/kg/d). Basal postsischemic neovascularization was reduced in SHR compared to WKY (P<0.05, n=8). Treatment with ACE inhibitor or angiotensin type 1 receptor blocker decreased blood pressure levels by 1.4- and 1.3-fold (P<0.001), respectively and restored vessel growth in SHR to WKY levels. Interestingly, 14 days after bone-marrow mononuclear cell (BM-MNC) transfusion, angiographic scores, capillary density, and foot perfusion were decreased by 1.4-, 1.5-, and 1.2-fold, respectively in SHR transfused with BM-MNCs isolated from SHR compared to those receiving BM-MNCs of WKY (P<0.05, n=6). Alteration in BM-MNCs proangiogenic potential was likely related to the reduction in their ability to mobilize into peripheral circulation, as revealed by the 2.9-fold decrease in number of circulating CD34+/CD117+ cells (P<0.001) and to differentiate into cells with endothelial phenotype, as revealed by the 2.1-fold reduction in percentages of DilLDL/BS-1 lectin positive cells (P<0.001). In addition, reactive oxygen species (ROS) levels were increased by 2.2-fold in SHR BM-MNCs compared to WKY BM-MNCs (P<0.01), as assessed by L-012 luminescence. Cotreatment with ACE inhibitor, angiotensin type 1 receptor blocker, or antioxidants (NAC 3 mmol/L, Apocynin 200 μmol/L) reduced ROS levels, improved the number of DilLDL/BS-1 lectin-positive cells by around 1.5-fold, and restored BM-MNCs proangiogenic effects in ischemic hindlimb. In conclusion, alteration in progenitor cell proangiogenic function may participate to the hypertension-induced impairment in postischemic revascularization. (Hypertension. 2008;51:1537-1544.)

Key Words: hypertension ■ angiogenesis ■ progenitor cells ■ angiotensin converting enzyme ■ angiotensin type I receptor

The revascularization process, including vasculogenesis, angiogenesis, and collateral growth, characterizes tissue repair and remodeling occurring in acute and chronic ischemic vascular diseases. In particular, postnatal vasculogenesis referred to the homing and differentiation of circulating progenitor cells from bone marrow or non–bone marrow origins1 into endothelial cells within sites of active neovascularization. In addition, circulating progenitor cells may deliver angiogenic growth factors to pathological tissues and contribute to neovascularization and tissue/vessel remodeling by paracrine effects.2-3

In most clinical settings, however, these natural adaptive responses to a compromised perfusion are insufficient to block the progression of ischemic diseases. Hence, certain cardiovascular risk factors including diabetes, aging, and hypercholesterolemia adversely affect postnatal vasculogenesis and revascularization in animals models of limb ischemia.4-7 In support of this view, patients with type I and II diabetes displayed a reduction in endothelial progenitor cell (EPC) number and angiogenicity.8,9

In most forms of clinical and experimental hypertension, increased arterial blood pressure is associated with microvascular rarefaction and increased peripheral vascular resistances.10 Similarly, postischemic reparative neovascularization is impaired in spontaneously hypertensive rats (SHR) as a function of progression of the hypertensive disease.11,12 Several molecular and cellular mechanisms may be involved in the hypertension-induced impairment in vessel growth. First, the angiogenic capacity of serum derived from SHR was less than that from normotensive animals in a chick embryo chorio-allantoic membrane model.13 Protein levels of key proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor are also reduced in hypertensive animals.11,14 Second, previous obser-
ventions that endothelial function is impaired in SHR as well as in patients with essential hypertension imply that the defective endothelial function may contribute to impaired angiogenesis in SHR.15,16,17 Alternatively, the participation of progenitor cell dysfunction to the pathogenesis of hypertension can be speculated. In adult subjects without history of cardiovascular diseases, the number of circulating EPCs was inversely correlated with the endothelial function and the Framingham risk score, which includes systolic blood pressure as a major component.18 Recently, accelerated senescence of EPCs was demonstrated in hypertensive animals and humans.19

We therefore hypothesized that hypertension may decrease bone marrow–derived mononuclear cell (BM-MNC) number and proangiogenic potential leading to abrogation in postischemic revascularization. We provide evidence that hypertension reduces circulating progenitor cells number and ability to differentiate into endothelial cells. Furthermore, we find that antihypertensive treatments using either an angiotensin converting enzyme (ACE) inhibitor (perindopril), an angiotensin II type I receptor (AT1R) blocker (losartan), or a nonspecific vasodilator hyaluridine could restore BM-MNC dysfunction by blocking the hypertension-induced oxidative stress.

