Chapter 5

Intracellular angiotensin II and cell growth of vascular smooth muscle cells.

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Chapter V

Summary
1. We recently demonstrated that intracellular application of angiotensin II (AngII\textsubscript{intr}) induces rat aorta contraction independent of plasma membrane AngII receptors. In this study we investigated the effects of AngII\textsubscript{intr} on cell growth in A7r5 smooth muscle cells.

2. DNA-synthesis was increased dose-dependently by liposomes filled with AngII as measured by [$^3$H]thymidine incorporation at high (EC\textsubscript{50}=27±6 pM) and low (EC\textsubscript{50}=14±5 nM) affinity binding sites with increases in E\textsubscript{max} of 58±4 and 37±4 % above quiescent cells, respectively. Cell growth was corroborated by an increase in cell number.

3. Extracellular AngII (10 pM-1\textsuperscript{M}) did not modify [$^3$H]thymidine incorporation.

4. Growth effects of AngII\textsubscript{intr} mediated via high affinity sites were inhibited by liposomes filled with 1 \textmu M of the non-peptidergic antagonists losartan (AT\textsubscript{1}-receptor) or PD123319 (AT\textsubscript{2}-receptor) or with the peptidergic agonist CGP42112A (AT\textsubscript{2}-receptor). E\textsubscript{max} values were decreased to 30±3, 29±4 and 4±2 %, respectively, without changes in EC\textsubscript{50}. The AngII\textsubscript{intr} effect via low affinity sites was only antagonised by CGP42112A (E\textsubscript{max}=11±3 %), while losartan and PD123319 increased E\textsubscript{max} to 69±4 %. Intracellular applications were ineffective in the absence of AngII\textsubscript{intr}.

5. Neither intracellular nor extracellular AngI (1 \textmu M) were effective.

6. The AngII\textsubscript{intr} induced growth response was blocked by selective inhibition of phosphatidyl inositol 3-kinase (PI-3K) by wortmannin (1 \textmu M) and of the mitogen-activated protein kinase (MAPK/ERK) pathway by PD98059 (1 \textmu M) to 61±14 and 4±8 % of control, respectively.

7. These data demonstrate that AngII\textsubscript{intr} induces cell growth through atypical AT-receptors via a PI-3K and MAPK/ERK -sensitive pathway.

Keywords: intracellular angiotensin II, growth, losartan, PD123319, CGP42112A, PI-3 kinase, MAP kinase, A7r5 cells

Abbreviations: AngII\textsubscript{intr}, intracellular angiotensin II; CGP42112A, nicotinic acid-Tyr-(N-benzoylcarbonyl-Arg)-Lys-His-Pro-Ile-OH; DMEM, Dulbecco’s Modified Eagle’s
Medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; losartan, (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2’-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole); MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3-kinase; PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PD123319, (s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylate

Introduction

It has been extensively documented that the renin-angiotensin system is a major factor in the regulation of cardiovascular homeostasis, including blood pressure, mineral balance and tissue remodelling (Weber, 1998). However, the beneficial effects of ACE inhibitors on tissue remodelling appear to be independent, at least in part, of their effects on blood pressure (Linz et al., 1996). In this respect, AngII can be generated either in the kidney and released in the circulation (circulating AngII) subsequently activating different plasma membrane receptors or it can be produced in different tissues to exert its effects at the place of production (local AngII; Danser & Schalekamp, 1996). To date, two different receptors have been cloned; namely the AT₁ and AT₂-subtype receptor (Griendling et al., 1996). These receptors are differently localised and have different functions, among which is modulation of cellular growth. The AT₁-receptor, which is prominent in adult tissues, stimulates cell growth (Matsukada & Ichikawa, 1997). In contrast, the AT₂-receptor, which is mostly abundant in fetal tissues, inhibits cell growth and promotes apoptosis (Xoriuchi et al., 1999). There is growing evidence for intracellular actions of AngII not related to activation of ‘classical’ plasma membrane receptors. We recently reported effects of intracellular AngII (AngII_{intr}) on rat aorta contraction, independent of activation of plasma membrane AngII receptors (Brailoiu et al., 1999). Intracellular AngII was reported to increase cytosolic [Ca²⁺] in vascular smooth muscle cells (Haller et al., 1996; 1999), to inhibit gap conductance in heart muscle (De Mello, 1996) and to affect L-type Ca²⁺ channel in a specific manner (De Mello 1998). Such changes in Ca²⁺ homeostasis are important for cell
growth, therefore we addressed the following questions in this investigation: 1) Is there a role for an AngII\textsubscript{intr} receptor in vascular smooth muscle cell growth, 2) Is the receptor similar to the known subtypes based on its pharmacological profile, and 3) Can we identify part of its signal transduction pathway leading to cell growth.

