Angiotensin II Inhibits Endothelial Cell Motility Through an AT₁-Dependent Oxidant-Sensitive Decrement of Nitric Oxide Availability

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Objective—The migratory capability of vascular endothelial cells plays a pivotal role in the maintenance of vessel wall integrity and is stimulated by nitric oxide (NO). Angiotensin II increases NAD(P)H oxidase activity in endothelial cells, thereby promoting reactive oxygen species (ROS) generation. Because ROS can both reduce NO synthase activity and increase NO breakdown, thus impairing NO availability in endothelial cells, we evaluated the effect of angiotensin II on human vascular endothelial cell (HUVEC) motility.

Methods and Results—Angiotensin II dose- and time-dependently reduced HUVEC migration. Besides inhibiting HUVEC motility, angiotensin II altered intracellular glutathione redox status. The generation of ROS by cultured HUVECs was significantly increased by angiotensin II. Furthermore, angiotensin II reduced NO metabolite concentrations in culture media. The angiotensin II type 1 receptor antagonist candesartan cilexetil attenuated the inhibitory action exerted by angiotensin II on HUVEC motility, reversed the angiotensin II-induced increase in intracellular oxidative stress, and restored NO availability. Similar effects were exerted by the flavonoid inhibitor diphenylene iodinium and the antioxidant agent N-acetyl-L-cysteine.

Conclusions—All together, our data demonstrate that angiotensin II inhibits HUVEC motility by reducing NO availability.

Key Words: angiotensin II □ endothelium □ migration □ oxidative stress □ glutathione

The rapid migration of proliferating endothelial cells to reconstitute a continuous endothelial monolayer is fundamental for the restoration of vessel wall integrity.1–3 Different pathologic conditions could be associated with abnormalities in the migratory capability of vascular endothelial cells, including human atherosclerosis and restenosis occurring after percutaneous transluminal coronary angioplasty either with or without arterial stenting.4

In this context, several studies have demonstrated that the angiotensin system modulates the response of vascular endothelial and smooth muscle cells to injury.5 With respect to endothelial cell motility, previous reports have shown that angiotensin II can influence endothelial cell migratory ability.6,7 Furthermore, an autocrine pathway leading to angiotensin II formation has been demonstrated in endothelial cells, where it modulates their migratory response to various stimuli.7

Mechanisms underlying the angiotensin II action on vascular endothelial cell motility are not fully understood. Angiotensin II strongly affects intracellular oxidative stress.5,8 Indeed, the peptide can activate membrane-bound NAD(P)H oxidase, with a consequent increment in the production of superoxide anion (·O₂⁻).9 In vascular endothelial cells, ·O₂⁻ can be converted by superoxide dismutase to hydrogen peroxide, which, if unscavenged by catalases or peroxidases, can deeply influence several endothelial functions, including cell proliferation and migration.5,9,10 In addition, ·O₂⁻ quenches nitric oxide (NO) to give peroxynitrite,11 one of the most potent endocellular oxidants,8,11 and thereby reduces NO availability. Peroxynitrite also oxidizes tetrahydrobiopterin, a critical cofactor for NO synthase, thus additionally decreasing NO production.12

The oxidant properties of angiotensin II have been suggested to influence several endothelial functions.5 In particular, an angiotensin II–mediated increment in intracellular oxidative stress participates in the upregulation of redox-sensitive genes coding for the synthesis of chemoattractant proteins as well as for the expression of adhesion molecules on the endothelial cell membrane.5,10 Such upregulation could promote atherogenesis by favoring leukocyte/monocyte adhesion to the luminal endothelial cell surface and the consequent transendothelial migration of these circulating cells into...
the vessel wall. In addition, the pro-oxidant properties of angiotensin II could lead to the oxidation of arachidonic acid and thereby induce the release of the potent vasoconstrictor substance 8-iso-prostaglandin F₂α.⁵,¹⁰

In the present study, we investigated the impact of angiotensin II–mediated changes in intracellular oxidative stress on human vascular endothelial cell motility by using a modified Boyden chamber system. We demonstrated that angiotensin II reduced vascular endothelial cell motility through angiotensin II type 1 receptor (AT₁) stimulation. We additionally investigated the underlying signaling pathway and showed that the inhibitory action exerted by angiotensin II on vascular endothelial cell motility was mediated by a reduction in NO availability. In turn, this latter reduction was attributable to an AT₁-dependent increase in intracellular oxidative stress.