Materials and Methods

Animal Model

Twelve-week-old male normotensive Wistar Kyoto rats (WKY) and SHR were separated into 8 groups (n=6 to 8) receiving the following treatment for 3 weeks in drinking water: placebo, ACE inhibitor (perindopril, 0.76 mg/kg per day, Servier), AT1R blocker (losartan, 30 mg/kg per day, MSD-Chibret), and hyaluridine (hyaluridine hydrochloride, 200 mg/L, drinking water, Sigma). Untreated WKY and SHR served as control groups. All the experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430).

Arterial Pressure Measurement

Systolic blood pressure was measured in conscious rats, by the tail-cuff method, between 9 AM and 12 AM (BP2000, Visitech system). Blood pressure was measured for 10 different consecutive cycles.

Ischemic Hindlimb Model

After 1 week of treatment, rats were anesthetized and right femoral artery was occluded (6-0 silk suture) by ligature. The ligature was performed on the femoral artery 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries, as previously described.20 After 2 weeks of ischemia, vessel density was determined by high-definition microangiography and capillary density analysis in hindlimb. Laser-doppler perfusion imaging was also used to assess in vivo tissue perfusion in the paw, as previously described.3,20

BM-MNC Isolation

Bone marrow was obtained by flushing tibia and femur, low-density mononuclear cells were then isolated by centrifugation on a Ficoll gradient, as previously described.3,4 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 flow analysis cytometry system (FACS). Flow Analysis Cytometry System (FACS) Analysis

10⁶ BM-MNCs or circulating MNCs were incubated for 15 minutes at 4°C with 5% SVF fetal bovine serum and then with 20 μL of fluorescein (FITC)-conjugated anti-CD34 antibody (Tebu bio) for 30 minutes at 4°C with PBS buffer. MNCs were also incubated with 20 μL of phycoerythrin/Cy5 (PE/Cy5)-conjugated anti-CD117 antibody (BD Biosciences, Pharmingen) for 30 minutes at 4°C with PBS buffer. After washing, the samples were centrifuged and the pellets suspended in 500 μL of PBS buffer. The percentage of CD34 and CD117-positive cells were analyzed using CellQuest software (Beckman Coulter EPICS-XL).

BM-MNC Differentiation Into Cells With Endothelial Phenotype

BM-MNC (2×10⁶ per 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). BM-MNCs were maintained in Endothelial basal medium for 7 days (EGM2; Bio-Whittaker) with or without antioxidant treatments (apocynin 1 mmol/L or N-acetyl-L-cysteine NAC 0.4%, Zambon France), ACE inhibitor (perindopril 10 μmol/L), or AT1R inhibitor (losartan 10 μmol/L). Nondifferented cells were then removed and adherent cells analyzed by immunochemical assay with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (Dil-LDL; Tebu Bio) and FITC-labeled BS-1 lectin (Sigma). Cells were incubated in EGM2 containing Dil-LDL at 37°C for 1 hour. Cells were then fixed in 2% parafomaldehyde and incubated with FITC-labeled BS-1 lectin. Endothelial cell phenotype was revealed with double-positive staining for both Dil-LDL and BS-1 lectin and by expression of endothelial specific markers including endothelial nitric oxide (eNOS) and von Willibrand Factor (vWF). Cell numbers were counted and expressed in cells per well by using epifluorescence microscopy, as previously described.4,9 Five replicates were counted for each treated or untreated rats. Results are expressed as percentages of total cell numbers.

Progenitor Cells Proangiogenic Potential

Five hours after hindlimb ischemia, SHR received intravenous injections of 1.10⁶ BM-MNCs isolated from treated or nontreated rats. Animals were euthanized 14 days after cell transfusion, and vascular density was determined by 3 different methods: high-definition microangiography, capillary density analysis, and laser-doppler perfusion imaging, as described above.

VEGF and eNOS Protein Expression

Gastrocnemius muscles from ischemic and nonischemic hindlimbs were thawed and homogenized in 500 μL of buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 1% Deoxycholate, pH 7.4) containing protease inhibitors (Boehringer-Mannheim). Proteins were separated in denaturing 9% polyacrylamide gels and then blotted onto a nitrocellulose sheet (Hybond ECL, Amersham). Antibodies directed against VEGF-A (Tebu Bio), eNOS (Cell signaling), and GADPH (Sigma) were used at a dilution of 1:1000. Specific protein was detected by chemiluminescent reaction (ECL+kit, Amersham).

