The influence of peptides from the angiotensin family on tyrosine kinase activity and cell viability in a human hormone-dependent prostate cancer line

Wpływ peptydów z rodziny angiotensyn na aktywność kinaz tyrozynowych i przeżywalność komórek hormonozależnej ludzkiej linii raka prostaty

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Abstract

Introduction: The results of many studies have reported that peptides from the angiotensin family are involved in the regulation of cell growth, proliferation, cell migration, apoptosis, inflammation, differentiation, and angiogenesis, which suggests that they might play an important role in carcinogenesis. The role of the renin–angiotensin system in supporting prostate cancer induction and progression has so far received little study.

Material and methods: The present study was to examine the influence of Ang II, Ang III, and Ang IV on a human hormone-dependent prostate cancer line (LNCaP). Using an isotopic method, we tested the effects of angiotensins on tyrosine kinase activity, and measured cell viability using an MTT Assay.

Results: The results showed that only Ang IV significantly reduced tyrosine kinase activity and cell viability in LNCaP cells. The process seemed to be mediated partly by AT2 and probably by another type of receptor with high affinity for Ang IV and low affinity for PD123319 and Losartan.

Conclusions: These findings suggest that components of the renin-angiotensin system, specifically angiotensin peptides and receptors (AT1, AT2) can modify prostate cancer cell viability.

Key words: hormone-dependent prostate cancer, LNCaP, cell viability, PTKs, losartan, PD123319

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Streszczenie

Wstęp: Wyniki wielu badań wskazują, że peptydy z rodziny angiotensyn są włączone w regulację cyklu komórkowego, apoptozę, różni-cowania się komórek, jak również odgrywają istotną rolę w procesach zapalnych i migracji komórek. Ich zaangażowanie w tak istotne procesy sugeruje, że mogą one odgrywać istotną rolę w procesie kancerogenezy. Stosunkowo mało wiadomo na temat roli systemu renina–angiotensyna w zapoczątkowaniu i późniejszym rozwoju raka stercza.

Materiał i metody: Poniższe wyniki przedstawiają wpływ Ang II, Ang III, Ang IV na linię komórkową ludzkiego hormonozależnego raka prostaty. Używając metody izotopowej, badano wpływ wybranych angiotensyn na aktywność kinaz tyrozynowych. Podczas gdy przeży-walność komórkowa była oznaczana za pomocą testu MTT.

Wyniki: Wyniki wskazują, że tylko Ang IV w sposób istotny statystycznie redukuje aktywność kinaz tyrozynowych i obniża przeżywalność komórek linii LNCaP. Proces ten był pośredniczony przez receptor AT2, a prawdopodobnie przez inny receptor, o wysokim powinnowo-wactwie dla Ang IV i niskim dla PD123319 i losartanu.

Wnioski: Otrzymane wyniki sugerują, że komponenty układu renina-angiotensyna, w tym szczególnie peptydy angiotensynowe oraz klasyczne receptory dla angiotensyn (AT1, AT2), mogą modyfikować przeżywalność komórek raka gruczołu krokowego.

Słowa kluczowe: hormonozależny rak gruczołu krokowego, LNCaP, przeżywalność komórkowa, PTKs, losartan, PD123319

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Introduction

Prostate cancer is the most commonly diagnosed invasive cancer in men. In 2007, an estimated 218,890 men were diagnosed with prostate cancer, and there were an estimated 27,050 deaths due to prostate cancer [1]. During the last decade, it has been established that the renin-angiotensin-aldosterone system (RAAS) is not only concerned with cardiovascular system and water-electrolyte balance. Angiotensin II (Ang II) can be locally produced and selectively regulated in many tissues and organs, for instance the adrenal, kidney, brain, breast, and prostate [2–4]. The expression of components of the local RAS, such as ACE and membrane receptors AT1 and AT2, has been found in human and rat prostate cells [5].

The first and most active multifunctional hormone of the renin-angiotensin-aldosterone system is angiotensin II. The existence of RAS in the prostate gland strongly supports the finding that human seminal plasma contains Ang II in concentrations from 3- to 5-fold higher than in circulating blood plasma. Therefore, it is appropriate to determine the source of this secreted angiotensin II [6]. The results of many studies have reported that Ang II is involved in the regulation of cell growth, proliferation, cell migration, apoptosis, inflammation, differentiation, and angiogenesis, which indicates a possible important role in cancer [7–9]. These various effects are often the consequence of Ang II-induced activation of many cytoplasmic tyrosine kinases and transactivation of membrane associated growth factor receptor kinases [10–12].

