Differential regulation of thrombospondin-1 and fibronectin by angiotensin II receptor subtypes in cultured endothelial cells

Jens W. Fischer\textsuperscript{a,*}, Monika Stoll\textsuperscript{a,1}, Alfred W.A. Hahn\textsuperscript{b}, Thomas Unger\textsuperscript{a}

\textit{a}Institute of Pharmacology, Christian-Albrechts-University Kiel, 24105 Kiel, Germany
\textit{b}Knoll AG, Ludwigshafen, Germany

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Abstract

Objectives: Angiotensin II (ANG II) can modulate cellular proliferation in various cell types via AT\textsubscript{1} and AT\textsubscript{2} receptors. In the present study, we investigated the effect of the angiotensin AT\textsubscript{1} and AT\textsubscript{2} receptors on DNA-synthesis as well as on the expression of the extracellular matrix (ECM) components, thrombospondin-1 (TSP-1) and fibronectin (FN) in endothelial cells (EC). Methods: The experiments were performed in microvascular EC derived from rat heart (CEC) and macrovascular EC derived from bovine aorta (BAEC). The experiments were performed in cells of the second and third passage and the expression of AT\textsubscript{1} and AT\textsubscript{2} receptors was verified by binding studies, Northern analysis or RT–PCR. Quiescent rat CEC and BAEC were stimulated to proliferate by the addition of 25 ng/ml bFGF, while ANG II (10^{-7} M) and the selective ANG II receptor antagonists, Losartan (10^{-8} M) and PD123177 (10^{-6} M) or the AT\textsubscript{2} agonist, CGP42112A (10^{-7} M) were added 16 h later. Results: ANG II induced a dose-dependent decrease of DNA-synthesis in BAEC measured by \textsuperscript{[3]}H-thymidine incorporation. This inhibitory effect of ANG II was prevented by the addition of the AT\textsubscript{1} receptor antagonist PD123177 (10^{-6} M), demonstrating, that the inhibition of DNA synthesis is mediated by the AT\textsubscript{1} receptor. In the presence of Losartan, stimulation of both, CEC and BAEC, with ANG II resulted in a marked increase of TSP-1 mRNA levels, which was maximal between 3 and 6 h in rat CEC and after 9 h in BAEC. In addition, TSP-1 was clearly induced by the AT\textsubscript{2} agonist CGP42112A. In contrast, blockade of the AT\textsubscript{2} receptor by the selective AT\textsubscript{2} antagonist, PD123177 (10^{-6} M), resulted in a pronounced down regulation of FN mRNA 9 h after the stimulation. Conclusions: The present results suggest that the ANG II receptor subtype AT\textsubscript{2} mediates growth inhibition in macrovascular EC similar to what has been shown before in microvascular rat EC and that AT\textsubscript{2} receptors mediates remodeling of the endothelial ECM by upregulation of TSP-1 expression in both macro- and micro-vascular endothelial cells. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The integrity of the endothelial lining is essential for the maintenance of the normal structure of the artery wall, whereas injury or dysfunction of the endothelial cell layer leads to the initiation of atherosclerotic disease or thrombosis. Proliferation and migration of endothelial cells are essential for repair and maintenance of the endothelial monolayer. Proliferation is controlled by growth factors, hormones and the adhesive interactions of endothelial cells (EC) with the pericellular matrix, which is mediated by integrin and non-integrin receptors. Control of EC prolifer-
Angiotensin II (ANG II), the main effector peptide of the renin-angiotensin system, exerts a remarkable diversity of physiological actions through two major classes of ANG II receptors, $A_1$ and $A_2$ receptors. There is extensive evidence that most of the known physiological effects of ANG II are attributable to $A_1$ receptors (for review see Ref. [2]). These include, among others, vasoconstriction, aldosterone release and the growth promoting effects on vascular smooth muscle cells (VSMC) and cardiomyocytes [3–7]. On the other hand, recent studies indicate an involvement of $A_2$ receptors in development, apoptosis and regeneration following injury [8–10]. Interestingly, in most of these studies $A_1$ and $A_2$ receptors have been shown to exert counteracting effects on cellular growth, differentiation and apoptosis [11–14]. However, the mechanisms by which the $A_2$ receptor exerts its actions and how it signals are still not completely understood. We showed previously that ANG II via the $A_2$ receptor inhibits bFGF induced proliferation of microvascular EC, which might of significance with respect to the development of endothelial lesions in situations where the renin–angiotensin-system is activated. The present study was designed to examine whether the antimitogenic effect of the $A_2$ receptor is paralleled by specific changes in the expression of the ECM components, thrombospondin-1 (TSP-1) and fibronectin (FN), known to be important modulators of proliferation and differentiation in vascular cells [15–18]. Furthermore, experiments were performed in cells from macrovascular origin, namely bovine aortic endothelial cells (BAEC), in order to investigate whether the antiproliferative effects and related changes in ECM composition are constrained to the microvascular endothelium or are rather a general feature of $A_2$ receptor stimulation in endothelial cells.