Reactive Oxygen Species Levels

Tissue and cellular reactive oxygen species (ROS) levels, reflecting a balance between oxidant production and removal by endogenous antioxidants, were also quantified using L-012 as described recently.21,22 BM-MNCs, isolated from WKY and SHR, were cultured in EGM2 medium with or without apocynin (1 mmol/L, NAC (0.4%), perindopril (10 μmol/L) or losartan (10 μmol/L), 7 days after cells culture, the adherent cells were lysed in 50 mmol/L Tris buffer (pH 7.5) containing protease inhibitors and centrifuged at 10 000 g for 15 minutes at 4°C. Supernatants were then incubated with L-012 100 μmol/L (Wako). Luminescence was counted (Topcount NXT; Perkin Elmer) during 20 seconds after a 10-minute interval, allowing for the plates to become dark-adapted.

Statistical Analysis

Results were expressed as mean±SEM. One-way analysis of variance ANOVA was used to compare each parameter. Posthoc
Bonferroni t test comparisons were then performed to identify which group differences accounted for the significant overall ANOVA. P<0.05 was considered significant.

### Results

#### Arterial Pressure

Systolic blood pressure was increased by 1.3-fold in 12-week-old SHR compared to WKY (P<0.001). As expected, administration of perindopril, losartan, and hydralazine reduced systolic blood pressure when compared to untreated SHR (P<0.001). Hindlimb ischemia did not affect systolic blood pressure when compared with groups of rats without right femoral artery ligation (see Table).

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#### Postischemic Revascularization

Angiographic score, capillary density, and paw perfusion were reduced by 1.5-, 1.6-, and 1.3-fold, respectively, in untreated SHR when compared to WKY (Figure 1). Interestingly, treatment with antihypertensive agents, perindopril, losartan, or hydralazine, abrogated the hypertension-induced impairment in postischemic revascularization.

We next attempted to define the molecular and cellular mechanisms associated with these changes in revascularization. We first analyzed VEGF-A protein content, a key angiogenic factor. There was not significant difference in VEGF-A expression in nonischemic groups. Under ischemic condition, VEGF-A protein levels were increased by 1.7-fold in ischemic muscle in reference to nonischemic muscle of WKY (P<0.05). VEGF-A protein content was decreased by 2.4-fold in the ischemic hindlimb of SHR compared to that of WKY (P<0.001). However, treatment with perindopril or losartan did not restore VEGF-A protein content (Figure 2).

VEGF-A has been shown, through Fli-1/KDR, to activate endothelial nitric oxide synthase (eNOS)-related pathways leading to NO production. We then determined eNOS expression in the different experimental groups. We showed that eNOS protein levels were not significantly different in nonischemic hindlimbs whatever the treatment. Fourteen days of hindlimb ischemia increased eNOS protein content by 1.7-fold in WKY but not in SHR (P<0.05). Interestingly, treatment with perindopril or losartan increased eNOS levels.

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### Table. Systolic Blood Pressure (mm Hg)

<table>
<thead>
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<th>14W</th>
<th>15W</th>
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<td>150±1</td>
<td>156±6</td>
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<tr>
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<td>162±3</td>
<td>158±3</td>
<td>156±8</td>
</tr>
<tr>
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<td>198±3†</td>
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<td>206±5†</td>
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<tr>
<td>SHR Isch</td>
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<td>193±4†</td>
<td>200±6†</td>
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<td>154±7§</td>
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<tr>
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<tr>
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<td>153±5§</td>
<td>157±5§</td>
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<td>SHR Isch + Los</td>
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<tr>
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<td>137±8§</td>
<td>135±9§</td>
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</tbody>
</table>

*P<0.01, †P<0.001 vs WKY; ††P<0.01, §P<0.001 vs SHR.

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Figure 1. Representative photomicrographs and quantitative evaluation of microangiography (a), capillary density (capillary appear in green) (b), and paw perfusion (c) in WKY and SHR with (Isch) or without (W/O Isch) hindlimb ischemia. Per indicates perindopril; Los, losartan; Hyd, hydralazine. *P<0.05, **P<0.001 vs WKY; †P<0.05, ††P<0.01, †††P<0.001 vs SHR.
in ischemic SHR compared to untreated SHR and restored eNOS protein expression to ischemic WKY levels.

