Angiotensin II Impairs Endothelial Progenitor Cell Number and Function In Vitro and In Vivo
Implications for Vascular Regeneration

Cathleen Endtmann, Talin Ebrahimian, Thomas Czech, Omar Arfa, Ulrich Laufs, Mathias Fritz, Kerstin Wassmann, Nikos Werner, Vasileios Petoumenos, Georg Nickenig, Sven Wassmann

See Editorial Commentary, pp 356–358

Abstract—Endothelial progenitor cells (EPCs) contribute to endothelial regeneration. Angiotensin II (Ang II) through Ang II type I receptor (AT1-R) activation plays an important role in vascular damage. The effect of Ang II on EPCs and the involved molecular mechanisms are incompletely understood. Stimulation with Ang II decreased the number of cultured human early outgrowth EPCs, which express both AT1-R and Ang II type 2 receptor, mediated through AT1-R activation and induction of oxidative stress. Ang II redox-dependently induced EPC apoptosis through increased apoptosis signal-regulating kinase 1, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase phosphorylation; decreased Bcl-2 and increased Bax expression; and activation of caspase 3 but had no effect on the low cell proliferation. In addition, Ang II impaired colony-forming and migratory capacities of early outgrowth EPCs. Ang II infusion diminished numbers and functional capacities of EPCs in wild-type (WT) but not AT1α-R knockout mice (AT1α−/−). Reendothelialization after focal carotid endothelial injury was decreased during Ang II infusion. Salvage of reendothelialization by intravenous application of spleen-derived progenitor cells into Ang II-treated WT mice was pronounced with AT1α−/− cells compared with WT cells, and transfusion of Ang II-pretreated WT cells into WT mice without Ang II infusion was associated with less reendothelialization. Transplantation of AT1α−/− bone marrow reduced atherosclerosis development in cholesterol-fed apolipoprotein E–deficient mice compared with transplantation of apolipoprotein E–deficient or WT bone marrow. Randomized treatment of patients with stable coronary artery disease with the AT1-R blocker telmisartan significantly increased the number of circulating CD34/KDR-positive EPCs. Ang II through AT1-R activation, oxidative stress, and redox-sensitive apoptosis signal-regulating kinase 1–dependent proapoptotic pathways impairs EPCs in vitro and in vivo, resulting in diminished vascular regeneration. (Hypertension. 2011;58:394-403.)

Key Words: angiotensin • endothelium • atherosclerosis • progenitor cells • oxidative stress

Activation of the angiotensin II (Ang II) type 1 (AT1) receptor by Ang II leads to several molecular and cellular events that are involved in atherogenesis, and, thus, AT1 receptor activation is considered to play an important role in endothelial damage and atherosclerosis development and progression. Diminished endothelial regeneration is considered to play an important role in the pathogenesis of atherosclerosis. Endothelial regeneration is not only accomplished by resident endothelial cells but also by endothelial progenitor cells (EPCs). EPCs are premature circulating cells that are mainly derived from the bone marrow (BM) and that are involved in postnatal vasculogenesis and reendothelialization after endothelial damage and in atherosclerosis-prone vascular areas. EPCs are defined according to surface markers and properties. One recent classification distinguishes between early outgrowth EPCs, which display paracrine actions, and late outgrowth EPCs, which are characterized by high proliferative potential, by differentiating between cellular functional capacities, time of appearance during cell culture, and the cell culture protocol used. Endogenous mobilization or systemic application of EPCs leads to enhancement of reendothelialization, improvement of angiogenesis and endothelial function, decreased neointima formation, and reduced development of atherosclerosis. Cardiovascular risk

Received December 31, 2010; first decision January 16, 2011; revision accepted July 5, 2011.
From the Medizinische Klinik und Poliklinik II (C.E., K.W., N.W., V.P., G.N., S.W.), Universitätsklinikum Bonn, Bonn, Germany; Lady Davis Institute for Medical Research and Division of Cardiology (T.E., O.A., S.W.), Jewish General Hospital, McGill University, Montréal, Québec, Canada; Klinik für Neurochirurgie (T.C.) and Klinik für Innere Medizin III (U.L., M.F.), Universitätsklinikum des Saarlandes, Homburg/Saar, Germany. C.E. and T.E. contributed equally to this study.
Correspondence to Sven Wassmann, Lady Davis Institute for Medical Research, Jewish General Hospital, 3755 Côte-Ste-Catherine Rd, Montréal, Québec H3T 1E2, Canada. E-mail sven.wassmann@mcmill.ca
© 2011 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.110.169193
factors and atherosclerosis are characterized by dysfunctional and numerically reduced EPCs, and decreased numbers and impaired function of circulating EPCs are associated with endothelial dysfunction and poor cardiovascular outcomes in patients with coronary artery disease.17,21–23

In this study, we investigated the impact of Ang II on number and function of EPCs, the mechanisms involved, and the relevance of this interplay for vascular damage and regeneration.

Methods
The Methods section of this article can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

Results
Characterization of Human Early Outgrowth EPCs
Human mononuclear cells (MNCs) were isolated from peripheral blood and cultured on fibronectin in supplemented endothelial basal medium. Fibronectin-adherent cells double positive for Dil-labeled, acetylated low-density lipoprotein (Dil-acLDL), and lectin staining were considered early outgrowth EPCs (Figure S1A of the online Data Supplement; please see http://hyper.ahajournals.org). After 7 days of culture, 71±7% of all 4′,6-diamidino-2-phenylindole–positive cells were Dil-acLDL/lectin double positive (n=6). Quantitative immunochemical analysis revealed that Dil-acLDL/lectin double-positive cells expressed the endothelial markers vascular endothelial growth factor receptor 2 (100±0%), phospho-endothelial NO synthase (100±0%), and CD31 (84±8%); the angiotensin receptors AT1 (76±8%) and Ang II type 2 (74±2%); and to some degree the stem cell marker CD34 (18±1%; n=3 to 4; Figure S1B).

Effect of Ang II on Early Outgrowth EPC Number and Function In Vitro
MNCs were cultured for 7 days as described above in the presence of Ang II or vehicle. Treatment with 10−10 to 10−6 mol/L of Ang II led to a significant reduction of early outgrowth EPC numbers compared with control, with a decrease of both 4′,6-diamidino-2-phenylindole–positive and Dil-acLDL/lectin double-positive cells (Figure 1 A and B), and without significant differences in the ratios of double-positive to 4′,6-diamidino-2-phenylindole–positive cells (data not shown). Coincubation experiments with Ang II, AT1, and Ang II type 2 receptor antagonists revealed that AT1 receptor activation but not Ang II type 2 receptor activation was essential for the Ang II–mediated effect on early outgrowth EPC numbers (Figure 1C).