**Methods**

**Cell culture**

A7r5 vascular smooth muscle cells were grown in 75cm\textsuperscript{2} flasks in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (50 µg ml\textsuperscript{-1}) and streptomycin (50 units ml\textsuperscript{-1}) at 37 °C in a humidified atmosphere (5 % CO\textsubscript{2}). The cells were subcultured at 95% confluency by trypsinization. Cell number was established by counting dispersed cells in a Bürker counter (Schreek, Germany). Experiments were performed in 6 well plates (Costar, 9.6 cm\textsuperscript{2} well\textsuperscript{-1}) at a density of 10\textsuperscript{5} cells well\textsuperscript{-1}, unless stated otherwise.

**Determination of DNA synthesis**

To obtain quiescent cells, the medium was replaced with DMEM containing 0.1% fetal calf serum 24 h after plating. The intracellular additions of AngII by liposomal delivery were performed one day after the switch to 0.1 % FCS containing DMEM and lasted for another 24 h. Extracellular addition of various compounds happened 1 min prior to liposomal addition. \[^{3}\text{H}]\text{thymidine}\ (0.5 \mu\text{Ci}, \text{specific activity:} 20 \text{Ci mmol}^{-1}) \text{ was added on each well during the final } 3 \text{ h of incubation. The medium was withdrawn at the end of incubation period and the cells were washed twice with ice-cold phosphate buffered saline (PBS). To remove non-genomic } \[^{3}\text{H}]\text{thymidine, the cells were incubated in the presence of } 400 \mu\text{l trichloroacetic acid for } 1 \text{ h on ice. Finally, the cells were digested with } 1 \text{ ml of } 1 \text{ M NaOH and the incorporated radioactivity was measured in a } \beta\text{-scintillation counter. Quiescent cells incorporated } 210 \pm 11 \text{ dpm well}^{-1} \text{ (mean } \pm
Intracellular AngII stimulates cell growth

s.e.mean, n=24), whereas 10% FCS stimulated cells incorporated 1078 ± 71 dpm well⁻¹ (n=24).

**Liposomes preparation**

Liposomes containing AngII or AngI and control liposomes containing 140 mM KCl were prepared as described (Brailoiu et al., 1999) from egg phosphatidylcholine, using 10 mg ml⁻¹ of solution to be incorporated. Dialysis against PBS solution was performed for 4 h in order to remove the non-incorporated compounds. To maintain sterile cell culture conditions the liposomes solution was filtrated (0.2 μm pore seize). Liposomes were added to the medium above the cells in a ratio of 1 to 20 (v v⁻¹). If other compounds were delivered intracellularly, they were encapsulated together with AngII. The amount of AngII delivered intracellularly was determined using ¹²⁵I-angiotensin II filled liposomes. The incorporation into liposomes after the filtration step was 7.2 ± 0.2 % (n=8) of the initial amount of radioactive AngII added to the cells. Recovery of incorporated ¹²⁵I angiotensin II into the cells after incubation for 30 min amounted to 5.6 ± 0.2 % (n=8) of the initial amount of radioactive AngII added to the cells.

