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Angiotensin II-mediated calcium signals and mitogenesis in human prostate stromal cell line hPCPs

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1 Western blots and immunocytochemistry were used to detect angiotensin 1 (AT_1) and angiotensin 2 (AT_2) receptors in human primary cultures of the prostate stromal compartment (hPCPs). Immunohistochemistry was performed on human prostate tissue-embedded paraffin. In addition, pharmacological tools were applied in combination with photometry experiments to characterize the physiological activity of AT_1 and AT_2 receptors in hPCPs cell culture. A proliferation assay was used to describe the mitogenic activity of angiotensin II (Ang II) on hPCPs cells.

2 Only the AT_1 receptor was detected in Western blot analysis. Immunocytochemistry of hPCPs cells showed that the AT_1 receptor is present in both the smooth muscle type and the fibroblastic type. In the stromal compartment of human prostate tissue, immunoreaction with antibodies against the AT_1 receptor was detectable.

3 Fura-2-loaded hPCPs cells showed an instantaneous and linear rise in free intracellular calcium ion concentration ($[Ca^{2+}]_i$) after local perfusion with Ang II in concentrations of 10 nM. Removing of external calcium or emptying intracellular calcium stores before Ang II application diminished or abolished this $[Ca^{2+}]_i$ response.

4 The response to Ang II was also diminished when hPCPs cells were perfused with the AT_1 receptor inhibitor losartan prior to Ang II application. No inhibition of the $[Ca^{2+}]_i$ increase was detectable after perfusion with PD 123319, a specific inhibitor of the AT_2 receptor.

5 hPCPs cells were stimulated with Ang II in various concentrations over a period of 2 days. The subsequently performed proliferation assay revealed a mitogenic effect of Ang II on hPCPs in concentrations starting at 10 nM. This effect could be inhibited by losartan.
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Abbreviations: ACE, angiotensin-converting-enzyme; Ang II, angiotensin II; $[Ca^{2+}]_0$, free extracellular calcium ion concentration; $[Ca^{2+}]_i$, free intracellular calcium ion concentration; CPA, cyclopiazonic acid; DMSO, dimethylsulfoxide; HBSS, HEPES-buffered salt solution; hPCPs, human primary culture of the prostate stromal compartment; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride membranes; SERCA, the sarcoendoplasmic reticulum Ca^{2+}-ATPase; TCA, trichloroacetic acid

Introduction

In recent years, it became clear that the action of angiotensin II (Ang II) is not limited to the regulation of body fluid volume in response to hypovolemia. Other physiological tasks of this peptide were discovered like the regulation of the activity of releasing hormones and pituitary hormones in pregnancy (Bing *et al*., 1996), neurotransmitter interactions of Ang II with catecholamines and other peptides (Diz *et al*., 2002) and mitogenic effects of Ang II on various cell types, for example, vascular smooth muscle cells (Sayeski & Ali, 2003), hepatic stellate cells (Zhang *et al*., 2003), fibroblasts (Qu *et al*., 2001), hematopoietic progenitor cells (Rodgers *et al*., 2000) and mesothelial cells (Kuwahara *et al*., 2000). Two receptor types are responsible for the action of Ang II, the angiotensin 1 (AT_1) and the angiotensin 2 (AT_2) receptor. The regulation of blood pressure and the mitogenic effects seem to be regulated by the AT_1 receptor, whereas the AT_2 receptor seems to play a role in developing tissues and antimitogenic effects (Huckle & Earp, 1994).

Benign prostatic hyperplasia is defined as a proliferative process of the stromal compartment of the prostate (Berry *et al*., 1984; Shapiro, 1990; Shapiro & Lepor, 1994; Shapiro *et al*., 1997). This pathological change is regarded as a stromal disease and occurs with a high frequency in men over 50 years of age (Kojima *et al*., 1997). Still not much is known about the factors that induce and regulate benign prostatic hyperplasia. Beside a change in the action of sex hormones, other mitogenic factors that could induce proliferation in the periurethral tissue of the prostate are under investigation (Bonkhoff & Remberger, 1998; Lee *et al*., 1999; Hellawell & Brewster, 2002). Recent studies suggested that angiotensin receptors (Dinh *et al*., 2001) and reasonable amounts of angiotensin-converting enzyme (ACE) (Dinh *et al*., 2002; Kaplan, 2002) are present in human prostate. The intriguing question is what role Ang II plays in prostate physiology and pathophysiology.