In addition, Ang II decreased functional capacities of early outgrowth EPCs. As shown in Figure 1D and 1E, incubation with Ang II reduced the number of colony forming units and migratory capacity. Both effects were inhibited by AT1 receptor blocker cotreatment.

Effect of Ang II on Early Outgrowth EPC Proliferation and Apoptosis In Vitro
The rate of proliferating cells, as determined by 5-bromodeoxyuridine incorporation, was very low in early outgrowth EPCs, and incubation with Ang II had no further effect on cell proliferation (5-bromodeoxyuridine–positive cells: control, 0.17±0.07%; Ang II, 0.11±0.05%), as shown in Figures 2A and S2. This was confirmed by immunocytochemistry for the proliferation marker Ki-67 in Dil-acLDL/lectin double-positive early outgrowth EPCs, which showed that only 1±0.5% of these cells were Ki-67 positive after 7 days of culture (Figure 2B). In contrast, control experiments with human coronary artery endothelial cells and human umbilical vein endothelial cells revealed proliferation rates of 71% and 64%, respectively (Figure S2).

Figure 2C demonstrates that Ang II significantly increased the rate of apoptosis in early outgrowth EPCs, which was inhibited by AT1 receptor blocker cotreatment. This was confirmed by immunocytochemistry for the apoptosis marker cleaved (active) caspase 3 in Dil-acLDL/lectin double-positive early outgrowth EPCs (Figure 2D and 2E).

To identify a potential signaling pathway underlying the Ang II–induced increase in early outgrowth EPC apoptosis, the apoptosis signal-regulating kinase 1 (ASK-1)–dependent proapoptotic signaling pathway was investigated. Western blot analyses revealed that Ang II increased phosphorylation of ASK-1, phosphorylation of c-Jun N-terminal kinase, and phosphorylation of p38 mitogen-activated protein kinase; decreased the expression of antiapoptotic Bcl-2; and increased the expression proapoptotic Bax, which subsequently initiates activation of caspases, such as caspase 3, and induction of apoptosis (Figures 2F to 2I and S3).

Effect of Ang II on Oxidative Stress in Early Outgrowth EPCs In Vitro
As shown in Figure 3A and 3B, Ang II significantly increased intracellular reactive oxygen species production in early outgrowth EPCs, which was inhibited by an AT1 receptor blocker and the antioxidant polyethylene glycol-conjugated superoxide dismutase. Coincubation with the antioxidants polyethylene glycol-conjugated superoxide dismutase or tiron completely abolished the Ang II–mediated reduction of early outgrowth EPC numbers, indicating that oxidative stress is involved in this action of Ang II (Figure 3C). As demonstrated in Figure 3D to 3G, both Ang II–induced activation of ASK-1, as determined by ASK-1 phosphorylation, as well as Ang II–mediated induction of apoptosis, as determined by active caspase 3 immunocytochemistry, were blocked by coincubation with the antioxidant polyethylene glycol-conjugated superoxide dismutase, indicating that Ang II–induced oxidative stress mediates proapoptotic ASK-1 signaling and, ultimately, apoptosis in early outgrowth EPCs.

Effect of Ang II on EPC Number and Function In Vivo
Wild-type (WT) and AT1+/− mice were treated with Ang II or vehicle via osmotic minipumps, and numbers and functional capacities of peripheral blood and spleen-derived EPCs were assessed. Ang II infusion led to a significant blood pressure increase in WT but not AT1+/− mice (systolic blood pressure: WT control, 122±2 mm Hg; WT Ang II, 168±2 mm Hg, P<0.05 versus WT control; AT1+/− control, 81±7 mm Hg; AT1+/− Ang II, 93±8 mm Hg, P value not significant versus AT1+/− control). As shown in Figure 4,
Figure 1. Effect of angiotensin II (Ang II) on early outgrowth endothelial progenitor cell (EPC) number and function in vitro. A, Human mononuclear cells were isolated and cultured in supplemented endothelial basal medium in the presence of 1 μmol/L of Ang II or vehicle (Con) for 7 days. Representative microscopic scans showing the number of 4',6-diamidino-2-phenylindole (DAPI)–positive nuclei, Dil–labeled, acetylated low-density lipoprotein (Dil-acLDL)/lectin double-positive early outgrowth EPCs and a merged image after 7 days of cell culture. Merge: green = lectin, blue = DAPI, red = Dil-acLDL. B, EPC numbers after stimulation with 10^-10 to 10^-6 mol/L of Ang II or vehicle (Con). Numbers per high-power field in percentage of control, mean ± SEM, n = 3, *P < 0.05 vs control. C, EPC numbers after stimulation with vehicle (Con), Ang II (Ang; 1 μmol/L), the Ang II type 1 (AT1) receptor antagonist irbesartan (ARB; 100 μmol/L), the Ang II type 2 (AT2) receptor antagonist PD123,319 (PD; 1 μmol/L), or combinations of the aforementioned. Numbers per high-power field in percentage of control, mean ± SEM, n = 4, *P < 0.05 vs control, #P < 0.05 vs Ang II. D, Number of endothelial cell-colony forming units (CFUs) after cell cultivation in the presence of vehicle (Con), 1 μmol/L Ang II, irbesartan (ARB; 100 μmol/L), or a combination. Numbers of CFUs per well in percentage of control, mean ± SEM, n = 3, *P < 0.05 vs control, #P < 0.05 vs Ang II. E, EPC migration after cell cultivation in the presence of vehicle (Con), 1 μmol/L of Ang II, irbesartan (ARB; 100 μmol/L), or a combination. Numbers of Dil-acLDL/lectin double-positive EPCs on the lower side of the filter per high-power field in percentage of control, mean ± SEM, n = 3, *P < 0.05 vs control, #P < 0.05 vs Ang II.

Effect of Ang II on Neointima Formation and Reendothelialization In Vivo

To assess functional effects of Ang II–mediated reduction of number and function of EPCs in vivo, mice were treated with Ang II or vehicle via osmotic minipumps, and focal carotid artery injury was induced. As shown in Figure 5A and 5B, treatment of WT mice with Ang II significantly enhanced neointima formation after carotid injury. This Ang II effect was abolished in Ang II–treated AT1^-/- mice. Consistently, treatment of WT mice with Ang II significantly decreased reendothelialization after electric carotid endothelial injury, as visualized by Evans blue stainings (Figure 5C).