2. Methods

2.1. Reagents

All chemicals were purchased from Sigma, if not stated otherwise. DMEM, FCS, $\gamma$-glutamine, sodium pyruvate, non-essential amino acids and antibiotics were obtained from Gibco BRL, Eggenheim, Germany. dATP, dCTP, dTTP, dGTP were from Boehringer, Mannheim, Germany, and Taq Polymerase was from Perkin Elmer, Norwalk, USA. Collagen A was supplied from Biochrom, Berlin, Germany. PD123177 was kindly provided by Dr D. Taylor, Parke Davis Pharmaceutical Research, Ann Arbor, USA. Losartan was a kind gift from Dr R. Smith, DuPont Merck Pharmaceutical Company, Wilmington, USA and CGP42112A was obtained from Ciba-Geigy Ltd., Basel, Switzerland. ANG II was purchased from Bachem, Bubendorf, Switzerland. [Methyl, $1',2',3'H$]-thymidine and [$^{33}P$]-dCTP were obtained from Amersham, Braunschweig, Germany. $^{125}I$-SarIleANG II was from NEN, Boston, MA, USA.

2.2. Cell culture

Care of rats used for the isolation of rat coronary endothelial cells (CEC) conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local animal welfare committee. CEC from male Wistar rats were characterized and cultured as described previously [12] and were used exclusively at passage 2. Bovine aortic endothelial cells (BAEC) were isolated according to Schor et al. [19]. The experimental protocol was identical to the one used in a previous study [12], in which we demonstrated that selective stimulation of the $A_2$ receptor inhibits proliferation of CEC.

Briefly, prior to addition of ANG II, CEC were serum-deprived for 48 h and stimulated with 25 ng/ml FGF for 16 h. AT$_1$ receptors were specifically blocked by Losartan ($10^{-5}$ M) and AT$_2$ receptors by PD123177 ($10^{-6}$ M). In addition, selective stimulation of AT$_2$ receptors was achieved by the application of CGP 42112A ($10^{-7}$ M). Losartan and PD123177 were added 30 min prior to the addition of ANG II to allow equilibrium binding to the receptor and activation of Losartan. mRNA was isolated according to the method of Chomcynski and Sacchi [20] 3, 6 and 9 h after stimulation with ANG II in the presence or absence of the receptor antagonists.

2.3. Angiotensin receptor binding studies

To verify the presence of both AT$_1$ and AT$_2$ receptors in rat CEC, angiotensin receptor binding studies were performed prior to the experiments. The binding studies were conducted as described previously [12].

2.4. Reverse transcription–polymerase chain reaction (RT–PCR)

The expression of AT$_1$ and AT$_2$ receptors was also demonstrated by RT–PCR. For this purpose the Superscript™ Preamplification Kit (Gibco BRL) was used for first strand synthesis. Subsequent PCR reactions with AT$_1$ and AT$_2$, receptor-specific primers were carried out under standard conditions. The PCR temperature profile was 94°C (5 min), followed by 30 cycles at 94°C (1 min), 60°C (1 min), 72°C (1 min) and lastly 5 min at 72°C. The following primer pairs were used: (1) rat AT$_1$ a, forward,
5’-GAGTCCTGTCCACCCCGATCAC-3’, reverse 5’-GGATGACGCCCAGCTGAATCAGCACATCC-3’; (2) rat AT2, forward, 5’-CAGCTTGGTGGTGATTGTC-3’, reverse, 5’-GCCATCGGTATTCCATAGC-3’; (3) human AT1 receptor, forward, 5’-TGTAAGATTGCTTC-3’, reverse, 5’-GCCATCGGTATTCCATAGC-3’; and (4) human AT2 receptor, forward: 5’-GCCATCGGTATTCCATAGC-3’. In case of the data shown in Fig. 4, sequences were blotted onto nylon membranes, hybridized to full length cDNA probes of human AT1 and AT2 receptors as described [12].