**Measurement of inositol (1,4,5) trisphosphate (Ins(1,4,5)P₃)**

Mass measurements of Ins(1,4,5)P₃ were performed as described earlier (Sipma et al, 1995), using an isotope dilution ligand binding assay. In brief, samples were assayed in 25 mM Tris/HCl (pH=9.0), 1 mM EDTA, 1 mg bovine serum albumin, [1⁻³H(N)]- Ins(1,4,5)P₃ (21.0 Ci mmol⁻¹, 2000 c.p.m. assay⁻¹) and 1 mg binding protein isolated from beef liver. Bound and free radioactivity was separated by centrifugation. The radioactivity in the pellet was determined by scintillation counting.
Measurement of intracellular Ca\(^{2+}\)

Intracellular [Ca\(^{2+}\)] was measured using Fura-2 fluorometry as described (Filipeanu et al., 1997). Cells were loaded with 5 \(\mu\)M Fura-2 acetoxyethyl ester at 22 °C, for 45 min in the dark. Fluorescence was measured at 37 °C.

Chemicals

All cell culture media were purchased from Gibco BRL, phosphatidyl choline type X-E, AngI, AngII, and wortmannin from Sigma Chemical Co, CGP 422112A (nicotinic acid-Tyr-(N-benzoylcarbonyl-Arg)-Lys-His-Pro-Ile-OH) from RBI, and PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) from Calbiochem. Fura 2-AM was obtained from Molecular Probes, losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2’ - (1H - tetrazol - 5-yl) biphenyl-4-yl )methyl]imidazole) from Merck, Sharpe and Dohme, PD123319 ((s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylate) from Park-Davis, [6-\(^3\)H]-thymidine from Amersham Int, [1-\(^3\)H(N)]-Ins(1,4,5)P\(_3\) from NEN Life Science Products, and all other agents from Merck.

Data analysis

Data are given as mean ± s.e.mean. The results of the growth experiments are expressed as percentage of the radioactivity incorporated by control quiescent cells. Independent measurements were performed in at least 2 different passages. Measurements were normalised against liposomes filled with 10\(^{-7}\) M AngII, present in every experimental protocol. Statistical significance was tested by one-way ANOVA followed by Bonferroni test. A value of \(P<0.05\) was considered statistically significant. Concentration response curves were fitted and the corresponding parameters calculated using Multifit (Dr. J. H. Proost, Dept. of Pharmacokinetics and Drug Delivery, University Centre for Pharmacy, University of Groningen). Curve fitting was based on the following sigmoidal model:

\[
Y = V_1 + V_2 x X^V_3 / (X^V_3 + V_4^V_3) + V_5 x X^V_6 / (X^V_6 + V_7^V_6).
\]

Fitting for a single binding site was performed after omission of the term.
containing the parameters V5, V6 and V7. To determine if the data were fitted significantly better with 1 or 2 binding sites the variance was calculated using the F-test.

**Results**

**Intracellular AngII stimulates cell growth in quiescent A7r5 cells**

Addition of AngII filled liposomes increases DNA synthesis in a dose-dependent fashion, as measured by [³H]thymidine incorporation into A7r5 cells (Fig 1). The first observation above the background was obtained with liposomes containing 10 pM AngII, whereas the maximum effect was reached with liposomes containing 0.1 μM AngII (doubling of [³H]thymidine incorporation compared to quiescent cells).

![Figure 1](image-url)  
*Figure 1.* Effects of AngII filled liposomes on A7r5 cell growth. Effect curves of AngII filled liposomes alone (n=12) and in the presence of liposomes encapsulating losartan, PD123319 or CGP42112A (all 1 μM, n=12). All results were reported as increases (%) above [³H]thymidine incorporation in quiescent cells. The maximal value (100 % at LAngII = 0.1 μM) corresponds to 448 ± 56 dpm well⁻¹, n=24). Lines were fitted according to the 2 binding site model given in Table 1.
Data analysis showed that the increases in $[^3]$Hthymidine incorporation were better described using a model with 2 binding site kinetics. Parameters of the dose-response curve are given in Table 1.