To further assess the functional in vivo relevance of Ang II stimulation and AT1 receptor expression on EPCs for reendothelialization capacities of these cells, splenectomized WT mice were treated with Ang II or vehicle, and cardiac electric injury was induced, spleen-derived MNCs from WT or AT1^-/- donor mice or saline were injected intravenously, and, subsequently, cardiac artery reendothelialization was assessed. Figure 5D demonstrates that the diminished reendothelialization during Ang II treatment was not affected by intravenous WT cell treatment, whereas reendothelialization was significantly rescued by intravenous treatment with AT1^-/- cells. As shown in Figure 5E, in another set of experiments, transfusion of WT cells pretreated in vitro with...
Figure 2. Effect of angiotensin II (Ang II) on early outgrowth endothelial progenitor cell (EPC) proliferation and apoptosis in vitro. A. Cell proliferation of early outgrowth EPCs was assessed by 5-bromodeoxyuridine fluorescence-activated cell sorter (FACS) analysis after cell cultivation in the presence of 1 μmol/L of Ang II or vehicle (Con) for 7 days and is expressed as the number of 5-bromodeoxyuridine–positive cells in percentage of all gated events, mean±SEM, n=6. B. Cell proliferation was assessed by Ki-67 immunocytochemistry in Dil-labeled, acetylated low-density lipoprotein (Dil-acLDL)/lectin double-positive early outgrowth EPCs. Representative microscopic scan. Merge: green=lectin, blue=Dil-acLDL, red=Ki-67. 4’,6-diamidino-2-phenylindole (DAPI) nuclear staining is shown in the separate image. C. Rate of apoptosis of early outgrowth EPCs was assessed by histone-DNA assay after cell cultivation in the presence of vehicle (Con), 1 μmol/L of Ang II, irbesartan (ARB; 100 μmol/L), or a combination for 7 days and is expressed as percentage of control, mean±SEM, n=3. *P<0.05 vs control, #P<0.05 vs Ang II. D. Apoptosis was assessed by cleaved (active) caspase 3 immunocytochemistry in Dil-acLDL/lectin double-positive early outgrowth EPCs after cell cultivation in the presence of 1 μmol/L of Ang II or vehicle (Con) for 7 days. Number of Dil-acLDL/lectin double-positive early outgrowth EPCs with positive staining for active caspase 3 in percentage of control, mean±SEM, n=4. *P<0.05 vs control. E. Representative microscopic scans showing cleaved caspase 3 immunostaining in Dil-acLDL/lectin double-positive EPCs. Merge: green=lectin, blue=Dil-acLDL, red=cleaved caspase 3. DAPI nuclear staining is shown in the separate images. F through I. Western blot analyses were performed with total proteins of early outgrowth EPCs after cell cultivation in the presence of 1 μmol/L of Ang II or vehicle (Con) for 7 days to detect phosphorylated and total forms of apoptosis signal-regulating kinase-1 (ASK-1), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38), and total forms of Bcl-2, Bax, and β-actin. Representative immunoblots.

Ang II into WT mice without Ang II infusion was associated with significantly less reendothelialization than transfusion of vehicle-treated WT or AT1−/− cells. These findings indicate the relevance of the Ang II/AT1 receptor interplay for impaired EPC function in vivo. Finally, the Ang II–induced impairment of reendothelialization was abolished in Ang II–treated AT1−/− mice, with Ang II–infused AT1−/− mice treated with WT cell transfusion showing the highest degree of reendothelialization (Figure 5E).

Role of AT1 Receptors in BM-Derived Cells for Atherogenesis

BM derived from AT1−/−, WT, and apolipoprotein (Apo) E−/− mice was transplanted into lethally irradiated ApoE−/− mice, resulting in AT1−/−ApoE+/+, AT1+/+ApoE−/+, and AT1+/+ApoE−/− BM in the recipient mice, respectively. Four weeks after BM transplantation (BMT), these mice were fed a high-fat, high-cholesterol diet for 7 weeks. There was no significant difference between the groups after treatment with respect to cholesterol levels in the aortic wall (AT1−/− BMT, 6.0±0.3 μg/mg; WT BMT, 5.9±0.7 μg/mg; ApoE−/− BMT, 7.2±0.4 μg/mg). Figure 6A and 6B shows that atherosclerotic lesion formation in the aortic root was significantly lower in ApoE−/− mice that received AT1−/− BM than in those that received ApoE−/− or WT BM, indicating the relevance of AT1 receptor expression on BM-derived cells for atherogenesis. Vascular oxidative stress, as determined by aortic reactive oxygen species production, tended to be lower in ApoE−/− mice that received AT1−/− BM than in mice that received ApoE−/− or WT BM (relative L-012 chemiluminescence per milligram of aortic tissue: ApoE−/− BMT, 100±28%; AT1−/− BMT, 65±12%, P value not significant versus ApoE−/− BMT; WT BMT, 117±20%, P value not significant versus ApoE−/− BMT).
Figure 3. Effect of angiotensin II (Ang II) on oxidative stress in early outgrowth endothelial progenitor cells (EPCs) in vitro. A. Representative microscopic scans of 2′,7′-dichlorofluorescein fluorescence microscopy visualizing intracellular reactive oxygen species (ROS) production in early outgrowth EPCs after cell cultivation for 5 days plus an additional 48 hours in the presence of 1 μmol/L of Ang II or vehicle (Con). B. Quantification of intracellular ROS production in early outgrowth EPCs after incubation with vehicle (Con) or Ang II (Ang; 1 μmol/L) for 4, 24, and 48 hours; the antioxidant polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD; 50 U/mL; 48 hours); irbesartan (ARB; 100 μmol/L; 48 hours); or combinations of the aforementioned with Ang II (48 hours). Relative fluorescence in percentage of control, mean±SEM, n=3, *P<0.05 vs control, #P<0.05 vs Ang II. C. Early outgrowth EPC numbers after cell cultivation for 5 days plus an additional 48 hours in the presence of vehicle (Con), 1 μmol/L of Ang II, the antioxidants PEG-SOD (SOD; 50 U/mL) and tiron (1 mmol/L), or combinations of the aforementioned. Numbers of Dil-labeled, acetylated low-density lipoprotein (Dil-acLDL)/lectin double-positive EPCs per high-power field in percentage of control, mean±SEM, n=3, *P<0.05 vs control, #P<0.05 vs Ang II. D. Western blot analyses were performed with total proteins of early outgrowth EPCs after cell cultivation for 5 days plus an additional 48 hours in the presence of vehicle (Con), 1 μmol/L of Ang II, PEG-SOD (50 U/mL), or a combination to detect phosphorylated and total forms of apoptosis signal-regulating kinase-1 (ASK-1). Representative immunoblot. E. Densitometric analysis of ASK-1 activation, expressed as ratio of phospho-ASK-1:total ASK-1 expression in percentage of control, n=4, mean±SEM, *P<0.05 vs control, #P<0.05 vs Ang II. F. Apoptosis assessment by cleaved caspase 3 immunocytochemistry in early outgrowth EPCs after cell cultivation for 5 days plus an additional 48 hours in the presence of vehicle (Con), 1 μmol/L of Ang II, PEG-SOD (50 U/mL), or a combination. Representative microscopic scans showing cleaved caspase 3 immunostaining in Dil-acLDL/lectin double-positive EPCs. Merged image: green=lectin, blue=Dil-acLDL, red=cleaved caspase 3. G. Number of Dil-acLDL/lectin double-positive early outgrowth EPCs with positive staining for cleaved caspase 3, expressed in percentage of control, mean±SEM, n=4, *P<0.05 vs control, #P<0.05 vs Ang II.
**Effect of AT₁ Receptor Antagonism on Circulating CD34/KDR-Positive EPCs in Patients With Coronary Artery Disease**