The greatest hindrance to developing experiments to understand the pathogenic process in benign prostatic hyperplasia is

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the severely limited number of animal models available. Here, we show the effect of Ang II on the cell line hPCPs (human primary cultures of the prostate stromal compartment cells). The hPCPs cell line was established in our lab from the stromal compartment of human prostate tissues and characterized in former studies (Janssen *et al*., 2000; Albrecht *et al*., 2002). The cultures consist of cells with fibroblast- and smooth muscle-like characteristics and currently serve as a model for the pathophysiological events during the development of benign prostatic hyperplasia. Besides investigating the presence of AT_1 and AT_2 receptors in hPCPs cells, we performed experiments to prove their Ca^{2+}-dependent physiological activity. Furthermore, it was an aim of this study to determine if Ang II has a mitogenic effect on stromal prostate cells.

Methods

Immunoblotting of AT_1 and AT_2 receptors

hPCPs cells grown in tissue flasks were washed three times with phosphate-buffered saline (PBS, 10 mM, pH 7.3) for 2 min. Thereafter, cells were incubated with lysis buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM Tris-HCl, pH 7.4) at 90°C and scraped of the flasks after 30 s. Using a commercial protein assay (BioRad™), the protein content of the samples was measured. Freeze-dried protein (250 μg) was dissolved in sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.006% bromophenol blue, 1.8% β-mercaptoethanol). After heating these protein extracts at 95°C for 5 min, samples were run in a 12% polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes (PVDF) with transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol). Prior to the incubation with primary antibodies, nonspecific bindings were blocked with PBS containing 10% Rotiblock™ for 2 h. Thereafter, blots were incubated with the AT_1 or AT_2 receptor antibody in dilutions of 1 : 1000 over night and than washed three times with 0.1% Tween in PBS for 3 min. Horseradish peroxidase-labeled secondary antibodies were diluted 1 : 2000 and blots were incubated in it for 1 h. Finally, blots were washed again three times with 0.1% Tween in PBS and treated with enhanced-chemiluminescence detection reagents for 5 min. As positive controls, we used whole-cell lysate of PC12 WCL cells for the AT_1 receptor and KNRK cells for the AT_2 receptor provided by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). As a negative control fibroblast lysate was used.

Immunocytochemistry

Cells plated on Petri dishes were rinsed with PBS three times before they were fixed in 4°C 100% methanol for 2 min. Fixed cells were washed with PBS for 3 min before and after they were treated with 0.01% Triton X-100 for 10 min. Primary antibodies against the AT_1 and AT_2 receptors and smooth muscle actin were diluted to 1 : 100 in PBS and applied overnight at 4°C in a humidified chamber. After additional three washings with PBS for 5 min, cells were incubated with the Cy3- or FITC-labeled secondary antibody for 1 h at room temperature in a moist chamber. Before cells were mounted in glycerol containing 1% propylgallate, they were washed in PBS for 5 min. As a control, we used sections that were incubated with non-immune serum or non-primary antibody. Stainings were evaluated on a Nikon invertoscope (TE 2000, Nikon, Düsseldorf, Germany). Confocal imaging was conducted on a Leica microscope (TCS SP/MP, Leica, Bensheim, Germany) at the Keck Imaging Facility at the University of Washington, Seattle, U.S.A.

Immunohistochemistry

Tissues samples of human prostate were fixed in 4% formaldehyde and embedded in paraffin before they were cut to a thickness of 5 μm and mounted on laminin-coated glass slides. To inhibit unspecific protein binding and endogenous peroxidase, sections were preincubated with 3% normal swine serum in PBS and 2% H_2O_2 in ethanol for 60 min. After washing the sections three times in PBS for 5 min, primary AT_1- or AT_2-receptor antibodies were applied at a dilution of 1 : 100 in a moist chamber overnight at 4°C. After three additional washing steps with PBS for 5 min, biotinylated secondary antibody was applied. Sections were again washed three times in PBS for 5 min and subsequently incubated with Vectastain™ ABC reagent for 30 min followed by another three washings with PBS for 5 min each. The slides were then incubated for a few minutes with a peroxidase substrate solution until desired staining was developed. Finally, sections were rinsed in tap water and counterstained with hematoxylin before they were mounted.

