Angiotensin II type 2 receptor vasoactivity in internal mammary arteries of patients with coronary artery disease

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Abstract

Objectives Several animal studies suggested that the angiotensin II type 2 (AT2) receptor subtype mediates vasodilation, yet in human arteries the results are less well-described and inconsistent. Therefore, we evaluated the role of the AT2 receptor stimulation on the vasotonus of human internal mammary arteries.

Methods and results Human internal mammary arteries were obtained from 50 patients undergoing coronary bypass surgery. The expression of angiotensin II type 1 receptor and AT2 receptor mRNA was determined by using real time polymerase chain reaction. In addition, angiotensin II and CGP42112A concentration-response curves (concentration range: 10-10 M to 10-6 M) were constructed in absence or presence of candesartan (10-5 M) and/or the AT2 receptor antagonist PD-123319 (10-6 M) and/or the a receptor antagonist phentolamine.

Both AT1 and AT2 receptor protein and mRNA were present in human internal mammary arteries, and higher AT2 receptor mRNA expression levels were associated with increased contractile response to angiotensin II. Angiotensin II caused vasoconstriction up to 41.1 ± 2.0% of the maximal response to phenylephrine, and PD123319 significantly reduced this response (28.6 ± 1.0%, p<0.001). Candesartan completely blocked the angiotensin II mediated response (1.4 ± 1.2%, p<0.001 versus control), and additional blockade of the AT2 receptor with PD123319 did not change this effect (1.8 ± 1.3%). Phentolamine (10-5 M) caused an attenuation and a rightward shift of the angiotensin II concentration response curves. The AT2 receptor agonist CGP42112A did not induce a significant response.

Conclusion Although AT2 receptor mRNA is present in human internal mammary arteries, AT2 receptor stimulation does not mediate vasodilation in these arteries.
Introduction

As the key effector peptide of the renin angiotensin system (RAS), angiotensin II exerts a potent role in the control of cardiovascular homeostasis. Over the last 15 years several receptors for angiotensin II have been identified of which the angiotensin II type 1 (AT1) receptor and the angiotensin II type 2 (AT2) receptor are the two most important subtypes. The classic hormonal actions on blood pressure and fluid homeostasis are being attributed to stimulation of the AT1 receptor. Moreover, through this receptor angiotensin II plays a part in inflammation and in cell proliferation. In contrast to the AT1 receptor, the effect of stimulation of the AT2 receptor remains less well-defined. In general, it is presumed that the AT2 receptor has effects opposing the AT1 receptor. AT2 receptors are abundantly expressed in foetal tissues and they predominantly disappear in most tissues after birth, which suggests a role in growth and development. In addition, the antiproliferative and apoptotic effects of the AT2 receptor have been well-established. Several in vitro and animal studies suggested that stimulation of the AT2 receptor mediates signalling pathways associated with vasodilation. Furthermore, a recent study in spontaneously hypertensive rats demonstrated that reduction of high blood pressure reversed AT2 receptor mediated vasoconstriction into vasodilation. Extrapolation of these data to the human species is highly questionable, and for that reason unwarranted. Data concerning the vasoactivity of AT2 receptor stimulation in humans are scarce and also inconsistent. Therefore, the aim of the present study was to examine the effect of AT2 receptor stimulation on the vascular tonus of the human internal mammary artery.

Methods

Human tissue collection
Segments of human internal mammary arteries (IMA) were obtained from 50 patients who underwent coronary bypass surgery (CABG) in St Antonius Hospital in Nieuwegein, or in the University Medical Center Groningen, the Netherlands. After removal, the tissue was stored in cold (4 °C) RPMI 1640 medium (GIPCO, Paisley, UK) and prepared for organ bath studies. In addition, IMA segments from 14 of the patients were immediately stored at -80 °C for mRNA determination. Patient receiving therapy with ACE-inhibitors or AT1 receptor antagonists at the time of surgery were excluded. The segments were processed within 24 hours after removal. Studies with excess human arteries are approved by the Ethics Review Committee, and conform with the principles outlined in the Declaration of Helsinki.
**Chapter 5**