To evaluate the relevance of AT₁ receptor activation for circulating EPC numbers in humans, 36 patients with stable coronary artery disease were treated with placebo, the angiotensin-converting enzyme inhibitor ramipril, or the AT₁ receptor antagonist telmisartan for 3 months in a randomized, double-blinded clinical study. Baseline characteristics did not differ between treatment groups (Table S1). Only telmisartan but not ramipril or placebo treatment led to a significantly increased number of circulating CD34/KDR-positive cells (primary study end point) in the peripheral blood (CD34/KDR-positive cells per 100 000 gated events, mean±SEM, n=4 to 5 per group, \(^{1}P<0.05\) vs WT control). Number of spleen-derived early outgrowth EPCs, expressed as Dil-labeled, acetylated low-density lipoprotein (Dil-acLDL)/lectin double-positive EPCs per high-power field, mean±SEM, n=4 to 5 per group, \(^{1}P<0.05\) vs WT control. Number of spleen-derived endothelial cell-colony forming units (CFU), expressed as CFU per well, mean±SEM, n=4 to 5 per group, \(^{1}P<0.05\) vs WT control. Migratory capacity of spleen-derived early outgrowth EPCs, expressed as number of Dil-acLDL/lectin double-positive EPCs on the lower side of the filter per high-power field, mean±SEM, n=4 to 5 per group, \(^{1}P<0.05\) vs WT control.

**Discussion**

There is ample evidence that AT₁ receptor activation by Ang II is involved in endothelial damage and atherogenesis via multiple mechanisms.\(^ {1,3–8}\) Diminished regeneration of damaged endothelium contributes to the atherosclerotic process. Vascular progenitor cells and especially EPCs have emerged recently as a pivotal cell type in endothelial homeostasis and replenishment and have been shown to substantially contribute to endothelial regeneration and to restoration of endothelial function.\(^ {10–12,15–18,24}\) We reasoned that some of the harmful consequences of Ang II could be mediated by impairment of EPCs. There is only limited knowledge about the interactions of AT₁ receptor stimulation and number and function of EPCs. Studies in humans and animals using angiotensin-converting enzyme inhibitors or AT₁ receptor blockers, which involved different pathological conditions and models, revealed conflicting results.\(^ {25–30}\) Importantly, there are only few data available concerning underlying mechanisms. Again, contrasting results were shown in experimental investigations using different models.\(^ {31–37}\) A thorough analysis of the effects of Ang II on multiple aspects of EPC regulation and function and especially involved molecular mechanisms has not been undertaken.

In this study, we demonstrate that Ang II consistently decreases the number of cultured early outgrowth EPCs in vitro and in vivo through activation of the AT₁ but not Ang II type 2 receptor and induction of apoptosis. The rate of proliferation is very low in early outgrowth EPCs and is not modulated by Ang II. In addition, Ang II impairs colony-forming and migratory capacities of early outgrowth EPCs. Importantly, reendothelialization after endothelial injury, which in part depends on functional EPCs, is decreased, and neointima formation, a process related not only to increased smooth muscle cell proliferation but also to decreased
reendothelialization, is enhanced during Ang II infusion, suggesting impairment of EPCs by Ang II in vivo. Indeed, our experiments investigating salvage of reendothelialization by intravenous application of AT1a−/− progenitor cells into Ang II–treated WT mice and transfusion of Ang II–pretreated cells into WT mice indicate the importance of the Ang II/AT1 receptor interplay for impaired vascular progenitor cell function in vivo. Our findings in AT1a−/− mice underline the pathophysiological relevance of AT1 receptor expression in both resident vascular cells and circulating vascular progenitor cells.

Our data indicate that Ang II induces increased reactive oxygen species production in early outgrowth EPCs and that this induction of oxidative stress accounts for the Ang II–mediated reduction of early outgrowth EPC numbers, because this effect can be blocked by cotreatment with an antioxidant. This finding is in agreement with other studies, indicating the importance of oxidative stress in EPCs during diabetes mellitus–associated impairment of reendothelialization capacities and Ang II–mediated acceleration of senescence of EPCs.24,31,34 However, the molecular mechanisms involved in Ang II–induced effects in EPCs are incompletely understood. Our data demonstrate for the first time that Ang II, through the AT1 receptor, activates the proapoptotic ASK-1 signaling pathway in early outgrowth EPCs. Ang II enhances phosphorylation of ASK-1, c-Jun N-terminal kinase, and p38-mitogen-activated protein kinase, decreases expression of antiapoptotic Bcl-2, and increases expression of proapoptotic Bax, leading to activation of caspase 3 and apoptosis (Figure 7). ASK-1 is a member of the mitogen-
activated protein kinase kinase family, which activates both c-Jun N-terminal kinase and p38-mitogen-activated protein kinase pathways.\(^{38}\) p38-mitogen-activated protein kinase inhibition in vitro and in vivo improves number and functional capacities of BM-derived MNCs and EPCs, which is associated with reduced atherosclerosis in atherosclerotic mice.\(^{39,40}\) ASK-1 constitutes a pivotal signaling pathway in stress-induced apoptosis, especially in the context of oxidative stress, and was demonstrated to be activated by the pro-oxidant NADPH oxidase, an enzyme known to be stimulated by Ang II.\(^{41,42}\) Consistently, our data show that Ang II–induced activation of ASK-1 and caspase 3, resulting in apoptosis, is mediated through induction of oxidative stress, because both effects are inhibited by coincubation with an antioxidant.

Although the influence of progenitor cells on experimental atherosclerosis is not clear at present,\(^{14,16,43,44}\) EPCs might reduce atherosclerotic lesion formation via continuous restoration of intact endothelium but are impaired by AT\(_1\) receptor activation. Disruption of AT\(_1\) receptors in the BM by BMT, leading to inhibition of Ang II/AT\(_1\) receptor influence on EPCs residing in this compartment, significantly reduced atherosclerosis development in cholesterol-fed ApoE\(^{-/}\) mice and resulted in a trend toward reduced vascular oxidative stress. These data support the above concept. However, it has to be acknowledged that this experimental approach is nonselective, and direct experimental evidence is lacking. No specific EPC-directed intervention was available because of the lack of EPC-specific marker proteins. Ang II affects the entire MNC pool, and other cell lineages, such as hematopoietic cells, are also deprived of AT\(_1\) receptors after BMT, and, thus, in addition to the beneficial effect on EPCs, other infiltrating cells, such as monocytes/macrophages, may have a less atherogenic phenotype after AT\(_1\) receptor disruption, contributing to decreased atherosclerosis development. Our finding is in agreement with other studies showing reductions of atherosclerosis after transplantation of AT\(_1\)-a receptor-deficient BM in ApoE\(^{-/}\) or low-density lipoprotein receptor-knockout mice.\(^{45,46}\) These studies used the model of Ang II–driven atherosclerosis with high doses of Ang II, which differs from the model of cholesterol-induced atherosclerosis, as used in our study.