Dye loading and Ca^{2+} photometry

Stromal cells of human prostate were isolated as primary cultures from surgical specimens of benign prostatic hyperplasia as described previously (Janssen *et al*., 2000). hPCPs cells grown as a monolayer on coverslips were used for the Ca^{2+} measurements. Cells were washed with HEPES-buffered salt solution (HBSS, pH 7.4; 10 mM HEPES, 135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM KCl, 0.8% dextrose). Fura-2 AM in dimethylsulfoxide (DMSO) was diluted from a 3 mM stock solution in an equal volume of Pluronic F-127 and adjusted with HBSS to a final concentration of 3 μM. This dilution was used to incubate cells for 30 min with the dye at 37°C with 5% CO_2 followed by three brief washings with HBSS to eliminate dispensable Fura-2 AM. Cells were allowed to sit an additional 30 min before recording fluorescent signals. Coverslips were placed in Attofluor® measuring chambers (Molecular Probes, Leiden, Netherlands) and examined with a $\times 40$ oil objective under a Nikon invertoscope (TE 2000). Fluorescence was monitored by a Till-Photonics photometry setup (Till-Photonics, Gräfeling, Germany). Emission intensities were monitored as a ratio (340/380 nm) at 510 nm with a photodiode. By applying the standard equation $[Ca^{2+}] = K_d(R-R_{min})/(R_{max}-R)$ (Grynkiewicz *et al*., 1985), the background corrected signal (R) was calibrated. R_{min} and R_{max} values were obtained from cells equilibrated in solutions fortified with ionomycin (10 μM) and containing 50 mM EGTA, 15 mM $CaCl_2$ or 20 mM EGTA with 15 mM $CaCl_2$. The solutions were applied with a solenoid-controlled, gravity-fed, multibarreled local perfusion system as described previously (Wennemuth *et al*., 1998; 2000; 2003).

For cuvette measurements of free intracellular calcium ion concentration ($[Ca^{2+}]_i$), cells were grown in cell culture flasks

and loaded with Fura-2 AM as described previously (Wennemuth *et al*., 2003). We took the threshold concentration to be the first value that differed significantly from lower concentrations. As the maximal value, we considered the concentration that did not lead to a statistically higher $[Ca^{2+}]_i$ than the next lower concentration used in the experiments.

Proliferation assay

hPCPs cells were grown in 96-well plates with RPMI medium containing 10% fetal calf serum at 1500 cells well^{-1} and allowed to attach overnight. Cells were washed with serum-free media and incubated in serum-free medium containing different concentrations of Ang II with PBS (vehicle) or Ang II in different concentrations with losartan or just losartan for 2 days. DNA synthesis was measured by [^{3}H]thymidine incorporation into the trichloroacetic acid (TCA) precipitable material. Cells were pulsed with 1 μCi well^{-1} [^{3}H]thymidine (5 Ci mmol^{-1}) during the last 10 h of culture. At the end of the incubation period, hPCPs were washed twice in PBS, trypsinized for 5 min at 37°C and finally collected. DNA was precipitated with 5% TCA at 4°C for 15 min. The precipitates were then washed twice with 95% ethanol, dissolved in 1 ml of NaOH and analyzed by liquid scintillation counting. Results are expressed as percent control unstimulated [^{3}H]thymidine incorporation (mean $\pm$ s.e.) of five independent experiments.

Data analysis

Statistical analyses were performed in Excel (Microsoft, Redmond, WA, U.S.A.) and Igor 4.0 (Wavemetrics, Lake Oswego, OR, U.S.A.). Automated correction, calibration and kinetic analysis of digital photometric records were performed in Igor 4.0 and Axoscope 9 (Axon Instruments, CA, U.S.A.). Calculation of the EC_{50} was carried out with the analysis software GraphPad Prism 4.01, GraphPad Software, San Diego, CA, U.S.A.

Materials

If not otherwise stated, chemicals were purchased from Sigma Chemicals (Deisenhofen, Germany). Fura-2 AM, DMSO and Pluronic F-127 were from Molecular Probes (Leiden, Netherlands). PD 123319 was from Research Biochemicals International (Natick, MA, U.S.A.), losartan from Merck (Whitehouse Station, NJ, U.S.A.). Vectastain ABC staining kit was purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Primary antibodies against AT_1 and AT_2 receptors were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), antibodies against smooth muscle actin from Sigma (Deisenhofen, Germany). Rotiblock™ was from Roth (Karlsruhe, Germany). Super Signal Western Blotting Kit and PVDF membranes were from Pierce (Bonn, Germany). Protein assays were purchased from BioRad (Munich, Germany).

Results

Immunoblotting of AT_1 and AT_2 receptors

Two polyclonal antibodies were used to detect AT_1 and AT_2 receptors in hPCPs cells. The AT_1 receptor antibody was raised against a peptide near the amino-terminus, the antibody against the AT_2 receptor against the carboxy-terminus, both of human origin. Whole-cell lysate of hPCPs cells was blotted and the AT_1 receptor was detected with a major band migrating to approximately 50 kDa (Figure 1). In addition, a minor band was visible at 40 kDa. No signal was detected with the AT_2 receptor antibody at the expected migrating range of 50 kDa.