In mammalian cells, Ang II mediates biological effects through binding to two classical angiotensin receptors. Interestingly, Ang II induces cell proliferation by activating AT1 receptors, but stimulation of the AT2 receptor inhibits cell growth in different cell types [13–15]. Analyses have shown that the AT1 mRNA level is significantly higher in prostate cancer tissue than in control normal prostate tissue [12].

The octapeptide Ang II is converted into the various shorter, active angiotensin fragments by aminopeptidases (Fig. 1). These other RA system peptides also possess important functions. The physiological actions of the bioactive angiotensin peptides depend on their chemical structure and their specificity for the receptors, the information transducers at the cellular level [16].

The aims of this study were to compare the effects of different concentrations of angiotensin peptides (Ang II, Ang III, Ang IV) on protein tyrosine kinases (PTKs) activity and viability of cells of the prostate cancer cell line, LNCaP.

Moreover, we examined the potential association of the effects of Ang II, III, IV with two types of angiotensin receptor using selective antagonists of AT1 (Losartan) and AT2 (PD 123319).

Material and methods

Cell Line

LNCaP cells were cultured in RPMI 1640 medium that was supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 1 mM Sodium Pyruvate, 10 mM Hepes Buffer, and antibiotics (Penicillin 50 U/ml; Streptomycin 50 µg/ml; Neomycin 100 µg/ml) at 37°C in a humidified atmosphere of 5% CO2 in air. These cell cultures were passaged once a week.

Reagents

Angiotensins II, III and IV were purchased from Bachem. Adamed donated a selective AT1 antagonist (Losartan). The nonpeptide AT2 antagonist, PD 123319, was from Sigma.

Phosphorylation Assay for PTK Activity

A modification of Hirano’s method for the determination of protein kinase activity was used. The most important advantage of this method is its high sensitivity.

Five-day-old LNCaP cultures were preincubated for 15 min at 37°C with angiotensin peptides (Ang II, Ang III, Ang IV) at the following concentrations: 0.05 nM,
0.5 nM, 5 nM. Combinations of angiotensins and 50 nM Losartan (specific AT1 antagonist) or 50 nM PD123319 (specific AT2 antagonist) were also tested.

Then the cells and drugs were incubated with 0.1% TRITON-X-100 for 15 min at 0°C. The standard phosphorylation assay medium contained: 20 mM TRIS/HCl pH-7.4, 20 mM MgCl2, 1 mM MnCl2, 1 mM EGTA, 0.5 mM EDTA, 0.1 M ouabain and 1 mM Na2VO4 as phosphatase inhibitors and 200 µM γ32P-ATP as a phosphate donor in the phosphorylation reaction. The reaction was started by adding treated cells to phosphorylation assay medium in the presence or absence (control group) of 200 µg/ml polyGlu, Tyr (4:1) as the substrate. After incubation in a water bath at 30°C for 7 min, the reaction was stopped by the addition of 20 µl of a mixture of 5 mM ATP and 0.25 mM EDTA. A 50 µl aliquot of reaction mixture was then spotted onto a Whatman paper disc. The discs were washed three times in cold 10% TCA with 5% sodium pyrophosphate. The 32P radioactivity was counted via Cerenkov radiation. The specific activities of PTKs were defined as pmol of radioactive 32P incorporation per mg of exogenous polyGlu,Tyr per min. The results were compared to basal activity of protein tyrosine kinase obtained from samples not containing any tested compounds (basal activity assumed to be 100%).

**MTT Assay for Cell Viability**

The principle of this assay is that MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced by the mitochondrial dehydrogenase of viable cells into dark blue formazan crystals that can be measured spectrophotometrically. The number of surviving cells is directly proportional to the level of the formazan product created.

For cell viability studies, LNCaP cells were plated at a density of 2 × 10^3 cells/well in 96-well plates and were maintained overnight in complete medium.

Briefly, LNCaP cells cultured in 96-well plates were incubated (24 h) with 5000 nM – 0.005 nM angiotensin peptides (Ang II, Ang III or Ang IV) in the presence and absence of 5000 nM Losartan or 5000 nM PD123319. The control cells were grown under the same conditions, without the addition of the compounds. After incubation, the medium was discarded and 100 µl of MTT (0.5 mg/ml PBS) was added to each well. The cells were then incubated at 37°C, in 5% CO2 for 2–3 hours. The MTT solution was removed from the wells by aspiration, and the formazan crystals were dissolved in 50–80 µl of DMSO. Plates were shaken for 2 minutes and absorbance was recorded at 620 nm. Cell survival (% of control) was calculated relative to untreated control cells.

**Statistical evaluation**

All data are expressed as mean ± SEM. Statistical significance was assessed by analysis of variance (On-Way ANOVA) for repeated measurements followed by the LSD post-hoc test to determine the extent of the differences using the StatGraphics Plus 6.0. A significance level of 5% was chosen (p < 0.05).