**AT1 and AT2 receptor mRNA isolation and real-time polymerase chain reaction**

For assessment of vascular gene expression, IMA samples were cleaned of surrounding tissue, quickly frozen in liquid nitrogen, and homogenized with a motorized homogenizer. RNA from the homogenates was isolated with RNA-clean according to the manufacturer’s protocol, and 1 µg aliquots were electrophoresed through 1.2% agarose/0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. 1 µg of the isolated total RNA was reverse transcribed using random primers and MMLV reverse transcriptase for 60 min at 42 °C and 10 min at 75 °C. The single stranded cDNA was amplified by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the TaqMan system (ABI-Prism 7700 Sequence Detection System, Applied Biosystems, Weiterstadt, Germany) using SYBR Green dye. For human AT1 receptors, the primers were 5’-CTG-GAG-GGC-ATA-ATT-ACA-TAT-TTG-TCA-3’ and 5’-GCC-ACA-GTC- TTC-ACG-TTC-ATA-TAA-AA-3’, for human AT2 receptors, the primers were 5’-TCC-CCT-TGT-TTG-GTG-TGT-GGC-C-3’ and 5’-CAC-TGC-GGA-GCT-TCT-GTT-GGA-A-3’, for GAPDH, the primers were 5’-ACC-ACA-GTC-CAT-GCC-ATC-3’ and 5’-TTC-ACC-ACC-CTG-CTG-TG-TA-3’.

For quantification, AT1 receptor and AT2 receptor mRNA expression were normalized to the expressed housekeeping gene GAPDH.

**Quantification of protein expression**

Frozen tissue samples were homogenized in lysis buffer and Western blot analysis was performed as described by Adams et al.\(^{16}\) To detect specific proteins the following antibodies were applied: AT1 receptor and AT2 receptor (both Santa Cruz Biotechnology, Heidelberg, Germany). This antibody was diluted 1:500 in Tris buffered salt solution containing Tween 20 (TTBS). The antibody was incubated overnight at 4 °C. The second antibody used was a goat anti-rabbit coupled to peroxidase (Dako, Hamburg, Germany), which was diluted 1:10.000. After an incubation time of 2 hours at room temperature, this peroxidase-coupled secondary antibody was detected by chemiluminesence. The amount of protein loaded on the gel (70 µl in each lane) was checked in two ways. First, equal loading was evaluated by Ponceau red (Sigma-Aldrich Chemie Gmbh, Munich, Germany) staining. Second, to control for loading differences the blots were reprobed with an antibody against GAPDH (Hytest, Turku, Finland).

**Organ chamber studies**

The vessels were dissected free, cleaned of surrounding tissues, and cut into several rings (± 2mm), while care was taken not to damage the endothelium.\(^{17}\) Rings were mounted in 15 ml organ baths, containing a buffer solution of the following (Krebs) composition (mM): NaCl (120.4), KCl (5.9), CaCl2(2.5), NaHCO3 (25.0), MgSO4 (1.2), and glucose (11.1).
MgCl₂(1.2), NaH₂PO₄(1.2), Glucose (11.5) and NaHCO₃ (25.0). The medium was continuously aerated with 95% O₂-5% CO₂ and kept at 37°C. The rings were connected to an isotonic displacement transducer by 5-0 braided, uncoated polyester suture. We performed isotonic measurements of vascular contraction; that is, vessel rings were subjected to a constant tension of 1.4 g and changes in vessel diameter were registered in μm. Both the isotonic transducer, the recording system, and the software were custom made and calibrated by the University Medical Center Groningen, the Netherlands.

In every single experiment, we studied several arterial rings from one donor in a parallel fashion. Two of the rings were always used to obtain control responses (incubated with vehicle) in each experiment. The artery rings were allowed to equilibrate for at least 60 minutes, during which regular washing periods were performed. Rings were primed and checked for viability by repeated stimulation (2-3 times) with 10 μM phenylephrine followed by in-between washing and stabilization periods. Rings that failed to reach a contractile response of at least 100 μm to phenylephrine were not included in the experiment. In experiments 2A, 2B and 4, results were excluded if control rings did not reach a contraction response to angiotensin II of 15% of the final response to 10 μM phenylephrine. In experiment 3, rings were stimulated with a single high concentration of 10 mM sodium nitrite at the end of the experiment. In these two experiments, vessel segments were excluded from analyses if the contractile response of control rings to 10 μM phenylephrine minus the dilatory response to 10 mM sodium nitrite was less than 100 μm. In order to exclude procedure-related endothelial damage, data of 187 consecutive CABG patients were analysed. In these vessel segments both endothelial dependent and endothelial independent were tested by constructing an acetylcholine (10⁻⁸-10⁻⁴ M) concentration-response curve and by a single bolus injection of sodium nitrite (10mM), respectively. Vessel segments were excluded from further analysis if the contractile response to 10 μM phenylephrine was less than 100 μm.