Immunocytochemistry

The same antibodies used in immunoblotting were used to depict the distribution of AT_1 and AT_2 receptors in hPCPs cells with immunocytochemistry. The secondary antibodies were Cy3 labeled and raised against goat- and rabbit-IgG for AT_1 and AT_2, respectively. To differentiate between the two characteristic cell types of hPCPs, more fibroblast or more smooth muscle like, we performed a double staining and used a second antibody against smooth muscle actin to show colocalization of AT_1 and/or AT_2 receptors with actin. The secondary antibody against the smooth muscle actin antibody was FITC labeled (green). As a counter staining, we used DAPI to depict the nuclei. Figure 2 shows a typical staining at a $\times 40$ magnification with the two different cell types found in hPCPs cell culture. As stated above, the smooth muscle-like hPCPs cells show the anticipated green stain; the remarkable aspect is the (red) immunoreaction revealing the presence of the AT_1 receptor. Owing to the fact that there are only two cell types present in the culture, which do not stain green are subsequently identifiable as the fibroblastic stromal cell type. Despite having no smooth muscle actin, these cells also show an immunoreaction with the AT_1 receptor antibody.

Immunohistochemistry

We used human prostate tissue embedded in paraffin to depict the distribution of angiotensin receptors *in vitro* by performing

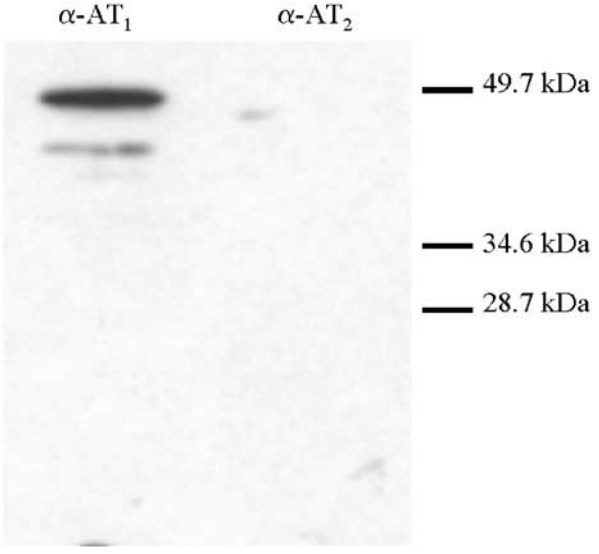

Figure 1 Anti-AT_1 and anti-AT_2 immunoblot of extracts from hPCPs. The bars on the right mark the migration of three different reference markers. The immunoblot on the right indicates two major immunoreactive protein bands near the 49 kDa marker. No immunoreaction was found with the antibodies specific to the AT_2 receptor. Both receptor proteins should migrate at a size of 50 kDa.

a DAB staining. Figure 3 is a representative, $\times 40$ magnification of the immunoreaction (brown) using the AT_1 antibody. Most of the immunoreaction is localized in the interstitial compartment of the tissue. There is only a light staining in the apical part of the epithelial cells of the gland tissue. Using the AT_2 receptor antibody did not reveal any positive immunoreaction in human prostate tissue.

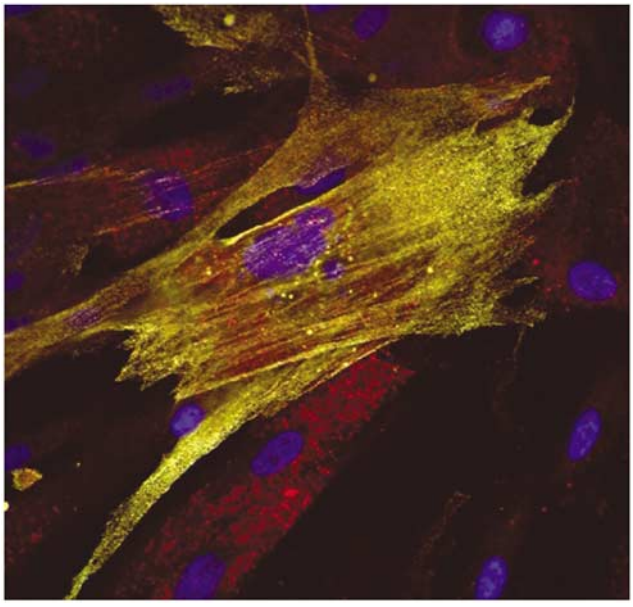

Figure 2 Triple-staining immunofluorescence of hPCPs cells with antibodies against the AT_1 receptor (red, Cy3), smooth muscle actin (green, FITC) and DNA (blue, DAPI). Smooth muscle-like hPCPs cell can be identified by the green (smooth muscle actin) and red (AT_1 receptor) fluorescence. Fibroblastic hPCPs cells show an immunoreaction with the AT_1 receptor antibody only. Magnification $\times 40$.