**Postischemic Vasculogenesis**

**Proangiogenic Potential of BM-MNC**

We next hypothesized that reduction in blood vessel growth may depend on the hypertension-induced BM-MNC dysfunction. Hypertension is associated with an increase in ROS levels.23 Increased oxidative stress constitutes an underlying pathogenic mechanism that affects both angiogenesis and vasculogenesis.4 We therefore evaluated the effects of hypertension-induced increased ROS levels on the ability of BM-MNCs to stimulate neovascularization.

Protein expression of NADPH oxidase subunits was detected in control BM-MNCs. Their levels were upregulated in SHR BM-MNCs versus WKY BM-MNCs (Figure 3a). ROS levels were also increased by 2.0-fold in SHR BM-MNCs compared to control BM-MNCs (P<0.001, Figure 3b). Treatment with NAC or apocynin reduced ROS levels in both control and SHR cells. Interestingly, treatments with perindopril or losartan also decreased by 1.6-fold the production of ROS levels (Figure 3b). Angiography score, capillary density, and foot perfusion were decreased by 1.4-, 1.5- (P<0.001), and 1.2-fold (P<0.05), respectively, in ischemic SHR receiving BM-MNCs isolated from untreated ischemic SHR compared to those transfused with BM-MNCs isolated from ischemic WKY. BM-MNCs from ischemic SHR treated with perindopril, losartan, or hydralazine displayed a restored proangiogenic potential when injected in untreated ischemic SHR. Interestingly, treatment with the antioxidant, NAC, also counteracted BM-MNC dysfunction (Figure 4). These results suggest that hypertension-induced oxidative stress hampers vasculogenesis, and subsequently postischemic neovascularization.

**BM-MNC Differentiation Into Cells With an Endothelial Phenotype**

BM-MNCs improve neovascularization of ischemic hind limbs and ischemic hearts through their capacity to integrate new blood vessels or secrete proangiogenic factors. We thus evaluated the ability of BM-MNCs to differentiate in vitro into cells with endothelial phenotype. Acquisition of endothelial cell phenotype was revealed with double-positive staining for both Dil-LDL and BS-1 lectin. The percentage of
double positive cells was lower by 1.3-fold in nonischemic SHR compared to nonischemic WKY ($P<0.05$). Administration of perindopril or losartan did not affect the percentage of double positive cells in SHR without hindlimb ischemia. After 2 weeks of ischemia, the percentage of double positive cells was increased by 1.6-fold in ischemic WKY in reference to nonischemic WKY ($P<0.001$). However, the number of double positive cells was reduced in ischemic SHR compared to ischemic WKY. Similarly, the number of cells positive for both BS1-lectin/eNOS or BS-1 lectin/vWF was lower by around 2-fold in SHR compared to WKY (Figure S1, available online at http://hyper.ahajournals.org). Interestingly, treatment with perindopril, losartan, and hydralazine increased the number of cells double positive for both DilLDL and BS-1 lectin by 1.7-, 1.6-, and 1.4-fold, respectively in SHR without hindlimb ischemia. Endothelial phenotype derived from SHR BM-MNCs was mediated by enhanced ROS levels.

**Number of Circulating Progenitor Cells**

Reduction in blood vessel growth may also depend on the hypertension-induced decrease in the mobilization of progenitor cells from the bone marrow and subsequently in the number of circulating progenitor cells. We therefore analyzed the number of vascular progenitor cells in peripheral blood by FACS analysis. A population of circulating vascular progen-
Diabetic hypertensive patients, and administration of ACE inhibitors was associated with high levels of these cells in patients with coronary artery disease. However, prolonged treatments with an ACE inhibitor, but not with AT1R antagonist, increased the number of circulating progenitor cells. Hydralazine also raised the number of circulating progenitor cells suggesting that blood pressure reduction is the principal mechanism leading to upregulation of double-positive cells in this setting. The discrepancy between perindopril and losartan administration may first depend on their blood pressure lowering effects. After 3 weeks of treatment, blood pressure tended to be lower in perindopril-treated SHR compared to losartan-treated SHR. Nevertheless, it is likely that antihypertensive drug treatment may have specific effects on proangiogenic and provasculogenic pathways. In support of this view, ACE inhibition, through activation of bradykinin signaling, upregulates VEGF and eNOS and promotes neovascularization in normotensive and hypertensive rats. VEGF and eNOS are also involved in progenitor cells mobilization from the bone marrow, BM-MNC differentiation, and progenitor cell proangiogenic function.