Table 1. Dose-response parameters of AngII$_{inr}$ induced $[^3]$Hthymidine incorporation

<table>
<thead>
<tr>
<th>Binding sites</th>
<th>Control (L$_{AngII}$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ ($V_2$)</td>
<td>$93.5 \pm 2.5$ (%)</td>
<td>58.3 $\pm 3.7$ (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hill-coefficient ($V_3$)</td>
<td>$0.46 \pm 0.04$</td>
<td>1.02 $\pm 0.16$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$E_{\text{max}}$ ($V_4$)</td>
<td>$2.2 \pm 0.5 \times 10^{-10}$ (M)</td>
<td>$2.7 \pm 0.6 \times 10^{-11}$ (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hill-coefficient ($V_6$)</td>
<td>-</td>
<td>36.9 $\pm 3.8$ (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$E_{\text{max}}$ ($V_7$)</td>
<td>-</td>
<td>1 (fixed)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

Dose-effect curves of liposomes filled with AngII (L$_{AngII}$) in the range of 1 pM to 1 µM were fitted using the equation: $Y = V_1 + V_2 x X^{V_3} / (X^{V_3} + V_4^{V_3}) + V_5 x X^{V_6} / (X^{V_6} + V_7^{V_6})$. The last term was omitted for the single site fit. Effects are expressed as increases (%) above $[^3]$Hthymidine incorporation in quiescent cells ($V_1=0$) and presented as mean $\pm$ s.e.mean, n=12 for each concentration. Significance level: * F-value = 23.8, $P < 0.0001$ vs single site model.

The simplest model with $V_6=1$ was used for further analysis since varying the Hill-coefficient ($V_6$) from 1 to 9 for the second binding site did not significantly alter the fitting results. Neither ‘empty’ control liposomes, filled only with 140 mM KCl, nor liposomes filled with the parent peptide AngI (1 µM) affected $[^3]$Hthymidine incorporation (102.7 $\pm$ 3.8 %, n=36 and 102.6 $\pm$ 0.8 %, n=12 of control cells, respectively).

The growth stimulating effect of liposomes containing 0.1 µM AngII was corroborated in experiments showing actual increases in cell number (Table 2). The growth stimulating effect of AngII filled liposomes (0.1 µM AngII) was unchanged by extracellular addition (1 µM) of the nonpeptidergic AT$_1$-type receptor antagonist losartan, the nonpeptidergic AT$_2$-type receptor antagonist PD123319, or the peptidergic AT$_2$-type receptor agonist CGP42112A (97.1 $\pm$ 4.0 %, 103.9 $\pm$ 2.8 %, and 91.7 $\pm$ 5.6 % of control cells, n=18, respectively).
Table 2. Effect of intracellular applied AngII on cell number

<table>
<thead>
<tr>
<th></th>
<th>Cell number flask⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$81 \pm 11 \times 10^3$</td>
</tr>
<tr>
<td>L_control</td>
<td>$75 \pm 6 \times 10^3$</td>
</tr>
<tr>
<td>L_AngII</td>
<td>$114 \pm 12 \times 10^3$ *</td>
</tr>
</tbody>
</table>

A7r5 cells were plated in 25 cm² flasks at a density of $2.10^3$ cells cm⁻². After 24 hr the medium containing 10 % FCS was replaced for 24 hr with medium containing 0.1 % FCS. Then either intracellular AngII (L_AngII) using liposomes filled with AngII (0.1 µM) or control liposomes (L_control) containing 140 mM KCl were applied. The cell number was counted 24 hr after application of the liposomes (mean ± s.e.mean, n=4, determination in triplicate). Significance level: * $P<0.05$ vs. L_control.