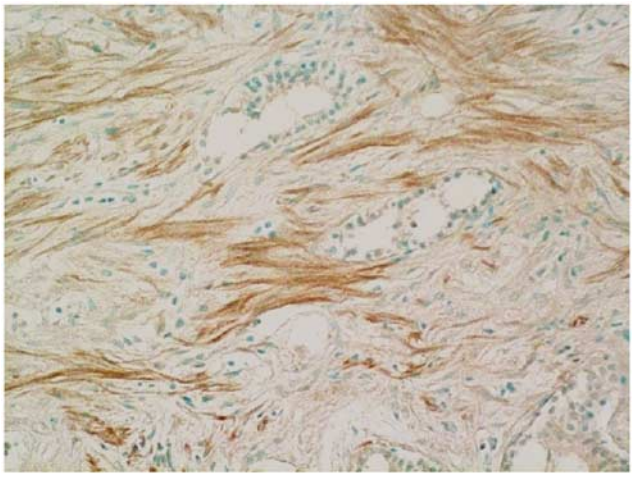

Figure 3 Human prostate material embedded in paraffin was used to show the distribution of AT_1 receptors in gland tissue. A hematoxylin counterstaining was performed to mark the nuclei. The brown DAB staining localizes the immunoreaction predominantly in the stromal compartment. Little immunoreaction is seen in the apical segment of epithelial cells.

Ang II induced $[Ca^{2+}]_i$ elevation in hPCPs cells

With local perfusion, we stimulated single hPCPs cells with Ang II in concentrations of 10 nM. The cells responded in 82% ($n=54$) of the cases with a linear, fast and reversible response to the drug application. Figure 4 shows a typical experiment where a single hPCPs cell was stimulated three times with Ang II. After the perfusion with Ang II ringer buffer was applied and the cells were allowed to recover from the calcium rise. No significant difference in the calcium increase over time or the maximum amplitude of $[Ca^{2+}]_i$ could be seen between the three stimuli in these experiments. The average amplitude of $[Ca^{2+}]_i$ after application of 10 nM Ang II was 323 mM (± 24 s.e.; $n=54$). Figure 5 shows the effect on $[Ca^{2+}]_i$ after long-term exposure of hPCPs to Ang II (10 nM). A single hPCPs cell was perfused for more than 3 min with 10 nM Ang II. After an initial rise in $[Ca^{2+}]_i$, the elevated ion concentration did not return to the baseline within 200 s.

Figure 6 depicts the average change $[Ca^{2+}]_i$ ($n=6$) as a function of Ang II, where $[Ca^{2+}]_i$ is defined as the maximal $[Ca^{2+}]_i$ response to Ang II minus the resting $[Ca^{2+}]_i$. The threshold concentration for stimulation was 100 pM and the maximal response was observed at 1 μM Ang II. The mean

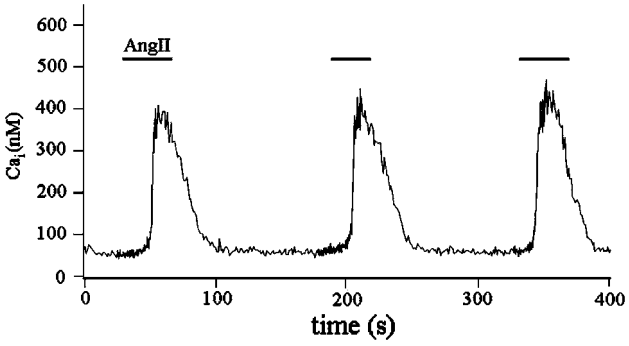

Figure 4 Fura-2-loaded hPCPs cells were stimulated three times briefly with Ang II at a concentration of 10 nM. The emission fluorescence signals measured above 510 nm show that intracellular calcium rises linearly and is reversible in these cells. The bars depict the duration of Ang II application. Between the stimuli, cells were perfused with ringer buffer.

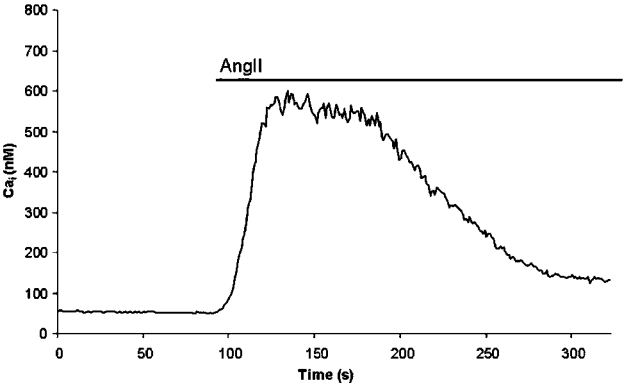

Figure 5 A typical recording ($n=6$) showing the effect of Ang II for a longer period of time (10 nM) on the increase in the intracellular calcium ion concentration in hPCPs cells. The bar shows the time over which cells were perfused with Ang II.