**Experiment 1**

In 14 IMA segments AT1 receptor mRNA and AT2 receptor mRNA expression was analysed. In addition, the contractile response to increasing concentrations (0.1 nM to 1 μM) of angiotensin II of segments from the same patients was determined.

**Experiment 2A**

In the second experiment, rings were preincubated (30 minutes) with either vehicle, an AT1 receptor antagonist (candesartan, 10 μM), an angiotensin II-AT2 receptor antagonist (PD123319, 1 μM), or a combination of both AT receptor antagonists. They were then stimulated with increasing concentrations (0.1 nM to 1 μM) of angiotensin II.


Experiment 2B
Rings were preincubated (30 minutes) with either vehicle, an AT1 receptor antagonist (candesartan, 10 µM), an AT2 receptor antagonist (PD123319, 1 µM), or a combination of both AT receptor antagonists. Furthermore, for each condition all the rings were additionally incubated with 10 µM of the α-receptor blocking agent phentolamine. Subsequently, rings were stimulated with increasing concentrations (0.1 nM to 1 µM) of angiotensin II.

Experiment 3
In the third experiment, after preincubation (30 minutes) with either vehicle, 10 µM candesartan, 1 µM PD123319, or a combination of both AT receptor antagonists, all rings were precontracted by administrating 10 µM phenylephrine. Then they were stimulated with increasing concentrations (0.1 nM to 1 µM) of angiotensin II.

Experiment 4
In the final experiment rings were preincubated (30 minutes) with either vehicle, an angiotensin II-AT1 receptor antagonist (candesartan, 10 µM), an AT2 receptor antagonist (PD123319, 1 µM), or a combination of both AT receptor antagonists. Then they were stimulated with increasing concentrations (0.1 nM to 1 µM) of the AT2 receptor agonist CGP42112A.

Drugs and Reagents
Candesartan was a gift from Astra Pharmaceutica BV (Zoetermeer, The Netherlands). PD123319 (1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid difluoroacetate), phenylephrine, sodium nitrite, phentolamine, and CGP42112A (nicotinic acid-Tyr-N-benzoxy-carbonyl-Arg-Lys-Pro-Ile-OH) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Angiotensin II was obtained from CIBA-Geigy ltd. (Basle, Switzerland). The drugs were dissolved in saline and freshly prepared each day from stock solutions.

Data Analyses
All data are expressed as means ± SEM. To avoid non-specific differences between subjects, we evaluated the effect of the inhibitors by comparing concentration-response curves obtained with parallel rings from the same patient. To control for non-specific differences between rings from the same patient, contractile responses of individual rings to angiotensin II are expressed as a percentage of the contractile response to 10 µM phenylephrine, whereas relaxant responses are expressed as a percentage of the difference between the response to 10 µM phenylephrine and 10 mM sodium nitrite. Comparisons between the complete
concentration-response curves were made by repeated measures analysis of variance. Analysis was performed according to recommendations by Ludbrook.\textsuperscript{20} To correct for multisample asphericity, the Huynh-Feldt adjustment was always made. Calculations were done using the GLM procedure of the SAS-system (SAS Institute Inc., Chicago, IL, USA, version 8.2). When maximum response was reached, results from each dose-response curve were fitted to Hill’s equation. The negative log of the concentration that would give 50% constriction or dilation (\(-\log \text{EC50}\)), and the maximum response (contraction or dilation, \(E_{\text{max}}\)) was calculated from this curve. If during an experiment no contraction or dilation was observed, \(-\log \text{EC50}\) could not be calculated (indicated as not determined, ND, in the tables). Shifts in \(-\log \text{EC50}\) and \(E_{\text{max}}\) between rings from different experiments were compared by 2-sided Student’s \(t\)-test. A probability level of <0.05 was considered to indicate statistical significance.