In contrast to intracellular delivered AngII, extracellular application of AngII (10 pM to 10 µM) did not change $[^3]$H]thymidine incorporation in quiescent A7r5 cells. A similar amount of radioactivity as in control cells was incorporated after 0.1 µM extracellular AngII compared to control cells (98.1 ± 3.2 %, n=12). A7r5 cells apparently lack functional AT₁-receptors which is also evident from the inability of extracellular AngII (10 µM) to change basal Ins(1,4,5)P₃ formation (2.3 ± 0.6 vs. 2.1 ± 0.8 pmol 10⁵ cells⁻¹, n=12) or basal intracellular Ca²⁺ concentration (57 ± 6 vs. 58 ± 7 nM, n=12), as reported before (Filipeanu et al., 1998a,b). Furthermore, extracellular application of AngI (1 µM, 102 ± 2.4 %, n=12), losartan (1 µM, 95.6 ± 4.7 %, n=24), PD123319 (1 µM, 98.7 ± 5.8 %, n=16) or CGP42112A (1 µM, 92.9 ± 4.8 %, n=18) did not affect $[^3]$H]thymidine incorporation.

**Pharmacology of intracellular AngII**

We next attempted to characterise pharmacologically the effects of AngII_intr. Addition of liposomes filled with losartan (1 µM), PD123319 (1 µM) together with various concentrations of AngII reduced $[^3]$H]thymidine incorporation (Fig 1). Abolition of the growth stimulating effect of AngII was obtained by liposomes filled with the AT₂-type receptor agonist CGP42112A (1 µM, Fig 1).
All 3 agents substantially reduced stimulation of the high affinity binding site. Non-competitive inhibition is this receptor site is likely involved in view of the decreasing $E_{\text{max}}$ value without changes in $E_{50}$. The following rank order of antagonist potencies was obtained: CGP42112A$>$PD123319$=$losartan (Fig 2, Table 3).

In contrast, at the low affinity binding site these compounds elicited opposite effects. Again strong inhibition was observed for CGP42112A, but losartan and PD123319 significantly increased $E_{\text{max}}$ as compared to control. It is noticeable that maximal values obtained at this site are comparable to the control value at the high affinity binding site (Fig 2, Table 3). To verify the nature of the antagonism by the compounds studied additional experiments were performed at

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**Figure 2.** Contribution of the different binding sites to growth induced by intracellular AngII in the absence or presence of antagonists. Dose-response curves were plotted using the data of Fig 1 according to the model as given in Table 1. AngII filled liposomes were in the absence (control) or in the presence of liposomes encapsulating losartan (los), PD123319 (PD) or CGP42112A (CGP). Values obtained for $E_{\text{max}}$ and $E_{50}$ are presented in Tabel 3.
other concentrations of the antagonists across a limited range of AngII concentrations (Table 3). Although one should be cautious not to over interpret the fitting results of the limited data, it is clear that no evidence of a parallel shift of the log dose-response relationship was observed for both binding sites.

Table 3. Effect of L\textsubscript{AngII} on [\textsuperscript{3}H]thymidine incorporation in the presence of various agents on dose-response parameters