Treatment of patients with stable coronary artery disease with the AT\(_1\) receptor blocker telmisartan for 3 months significantly increased the number of circulating CD34/KDR-positive cells, which supports the hypothesis that AT\(_1\) receptor activation by Ang II decreases EPC numbers in humans and especially in the setting of atherosclerotic disease. Our findings are in agreement with an AT\(_1\) receptor blocker study in diabetics but in contrast with studies in patients with hypertension and acute coronary syndromes.\(^{25,30,47}\) Treatment durations differed, and our study enrolled patients with stable coronary artery disease, which resembles a different pathological setting. Treatment with the angiotensin-converting enzyme inhibitor ramipril had no significant effect on the number of circulating CD34/KDR-positive cells, which is in contrast with previous studies.\(^{47,48}\) It can only be speculated why the angiotensin-converting enzyme inhibitor did not exert a comparable effect in our study. First, the number of enrolled patients was limited. Second, the time course of action may be different between the drugs. Third, it was demonstrated that telmisartan increases EPC numbers in vitro through a peroxisome proliferator-activated receptor-\(\gamma\)-dependent mechanism independent of blocking Ang II.\(^{49}\) Given

---

**Figure 6.** Role of angiotensin II type 1 (AT\(_1\)) receptors in bone marrow (BM)–derived cells for atherogenesis. Atherosclerosis development in apolipoprotein E knockout (ApoE\(^{-/}\)) mice after BM transplantsations with BM derived from wild-type (WT), AT\(_1\)a receptor knockout (AT1a\(^{-/-}\)), and ApoE\(^{-/}\) mice and subsequent high-cholesterol diet for 7 weeks. A, Representative histological cross-sections of the aortic root after oil red O staining. B, Quantification of atherosclerotic plaque formation, expressed as plaque area in percentage of total surface area, mean±SEM, \(n=5\) to 9 per group, \(*P<0.05\) vs ApoE in ApoE.

---

**Figure 7.** Angiotensin II (Ang II)–induced signaling in endothelial progenitor cells (EPCs). Ang II through Ang II type 1 (AT1) receptor (AT1-R) activation, induction of oxidative stress (reactive oxygen species [ROS]), and activation of redox-sensitive apoptosis signal-regulating kinase-1 (ASK-1)–dependent proapoptotic signaling pathways increases EPC apoptosis and decreases EPC numbers. Impaired EPC number and function by Ang II results in profoundly diminished endothelial regeneration. JNK indicates c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.
this additional mechanism, telmisartan might have a selectively pronounced effect on EPCs compared with ramipril within a given treatment period. Our clinical trial was designed as a translational study to obtain mechanistic insight and to confirm our cell culture and animal data in humans. While corroborating our preclinical findings regarding EPC counts, the study is clearly limited by the small sample size and short duration of treatment. Therefore, effects on EPCs cannot be linked with clinical outcome, and conclusions about clinical impact on atherosclerosis and cardiovascular events are not possible.

**Perspectives**

Pharmacological blockade of the renin-angiotensin system, leading to diminished actions of Ang II, was demonstrated to be vasculoprotective in experimental studies and clinical trials. Our findings show that Ang II, through activation of the AT₁ receptor, oxidative stress, and involvement of redox-sensitive ASK-1-dependent proapoptotic pathways, impairs number and function of EPCs in vitro and in vivo, resulting in diminished vascular regeneration (Figure 7). This demonstrates an important pathological effect of the renin-angiotensin system that contributes to impaired endothelial regeneration and possibly atherogenesis and may potentially represent a vasculoprotective treatment target, especially in future cell-based therapies.

**Acknowledgments**

The excellent technical assistance of Isabel Paez-Maletz, Annika Bohner, Katrin Paul, Sybille Richter, and Bianca Klöckner is greatly appreciated. We thank Dieter Lütjohann, University of Bonn, Bonn, Germany, for measurements of cholesterol concentrations.

**Sources of Funding**

This study was supported by the Deutsche Forschungsgemeinschaft, Canada Foundation for Innovation, Canada Research Chairs, Canadian Institutes of Health Research, and by an unrestricted research grant from Bayer Vital Germany.

**Disclosures**

G.N. received an unrestricted research grant from Bayer Vital Germany.

**References**


Angiotensin II Impairs Endothelial Progenitor Cell Number and Function In Vitro and In Vivo: Implications for Vascular Regeneration

Cathleen Endtmann, Talin Ebrahimian, Thomas Czech, Omar Arfa, Ulrich Laufs, Mathias Fritz, Kerstin Wassmann, Nikos Werner, Vasileios Petoumenos, Georg Nickenig and Sven Wassmann

*Hypertension*. 2011;58:394-403; originally published online August 8, 2011;
doi: 10.1161/HYPERTENSIONAHA.110.169193

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/58/3/394

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/08/05/HYPERTENSIONAHA.110.169193.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Angiotensin II impairs endothelial progenitor cell number and function in vitro and in vivo: implications for vascular regeneration

Short title: Angiotensin II and endothelial progenitor cells

Cathleen Endtmann1*, MD; Talin Ebrahimian2*, PhD; Thomas Czech3, MD; Omar Arfa2, MSc; Ulrich Laufs4, MD; Mathias Fritz4, MD; Kerstin Wassmann1, MD; Nikos Werner1, MD; Vasileios Petoumenos1, MD; Georg Nickenig1, MD; Sven Wassmann1,2, MD

1Medizinische Klinik und Poliklinik II, Universitätsklinikum Bonn, Germany
2Lady Davis Institute for Medical Research and Division of Cardiology, Jewish General Hospital, McGill University, Montréal, QC, Canada
3Klinik für Neurochirurgie and 4Klinik für Innere Medizin III, Universitätsklinikum des Saarlandes, Homburg/Saar, Germany
*Both authors contributed equally to this study

Corresponding author:
Dr. Sven Wassmann
Lady Davis Institute for Medical Research, Jewish General Hospital
3755, Côte-Ste-Catherine Rd, Montréal, QC, H3T 1E2, Canada
e-mail: sven.wassmann@mcgill.ca
Methods

Materials
Angiotensin II, PD123319, PEG-SOD, tiron, DCF, oil red O solution, Evans blue, salts, and other chemicals were purchased from Sigma. L-012 was purchased from Wako Chemicals. Irbesartan was obtained from Sanofi-Aventis.