2.5. Northern blot analysis and cDNA probes

Northern blot analysis of AT1 and AT2 receptors was performed by using the cDNA of the full length coding regions of the human AT1 and rat AT2 receptors. The cDNA probes of rat TSP-1 and rat FN as well as the cDNA probe of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described earlier [21,22]. RNA (20 μg/lane) was separated by electrophoresis on a 1.2% agarose gel containing 2 M formaldehyde and blotted onto a nylon membrane (Amersham, Hybond N). After addition of 1–5×10⁷ cpm of 32P-labeled cDNA the hybridization was carried out using QuickHyb™ hybridization solution (Stratagene) following the instructions of the manufacturer. Subsequent washing and autoradiography were performed according to standard procedures [23]. TSP-1- and FN-signals were quantitated by densitometric scanning, corrected for RNA-loading by means of the respective GAPDH-signal and normalized to the bFGF-response. Statistical analysis was performed by a nonparametric ANOVA (Kruskal–Wallis) and Dunn’s post test, P<0.05 was considered statistically significant.

2.6. DNA-synthesis

The relative rate of DNA-synthesis was determined by measurement of [3H]-thymidine incorporation following the method of Glaser et al. [24] with minor modifications. Briefly, 4 h before the end of the respective experimental protocol 1 μCi/well [methyl, 1’,2’,3’-H]-thymidine was added to the culture medium of cells grown in 96-well plates. Subsequently, cells were rinsed twice with cold phosphate buffered saline (PBS) and fixed twice (3 and 20 min) in PBS containing ethanol (35%) and acetic acid (15%) at 37°C and were covered with 0.3 M perchloric acid for 10 min, followed by a thorough rinse with distilled water. DNA was then solubilized by 100 μl 0.25 N NaOH (20 min at room temperature) and 100 μl of distilled water was added. The relative amount of incorporated [3H]-thymidine was determined by scintillation counting.

3. Results

The experiments were performed in 2nd passage rat CEC and the expression of angiotensin receptor subtypes, AT1 and AT2, were verified by Northern analysis (Fig. 1A) and RT–PCR (Fig. 1B). In addition, binding studies were performed using 125I-SarIleANG II as ligand for AT1 and AT2 receptors. The binding experiments revealed that rat CEC expressed about 20–30% AT2 and 70–80% AT1 receptors (Table 1) which is in accordance with our previous observation on AT1 and AT2 receptor distribution [12].

CEC were rendered quiescent by serum deprivation and induced to proliferate for 16 h by the addition of bFGF (25
Table 1
Characterization of the expression of angiotensin receptor subtypes in rat CEC. Representative data from receptor binding studies in two different isolations of rat CEC at passage 2. Binding studies were performed in serum-deprived (24 h), subconfluent CEC growing in 24-well plates. 125I-SarIleANG II (2.5×10^{-10} M) was used as tracer and ANG II, Losartan, PD 123177 and CGP 42112A as competing ligands.