**Methods**

**Cell Culture**

Human vascular endothelial cells (HUVECs) were harvested from fresh human umbilical cord veins and cultured until the third passage, as previously described.¹³ Purity of the endothelial cell monolayer was confirmed by the cobblestone morphological pattern and by cell staining with a monoclonal antibody specific for von Willebrand factor.¹⁴ Newly confluent cells were lifted with trypsinization; the trypsin was inhibited with 20% FCS, and cells were washed in culture medium. After centrifugation (1100 rpm over a period of 10 minutes at +20°C), the supernatant was removed and HUVECs were resuspended in culture medium (3 mL). Finally, HUVECs were used for measurements of motility, NO production, glutathione (GSH) redox status, and oxidative stress. All of the above parameters were evaluated after 18 hours incubation with angiotensin II (10⁻⁶ mol/L). Angiotensin II was added to culture media alone or with either the AT₁ receptor antagonist candesartan cilexetil (10⁻⁷ mol/L), the AT₂ receptor antagonist PD 123319 (10⁻³ mol/L), the phospholipase D (PLD) inhibitor dihydro-D-erythro-sphingosine (SPH, 10⁻⁷ mol/L), or the antioxidant N-acetyl-cysteine (NAC, 10⁻⁴ mol/L). In addition, experiments, the effects of angiotensin II on HUVEC migratory ability, NO production, GSH redox status, and oxidative stress were also evaluated after either 3 or 18 hours of incubation with angiotensin II, in both cases by using different angiotensin II concentrations (10⁻⁵, 10⁻⁶, or 10⁻⁷ mol/L).

Angiotensin II was purchased from Clinalfa. Candesartan cilexetil was kindly donated by Takeda Spa, Italy. All other reagents were purchased from Sigma Chemical Co.

**Endothelial Cell Migration Assay**

In vitro endothelial cell migration assay was performed according to the modified Boyden chamber system. Briefly, cell culture inserts (diameter, 10 mm; 8.0-µm pore size gelatinized polycarbonate membrane, NUNC) were placed into a well of a 24-well multidish (NUNC) were placed into a well of a 24-well multidish and stained by Giemsa. Then each insert was disassembled, and the mean ± SD of experiments conducted on 2 different occasions, each time in triplicate.

**HUVEC Proliferation Assay**

To avoid bias attributable to angiotensin II–induced HUVEC proliferation, this latter was measured after HUVECs were incubated for 18 hours with increasing angiotensin II concentrations (10⁻⁴, 10⁻⁵, or 10⁻⁶ mol/L). For this purpose, HUVECs were cultured on 96-well flat-bottom microtiter plates and proliferation was assessed by using the MTS colorimetric assay (Cell Titer 96 Aqueous Non Radioactive Cell Proliferation Assay from Promega), according to manufacturer’s instructions.

**Statistical Analysis**

Changes in migration were analyzed by ANOVA followed by Bonferroni’s test. Multiple comparisons were analyzed by ANOVA followed by post-hoc analysis to adjust the significance level. Statistical significance was considered as *P*<0.05. Data are given as the mean±SD of experiments conducted on 2 different occasions, each time in triplicate.

**Results**

**Angiotensin II Inhibited HUVEC Migration by Stimulating the AT₁ Receptor**

HUVEC migration was inhibited by angiotensin II in dose- and time-dependent manners (Figure 1A). The AT₁ receptor antagonist candesartan cilexetil attenuated the effects of angiotensin II on HUVEC motility (Figure 1B). Candesartan cilexetil also reduced spontaneous endothelial cell migratory ability. In contrast, the angiotensin II type 2 (AT₂) receptor inhibitor PD 123319 was unable to modify both spontaneous HUVEC motility and the angiotensin II actions on HUVEC migratory ability (Figure 1B).

**NAD(P)H Oxidase–Related Induction of Intracellular Oxidative Stress Was Involved in the Inhibitory Actions Exerted by Angiotensin II on HUVEC Motility**