Preparation of mononuclear cells
Mononuclear cells (MNCs) were isolated from 20 ml sodium citrate-buffered peripheral blood from healthy subjects or buffy-coat preparations derived from healthy blood donors (both: age 20-35 years, male and female, no known disease or medication) using a Ficoll density gradient (Biocoll Separating Solution; Biochrom) according to standard protocols, as previously described. For mouse MNCs, spleens were explanted, mechanically minced, and MNCs were isolated using a Ficoll gradient (Lympholite-M, Cedarlane).

Early outgrowth endothelial progenitor cells
Human or mouse MNCs (1x10^6) were seeded on fibronectin-coated (Sigma) 24-well plates in endothelial basal medium (EBM) with supplements (Promocell), as previously described. After 7 days of culture, cells were washed and incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil)-labeled acetylated low-density lipoprotein (acLDL; CellSystems) and stained with FITC-labeled Ulex europaeus agglutinin (lectin; Sigma) for human early outgrowth endothelial progenitor cells (EPCs), and FITC-labeled Giffonia (bandeiraea) simpliciforia lectin I (Vector Laboratories) for mouse EPCs, respectively. Cells double-positive for acLDL and lectin staining were considered to be early outgrowth EPCs.

Immunocytochemistry was performed to further characterize these human early outgrowth EPCs. After 7 days of culture, fibronectin-adherent cells were stained with acLDL and lectin, washed and incubated with BSA (3%), primary antibodies against VEGF receptor-2, CD31, CD34, P-eNOS (Abcam), AT1 receptor or AT2 receptor (Santa Cruz), respectively, and a CY5-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI (Sigma). Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm), FITC (488 nm), CY3 (555 nm) and CY5 (647nm) filters, respectively (Leica Microsystems).

MNCs were cultured in supplemented endothelial basal medium in the presence of vehicle, angiotensin II and different inhibitors, respectively, for the indicated time points. Culture medium and substances were exchanged every other day, unless indicated differently.

Cell proliferation
Cell proliferation was assessed by BrdU incorporation and quantified by BrdU FACS analysis, as previously described. Briefly, MNCs were cultured as described above. BrdU (20 µmol/l; Beckton Dickinson) was added during the final 48h. Cells were fixed and stained with an anti-BrdU monoclonal antibody (Beckton Dickinson) and
subsequently with propidium iodide in the presence of DNase-free RNase A (Roche). Measurements of the immunofluorescent cells were performed using a FACS Calibur analyzer and analyzed with CellQuestPro software (Becton Dickinson). Cell doublets were discriminated from G2 cells based on the difference in pulse shape. At least 20,000 cells were analyzed per sample.

As an additional approach, Ki67 immunocytochemistry was performed. MNCs were cultured for 7 days as described above. Fibronectin-adherent cells were stained with acLDL and lectin, washed and incubated with BSA (3%), Ki67 primary antibody (Abcam) and a CY5-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI. Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm), FITC (488 nm), CY3 (555 nm) and CY5 (647nm) filters, respectively (Leica Microsystems). As a positive control, subconfluent cultured human umbilical vein endothelial cells were used.

**Apoptosis**

To determine early outgrowth EPC apoptosis, the Cell Death Detection ELISA Kit (Roche) was used according to the instructions of the manufacturer, as previously described. Briefly, after cell culture, cells were pelleted, lysed, and transferred to a streptavidin-coated microplate well. Anti-histone (biotin-labelled) and anti-DNA (peroxidase-conjugated) antibodies were added leading to the binding of nucleosome complexes to streptavidin. Samples were incubated with peroxidase substrate (ABTS) and the coloured product was measured spectrophotometrically.

As an additional approach, cleaved (active) caspase-3 immunocytochemistry was performed. MNCs were cultured for 7 days as described above. Fibronectin-adherent cells were stained with acLDL and lectin, washed and incubated with BSA (3%), cleaved caspase-3 primary antibody (Abcam) and a CY5-coupled secondary antibody (Invitrogen). Nuclei were stained with DAPI. Fluorescence was visualized with a DM2000 microscope with DAPI (358 nm), FITC (488 nm), CY3 (555 nm) and CY5 (647nm) filters, respectively (Leica Microsystems).

**Endothelial cell-colony forming units**

For endothelial cell-colony forming units (EC-CFU), also called CFU-Hill, 5x10^6 human MNCs were isolated and sub-cultured on fibronectin-coated plates for 48h, as described above. 1x10^6 cells derived from the supernatant were re-seeded per well and cultured for additional 7 days in EBM with supplements on human fibronectin pre-coated wells with change of medium every second day, as previously described. The number of colonies per well was manually counted. The number of mouse EC-CFU was determined accordingly using 5x10^6 spleen-derived MNCs.

**Migration**

Early outgrowth EPC migratory function was evaluated using a modified Boyden chamber (Becton Dickinson). A polycarbonate filter with 8-µm pore size (Becton Dickinson) was placed between the upper and lower chambers. After 4 days of initial culture, cell suspensions (1x10^5 cells per well) were placed in the upper chamber, and the lower chamber was filled with EBM containing 50 ng/ml of human recombinant stromal cell-derived factor-1 (SDF-1; R&D Systems). Cells were then incubated for
additional 24h. The migrated cells on the lower side of the filter were fixed and stained for acLDL/lectin. Migration activity was evaluated by the mean number of acLDL/lectin-positive cells counted in at least 3 high-power fields per filter.

**Western blotting**

MNCs were cultured for 7 days as described above. Fibronectin-adherent cells were collected and total proteins (15-20 μg) were extracted, separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight at 4°C with primary antibodies against phosphorylated and total forms of ASK-1 (Abcam), p38 MAPK, JNK (Cell Signaling Technology), and total forms of Bcl-2 (Abcam) and Bax (Santa Cruz), respectively. After incubation with secondary antibodies, signals were revealed by chemiluminescence (Western Lightning Plus ECL, Perkin Elmer) with the Molecular Imager Chemidoc XRS system (Bio-Rad) and quantified by densitometry using Quantity One software (Bio-Rad). Membranes were subsequently stripped and re-probed with a β-actin antibody (Sigma) to verify equal loading.