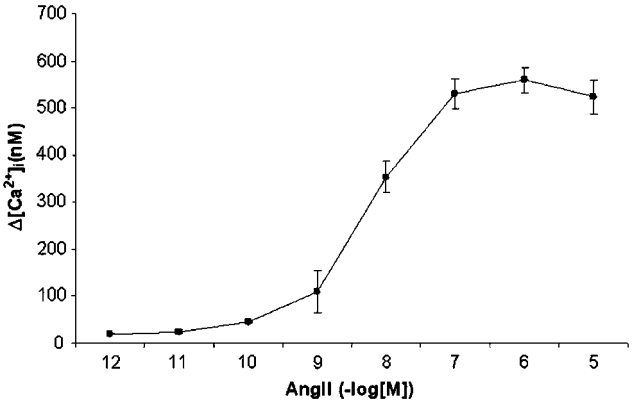

Figure 6 Effect of increasing concentrations of Ang II on $[Ca^{2+}]_i$ in hPCPs cells. Ang II concentrations of 100 mM to 1 pM were used to stimulate hPCPs cells in a cuvette-based system. The basal $[Ca^{2+}]_i$ was subtracted from the maximal $[Ca^{2+}]_i$ response ($\Delta[Ca^{2+}]_i$). Six experiments were averaged for each Ang II concentration and are shown with their s.e.m.

effective concentration (EC_{50}) calculated from the dose–response curve was 8.1 nM.

To show the dependence of the increase of $[Ca^{2+}]_i$ on external Ca^{2+}, cells were perfused with nominal Ca^{2+}-free ringer buffer ($[Ca^{2+}]_0$) approximately 20 s prior the Ang II (10 nM) stimulus. During the Ang II perfusion, no Ca^{2+} in the external milieu was present either. The response to Ang II under calcium-free conditions was diminished and showed an averaged maximum amplitude of 98 nM ($\pm$9 s.e.; $n=9$). Two control stimuli with Ang II were applied before and after $[Ca^{2+}]_0$ free conditions. Those averaged maximum amplitudes showed no significant difference 341 nM ($\pm$19 s.e.; $n=11$) and 302 nM ($\pm$12 s.e.; $n=9$) to each other (Figure 7). Cyclopiazonic acid (CPA), a reversible inhibitor of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), was applied in the same way. Between two control stimuli, cells were perfused with CPA at a concentration of 25 μM. During CPA application, a transient rise in $[Ca^{2+}]_i$ occurred. After approximately 3 min of CPA perfusion, the Ang II perfusion was applied simultaneously. As shown in the typical experiment in Figure 8, Ang II failed to increase $[Ca^{2+}]_i$. To prove that the cells were still able to respond to Ang II, ringer buffer was applied for approximately 5 min followed by a third Ang II stimulus. All cells responded with an elevation of $[Ca^{2+}]_i$ and no significant difference in the amplitude compared to the first stimulus was seen (Figure 8).

To specify the action of Ang II on angiotensin receptors, specific blockers were used in local perfusion protocols. As pharmacological tools, losartan, an inhibitor of the AT_1 receptor, and PD 123319, a specific inhibitor of the AT_2 receptor, were used. With local perfusion, a control stimulus with Ang II was applied to hPCPs cells. After recovery from $[Ca^{2+}]_i$ increase, cells were perfused with either one of the blockers for approximately 100 s followed by a second Ang II stimulus together with the inhibitor. In case of losartan, the increase of $[Ca^{2+}]_i$ was dramatically decreased (Figure 9) using the compound in a 1 μM concentration. The average value for the maximum amplitude of $[Ca^{2+}]_i$ was 86 mM ($\pm$13 s.e.; $n=6$), whereas for the first control stimulus, the values were 245 mM ($\pm$22 s.e.; $n=6$). After treatment with the inhibitor, cells were perfused with ringer buffer for another 360 s before another control stimulus with Ang II was applied. In the same way, experiments with PD 123319 were performed. However, the AT_2 receptor inhibitor had no effect on the increase of $[Ca^{2+}]_i$ in hPCPs after Ang II stimulation.

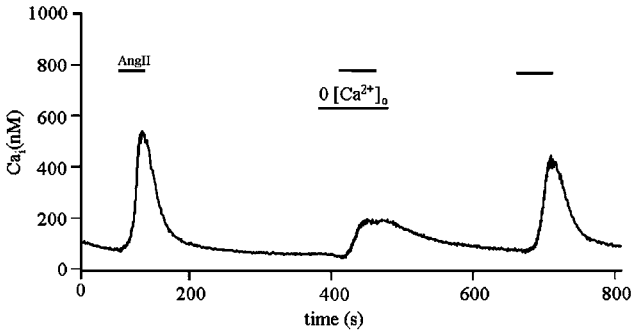

Figure 7 Ca^{2+} responses from cells transferred to and perfused with ringer buffer, stimulated with Ang II (10 nM) and then perfused again with ringer buffer. After recovery, nominally Ca^{2+}-free ringer buffer was applied for a few seconds before a second stimulus of Ang II under Ca^{2+}-free conditions was applied. The bars in the figure mark the period of exposure. Following recovery in Ca^{2+}-containing buffer, a third stimulus with Ang II was applied.

