Angiotensin II modulates VEGF-driven angiogenesis by opposing effects of type 1 and type 2 receptor stimulation in the microvascular endothelium

Javier Carbajo-Lozoya [a], Susanne Lutz [a,b], Yuxi Feng [a,c], Jens Kroll [d], Hans-Peter Hammes [c], Thomas Wieland [a,*]

[a] Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, University of Heidelberg, Maybachstrasse 14, D-68169 Mannheim, Germany
[b] Dept. of Pharmacology, University of Göttingen, Robert-Koch-Strasse 40, D-37075, Göttingen, Germany
[c] 5th Medical Department, Medical Faculty Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3 D-68167 Mannheim, Germany
[d] Division Vascular Biology of the Center for Biomedicine and Medical Technology Mannheim (CBTM), Medical Faculty Mannheim, University of Heidelberg, Ludolf-Krehl-Strasse 13-17 D-68167 Mannheim, Germany

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a main stimulator of pathological vessel formation. Nevertheless, increasing evidence suggests that Angiotensin II (Ang II) can play an augmentory role in this process. We thus analyzed the contribution of the two Ang II receptor types, AT_1R and AT_2R, in a mouse model of VEGF-driven angiogenesis, i.e. oxygen-induced proliferative retinopathy. Application of the AT_1R antagonist telmisartan but not the AT_2R antagonist PD123,319 largely attenuated the pathological response. A direct effect of Ang II on endothelial cells (EC) was analyzed by assessing angiogenic responses in primary bovine retinal and immortalized rat microvascular EC. Selective stimulation of the AT_1R by Ang II in the presence of PD123,319 revealed a pro-angiogenic activity which further increased VEGF-driven EC sprouting and migration. In contrast, selective stimulation of the AT_2R by either CGP42112A or Ang II in the presence of telmisartan inhibited the VEGF-driven angiogenic response. Using specific inhibitors (pertussis toxin, RGS proteins, kinase inhibitors) we identified $G_{12/13}$ and G_i dependent signaling pathways as the mediators of the AT_1R-induced angiogenesis and the AT_2R-induced inhibition, respectively. As AT_1R and AT_2R stimulation displays opposing effects on the activity of the monomeric GTPase RhoA and pro-angiogenic responses to Ang II and VEGF requires activation of Rho-dependent kinase (ROCK), we conclude that the opposing effects of the Ang II receptors on VEGF-driven angiogenesis converge on the regulation of activity of RhoA-ROCK-dependent EC migration.

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1. Introduction

Angiotensin II (Ang II), the main effector peptide of the renin–angiotensin system (RAS), is one of the most important regulators in the cardiovascular system. It is generally associated with the maintenance of vasotonus and vascular homeostasis. The biological effects of Ang II are mediated by G protein coupled Ang II receptors, of which two major mammalian types, AT_1R and AT_2R, have been identified [1–3]. The AT_1R is widely expressed in vascular tissue [4]. Most of the effects of Ang II are attributed to this type. In contrast to the AT_1R, the AT_2R is expressed at low density in adults and it was frequently shown that the AT_2R counteracts AT_1R-induced effects in many systems such as endothelial, vascular smooth muscle and neuronal cells [5–7]. The role of Ang II as an important regulator in

vascular homeostasis is well established [8]. Meanwhile, increasing evidence has presented that Ang II additionally regulates angiogenesis, the formation of blood vessels from pre-existing ones. This crucial process occurs during vascular development and in a number of pathological conditions, such as wound healing and new blood vessel formation in tumors. Angiogenesis is strongly dependent on vascular endothelial growth factor (VEGF), an endothelial-specific growth factor that induces endothelial cell proliferation, migration and sprouting [9]. VEGF activates multiple signaling cascades, such as the ERK pathway by its binding to the VEGF receptors Flt-1 (VEGFR1) and KDR (VEGFR2) [10]. Recent data suggested that VEGF-driven angiogenesis requires the activation of RhoGTPases by VEGFR2 and thus inhibition of RhoA disrupts tube formation in three-dimensional matrices [11–13]. Nevertheless, it has been shown that progressive diabetic retinopathy (PDR), a process which threatens vision and is driven by extensive VEGF-induced angiogenesis is influenced by Ang II [14]. Consequently, although the underlying molecular mechanisms are only partially understood, the use of the Ang II receptor type I (AT_1R) antagonist candesartan was already tested in recent

* Corresponding author at: Institute for Experimental and Clinical, Pharmacology and Toxicology, Medical Faculty Mannheim, University of Heidelberg, Maybachstrasse 14, 68169 Mannheim, Germany. Tel.: +49 621 383 9610; fax: +49 621 383 9611.
E-mail address: thomas.wieland@medma.uni-heidelberg.de (T. Wieland).

clinical trials as a potential therapy in treatment of PDR [15,16]. Candesartan reduced the incidence of retinopathy in normoalbuminuric normotensive type 1 diabetes and enhanced regression of retinopathy in type 2 diabetes. Recent reviews similarly highlighted the emerging role of the RAS and Ang II in tumor angiogenesis and AT$_1$R antagonist application reduced tumor progression and vascularization in several experimental cancer models [17–19]. Most of the anti-angiogenic properties of AT$_1$R antagonists have been attributed to the inhibition of an AT$_1$R-mediated increase in the local expression of VEGF and other angiogenic factors, but evidence for direct modulatory effects of the AT$_1$R as well as AT$_2$R in the endothelial cells (EC) is increasing. Based on a study, in which we found that physiological concentrations of Ang II inhibited VEGF-induced migration of human vascular EC by stimulation of the AT$_2$R [20], we studied the influence of selective inhibition and stimulation of the AT$_1$R and AT$_2$R in the mouse model of oxygen-induced retinal vascularization (OIR) and on angiogenic in vitro responses in cultured retinal and microvascular EC. We report here that Ang II via AT$_1$R modulates VEGF-driven sprouting angiogenesis by inducing EC migration. In contrast, selective stimulation of the AT$_2$R causes inhibition of angiogenic responses. Apparently, VEGF- and Ang II-induced signaling pathways in EC converge on the regulation of RhoA/Rho dependent kinase (ROCK) mediated responses.