<table>
<thead>
<tr>
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<th>Bound 125I-SarIleANG II (25 nmol)</th>
<th>Bound 125I-SarIleANG II (25 nmol)</th>
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<tr>
<td>Total binding</td>
<td>4900±651 cpm (100%)</td>
<td>1598±140 cpm (100%)</td>
</tr>
<tr>
<td>ANG II (10^{-7} M)</td>
<td>1515±119 cpm (100%)</td>
<td>540±25 cpm (100%)</td>
</tr>
<tr>
<td>Losartan (10^{-7} M)</td>
<td>2000±54 cpm (100%)</td>
<td>740±50 cpm (100%)</td>
</tr>
<tr>
<td>PD123177 (10^{-7} M)</td>
<td>3993±157 cpm (100%)</td>
<td>1194±106 cpm (100%)</td>
</tr>
<tr>
<td>CGP42112A (10^{-7} M)</td>
<td>3993±109 cpm (100%)</td>
<td>1214±95 cpm (100%)</td>
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Subsequent stimulation with ANG II (10^{-7} M) exerted a marked antiproliferative effect as reported previously [12] (data not shown). As shown in Fig. 2A, the expression of the ECM glycoproteins FN and TSP-1 was modestly increased in cells stimulated by bFGF plus ANG II compared to quiescent controls while stimulation with bFGF (Fig. 2B/C) alone had no effect on TSP or FN mRNA expression. In the presence of the selective AT1 receptor antagonist, Losartan (10^{-5} M), markedly increased TSP-1 expression was observed in bFGF plus ANG II stimulated cells, while FN mRNA levels were not affected by blockade of AT1 receptors. In contrast, blockade of the AT2 receptor by the selective AT2 antagonist,
PD123177 (10⁻⁶ M), had no effect on TSP-1 expression but resulted in a pronounced down regulation of FN mRNA suggesting an AT₁ receptor-mediated down regulation of FN mRNA levels (Fig. 2A, C). These effects were prevented in the presence of both Losartan and PD123177 demonstrating the specificity of ANG II actions on TSP-1 and FN mRNA expression (Fig. 2, last lane). Fig. 2B and C show quantitative data for TSP-1- and FN-mRNA expression 6 h after addition of ANG II in the presence or absence of the receptor antagonists. To further investigate the possibility of an AT₂ receptor mediated upregulation of TSP-1, CEC were stimulated with bFGF plus CGP42112A (10⁻⁷ M), an AT₂ receptor ligand known to exert agonistic properties at the concentration chosen [25]. CGP42112A induced a marked upregulation of TSP-1 mRNA similar to the effects observed in the presence of bFGF, ANG II plus Losartan (Fig. 3A, B), while the expression of FN was not affected by CGP42112A (data not shown).

To extend our findings from microvascular EC to cells from macrovascular origin, we examined whether ANG II has antiproliferative effects in BAEC as well, and whether FN and TSP-1 are differentially regulated by AT₁ and AT₂ receptors in these cells. BAEC were used in the 2nd and 3rd passage, since these cells lose expression of the AT₂ receptor at higher passages. RT–PCR and subsequent Southern blotting confirmed the presence of AT₁ and AT₂ receptor transcripts (Fig. 4).

Addition of ANG II to bFGF-stimulated BAEC resulted in a dose-dependent inhibition of DNA-synthesis (Fig. 5A). Maximal inhibition of DNA-synthesis was observed with ANG II at a concentration of 10⁻⁷ M (Fig. 5A). Pretreatment with the selective AT₂ receptors antagonist, PD123177 (10⁻⁶ M), abrogated the inhibitory effect of ANG II on DNA-synthesis while the selective AT₁ receptors antagonist, Losartan (10⁻⁷ M), had no effect (Fig. 5B).

Both FN and TSP-1 mRNA were abundant and therefore easily detectable in BAEC (Fig. 6) using Northern hybridization. In the presence of the AT₁ receptor antagonist, Losartan, ANG II induced TSP-1 mRNA after 9 h. In BAEC FN mRNA was upregulated after addition of Losartan as well. Furthermore, a marked downregulation of FN mRNA was observed 9 h after addition of the AT₂ receptor antagonist, PD123177. Thus, these data suggest that ANG II can mediate upregulation of TSP-1 and FN via the AT₂ receptor in BAEC, whereas the AT₁ receptor mediates downregulation of FN. The differential regulation of TSP-1 and FN by AT₁ and AT₂ receptors appears to be common for both, microvascular and macrovascular EC and coincides with the antiproliferative effect of AT₂ receptors in these cells.