**Results**

**PTKs activity**

The exposure of LNCaP cells to Ang IV decreased PTK activity (Fig. 2). The activity of tyrosine kinases was significantly lower than in controls, amounting to 73.3%, 74% and 73.7% of the control value for respective concentrations: 0.05 nM, 0.5 nM, and 5 nM. In contrast, Ang II was ineffective at all tested concentrations. The effect of Ang III on kinase activity depended on concentration. This peptide slightly inhibited the tested enzyme at a concentration 0.05 nM whereas higher concentrations were ineffective.

Losartan alone (AT1 antagonist) was ineffective on Ang IV-induced changes in PTK activity (Fig. 3). In contrast, when Losartan was added together with Ang IV, the degree of phosphorylation was lower (0.05 nM) or similar (0.5 nM, 5 nM) to the effect of Ang IV alone (Fig. 3). PD123319 (AT2 antagonist) alone slightly decreased PTK activity. The combination of PD123319 and Ang IV partly reversed the inhibitory effect of Ang IV at concentrations of 0.05 nM and 0.5 nM but not 5 nM (Fig. 4).
Cell Viability

To assess the reduced enzyme activity of the cells, we used the MTT assay to evaluate the effect of angiotensin peptides on viability. Human androgen-dependent LNCaP cells were treated for 24 hours with various concentrations of Ang II, Ang III, and Ang IV (5000–0.005 nM).

The incubation of LNCaP cells with Ang IV resulted in a significant decrease in cell viability, at all tested concentrations (Fig. 5). The maximum suppression of viability relative to control cells was observed at concentrations of 5 nM and 5000 nM Ang IV, which reduced viability to 76%. The effect of Ang II and Ang III on cell viability was generally similar to the control, with no statistically significant effect on viability observed, at any concentration.

Exposure of LNCaP cells to Losartan alone (AT1 antagonist; 5000 nM) and together with Ang IV resulted in similar effects on cell growth (Fig. 6). PD 123319 (AT2 antagonist) alone decreased cell survival to 82% of the control value. The AT2 receptor inhibitor given together with Ang IV promoted survival of LNCaP cells in comparison with treatment with Ang IV alone or PD 123319 alone (Fig. 7).

Discussion

The LNCaP cell line is one of the few lines that have been most commonly used for the majority of published research on prostate cancer. This line was isolated...
Ang IV may transmit signals through tyrosine kinase domains in both normal and cancer cells [10–12]. Previous studies show that Ang II and Ang IV can modulate PTK activity in pituitary tumour cells [20]. Therefore, we decided to evaluate the effects of angiotensin peptides on PTK activity in the LNCaP cell line. The protein tyrosine kinases are a large family, which catalyze phosphorylation of specific tyrosine residues in target proteins, using ATP. These enzymes play significant roles in the development of many disease states, including cancer [21]. Furthermore, tyrosine kinase inhibitors have recently been reported as novel anticancer drugs [22].

Using an isotopic method (Hirano) we tested three concentrations of angiotensin peptides on PTK activity in LNCaP cells. In this study, we focused on rapid effects of Ang II, III, and IV, evident after a few minutes of treatment (7 min). The data revealed that only Ang IV inhibits tyrosine kinase activity at all tested concentrations. Earlier studies demonstrated that Ang IV stimulates PTK activity in pituitary tumour cells, but in normal pituitary tissue this effect was dependent on concentration [20, 23]. The Hirano technique provides global information on the level of activation of a large pool of non-receptor tyrosine kinases, for example Jak, Fak, Pyk2, and Abl. Because transcription changes usually take hours, our results indicate that angiotensins have non-genomic actions on PTK activity.

The tissue actions of angiotensins are mediated via interaction with at least two specific membrane receptor subtypes: AT1 and AT2. These classic angiotensin receptors belong to the superfamily of G-protein-coupled receptors. It is well-established that GPCRs and tyrosine kinases can cross-talk and modulate each other [24]. Here, experiments with specific inhibitors were performed to investigate the influence of Losartan (AT1 inhibitor) and PD123319 (AT2 inhibitor) on Ang IV-induced effects on PTK activity. In this study, we observed that AT1 receptor blocker PD123319 partly abolished the suppressive effect of Ang IV on PTK activity, but this effect was observed only for lower concentrations of Ang IV (0.05 nM and 0.5 nM). In contrast, a combination of Losartan with the lowest concentration of Ang IV increased the Ang IV-induced inhibitory effect. This observation suggests that Ang IV effects on PTK activity did not occur via AT1 receptor.