**Real-time reverse transcription–polymerase chain reaction**

Total RNA was isolated from human MNCs and cultured early outgrowth EPCs and from bone marrow extracted from mouse femurs, reverse transcribed and amplified by real-time RT-PCR using SYBR Green dye, as previously described. The following primers were used: AT1 receptor (mouse), 5’-GGG-TGG-ACA-ATG-GCC-AGG-TAG-3’ (sense), 5’-CTC-GCC-CTG-GCT-GAC-TTA-TGC-3’ (antisense); AT1 receptor (human), 5’-CTG-GAA-GGC-ATA-ATT-ACA-TAT-TTG-TCA-3’ (sense), 5’-GCC-ACA-GTC-TTC-ACG-TTC-ATA-TAA-AA-3’ (antisense); AT2 receptor (human), 5’-TCC-CCT-TGT-TTG-GTG-TAT-GGC-C-3’ (sense), 5’-CAC-TGC-GGA-GCT-TCT-GTT-GGA-A-3’ (antisense); 18s rRNA, 5’-TTG-ATT-AAG-TCC-CTG-CCC- TTG-GT-3’ (sense), 5’-CGA-TCC-GAG-GGC-CTC-ACT-A-3’ (antisense); GAPDH, 5’-CCT-GGA-CCA-CCC-AGC-CCA-GCA-3’ (sense), 5’-TGT-TAT-GGG-GTC-TTG-GAT-GGA-3’ (antisense). For quantification, mRNA expression of the target gene was normalized to the expressed housekeeping gene 18s rRNA or GAPDH.

**Flow cytometry**

Flow cytometry to enumerate EPC numbers in mice and humans was performed as recently described by our group. Mouse blood was analyzed as previously described. The viable lymphocyte population was analyzed for sca-1-FITC (Becton Dickinson) and flk-1-PE (Becton Dickinson). Isotype-identical antibodies served as controls in every experiment (Becton Dickinson). Ficoll-concentrated MNCs were used for analysis of human EPCs. Blood samples were processed with the fluorescent-conjugated antibodies CD34-FITC (Becton-Dickinson) and KDR-PE (Miltenyi). For identification of KDR-positive cells, indirect immunolabeling was performed using a biotinylated goat mononuclear antibody against the extracellular domain of human KDR (R&D Systems). IgG 2a-FITC and IgG 2a-PE (Pharminen) served as negative controls. Cell fluorescence was measured immediately after staining using a FACS Calibur instrument (Becton Dickinson). Data were analyzed using CellQuestPro software (Becton Dickinson). Units of all measured components are absolute cell counts obtained after measuring of 50,000 events.
(mouse) and 100,000 events (human), respectively, in a pre-specified lymphocyte gate during FACS analysis.

**Measurement of reactive oxygen species**

Reactive oxygen species (ROS) release in intact aortic segments was determined by L-012 chemiluminescence, as previously described. ROS release is expressed as relative chemiluminescence per mg aortic tissue. Intracellular ROS production in early outgrowth EPCs was measured by 2', 7'-dichlorofluorescein (DCF; 10 µmol/L) fluorescence microscopy, as previously described. The relative fluorescence intensity is the average value of all experiments.

**Animals and procedures**

**Animals, diet, cholesterol, blood pressure**

12-week-old male C57BL/6J (wild-type) mice, 12-week-old male apolipoprotein E-deficient (ApoE-/-) mice (C57BL/6J genetic background; both Charles River, Sulzfeld, Germany) and 12-week-old male AT1a receptor knock-out (AT1/-) mice (C57BL/6J genetic background; initially provided by Dr. Coffman, University of North Carolina, Chapel-Hill, NC) were used for this study, as previously described. Animals were maintained in a 22°C room with a 12-hour light/dark cycle and received rodent chow and drinking water ad libitum. ApoE-/- mice were fed a high-fat, cholesterol-rich diet for 7 weeks that contained 21% fat, 19.5% casein, and 1.25% cholesterol (Ssniff, Soest, Germany), as previously described. Cholesterol concentrations in aortic tissue were determined by gas–liquid chromatography–mass spectrometry. Arterial blood pressure was measured with a computerized tail-cuff system (CODA 6, Kent Scientific) in conscious animals, as previously described.

**Bone marrow transplantation**

Bone marrow transplantation was performed as previously described. Recipient mice were lethally irradiated (9 Gy). 24h later, 4x10^6 donor bone marrow cells were transplanted intravenously by tail vein injection. Successful reconstitution of the bone marrow was validated by survival of the mice and peripheral blood counts, and transplantation success was assessed by genotyping and quantitative AT1 receptor real-time RT-PCR in bone marrow homogenates (AT1 receptor quantity: AT1/- BMT, 0.0001±0.00005 ng; WT BMT, 0.0032±0.00038 ng, P<0.05 vs AT1/-; ApoE-/- BMT, 0.0022±0.00033 ng, P<0.05 vs AT1/-). Four weeks after bone marrow transplantation and reconstitution of hematopoiesis, these mice were fed a high-fat, high-cholesterol diet for 7 weeks, as described above.

**Anesthesia**

For all invasive procedures, mice were anesthetized with 150 mg/kg body weight ketamine hydrochloride (Ketanest, Pharmacia) and 0.1 mg/kg body weight xylazine hydrochloride (Rompun 2%, Bayer) i.p.

**Osmotic minipumps, splenectomy, cell treatment**

Osmotic minipumps (Alzet) containing saline or angII were implanted subcutaneously at the dorsal neck. AngII was delivered subcutaneously at a constant rate of 0.7 mg/kg body weight per day. Splenectomy of recipient mice was performed as previously described. Animals were allowed to recover for 7 days before further treatment was
performed. Cell treatment of mice was performed as previously described\(^6\). Spleens from WT and AT1-/- donor mice were explanted, mechanically minced, and MNCs were isolated as described above. After implantation of osmotic minipumps and induction of carotid artery electric injury, splenectomized WT mice received 1x10\(^6\) MNCs in 200 µl normal saline solution by intravenous tail vein injection on 3 consecutive days.

**Carotid artery injury**
Carotid artery injury was induced by two protocols, as previously described\(^2, 9\). For assessment of neointima formation, the bifurcation of the left carotid artery was exposed, and after arteriotomy, a curved flexible wire (0.13 mm in diameter) was passed along the common carotid artery in a rotating manner for three times. Neointimal area was determined at day 10 after injury in histological cross-sections after staining with hematoxylin. The presence of alpha-smooth muscle actin-positive cells in the neointima was confirmed immunohistochemically (fig. S5 of the data supplement). For assessment of reendothelialization, the common carotid artery was exposed and submitted to an electric injury starting at the bifurcation and continuing to the proximal artery with a total denudation length of 4mm (each denudation with 2W for 5s, 2mm-tip bipolar coagulation forceps, Erbotom ICC 50 CF electrosurgical generator, Roeser Medical, Mulheim, Germany). The denuded area was determined at day 5 after injury in an en-face preparation of the vessel after intravenous injection of 50µl Evans blue 5%. All sections and vessel preparations were examined under a Zeiss Axiovert 200M microscope. Neointimal area, Evans blue-stained denuded area and total vessel area were measured using AxioVision version 4.5.0 software.