Compared with untreated HUVECs, angiotensin II–stimulated ones showed significant changes in redox status. In
Figure 1. A, Effects of incubation (3 and 18 hours) with increasing angiotensin II (Ang) concentrations (10⁻⁶, 10⁻⁷, and 10⁻⁸ mol/L) on HUVEC migration toward a 8.0-mm-pore gelatinized polycarbonate membrane. White columns represent control (ie, no added Ang). B, Effects of 18-hour incubation with either the medium alone or the Ang type 1 receptor antagonist candesartan cilexetil (AT1a, 10⁻⁷ mol/L), the Ang type 2 receptor antagonist PD123319 (AT2a, 10⁻⁷ mol/L), the phospholipase D inhibitor SPH (10⁻⁷ mol/L), the flavonoid inhibitor DPI (10⁻⁷ mol/L), or the antioxidant NAC (10⁻⁷ mol/L) on intracellular oxidant/antioxidant balance (Figures 3A through 3D). The flavoprotein inhibitor DPI almost completely abolished the effects of angiotensin II on intracellular oxidative status (Figures 3A through 3D). The flavoprotein inhibitor DPI almost completely abolished the effects of angiotensin II on GSH redox status and DCF content (Figures 3A through 3D). By contrast, the AT₁ receptor inhibitor PD 123319 was completely ineffective in counteracting angiotensin II–related changes in oxidant/antioxidant balance (Figures 3A through 3D). Preincubation of HUVECs with the PLD inhibitor SPH partially reversed the effects of angiotensin II on intracellular oxidative status (Figures 3A through 3D). The flavoprotein inhibitor DPI almost completely abolished the effects of angiotensin II on GSH redox status and DCF content (Figures 3A through 3D). These data suggest that angiotensin II–induced changes in intracellular oxidant/antioxidant balance were related to an AT₁ receptor–dependent activation of NAD(P)H or similar flavoprotein-dependent oxidase, which was only partially mediated by the PLD pathway.

When HUVECs were preincubated with DPI, the inhibitory action of angiotensin II on endothelial cell motility was significantly attenuated. The PLD inhibitor SPH only partially blocked the antimigratory effect of angiotensin II (Figure 1B). These data suggest that angiotensin II–related changes in intracellular oxidative stress were involved in the angiotensin II–induced changes in HUVEC motility. In keeping with this, the antioxidant NAC attenuated the effects of angiotensin II on endothelial cell motility (Figure 1B) and completely prevented angiotensin II–induced changes in intracellular oxidant/antioxidant balance (Figures 3A through 3D). DPI and NAC had no additive inhibitory actions on the angiotensin II effects on HUVEC motility (data not shown). Similarly, addition of either DPI or NAC to candesartan cilexetil did not additionally potentiate the inhibitory effects of AT₁ receptor blockade (data not shown). Finally, SPH did not additionally increase HUVEC motility after incubation with either candesartan cilexetil, DPI, or NAC (data not shown).

Figure 2. Effects of 18-hour incubation of human vascular endothelial cells with either no additive (medium) or the angiotensin II (Ang) type 1 receptor antagonist candesartan cilexetil (AT1a, 10⁻⁷ mol/L), the Ang type 2 receptor antagonist PD123319 (AT2a, 10⁻⁷ mol/L), the phospholipase D inhibitor SPH (10⁻⁷ mol/L), the flavonoid inhibitor DPI (10⁻⁷ mol/L), or the antioxidant NAC (10⁻⁷ mol/L) on intracellular GSH (A) and GSSG/GSH ratio (C), and DCF (D) contents. White columns represent control (ie, no added Ang). a, P=0.002 vs baseline; b, P=0.02 vs 3 hours.
Impaired NO Availability Was Responsible for the Inhibitory Effect of Angiotensin II on HUVEC Motility

NO plays a fundamental role in regulating endothelial cell migratory capability.\textsuperscript{21} Angiotensin II–related changes in oxidative stress could affect NO availability by increasing NO breakdown\textsuperscript{22} and reducing NO synthase activity.\textsuperscript{12} In our experiments, angiotensin II dose- and time-dependently reduced NO availability, as evaluated by assessing NOx concentrations in culture media (Figure 4A). Previous inhibition of AT\textsubscript{1} but not of AT\textsubscript{2} receptors prevented angiotensin II–induced changes in NO production by cultured HUVECs (Figure 4B). SPH partially counteracted the angiotensin II action on NO production by cultured HUVECs (Figure 4B). Finally, both DPI and NAC completely prevented the effects of angiotensin II on NO availability (Figure 4B).