**Results**

**Functional measurements.**

Internal mammary arteries were obtained from 50 patients undergoing coronary bypass surgery. The patient population was a representative group of patients undergoing coronary bypass surgery with a mean age of 65.0 years (range 44-82); patient characteristics are shown in Table 1.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>65.0 ± 1.3</th>
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<tr>
<td>Male gender (%)</td>
<td>74.0</td>
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<tr>
<td>Body mass index (kg/m2)</td>
<td>27.4 ± 0.5</td>
</tr>
<tr>
<td>NYHA functional class</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>12.0</td>
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<tr>
<td>Family history of CAD (%)</td>
<td>48.0</td>
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<tr>
<td>Myocardial Infarction (%)</td>
<td>32.0</td>
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<tr>
<td>Current smoker (%)</td>
<td>20.0</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>147 ± 3</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>82.0</td>
</tr>
<tr>
<td>β-blocker (%)</td>
<td>76.0</td>
</tr>
<tr>
<td>Calcium antagonist (%)</td>
<td>52.0</td>
</tr>
<tr>
<td>Lipid lowering drugs (%)</td>
<td>52.0</td>
</tr>
<tr>
<td>Nitrates (%)</td>
<td>58.0</td>
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</table>

**Table 1.** Baseline characteristics

Values are mean ± SEM or percentage. Aspirin was discontinued 1 week before surgery.

Abbreviations: CAD, coronary artery disease; NYHA, New York Heart Association; DBP, diastolic blood pressure; SBP, systolic blood pressure
AT1 and AT2 receptor mRNA expression and contraction to angiotensin II

Both AT1 and AT2 receptor mRNA were identified in human internal mammary arteries of 14 different patients. AT1 receptor mRNA expression (ratio versus GAPDH expression) was significantly higher than the expression of AT2 receptor mRNA (3.91 ± 0.65 versus 1.72 ± 0.42, p< 0.001).

Median AT2 receptor mRNA level was used to stratify these 14 patients into a low and a high AT2 receptor mRNA expression group (mean AT2 receptor mRNA expression ratio 0.48 ± 0.23 versus 2.95 ± 1.31 respectively, p< 0.001). The contractile response to increasing concentrations of angiotensin II was significantly higher in the high AT2 receptor mRNA expression group (Emax: 34.4 ± 3.3% versus 7.7 ± 1.5%, p<0.001), while -log EC50 values were comparable in both groups (-7.6 ± 0.3 mol/L versus -8.0 ± 0.2 mol/L, p=0.341). Comparable results were found for AT1 receptor mRNA expression. Furthermore, a linear relation between AT2 receptor mRNA expression and angiotensin II mediated maximal vasoconstriction was observed (r=0.62, p=0.018), whereas the correlation between contraction and AT1 receptor mRNA expression almost reached statistical significance (r=0.46, p=0.098, Figure 1).

AT1 and AT2 receptor protein expression

Both AT1 and AT2 receptor protein expression were determined in segments from 4 different patients. AT1 and AT2 receptor protein expression did not differ significantly in these patients (ratio versus GAPDH). The results of the Western blot analysis are shown in Figure 2.
Endothelial dependent and independent relaxation

Of the 187 patients analysed, segments of 86 patients (mean age 63.3 ± 1.2, 86.7% male) showed a contractile response to 10 μM phenylephrine. In these vessel segments endothelium dependent and independent relaxation was tested. The acetylcholine concentration-response curve in Figure 3 shows that acetylcholine cause a maximum dilation of 34.2% when expressed as percentage of the maximum response to phenylephrine. By subsequently exposing the vessel segments to 10 mM sodium nitrite an additional dilation was observed. Maximum endothelium independent relaxation was determined to be 91.1% (± 4.2%).

Effect of AT1 and AT2 receptor blockade on angiotensin II induced responses in arteries under baseline conditions

Organ bath experiments demonstrated that in control vessels, increasing doses of angiotensin II caused vasoconstriction up to 41.1% of the maximal response to 10 μM phenylephrine (Figure 4 and Table 2). Presence of 1 μM PD123319 significantly reduced maximal contraction and shifted the concentration-response curve to the right (although repeated measures analysis of variance did not reveal a significant difference), but did not abolish the response to angiotensin II. In contrast, contractions to angiotensin were almost completely abolished in the presence of 10 μM candesartan. Presence of PD123319 did not affect this effect of candesartan.