The mice were killed after the indicated treatments and tissue samples and blood were collected immediately. All animal experiments were performed in accordance with institutional guidelines and the German animal protection law.

**Staining of atherosclerotic lesions and histological analysis**
Hearts with ascending aortas were embedded in Tissue Tek OCT embedding medium (Miles), snap frozen and sectioned on a Leica cryostat (9 µm), starting at the apex and progressing through the aortic valve area into the ascending aorta. For detection of atherosclerotic lesions, cryosections were fixed with 3.7% formaldehyde and stained with oil red O solution and hematoxylin, as previously described\(^7\). All sections were examined under a Zeiss AxioVert 200M microscope. AxioVision version 4.5.0 software was used to measure lipid-staining plaque area and total area of serial histological sections. Atherosclerosis data are expressed as plaque area in percent of total surface area.

The presence of smooth muscle cells in neointima was validated by alpha-smooth muscle actin immunohistochemistry. Carotid arteries were embedded in Tissue Tek OCT embedding medium (Miles), snap frozen and stored at –80°C. Samples were sectioned on a Leica cryostat (7 µm) and placed on poly-L-lysine (Sigma) coated slides. Cryosections were postfixed in 5% PFA for 1h and then incubated with 0.2% Triton-X for 10min. Slides were preincubated with normal goat serum for 20min. The primary antibody (monoclonal mouse anti-mouse alpha-smooth muscle actin antibody, clone 1A4, Sigma) was applied for 2h at room temperature. Slides were then incubated with a TRITC-conjugated secondary antibody (goat anti-mouse, Sigma) for 1h. Nuclear staining
was performed using DAPI. Isotype-specific antibodies were used for negative controls. Sections were washed and mounted with fluorescent mounting medium (Dako) for fluorescent microscopic analysis. All sections were examined under a Zeiss Axiovert 200M fluorescence microscope using AxioVision version 4.5.0 software.

**Study subjects and protocol**

36 patients with angiographically defined, chronic stable coronary artery disease were enrolled in a double-blinded clinical study. Patients were randomized (1:1:1) to receive placebo (n=12), the ACE inhibitor ramipril (10 mg/d; n=12) or the AT1 receptor antagonist telmisartan (80 mg/d; n=12) for 3 months in addition to standard care medical treatment for coronary artery disease that included ASA, beta-blockers and statins. All study participants gave written informed consent, and the study protocol was approved by the ethics committee of the University of Homburg, Germany. The study was performed in accordance with the Declaration of Helsinki. Patients were excluded if they had evidence of unstable medical condition, changes in concomitant therapy, chronic kidney disease, intolerance of or contraindications for the study drugs, or any other condition interfering with the participation in the study. The number of circulating CD34/KDR-positive cells in peripheral blood was assessed before and after treatment by flow cytometry (FACS analysis). The primary endpoint of the study was the number of circulating CD34/KDR-positive EPCs in peripheral blood after 3 months of treatment.

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM). For statistical analysis, 2-tailed, unpaired Student’s t-test and ANOVA for multiple comparisons were employed where applicable. Post-hoc comparisons were performed with the Neuman-Keuls test. p<0.05 indicates statistical significance.


<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Placebo (n=12)</th>
<th>Ramipril (n=12)</th>
<th>Telmisartan (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60±2</td>
<td>56±3</td>
<td>56±2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>5/7</td>
<td>2/10</td>
<td>2/10</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26±1</td>
<td>29±1</td>
<td>28±2</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>127±4</td>
<td>129±3</td>
<td>134±3</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>63±4</td>
<td>64±2</td>
<td>67±2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>189±9</td>
<td>171±11</td>
<td>192±15</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>104±12</td>
<td>95±10</td>
<td>117±12</td>
<td>NS</td>
</tr>
<tr>
<td>Medication, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>12/12</td>
<td>12/12</td>
<td>12/12</td>
<td>NS</td>
</tr>
<tr>
<td>Statin</td>
<td>12/12</td>
<td>11/12</td>
<td>12/12</td>
<td>NS</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>12/12</td>
<td>12/12</td>
<td>11/12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table S1. Baseline characteristics of patients with angiographically documented coronary artery disease receiving placebo, ramipril or telmisartan treatment.
Values are expressed as mean±SEM, where appropriate. LDL, low-density lipoprotein; ASA, acetylsalicylic acid; NS, non significant.
Fig. S1: Characterization of human early outgrowth EPCs. MNCs were cultured for 7d in endothelial basal medium. Fibronectin-adherent cells were stained with acetylated LDL (Dil-LDL) and lectin. Nuclei were stained with DAPI. A: Cells double-positive for Dil-LDL and lectin staining were considered early outgrowth EPCs. Merge: green= lectin, blue= DAPI, red= Dil-LDL. B: Immunocytochemistry for VEGF receptor-2 (VEGF-R2), Phospho-eNOS (P-eNOS), CD31, CD34, AT1 (AT1-R) and AT2 receptor (AT2-R) in Dil-LDL/lectin double-positive cells. Inserts show magnified images of representative cells. Merge: green= lectin, blue= Dil-LDL, red= protein of interest. DAPI staining is shown in a separate image.
Fig. S2: Cell proliferation of human early outgrowth EPC and endothelial cells (EC). Representative BrdU FACS and Ki67 immunocytochemistry analyses of EPC (A,B) and EC (C,D). HCAEC, human coronary artery EC; HUVEC, human umbilical vein EC.
Fig. S3: Activation of the apoptosis signal-regulating kinase-1 (ASK-1)-dependent pro-apoptotic signaling pathway by angiotensin II (Ang II) in human early outgrowth EPCs. Ang II significantly increased ASK-1, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) phosphorylation, decreased anti-apoptotic Bcl-2 and increased pro-apoptotic Bax expression. A-D: Representative Western blots and respective densitometric analyses, n=3-8, mean±SEM, *P<0.05 vs control (Con).
**Fig. S4**: Effect of AT1 receptor antagonism and ACE inhibition on circulating CD34/KDR-positive EPCs in patients with coronary artery disease.

36 patients with stable coronary artery disease were randomized to treatment with placebo, the ACE inhibitor ramipril (10 mg/d) or the angiotensin receptor blocker telmisartan (80 mg/d) for 3 months. The number of circulating CD34/KDR-positive cells in peripheral blood was assessed before and after treatment by FACS analysis and is expressed as CD34/KDR-positive cells per 100,000 gated events, mean±SEM, n=12 patients per group, *P<0.05 vs telmisartan pretreatment value.
**Fig. S5**: Alpha-smooth muscle actin expression in neointima as determined by immunohistochemistry (red fluorescence). Representative histological cross-section showing neointima formation with alpha-smooth muscle actin-positive cells 10d after carotid artery wire injury.