<table>
<thead>
<tr>
<th>L\textsubscript{AngII}</th>
<th>E\textsubscript{max} (h) (%)</th>
<th>E\textsubscript{max} (l) (%)</th>
<th>EC\textsubscript{50} (h) (M)</th>
<th>EC\textsubscript{50} (l) (M)</th>
<th>Data (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{los7.5}</td>
<td>68 ± 8</td>
<td>42 ± 10</td>
<td>3 ± 2 \textsuperscript{-11}</td>
<td>2 ± 2 \textsuperscript{-8}</td>
<td>24</td>
</tr>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{los6}</td>
<td>30 ± 3 *</td>
<td>69 ± 4 *</td>
<td>3 ± 1 \textsuperscript{-11}</td>
<td>1.5 ± 0.3 \textsuperscript{-8}</td>
<td>84</td>
</tr>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{PD6}</td>
<td>29 ± 4 *</td>
<td>69 ± 4 *</td>
<td>2 ± 1 \textsuperscript{-11}</td>
<td>1.0 ± 0.2 \textsuperscript{-8}</td>
<td>84</td>
</tr>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{PD4.5}</td>
<td>40 ± 8 *</td>
<td>61 ± 10 *</td>
<td>5 ± 4 \textsuperscript{-11}</td>
<td>2 ± 1 \textsuperscript{-8}</td>
<td>24</td>
</tr>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{los6} + L\textsubscript{PD6}</td>
<td>17 ± 2 *</td>
<td>65 ± 3 *</td>
<td>1.5 ± 0.9 \textsuperscript{-11}</td>
<td>3.2 ± 0.7 \textsuperscript{-8}</td>
<td>84</td>
</tr>
<tr>
<td>L\textsubscript{AngII} + L\textsubscript{CGP6}</td>
<td>4 ± 2 *</td>
<td>11 ± 3 *</td>
<td>0.5 ± 1.2 \textsuperscript{-11} *</td>
<td>0.1 ± 0.1 \textsuperscript{-8}</td>
<td>84</td>
</tr>
</tbody>
</table>

Liposomes were filled with AngII (L\textsubscript{AngII}) in the range of 1 pM to 1 \mu M and added in the presence of losartan (L\textsubscript{los}), PD123319 (L\textsubscript{PD}) or CGP42112A (L\textsubscript{CGP}) at concentrations as indicated by its anti-log. Effects are expressed as increases (%) above [\textsuperscript{3}H]thymidine incorporation in quiescent cells. Parameters were obtained after fitting the 2 binding site model as given in Table 1, with the constraints V\textsubscript{1}=0, V\textsubscript{3}=V\textsubscript{6}=1 and presented for the high (h)- and low (l) affinity sites as mean ± s.e.mean. Datapoints were obtained from either 7 or 4 different concentrations with n=12 or 6 each. Significance level: * P < 0.05 vs L\textsubscript{AngII} (Table 1).

The lowest antagonist concentration used (30 nM losartan) was insufficient to induce inhibition. At high antagonist concentrations (e.g. 30 \mu M PD123319) further increases were not observed in the E\textsubscript{max} of the low affinity binding site. This was also concluded from the experiment in which losartan and PD123319 (both 1 \mu M) were given simultaneously, and from an experiment using losartan (10 \mu M) or PD123319 (10 \mu M) at a single dose of AngII (100 nM), showing maximal [\textsuperscript{3}H]thymidine incorporation was maintained (98.2 ± 4.1 %, n=6 and 109.0 ± 6.5 %, n=6 for losartan- and PD123319 filled liposomes, respectively).
In the absence of AngII filled liposomes, losartan-, PD123319-, and CGP4112A-filled liposomes did not modify basal $[^{3}H]$thymidine incorporation into quiescent cells ($n=12$; $99.1 \pm 2.9 \%$, $101.5 \pm 2.4 \%$, $98.3 \pm 5.5 \%$ of control, respectively).

**Signal transduction of intracellular AngII effects**

Extracellular signals often use multiple pathways to modify cell growth. In order to gain insight in the mechanism involved in growth stimulation by AngII$_{\text{intr}}$ two of these pathways were tested. Intracellular AngII induced cell growth was totally abolished by inhibition of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway by co-incubation of the cells with extracellular PD98059 (1 μM, Fig 3). Growth stimulation was also reduced, but to a lesser extent by inhibition of the phosphatidyl inositol 3-kinase (PI-3K) pathway with wortmannin (1 μM, Fig 3).