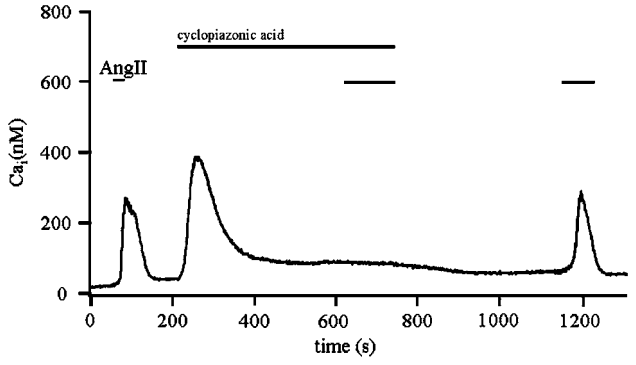

Figure 8 Cells transferred to ringer buffer were stimulated three times with Ang II (10 nM). The first and third applications were used as control stimuli. Before and during the second stimulation with Ang II, cells were perfused with CPA (25 μM) for 300 s to stop SERCA pumps reversibly and to empty intracellular Ca^{2+} stores.

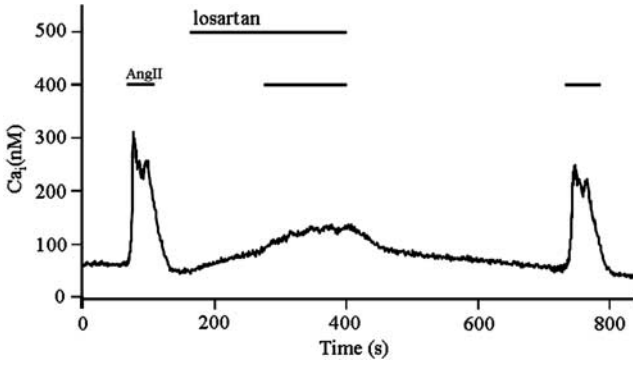

Figure 9 Three Ang II (10 nM) stimuli were applied to Fura-2-loaded hPCPs cell in this protocol. The first and the last perfusion with Ang II served as a control. Before the second stimulation with Ang II hPCPs cells were exposed to losartan (1 μM) for 100 s to block AT_1 receptors reversibly, followed by perfusion of losartan and Ang II together.

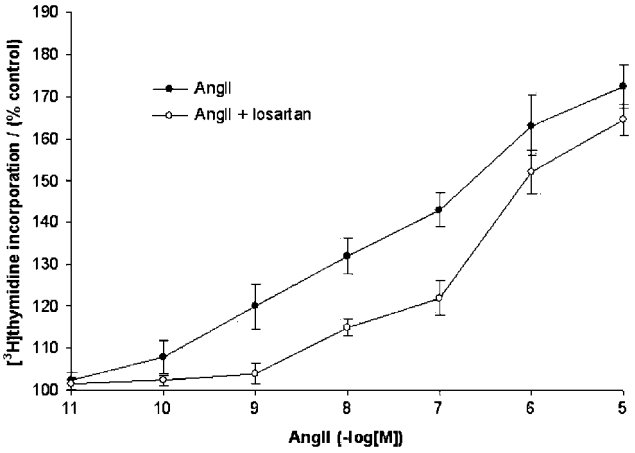

Figure 10 hPCPs cells were stimulated for 2 days with Ang II in varying concentrations and the mitogenic effect was measured by the [^{3}H]thymidine incorporation assay. The percentage of increased proliferation compared to the untreated control on day 2 is shown as a function of different Ang II concentrations (Ang II). The second graph depicts the percentage of change of proliferation with 1 μM losartan (Ang II + losartan) present during the Ang II stimulation compared to the untreated control on day 2 (100%).

Proliferation assay

To determine the mitogenic effect of Ang II on hPCPs cells, the [^{3}H]thymidine incorporation assay was used. Cells were stimulated 2 consecutive days with Ang II in concentrations between 100nM and 100 mM. Figure 10 shows the proliferation rate after 2 days of Ang II stimulation at different concentrations of the drug. The values are expressed as the percentage of [^{3}H]thymidine incorporation compared to cells treated with vehicle (PBS) only. Ang II in a concentration of 10 nM increased proliferation by 43% ($\pm$4.2 s.e.; $n = 5$). In addition, we used losartan and PD 123319 to see their abilities to inhibit the mitogenic effect of Ang II. Losartan decreased the effect of Ang II (10 nM) by 21%. On day 2, the increase in proliferation with losartan (1 μM) and Ang II (10 nM) present in the hPCPs culture was 22% ($\pm$5 s.e.; $n = 5$) compared to the control cells treated with Ang II and vehicle (PBS) alone. PD 123319 or treatment of cells with losartan alone in the absence of Ang II showed no inhibitory effect in these experiments (not shown).