Angiotensin II–related changes in NOx concentrations in culture media were comparable with those induced by the NO synthase inhibitor N\textsubscript{o}-nitro-L-arginine methyl ester (L-NAME) (Figure 4C). L-NAME also reduced HUVEC motility at a similar level as the one achieved after incubation with angiotensin II (Figure 4D). When HUVECs were incubated with L-NAME, angiotensin II failed to additionally affect both NOx concentration in culture media and HUVEC migratory ability (Figures 4C and 4D, respectively). The NO donor S-nitroso-N-acetyl-penicillamine (SNAP, 10\textsuperscript{-7} mol/L), which is known to stimulate endothelial cell motility,\textsuperscript{23} completely reversed L-NAME–related changes in NOx concentrations (Figure 4C). In contrast, SNAP was unable to counteract the angiotensin II effect on NO availability (Figure 4C). These data suggest that the angiotensin II actions on NO availability were attributable to increased NO inactivation rather than to reduced NO production. In keeping with this, SNAP also completely blocked the effect of L-NAME on HUVEC motility (Figure 4D) while failing to blunt the inhibitory action exerted by angiotensin II on HUVEC migratory ability (Figure 4D). However, when the SNAP dose was increased (2×10\textsuperscript{-6} mol/L) to obtain a significant rise in NOx concentration (after 18 hours, 94.7±3.5\% of control; NS versus control and P<0.0001 versus angiotensin II 10\textsuperscript{-7} mol/L, respectively) in angiotensin II–treated cells, a concomitant significant increase in HUVEC motility was observed (after 18 hours, 91.3±3.8\% of control; P<0.02 versus control and P<0.0001 versus angiotensin II 10\textsuperscript{-7} mol/L, respectively). Taken together, these data suggest that angiotensin II reduced NO availability by acting on an oxidant-sensitive pathway. In turn, the reduced NO availability was responsible for the inhibitory action exerted by angiotensin II on HUVEC motility.

Effects of Angiotensin II on HUVEC Proliferation

Using a colorimetric assay for cell proliferation, angiotensin II at 3 different concentrations (10\textsuperscript{-8}, 10\textsuperscript{-7}, and 10\textsuperscript{-6} mol/L) had no effects on HUVEC proliferation (after 18 hours of incubation, +1.1±1.9\%, +1.5±0.7\%, and +2.6±2.3\%, respectively; NS versus control), thus excluding a spurious effect of stimulated proliferation on the evaluation of HUVEC migration.

Discussion

The principal effector of the renin-angiotensin-aldosterone system angiotensin II, either generated in plasma or locally formed by tissue angiotensin-converting enzyme, has been claimed to induce several detrimental endothelial effects.\textsuperscript{5}
Different intracellular pathways can be activated by angiotensin II and lead to vascular endothelial damage. In particular, one of the most important mechanisms involved in angiotensin II–related vascular endothelial cell toxicity is the increased generation of $\cdot O_2^-$, which, in turn, is attributable to the AT$_1$-dependent activation of membrane NAD(P)H oxidase. Once produced, $\cdot O_2^-$ acts as a key determinant in altering vascular endothelial cell redox status. Indeed, $\cdot O_2^-$ facilitates or induces the formation of other oxidants, such as H$_2$O$_2$, and quenches NO to produce peroxynitrite, i.e., one of the most potent intracellular oxidants. In the vascular endothelial cell, the unbalance between NO and $\cdot O_2^-$ productions impairs endothelium-dependent vasodilation, increases smooth muscle cell proliferation, inhibits fibrinolysis, activates prothrombotic pathways, upregulates redox-sensitive genes, and mediates the expression of chemoattractant proteins and adhesion molecules, and decreases the inactivation of vasotoxic agents such as homocysteine.

The present study indicates a novel vascular action mediated by the angiotensin II–related increment in oxidative stress, i.e., the inhibition of vascular endothelial cell migration.

In our experiments, angiotensin II reduced HUVEC migration in dose- and time-dependent manners. The Boyden chamber system cannot exclude whether angiotensin II–related changes in HUVEC proliferation contributed to the observed modifications in HUVEC motility. Thus, we analyzed the effects of various concentrations of angiotensin II on HUVEC proliferation. This latter was not affected by angiotensin II over 18 hours of incubation, independently of the tested concentration. Thus, our results strongly suggest that angiotensin II was critical for inhibiting HUVEC migration rather than stimulating HUVEC proliferation.

The inhibitory effect of angiotensin II on HUVEC migration was attenuated by candesartan cilexetil, a selective inhibitor of the AT$_1$ receptor, but not by PD123319, a selective inhibitor of the AT$_2$ receptor. As candesartan cilexetil, both the flavoprotein inhibitor DPI and the antioxidant NAC increased spontaneous HUVEC migration and attenuated the antimigratory effect of angiotensin II. DPI was unable to additionally increase the inhibitory action of candesartan cilexetil on the antimigratory action of angiotensin II, thereby suggesting that an AT$_2$-mediated activation of endothelial NADPH oxidase was almost solely responsible for the actions exerted by angiotensin II on oxidant generation by HUVECs and HUVEC motility.