![Figure 3](image-url)  
*Figure 3.* Involvement of MAPK and PI-3K in AngII induced cell growth. Cell growth induced by AngII filled liposomes (L$_{\text{AngII}}$, 0.1 μM) was inhibited by simultaneous extracellular treatment with PD98059 (1 μM, $n=16$) or wortmannin (1 μM, $n=16$).
Discussion

In the present study, we demonstrate that AngII\textsubscript{intr} stimulates cell growth in quiescent A7r5 cells. Although ‘classical’ plasma membrane AngII receptors are involved in growth and apoptotic processes underlying cardiovascular remodelling (Dzau & Horiuchi, 1998) our results suggest additional targets for AngII at sites which have not previously been recognised. The growth response was characterised by the presence of two distinct binding sites for AngII\textsubscript{intr}. The high affinity site (picomolar range) was sensitive to intracellular delivered antagonists in the rank order of potencies, CGP42112A > PD123319 = losartan. In contrast, only the peptidergic antagonist CGP42112A could inhibit the low affinity site (nanomolar range). The other compounds even increased the maximal effect to the value obtained by stimulation of the high affinity site, indicating that both sites are possibly closely involved in the growth response. Also important is the observation that all three compounds are ineffective in the absence of AngII\textsubscript{intr}, showing that occupation of the receptor site by AngII is needed for their action. Although the nature and physiological significance of these distinct sites needs further investigation, it is unlikely that ‘classical’ AT\textsubscript{1}- or AT\textsubscript{2}-receptors (De Gasparo \textit{et al.}, 1998) mediate the AngII\textsubscript{intr} effect on \[^{3}H\]thymidine incorporation in view of the different potencies and the lack of competitive inhibition observed, and the growth inhibitory action of both CGP42112A and PD123319. These compounds were described as agonist and antagonist of the anti-proliferative AT\textsubscript{2}-receptor subtype, respectively (Timmermans \textit{et al.}, 1992; De Gasparo \textit{et al.}, 1998). Antagonist activity of CGP42112A on AT\textsubscript{1}- or AT\textsubscript{2}-receptors has only been reported for extracellular AngII mediated phospholipase A2 activation (Lokuta \textit{et al.}, 1994). The presence of an unusual type of receptor in A7r5 cells becomes also apparent from the lack of the growth response by extracellular addition of AngII and the ineffectiveness of extracellular addition of AT\textsubscript{1}-or AT\textsubscript{2}-receptor antagonists. No functional plasma membrane AT-receptors seems to be present in A7r5 cells, also in view of the absence of other cellular responses to extracellular AngII like Ins(1,4,5)P\textsubscript{3} formation and cytosolic $[\text{Ca}^{2+}]$ elevation. Therefore, the growth response elicited
by AngII_{intr} is likely mediated by atypical AT-receptors with distinct pharmacology from AT_1- or AT_2-receptors.

Liposomes filled with the parent peptide AngI did not affect DNA synthesis. In contractility studies of adult rat aorta, we observed that both AngI and AngII filled liposomes induced contraction (Brailoiu et al., 1999). Either the contractile and growth responses are not intimately related or different pharmacological profiles of AngII_{intr}-receptors are present in different cell types. Differences in pharmacological receptor profiles among different cell types is supported by recent observations that AngII_{intr} inhibited inward Ca^{2+} current in rat cardiac myocytes, but stimulated this current in hamster cardiac myocytes (De Mello, 1998).

The presence of various atypical angiotensin receptors was reported previously (Noble et al., 1993, 1996; Smith, 1995; Regitz-Zagrosek et al., 1996; Li et al., 1998; Moriuchi et al., 1998). The pharmacological profile of one of those atypical angiotensin receptors resembles the profile obtained in A7r5 cells. Although observed in another species, this receptor mediates microvascular network formation in chick embryo, has a low affinity for losartan and PD123319 and is antagonised by CGP42112A (Noble et al., 1993, 1996). Further studies are necessary to elucidate if the receptors activated by AngII_{intr}, as observed by us, are related to one of those atypical receptors, and to establish their existence and binding profiles in other tissues.