Discussion

Although many published studies address the mitogenic effects of Ang II (Kunert-Radek & Pawlikowski, 1992; Natarajan *et al*., 1992; Ray *et al*., 1994; McEwan *et al*., 1999), and other work concentrates on the role hormones play in the pathophysiology of benign prostatic hyperplasia (Sciarra & Toscano, 2000; Gooren, 2003; Zhu *et al*., 2003; Yokota *et al*., 2004), there is little work on the physiological and mitogenic effects of Ang II on prostate cells. One major limitation is the availability of adequate animal models. Dinh *et al*. (2001) showed, on a molecular level, the existence of angiotensin receptors type 1 in normal human prostate and benign prostatic hyperplasia. AT_1 receptors were also found in prostate cancer cells (Uemura *et al*., 2003). Other work showed that, in benign prostatic hyperplasia, elevated levels of local ACE are present and that the expression of the AT_1 receptor might be affected by this (Nassis *et al*., 2001; Dinh *et al*., 2002). In this study, we introduce hPCPs cells as a model to study the effects of growth factors like Ang II on the stromal cells of the human prostate. We have demonstrated in previous work that hPCPs cells have a homogeneous morphology and defined growth characteristics. In early passages, hPCPs cells show a normal genetic constitution and mainly myoid characteristics, but they differentiate to fibroblastic cells in later stages (Janssen *et al*., 2000). Here, we show with immunocytochemistry and Western blot analysis that hPCPs cultures express the AT_1 but not the AT_2 receptor. The AT_1 receptor is present in the cells with myoid and fibroblastic characteristics. To prove that AT_1 receptors are physiologically active in hPCPs cells, we demonstrated that physiological concentrations of Ang II lead to a rise in intracellular calcium concentration. Furthermore, we showed that hPCPs cells have to deplete internal stores as a response to Ang II to induce a maximal Ca^{2+} rise by influx of Ca^{2+} from the outside to the inside of the cell. The diminished rise by depleting internal calcium stores with CPA and the inhibition of the Ang II response by removing external Ca^{2+} is an indicator for the presence and the opening of calcium release activated channels (I_{crac}).

Experiments with specific inhibitors were performed to demonstrate the specificity of the Ang II action on hPCPs cells. Losartan is known and well characterized as a specific inhibitor of the AT_1 receptor (Timmermans *et al*., 1995), whereas PD 123319 is a specific blocker of the AT_2 receptor (Blankley *et al*., 1991). Only losartan was able to inhibit the Ca^{2+} response to Ang II; PD 123319 failed to have any effect on the Ca^{2+} rise in hPCPs cells. These results, together with those from immunocytochemistry, suggest that all effects on hPCPs cells triggered by Ang II are mediated exclusively by AT_1 rather than AT_2 receptors. Increases in $[Ca^{2+}]_i$ mediated by AT_1 receptors have been shown in many other cells, like breast cells (Greco *et al*., 2002), mesothelial cells (Kuwahara *et al*., 2000) and coronary smooth muscle cells (Hafizi *et al*., 1999). Common to all of these different cell types is the observation that Ang II not only induces Ca^{2+} signaling but also has a mitogenic effect that is mediated by the AT_1 receptor. Moreover, we show here that hPCPs cells respond with a much higher proliferation rate after stimulation with Ang II at specific concentrations for 2 consecutive days. This effect was dramatically inhibited by losartan but not by PD 123319. This reveals that in addition to inducing Ca^{2+} signals, Ang II, mediated by AT_1 receptors, affects mitogenesis also.

Conclusions

Our study provides several significant and exciting findings, which may turn out to be important in understanding of the effects of Ang II on the pathophysiological process of benign prostatic hyperplasia. This we have shown: at certain concentrations, Ang II has a mitogenic effect on the stromal compartment of human prostate tissue; this effect is mediated by AT_1 receptors and can be inhibited by losartan. We can only speculate the extent to which Ang II influences the development of benign prostatic hyperplasia because there are, indeed, multiple factors causing the proliferation of prostate stroma. Consideration of losartan as a protective drug for Ang II-induced proliferation in prostate tissue is the next step that has to be evaluated. Another intriguing question is to what

extent antihypertensive drugs concentrate in living tissue. Finally, we find that hPCPs cells serve as a good model to test physiological compounds and drugs and their impact on the stromal compartment of human prostate tissue.

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