Figure 2. Protein expression of AT1R and AT2R subtypes in left internal mammary arteries (n=4). To determine total protein content of AT1R and AT2R western blot analyses was performed. Top; representative western blot results

Figure 3. Dilations of human internal mammary artery rings to increasing doses of acetylcholine to test endothelial dependent relaxation. Responses (mean ± SEM, n = 86) are expressed as a percentage of the response to 10 μM PE.
Figure 4. Contractions of human internal mammary artery rings to increasing doses of angiotensin II in the presence and absence of 10 µM candesartan and/or 1µM PD123319. Data are mean ± SEM, (n = 7 to 10). Concentration response curves were compared using repeated measures analysis of variance.

Effect of α-receptor blockade on angiotensin II induced contraction

Under control conditions, blockade of the α-receptors by phentolamine caused a significant rightward shift of the concentration-response curves to angiotensin II (-log EC50 8.26 ± 0.07 mol/L vs 7.34 ± 0.14 mol/L for vehicle vs phentolamine, p<0.001), as well as a significant reduction in maximal contraction (Emax 41.1 ± 2.0% vs 27.7 ± 2.6% for vehicle vs phentolamine, p<0.001, Figure 5A).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>-log EC50 ± SEM</th>
<th>p*</th>
<th>Emax ± SEM</th>
<th>p*</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>10</td>
<td>8.26 ± 0.07</td>
<td>41.1 ± 2.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM candesartan</td>
<td>8</td>
<td>ND</td>
<td>1.4 ± 1.2%</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>1 µM PD123319</td>
<td>7</td>
<td>7.89 ± 0.03</td>
<td>&lt;0.001</td>
<td>28.6 ± 1.0%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 µM candesartan +</td>
<td>7</td>
<td>ND</td>
<td>1.8 ± 1.3%</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>1 µM PD123319</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. Concentration response characteristics of human internal mammary arteries to increasing concentrations of angiotensin II in the presence or absence of 10 µM candesartan and/or 1 µM PD123319

* versus control
• p<0.001 versus PD123319

Data are expressed as mean ± standard error of the mean (SEM).

Abbreviations: -log EC50, concentration at which 50% of the maximum response was reached; Emax, maximum contraction response; ND, not determined
In contrast, under conditions of AT2 receptor blockade the main effect of phentolamine seemed to be a reduction in maximal contraction (Emax 28.6 ± 1.0% vs 19.6 ± 0.8% for vehicle vs phentolamine, p<0.001) rather than a change in sensitivity to angiotensin II (-log EC50 7.98 ± 0.03 mol/L vs 7.65 ± 0.07 mol/L for vehicle vs phentolamine, p=0.008, Figure 5B). It should be reminded, however, that sensitivity and maximal contraction to angiotensin II already are reduced to some extend during conditions of per se AT2 receptor blockade (Figure 5 and Table 2). As a result, therefore, it appears that the sensitivity and maximal contraction to angiotensin II are similar during conditions of α-receptor blockade with phentolamine, irrespective of additional AT2R blockade (Table 3). Finally, under conditions of AT1 receptor blockade with candesartan the responses to angiotensin II were virtually abolished, and this remained unchanged by phentolamine (data not shown).

**Figure 5.** Effect of α-receptor blockade with 1 µM phentolamine on angiotensin-induced contraction in isolated human internal mammary artery rings under control conditions (panel A) and under conditions of AT2 receptor blockade with 1 µM PD123319 (panel B). Data are mean±SEM.