Extracellular AngII induces several effects commonly evoked by growth factor receptor stimulation, such as tyrosine phosphorylation or activation of the Ras/ERK pathway ultimately leading to protein synthesis and cell cycle progression (Berk, 1999; Eguchi et al., 1999; Inagami et al., 1999). Stimulation of plasma membrane AT_1-receptors activate the MAPK cascade in vascular smooth muscle cells other than A7r5 cells (Ge & Anand-Srivastava, 1998; Li et al., 1998; Moriuchi et al., 1998) and this pathway is inhibited by PD98059 (Servant et al., 1996; Ushio-Fukai et al., 1998). Our experiments showed that inhibition of this pathway by PD98059 effectively blocked the growth response to AngII_{intr} administration. Activation of the MAPK cascade can be achieved
Intracellular AngII stimulates cell growth via the PI-3K pathway, but a redundant pathway stimulates MAPK when large numbers of receptors are activated (Duckworth & Cantley, 1997). Interestingly, wortmannin only partially inhibited our AngII\textsubscript{intr} effect, a finding also reported for extracellular AngII induced growth (Berk, 1999) and for other stimuli or cell types activated (Balla \textit{et al}. 1998; Gutkind, 1998). This indicates that a strong signal is evoked by the AngII\textsubscript{intr} mediated stimulation, comparable to activation of large number of ‘classical’ plasma membrane receptors.

The obvious physiological candidate to stimulate the AngII\textsubscript{intr}-receptor is AngII. Intracellular trafficking of AngII might be important for directing AngII to certain cellular locations to fully express its biological response. Several studies have demonstrated that AngII is internalised into the cells via an AT\textsubscript{1}-but not AT\textsubscript{2} -mediated process (Anderson \textit{et al}.., 1993; Hein \textit{et al}.., 1997). Intracellular pools of AngII were noticed in cardiomyocytes (Sadoshima \textit{et al}.., 1993) and recently angiotensin peptides, ACE-activity and AT\textsubscript{1}-receptors were detected in a renal endosomal fraction (Imig \textit{et al}.., 1999). The functional targets for AngII\textsubscript{intr} are still unclear, but nuclear binding-proteins were reported for AngII (Booz \textit{et al}.., 1992; Tang \textit{et al}.., 1992; Jimenez \textit{et al}.., 1994).

Interaction of AngII\textsubscript{intr} with proteins at the cytosolic side of the plasma membrane also occurs in view of the results of AngII\textsubscript{intr} on Ca\textsuperscript{2+} channels and gap junctions (De Mello 1996, 1998; Haller \textit{et al}., 1996, 1999). This is possibly only a secondary related phenomenon, since the MAP-kinase pathway shown to be activated by AngII\textsubscript{intr} in the present paper, modulates the opening of L-type Ca\textsuperscript{2+} channels in cardiomyocytes (Murata \textit{et al}.., 1999).

In conclusion, these data demonstrate that intracellular delivered AngII induces cell growth in A7r5 cells. Atypical AT-receptors are involved in view of the ineffectiveness of extracellular addition and the rank order of antagonist potencies obtained by intracellular application. The AngII\textsubscript{intr} induced growth response is mediated \textit{via} a PI-3K and MAPK/ERK -sensitive pathway. AngII\textsubscript{intr} actions, inaccessible for common treatment, might open new views in understanding and treatment of cardio-vascular related diseases.
Acknowledgements
Catalin M. Filipeanu is a recipient of an Ubbo Emmius fellowship from Groningen University Institute of Drug Exploration (GUIDE). Disposition of the Multifit software by Hans Proost and additional help with the fitting procedures is greatly appreciated.

References


Intracellular AngII stimulates cell growth


Chapter V