In contrast to the other tested inhibitors and to the antioxidant NAC, SPH incompletely blocked the antimigratory action exerted by angiotensin II and did not potentiate the effects of candesartan, DPI, or NAC. In addition, SPH incompletely inhibited the effects of angiotensin II on intracellular oxidative stress and GSSG/GSH ratio as well as NO generation by cultured HUVECs. In contrast to our data, PLD has been recently suggested to be the enzyme responsible for AT$_1$-dependent NAD(P)H oxidase activation in vascular smooth muscle cells. However, recent elegant experiments by Touyz and Schiffrin indicate that PLD cooperates with other intracellular enzymes, such as protein kinase C, to activate NAD(P)H oxidase also in vascular smooth muscle cells. Accordingly, replication of such experiments in HUVECs demonstrated that coincubation with a selective inhibitor of protein kinase C plus sphinganine completely abolished the increase in oxidative stress attributable to angiotensin II (G. Desideri, unpublished data, 2002). Therefore, PLD seems to be important but not solely responsible for the angiotensin II–related increment of oxidant generation by cultured HUVECs.

The intracellular pathway responsible for the inhibitory action exerted by angiotensin II on HUVEC motility also involved NO availability. Angiotensin II time- and dose-dependently reduced NO concentrations in culture media, suggesting that a reduction in NO availability did occur in cultured HUVECs after angiotensin II stimulation. Because endogenous NO plays a fundamental role in regulating vascular endothelial cell migratory ability, it is intriguing to speculate that angiotensin II inhibited HUVEC motility by reducing NO availability. According to such interpretation, the effects of angiotensin II on NO availability as well as on HUVEC motility were comparable with those induced by the NO synthase inhibitor L-NAME. In addition, when HUVECs were incubated with L-NAME, angiotensin II failed to additionally affect both NO generation by cultured HUVECs and HUVEC migratory ability. These data strongly support the existence of a causal link between the actions of angiotensin II and NO levels. Of note, the NO donor SNAP, which is known to stimulate endothelial cell migration, did not affect angiotensin II–induced changes in NO availability and endothelial cell migration. By contrast, SNAP restored NO availability and completely abolished the antimigratory effect of L-NAME on endothelial cells. However, when the SNAP dose was increased to obtain a significant rise in NO concentration in angiotensin II–treated cells, a concomitant significant increase in HUVEC motility was observed. These findings suggest that the angiotensin II–related decrement in NO availability was attributable to increased NO breakdown rather than reduced NO production. In this context, it is interesting to observe that the antimigratory action exerted by angiotensin II was strictly dependent on its pro-oxidant capability. Indeed, angiotensin II simultaneously inhibited HUVEC migration and deeply altered the cell redox status, with an increment in intracellular oxidative stress and a decrement in intracellular reduced GSH content. Because ROS are able to inactivate NO, it is intriguing to speculate that angiotensin II inhibited HUVEC motility by increasing ROS production and thereby favoring NO breakdown. According to this, the antioxidant NAC restored NO availability and attenuated the effect of angiotensin II on HUVEC motility. Interestingly, a ROS-related reduction in NO availability also represents the main mechanism underlying the inhibitory action exerted by CD40L, i.e., a peptide that is deeply involved in the pathobiology of vascular diseases, on vascular endothelial cell motility.

Migration assays by the widely used Boyden chamber system may be affected by cellular events that are only indirectly related to cell migration. Angiotensin II may induce rearrangements of the cytoskeleton, alterations in cell adhesion or spreading, or change of cell size or shape. All these phenomena could mimic an apparent HUVEC migration...
through the polycarbonate membrane. To avoid this bias, we counted endothelial cells under a ×20 magnification, which allowed us to clearly identify each endothelial cell that migrated through the pores of the inserted membrane. In addition, we counted endothelial cells that were lost in culture media after migration and failed to find significant differences both in the presence and absence of different concentrations of angiotensin II (data not shown). Therefore, it is extremely unlikely that angiotensin II–related changes in HUVEC size, shape, or adhesion significantly affected our results. In contrast, the small number of HUVECs observed in the lower area of the polycarbonate membrane after migration and failed to find significant differences of angiotensin II (data not shown). Therefore, it is extremely unlikely that angiotensin II

In conclusion, angiotensin II, the main effector of the renin-angiotensin system, is known to play a fundamental role in the regulation of various endothelial functions. Endothelial cell migration is a critical event in atherogenesis and vascular response to injury. Furthermore, abnormalities in vascular endothelial cell migratory ability have been suggested to be essential in restenosis after percutaneous transluminal coronary angioplasty.

The above actions of angiotensin II are likely to play a physiological role in modulating vascular endothelial cell motility. Nevertheless, the antiangiogenic properties of angiotensin II have all the potential to be of particular relevance in human pathologic conditions, implying an abnormal vascular endothelial cell response to injury.

References


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