Conversely, BM-MNCs isolated from mice lacking AT1R blockade display alterations in their proangiogenic functions. Therefore, non–blood pressure–dependent actions of perindopril and losartan may also be involved in their effect on the different steps of vasculogenesis.

Hypertension is associated with an increase in ROS levels. Increased oxidative stress constitutes an underlying pathogenic mechanism that affects both angiogenesis and vasculogenesis. In particular, we have shown that diabetes-induced increases in ROS enhanced p38MAPK phosphorylation in BM-MNCs, reduced BM-MNC differentiation into EPCs in vitro, and impaired their proangiogenic potential in vivo. We found that ROS levels were higher in BM-MNCs isolated from hypertensive rats, in association with the upregulation of different subunits of NADPH oxidase. We also demonstrated that hypertension-induced increases in ROS levels decreased BM-MNC differentiation into cells with endothelial phenotype in vitro and hampered their therapeutic in vivo effect. Interestingly, antihypertensive agents and the scavenging of ROS similarly restored progenitor cells proangiogenic potential. In line with these findings, ACE inhibitor and AT1R blocker have been shown to suppress ROS generation in heart of hypertensive animals with diastolic heart failure. In addition, both AT1R antagonist and ACE inhibitor inhibited vascular remodeling and reduced ROS in stroke-prone spontaneously hypertensive rats via not only a reduction in NAD(P)H oxidase but also an upregulation of Cu/Zn superoxide dismutase.

Alternatively, several functions of progenitor cells might be impaired by cardiovascular risk factors. Hence, impairment in progenitor cell adhesion to endothelium might also participate to progenitor cells dysfunction in the setting of hypertension. In this view, the clonogenic and adhesion capacity of cultured EPCs was significantly lower in diabetic patients with peripheral arterial disease versus patients without. Diabetic EPCs had normal adhesion to fibronectin, collagen, and quiescent endothelial cells but a decreased adherence to human umbilical vein endothelial cells activated by tumor necrosis factor (TNF)-α. Notably,
thrombospondin-1 mRNA expression was significantly up-regulated in diabetic EPCs, associating with the decreased EPC adhesion activity in vitro and in vivo. In addition, adhesion to mature endothelial cells after activation with TNF-α was enhanced only in controls but not in patients with rheumatoid arthritis. The functional activities of isolated EPCs, such as proliferative, migratory, adhesive, and in vitro vasculogenesis capacity, were also impaired in patients with hypercholesterolemia. Finally, in SHR as well as in patients with essential hypertension endothelial function is impaired and may contribute to reduced vessel regeneration in this setting.

In addition, AT1R blocker and ACE inhibitor have been shown to ameliorate vascular function in SHR, suggesting that restoration of endothelial function may participate at least in part to the beneficial effects of AT1R blocker and ACE inhibitor in hypertension.

In conclusion, the decrease in the number of progenitor cells and their proangiogenic function likely participates to the hypertension-induced abrogation of postischemic vessel growth. ACE inhibitor and AT1R blocker restored postnatal vasculogenesis in this setting.

Perspectives

Despite the excitement regarding the possible clinical use of EPCs, recent studies have shown that age and other risk factors for cardiovascular diseases reduce the availability of EPCs and impair their function to varying degrees. Our study suggest that hypertension may also limit the therapeutic usefulness of vascular progenitor cells. The close interaction between hypertension and blood vessel growth might also constitute a new physiopathological view of arterial hypertension and could have important consequences in the understanding of hypertension after antiangiogenic treatment. This new hypothesis is strengthened by recent studies highlighting that appearance of hypertension in patients with metastatic renal cell carcinoma receiving antiangiogenic treatment.

Finally, activation of vasculogenesis after antihypertensive drugs therapy may participate to the reduction in major macrovascular and microvascular events observed in patients with stroke or type 2 diabetes.

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Disclosures

None.

References

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Hypertension impairs post-natal vasculogenesis: role of anti-hypertensive agents

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BM-MNC differentiation into cells with endothelial phenotype: Expression of endothelial specific markers: eNOS and vWF

**Figure S1.** Representative images and quantification of the number of cells with endothelial phenotype derived from BM-MNC of WKY and SHR with hindlimb ischemia. Endothelial phenotype was revealed by double-positive staining for BS-1 lectin and eNOS (a) or BS-1 lectin and vWF (b), after 7 days of culture. *p<0.05 versus WKY. n=4 per group.