**Effect of AT1 and AT2 receptor blockade on angiotensin II induced responses in precontracted arteries**

The mean absolute levels of precontraction did not differ significantly between the 4 groups; 330±63, 325±66, 289±84, and 308±64 µm (for the saline, candesartan, combination, and PD123319 group, respectively). After precontraction of the control rings, increasing doses of angiotensin II caused a non-significant further increase in contraction (Emax 10.5±1.5%, Figure 6). This response was significantly reduced by 1 µM PD123319 (Emax 1.7±1.1%, p<0.001 versus control; although repeated measures analysis of variance did not reveal a significant difference), and reversed into relaxation in the presence of 10 µM candesartan (Emax -12.9±0.5%, p<0.001 versus control).
After blockade of both the AT1 and AT2 receptor, angiotensin II administration induced a similar response compared to AT1 receptor blockade only (Emax = 10.3±0.6%, p=ns versus candesartan only).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>-log EC50 ± SEM</th>
<th>p*</th>
<th>E_max ± SEM</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>7.51 ± 0.18</td>
<td></td>
<td>27.6 ± 2.9%</td>
<td></td>
</tr>
<tr>
<td>10 µM candesartan</td>
<td>7</td>
<td>ND</td>
<td></td>
<td>-3.7 ± 0.4%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 µM PD123319</td>
<td>7</td>
<td>7.74 ± 0.08</td>
<td>0.260</td>
<td>21.3 ± 0.8%</td>
<td>0.048</td>
</tr>
<tr>
<td>10 µM candesartan + 1 µM PD123319</td>
<td>7</td>
<td>ND</td>
<td></td>
<td>-4.4 ± 0.5%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3. Concentration response characteristics of human internal mammary arteries to increasing concentrations of angiotensin II in the presence or absence of 10 µM candesartan and/or 1 µM PD123319, after addition of 10µM phentolamine

* versus control
# maximal dilation was used

Data are expressed as mean ± standard error of the mean (SEM).

Abbreviations: -log EC50, concentration at which 50% of the maximum response was reached; Emax, maximum contraction response; ND, not determined

AT2 receptor stimulation in absence and presence of AT1 and AT2 receptor blockade

Increasing concentrations of CGP42112A did not induce significant dilation or contraction in any of the groups, irrespective of the antagonist used. Data not shown.

Discussion

In this study we demonstrated that the AT2 receptor is present in human internal mammary arteries, and did not mediate vasodilation in our in vitro model. To our knowledge, the vasoactivity of the arterial AT2 receptor has been the focus of a human in vitro study only once before, when Batenburg et al. demonstrated that AT2 receptor mediated vasodilation in human coronary microarteries. An earlier publication from the same group did not specifically focus on the role of AT2 receptor, but in that study addition of PD123319 did not influence the angiotensin II induced concentration response curve in larger human coronary arteries. Unfortunately, quantification of the AT2 receptor was not part of the protocol in the latter study. Both studies used tissue that was collected from young heart-beating donors who died of non-cardiac causes. Furthermore, Borland et al. already demonstrated that stimulation of the AT2 receptor neither induces vasoconstriction, nor dilation in isolated human saphenous vein segments. In the present study, larger non-resistance arteries were studied and the segments were obtained from older patients with severe coronary
AT2 Receptors in Human Arteries

Figure 6. Response of phenylephrine-preconstricted human internal mammary arteries to increasing concentrations of angiotensin in the absence or presence of absence of 1 µM PD123319, 10µM candesartan or a combination of both agents (n = 10 to 14). Concentration response curves were compared using repeated measures analysis of variance.

disease. In previous studies the distribution of the AT2 receptor appeared to be species-, tissue-, and disease specific, and the developmental stage of the organism studied also seemed to play a role. In various pathological states of the cardiovascular system the expression of the AT2 receptor is increased, but even under pathological conditions the AT1 receptor subtype seems to outnumber the AT2 receptor subtype. Although the AT2 receptor mRNA levels are lower than the AT1 receptor mRNA levels in the arterial segments we analysed, the AT2 receptor protein levels seem to be comparable to the AT1 receptor protein levels. Furthermore, Adams et al. identified both AT2 receptor mRNA and protein expression in human internal mammary arteries, and they demonstrated that expression increased after a regular preoperative physical activity program. In this study, the authors found a blunted response to high concentrations of angiotensin II in human internal mammary vessels. They speculated that the blunted response was caused by a shift in AT1/AT2 receptor expression, since they found a linear correlation between receptor protein expression ratio and the maximal angiotensin II induced vasoconstriction. We found a correlation between AT2 receptor mRNA expression and maximal angiotensin II induced constriction.