Because cell-to-cell signals regarding growth, differentiation, adhesion, and cell death are frequently transmitted by tyrosine kinases, we hypothesized that angiotensins (especially Ang IV) might influence cell proliferation. Lawnicka et al. reported that Ang II, as well as its smaller fragments, could decrease the growth of human hormone-independent prostate cancer cells (DU-145) in vitro [25]. Using a colourimetric method (MTT assay), we tested the effect of seven concentrations of angiotensin peptides on viability of LNCaP cells (Fig. 5). As we hypothesized, Ang IV inhibited not only the enzyme activity tested but also the viability of LNCaP cells. The molecular mechanism by which the angiotensin IV lowered the survival of these hormone-dependent prostate cancer cells remains unclear. It seems that PTKs could play an important role in supporting this process because the inhibitory effects of Ang IV on cell viability paralleled the reduced PTK activity by Ang IV in LNCaP cells. The next question is regarding the type of angiotensin receptor involved in Ang IV effects on prostate cancer cell viability. Ang IV interacts with AT1 and AT2 receptors, but only with low affinity [26]. Unexpectedly, both Losartan and PD123319 did not significantly block the inhibitory effect of Ang IV, so it seems that Ang IV effects did not occur via classic angiotensin receptors. However, the AT2 receptor antagonist PD 123319 reduced the inhibitory effect of Ang IV, but it also reduced cell viability when added alone. Hence, it is possible to speculate that the inhibitory effect of Ang IV on cell viability is exerted partly via AT1, but probably also via another subtype of angio-

![Graph showing the influence of PD123319 on Ang IV-induced effect on cell viability of prostate cancer cells LNCaP. X ± SEM](image_url)

**Figure 7.** The influence of PD123319 on Ang IV-induced effect on cell viability of prostate cancer cells LNCaP. X ± SEM

**Rycina 7.** Wpływ PD123319 na oddziaływanie Ang IV na żywotność komórek raka prostaty z linii LNCaP. X ± SEM
tensin receptor, exhibiting high sensitivity to Ang IV. Recent evidence has suggested that the high affinity binding sites for this peptide (AT_4 receptor) may be the insulin-regulated aminopeptidase (IRAP). The AT_4 binding site has been found in a variety of mammalian tissues including heart vascular smooth muscle, kidney, colon, adrenal gland, brain, and prostate. The role of the AT_4 receptor is poorly understood but it has been suggested that it may regulate blood flow, memory retention, and neuronal development [26, 27].

Pawlikowski et al. tested proliferation of prostatic epithelium cells after treatment with Ang II and Ang IV. This group of authors has also reported that angiotensins are involved in the control of prostate cell growth, acting via receptors different from AT_1. However, they observed a stimulatory effect of angiotensins on prostate cell proliferation. These findings suggest that biological properties of peptides from the angiotensin family can be different in physiological and pathological prostate tissue [28].

In the present study, we also focused on the biological role of Ang II and Ang III on cell viability in prostate cancer cells. Similar to actions on PTK activity, we did not observe significant changes in cell growth after treatment with these peptides.

Conversely, several recent reports indicate that Ang II and Ang III stimulate proliferation of prostate cells via AT_4 [11, 29–31]. It is possible to explain the lack of effect of Ang II in our study as follows. Firstly, MTT reduction in a homogenous sample of cells like the LNCaP cell line is frequently used as an indicator of cell proliferation; however, MTT reduction is proportional to the number of metabolically active cells. Therefore, in interpreting our results obtained with MTT assay we draw conclusions regarding the effects on viability rather than proliferation, because the method is based on mitochondrial function and not on DNA synthesis. Secondly, the time of incubation of LNCaP cells with Ang II was shorter than in other published studies. Uemura et al. cultured LNCaP cells in the presence of Ang II for 5 days [11], and Chow et al. for 3 days [29], whereas the incubation time in our study was only 24 hours. Alternatively, our results obtained with LNCaP cells, after treatment with Ang II and Ang III, are consistent with results for human lung cancer cells. Gallagher and Menon et al. showed that both Ang II and Ang III did not significantly affect cell proliferation in SK-LU-1 cells when treated for 24 hours [32].

Conclusions

In summary, these experimental results demonstrate that only Ang IV significantly reduced cell viability in a human hormone-dependent prostate cancer cell line after a 24-hour incubation period. Unexpectedly, both of the longer peptides (Ang II and Ang III) were ineffective. Moreover, our observations suggested that Ang IV effects did not occur via AT_1. The process seems to be mediated partly by AT_4, and probably by another type receptor with high affinity for Ang IV and low affinity for PD123319 and Losartan. At this point, we can only speculate about the mechanism by which Ang IV inhibits cell viability in LNCaP cells. However, it is possible that the tyrosine kinases are involved in the early signalling pathway of this process. Future investigations are necessary to extend these results.

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