Fig. 3. TSP-1 mRNA expression 6 h after stimulation of rat CEC with ANG II (10⁻⁸ M) and the AT₂ receptor agonist CGP 42112A (10⁻⁷ M). (A) representative Northern blot, (B) Quantitation of TSP-1 mRNA signals derived from three experiments, 6 h after addition of CGP42112A. To correct for loading, data were expressed as a ratio of TSP-1 and GAPDH signals and normalized to the bFGF-response. TSP-1 mRNA was markedly increased in the presence of CGP 42112A. Data represent mean±S.E.M. (n=3), * P<0.05.

Fig. 4. Characterization of AT₁ and AT₂ receptor expression in BAEC. Southern blotting of RT–PCR reactions using primers specific for the human AT₁ receptor (upper lane) and human AT₂ receptor (bottom lane). Blot was hybridized with ³²P labeled human AT₁ and AT₂ receptor cDNAs. Lane 1: negative control (H₂O). Lane 2: kidney derived mRNA as positive control for AT₁ receptor (upper panel) and pancreas derived mRNA as positive control for AT₂ receptor (lower panel). Lanes 3 and 4: samples derived from BAEC (2nd and 3rd passage, respectively).
Fig. 5. Inhibition of DNA synthesis in proliferating BAEC by ANG II via the AT<sub>2</sub> receptor. BAEC were stimulated by addition of 25 ng/ml bFGF for 16 h prior to addition of ANG II at the indicated concentrations (A) ANG II dose–response relationship, (B) effects of the AT<sub>2</sub> receptor antagonist, Losartan (10<sup>-5</sup> M) and the AT<sub>1</sub> receptor antagonist, PD123177 (10<sup>-6</sup> M). Data were expressed as percentage of the bFGF-stimulated controls. Data represent mean±S.E.M. (n=3).

4. Discussion

Antiproliferative effects of ANG II via the AT<sub>2</sub> receptor have been demonstrated for microvascular endothelial cells. The present study extends the knowledge about the antiproliferative function of the AT<sub>2</sub> receptor to macrovascular EC, which might be of significance for the role of ANG II in endothelial regeneration and atherosclerosis. In addition, the present study shows that ANG II differentially modulates the expression of the pericellular matrix through AT<sub>1</sub> and AT<sub>2</sub> receptors. The pericellular matrix of endothelial cells is composed of a variety of secreted macromolecules including proteoglycans and glycoproteins such as laminin, collagen, TSP-1 and FN. EC express integrin and non-integrin receptors for the components of the pericellular matrix that are coupled to intracellular signaling pathways. These receptors generate intracellular signals that modulate the response to other stimuli, such as growth factors, cytokines and agonists of G-protein coupled receptors [26]. An alternative mechanism by which the pericellular matrix influences EC behavior and phenotype is the interaction with growth factors resulting in sequestration, inactivation or activation [27,28]. It has previously been demonstrated that ANG II can play an important role in ECM remodeling in a variety of experimental systems [29,30]. For example, ANG II regulates via the AT<sub>1</sub> receptor the expression of ECM molecules in SMC and cardiac fibroblasts [31,32]. However, the role of ANG II in the regulation of the pericellular matrix of EC is not yet clear, particularly the potential influence of AT<sub>2</sub> receptors on ECM expression in the context of growth inhibition has not been investigated. In the present study, we characterized ANG II-induced changes in the expression of ECM molecules in microvascular and macrovascular EC under conditions, where ANG II via the AT<sub>2</sub> receptor exerts antiproliferative activity [12]. Stoll et al. (1995) showed that ANG II can inhibit CEC-proliferation via the AT<sub>2</sub> receptor, if the pro-proliferative effects of the AT<sub>1</sub> receptor are blocked by Losartan [12]. Thus, in this setting Losartan can be used to demonstrate the anti-proliferative effects of the AT<sub>2</sub> re-
ceptor that would otherwise be counteracted by the AT$_1$ receptor and therefore be undetectable. In the present study a similar phenomenon was observed with respect to TSP-1 expression, since blocking of AT$_1$ receptors by Losartan induced TSP-1 mRNA expression in both, microvascular and macrovascular EC, whereas ANG II alone had no effect. The conclusion that the AT$_2$ receptor mediates the TSP-1 upregulation was confirmed by the finding that AT$_2$ receptor agonist, CGP-42112A, strongly induced TSP-1 mRNA. This finding appears to be of significance, since TSP-1 has been shown to modulate the behavior and phenotype of EC. In the majority of reports, TSP-1 has been shown to inhibit EC proliferation and angiogenesis [33,34]. The effects of TSP-1 on endothelial cell behavior are thought to be multifactorial involving binding to cell surface receptors for TSP-1 such as CD36 and possibly the α3β1 integrin [35,36], as well as binding to heparan sulfate proteoglycans [37]. In addition, TSP-1 competes with surface binding of bFGF [38] and activates TGFβ1 [39], which by itself is an inhibitor of EC proliferation and migration in vitro [40,41]. Furthermore, it has been demonstrated that the third type 3 repeat of the TSP-1 core protein specifically inhibits bFGF induced mitosis in EC [42]. It is therefore conceivable that the antiproliferative effect of the AT$_2$ receptor in bFGF-stimulated EC is at least partially mediated or supported by upregulation of TSP-1. In line with this hypothesis is the finding that an experimental inhibitor of angiogenesis, SR 25989, also induces increased levels of TSP-1 in human vascular EC [43].