Besides its direct vasoconstrictor effects, angiotensin II also facilitates norepinephrine release from norepinephrine stores in presynaptic nerve terminals through AT1 receptor stimulation. As a result postsynaptic alfa-1
receptors are stimulated, mediating vasoconstriction.\textsuperscript{26} With the intention of eliminating these indirect vasoconstrictor effects of angiotensin II, we repeated the first experiment after antagonizing α-receptors with phentolamine. This resulted in a rightward shift of the concentration response curve and into a reduction of E\textsubscript{max}, suggesting that a significant percentage of the response to angiotensin II is actually an indirect norepinephrine-mediated effect. Despite attenuation of the angiotensin II induced response that occurs after elimination of α-mediated effects, blockade of the AT2 receptor still seems to affect the concentration-response curve when compared to vehicle-treated rings. Unfortunately, in the absence of nerve stimulation, it is unclear how much alpha adrenergic nerve firing is occurring in isolated vessels. In the present study, the differences between concentration response curves are subtle, and consequently repeated measures analyses of variance can not uncover small changes. In contrast to common views, when we analyse E\textsubscript{max} and -log EC\textsubscript{50} values of the concentration response curves, our findings even suggest an AT2 receptor-mediated vasoconstriction in human internal mammary arteries. Several explanations can be proposed. First, due to lack of selectivity, PD123319 in a concentration of 1µM might have partly blocked AT1 receptors. The relatively high concentration of this AT2 receptor antagonist may have induced aspecific effects, although previous studies suggest that PD123319 is highly selective.\textsuperscript{18,27} Second, stimulation of the AT2 receptor could directly induce vasoconstriction. AT2 receptor-mediated vasoconstriction has been reported before in cerebral arteries,\textsuperscript{28} and in renal arteries.\textsuperscript{29} In addition, experiments performed by Touyz et al. using small mesenteric arteries of young spontaneously hypertensive rats, demonstrated that blockade of the AT2 receptor reduces angiotensin II-induced contractile response.\textsuperscript{21} A recent study performed by You et al. demonstrated that AT2 receptor stimulation induced a vasoconstriction in untreated spontaneously hypertensive rats resistance vessels associated with a decrease in AT2 receptor expression.\textsuperscript{12} They also found that treatment of hypertension restored both AT2 receptor expression and its vasodilator function. However, in our experiments direct stimulation of the AT2 receptor using the AT2 receptor agonist CGP42112A did not provoke dilation, nor contraction. Furthermore, in the curves constructed after precontraction with phenylephrine, no difference was found between the angiotensin II response in the presence of candesartan, and in the presence of the combination of candesartan and PD123319. This makes a direct contractile effect of the PD123319-sensitive receptor less likely.

A third explanation was proposed by Hong et al.\textsuperscript{30} They already speculated that the sensitivity of the AT1 receptor is revealed when the AT2 receptor is blocked. This hypothesis advocates the existence of a dynamic cross-talk between the AT1 and AT2 receptor. The nature of this receptor-receptor interaction ought to
be present at the second messenger system level, or at the mRNA and protein level. In the first case, an intermediate enzyme of the AT2 receptor secondary messenger system interferes with the secondary messenger system of the AT1 receptor. In the second case, treatment with PD123319 would result in a decreased expression of the AT1 receptor, giving rise to a reduction of Emax. Preceding studies demonstrated that in vascular smooth muscle cells angiotensin II increased AT2 receptor protein levels. This effect was suppressed by the AT1 receptor antagonist losartan but not by the AT2 receptor antagonist PD123319, suggesting that angiotensin II influences AT2 receptor expression through the AT1 receptor. Additionally, Andresen et al. report that AT2 receptor cross-talk with AT1 receptors through a nitric oxide- and RhoA-dependent mechanism in a rodent preglomerular smooth muscle cell culture. These studies illustrate the complexity of the interplay between angiotensin II receptor subtypes.

Limitations

It has been reported that adrenergic response can be affected by atherosclerotic disease. The contractile response to potassium appears not to change with stage of disease. In this study, we found a strong correlation (r=0.83, p<0.001) between contractile response to phenylephrine and to potassium. Therefore, we feel we can express the response to angiotensin II as a percentage of the contractile response to phenylephrine. Technically and logistically, it was not possible to perform the experimental protocols in all segments. Consequently, it was not possible to link endothelial function to other functional tests. However, it is important to notice that results from this paper and from previous other publications from our laboratory demonstrate that it is unlikely that manipulation of the arterial segments affects the functionality of the endothelium.

Conclusion

The present study demonstrated that although AT2 receptor is present in human internal mammary arteries, they do not mediate vasodilation in these arteries.

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Chapter 5

References