2. Materials and methods

2.1. Oxygen-induced retinopathy

The oxygen-induced retinal vascularization model (OIR model) was described by Smith et al. [21]. Neonatal mice (C57BL/6J) were obtained from breeding colonies maintained at the Medical Research Center, Mannheim Medical Faculty, University of Heidelberg. Briefly, 7-day-old (p7) mice were exposed to 75% oxygen for 5 days in an incubator (Stuart Scientific, Redhill, UK) with their nursing mothers. At p12, mice were returned to room air. From p12 to p17, mice were injected subcutaneously with Ang II (2 mg/kg, n = 4), AT$_1$R antagonist telmisartan (Telmi, 3 mg/kg, n = 8) or control substance DMSO alone (n = 13) once daily. Alternatively, Ang II combined with telmisartan (Ang II + Telmi, n = 6) or with the non-peptide selective AT$_2$ receptor antagonist PD123,319 (10 mg/kg, Ang II + PD, n = 6) was used. Mice were sacrificed and retinal angiogenesis was analyzed at p17.

2.2. Quantification of neovascularization

Eyes were fixed in 4% paraformaldehyde in PBS for at least 24 h, and embedded in paraffin. To quantify retinal neovascularization, serial sections (6 μm) were stained with Periodic Acid-Schiff (PAS) and hematoxylin. 10 sections at the optic nerve were evaluated. All retinal angiogenesis anterior to the inner limiting membrane was counted in each section by an observer blinded to the protocol. The mean of the 10 counted sections represented the average neovascular nuclei per section per eye. No neovascular cell nuclei anterior to the inner limiting membrane were observed in mice which were not subjected to the OIR model.

2.3. Adenoviral constructs

The cDNA for the catalytic PP2A subunit (NP_002706, kindly provided by S. Dimmeler, Frankfurt, Germany), was subcloned into the pAdTrack-CMV vector and a recombinant adenovirus serotype 5 was generated as described [22]. The adenoviruses encoding RGS4, RGS-LSC, transducin α (TDα) and Gβγ have been described previously [23–25].

2.4. Culture of primary bovine retinal EC

Endothelial cells (BREC, a gift from U. Schubert, Giessen, Germany) were isolated from bovine retinal capillaries as previously described [26]. BRECs were cultured on gelatin-coated dishes in EC medium (PromoCell) supplemented with 10% fetal calf serum (FCS), 0.4% ECGS/H, 0.1 ng/ml EGF, 1 μg/ml hydrocortison, and 1 ng/ml bFGF, at 37 °C in a humidified atmosphere containing 5% CO$_2$. For all experiments, cells were used at passage numbers between 4 and 8. All experiments with BREC were conducted in serum-reduced EC medium (2.5% FCS).

2.5. Culture of microvascular rat fat pad endothelial cells

Spontaneously immortalized endothelial cells primarily isolated from the capillaries of rat epididymal fat tissue (RFPEC, a kind gift from A. Horowitz, Dartmouth Medical School, Lebanon, New Hampshire, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS [27]. All experiments with RFPEC were conducted in serum-reduced DMEM (0.5% FCS).

2.6. Sprouting assay

In vitro sprouting angiogenesis assays were performed as described [28]. Briefly, spheroids generated for 24 h with 400 cells were embedded into a 3D collagen-based gel placed in a 48-well plate. They were stimulated under the indicated conditions for 24 h, and the cumulative sprout length was measured.

2.7. Migration assay

Trans-well cell migration assays were performed using 96-well Boyden chemotaxis apparatus (Neuro Probe) as previously described [20]. Briefly, polycarbonate filters with 8-μm pore size (Neuro Probe) were coated with 100 μg/ml type I collagen overnight. 5000 cells per well were incubated with test agents for 4 h, and thereafter, the filter was fixed with methanol and stained with Giemsa.

2.8. Proliferation assay

The incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was used to assess cell proliferation. Briefly, 5000 cells per well were seeded in a 96-well plate and cultured for 48 h before incubation with indicated test agents for an additional 24 h. MTT (5 mg/ml) was added, and the medium was removed 4 h later. The colored formazan product was resolved in 0.04 N HCl/isopropanol and quantified by absorbance measurements at 570 nm.

2.9. Precipitation of activated RhoA (effector-pull-down assay)

The cellular levels of GTP-loaded RhoA were determined using a GST fusion protein containing the RhoGTPase-binding domain of Rhotekin (GST-RBD) as described previously [29]. Briefly, cells starved overnight were stimulated as indicated. Ice-cold GST-fish buffer (10% (v/v) glycerol, 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM MgCl$_2$) was used for immediate cell lysis and cellular debris was removed by centrifugation at 4 °C. The GTPases-containing supernatant was incubated for 1 h at 4 °C with GST-RBD bound to glutathione Sepharose beads, expressed and purified from *Escherichia coli*. Protein complexes were washed with cold GST-fish buffer, eluted in sample buffer, separated by 15% SDS-PAGE and detected by immunoblotting.