Whereas the upregulation of TSP-1 by the AT$_2$ receptor might be related to the antiangiogenic effects of AT$_2$ receptor, the AT$_1$ receptor-mediated down-regulation of FN mRNA expression in both micro- and macrovascular EC is likely to be unrelated to endothelial proliferation under the experimental conditions used. This assumption is based on the observation, that the mitogenic effect of the AT$_1$ receptor is small in the present model: In quiescent EC stimulation of AT$_1$ receptors increased proliferation only when the AT$_2$ receptor is blocked and does not have any effect on growth or DNA-synthesis in bFGF stimulated CEC [12]. In addition, in BAEC the ANG II effects on growth were solely mediated by the AT$_1$ receptor and not counteracted by the AT$_2$ receptor. It is, however, interesting to speculate that the mechanism of AT$_1$ mediated downregulation of FN in EC plays a role for the proliferative response under different circumstances. FN is a ligand of endothelial integrin receptors, such as α5β1 and αvβ3, and is thought to support the proliferative and migratory phenotype, as well as angiogenesis and survival [17,18,44,45]. On the other hand, it needs to be considered that FN can also inhibit proliferation and migration under certain conditions. For example, aminoterminal fragments of fibronectin stabilize cell shape via effects on the cytoskeleton and inhibit cell cycle progression [46]. In addition, extensive deposition of fibrillar FN matrix [47] results in decreased endothelial proliferation. It is therefore difficult to speculate as to whether the AT$_1$ receptor mediated downregulation of FN supports or counteracts EC proliferation and remains to be elucidated in future studies.

The effect of ANG II receptor subtypes on the expression of TSP-1 and FN did not become visible unless one of the receptor subtypes was pharmacologically blocked by a specific antagonist, suggesting that AT$_1$ and AT$_2$ receptors counteract each other also with respect to matrix specific gene expression. This observation is in accord with other studies involving concomitant activation of both receptor subtypes, where the AT$_2$ receptor was shown to counteract actions mediated by the AT$_1$ receptor irrespective of whether these effects were associated with cell growth, differentiation or cardiovascular regulation [12,14,48,49]. However, it is interesting to note that with respect to matrix-specific gene expression it is the AT$_1$ receptor that down-regulates AT$_2$ induced gene expression, while in all previous studies it was the AT$_2$ receptor inhibiting AT$_1$ actions.

In conclusion, both AT$_1$ receptors and AT$_2$ receptors are capable of altering the expression of two different ECM-molecules that are known to generate growth-modulating signals in EC. This gives rise to the hypothesis that remodeling of the pericellular matrix into a less growth permissive form could support or even mediate the anti-proliferative effects of ANG II via the AT$_2$ receptor in EC. AT$_2$ receptor mediated ANG II effects on ECM remodeling, that would normally be counteracted by the AT$_1$ receptor, are possibly of clinical relevance, since AT$_1$ receptors antagonists are increasingly used to treat hypertension.

References


