Angiotensin I and II Stimulate Cell Invasion of SARS-CoV-2: Potential Mechanism via Inhibition of ACE2 Arm of RAS

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Summary

Angiotensin-converting enzyme 2 (ACE2), one of the key enzymes of the renin-angiotensin system (RAS), plays an important role in SARS-CoV-2 infection by functioning as a virus receptor. Angiotensin peptides Ang I and Ang II, the substrates of ACE2, can modulate the binding of SARS-CoV-2 Spike protein to the ACE2 receptor. In the present work, we found that co-incubation of HEK-ACE2 and Vero E6 cells with the SARS-CoV-2 Spike pseudovirus (PVP) resulted in stimulation of the virus entry at low and high micromolar concentrations of Ang I and Ang II, respectively. The potency of Ang I and Ang II stimulation of virus entry corresponds to their binding affinity to ACE2 catalytic pocket with 10 times higher efficiency of Ang II. The Ang II induced mild increase of PVP infectivity at 20 μM; while at 100 μM the increase (129.74±3.99 %) was highly significant ($p<0.001$). Since the angiotensin peptides act in HEK-ACE2 cells without the involvement of angiotensin type I receptors, we hypothesize that there is a steric interaction between the catalytic pocket of the ACE2 enzyme and the SARS-CoV-2 S1 binding domain. Oversaturation of the ACE2 with their angiotensin substrate might result in increased binding and entry of the SARS-CoV-2. In addition, the analysis of angiotensin peptides metabolism showed decreased ACE2 and increased ACE activity upon SARS-CoV-2 action. These effects should be taken into consideration in COVID-19 patients suffering from comorbidities such as the over-activated renin-angiotensin system as a mechanism potentially influencing the SARS-CoV-2 invasion into recipient cells.

Key words

Angiotensin peptide I/II (Ang I/Ang II) • Pseudoviral particles (PVP) • Angiotensin-converting enzyme 2 (ACE2) • Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

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Introduction

ACE2, a zinc metallopeptidase, plays a crucial role in SARS-CoV-2 infection by functioning as a divergent molecule [1]. On the one hand, it represents the viral receptor facilitating the invasion of SARS-CoV-2. On the other hand, the ACE2 generates angiotensin 1-7 (Ang 1-7) which binds to the Mas receptor and counter-regulates the angiotensin II (Ang II) - stimulated harmful arm of renin-angiotensin system (RAS) [2-4]. ACE2 splits Ang II, a potent vasoconstrictor, to produce the vasodilatory, antifibrotic, and anti-inflammatory peptide Ang 1-7 [5-7]. ACE2 also cleaves angiotensin I (Ang I), a precursor of Ang II, however with much lower efficiency due to its 300-fold reduced binding affinity to Ang I compared to Ang II [8].

The binding of SARS-CoV-2 S1 *via* RBD seems to be localized above the cleft of the catalytic pocket of the ACE2 [9-11]. *In vitro*, binding of the SARS-CoV-2 S protein to the extracellular catalytic region of human ACE2 enhanced the enzymatic activity [12]. Comparing the structure of ACE2 in the presence and absence of S protein revealed that the S protein initiated a conformation change to energetically facilitate ACE2 catalysis. One of the reasons is that binding of SARS-CoV-2 RBD to ACE2 moves several amino-acid residues in the substrate binding pocket closer to the aligned Ang II substrate [12].

It is known, that patients with COVID-19 have an unbalanced RAS with increased plasma ACE2, elevated or decreased concentration of Ang 1-7 and elevated or decreased concentration of Ang II depending on the phase of infection [13-16]. Despite that, Alberto Zangrillo's group [17] published a paper dealing with the use of Ang II infusion in patients with COVID-19-associated vasodilatory shock. Zangrillo hypothesized that after Ang II binds to ACE2, it causes such conformational changes in the enzyme molecule that reduce the binding of SARS-CoV-2 S1 to the cell [18]. In addition, the binding of Ang II itself to the catalytic domain of ACE2 might sterically prevent access of the S1 subunit of S protein to ACE2 and delay the virus invasion.

The hypothetical interaction between Ang II binding to the catalytic domain of ACE2 and SARS-CoV-2 infection was not investigated yet. Therefore, we have studied the effect of Ang I and Ang II on the invasion of SARS-CoV-2 using SARS-CoV-2 Spike Pseudotyped viruses (PVP) into Vero E6 and HEK-ACE2 cells. Our specific aim was to test whether or not the Ang I and Ang II peptides are able to stimulate the virus invasion into cells at micromolar concentrations. We also studied the effect of PVP on Ang I and Ang II metabolism and activation/inhibition of ACE2/ACE arms of RAS *in vitro*.

Methods

Cell lines

In order to prepare an efficient system for PVP production we have established cell line constitutively expressing Spike protein. The HEK-293T/17 cells (ATCC, Cat. no.: CRL-11268) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS) (GIBCO), Penicillin-Streptomycin-Amphotericin B Solution (10 ml/l, Lonza). The cells were transfected with the gene encoding for Spike protein in the expression plasmid pCMV3-SARS-CoV-2 Spike (Creative Biolabs, Cat. No.: VPLd-Wyb076) using Lipofectamine 3000 (Thermo Fisher Scientific). A few days after the transfection and the selection pressure (100 µg/ml hygromycin B, Invitrogen) we picked up several clones and screened for those with the highest expression of S protein, *via* Western blot using primary anti-S polyclonal antiserum (GeneTex Cat. No.: GTX632604) and secondary polyclonal goat anti-mouse Immunoglobulins/HRP (DAKO, Glostrup, Denmark). The same procedure was used for the generation of the ACE2 expressing cell line, specifically, the HEK293T/17 cells were transfected with pDUO2-hACE2-TMPRSS2a (InvivoGen) containing the hygromycin resistance gene. The colonies of stable transfectants (HEK-ACE2) were isolated and screened for the expression of the ACE2 protein by Western blotting using the antibody against ACE2 as described previously in Kovacech *et al.* [19].

The Vero E6 cells (ECACC Cat. No. 85020206) expressing ACE2 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5 % FBS (GIBCO) and Penicillin-Streptomycin-Amphotericin B Solution (10 ml/l, Lonza).

To visualize the ACE2 expression in situ, the HEK-ACE2 cells were plated on cover glass pre-coated with rat-tail collagen, type I (Sigma-Aldrich), fixed with 4 % paraformaldehyde and treated with rabbit monoclonal antibody against human ACE2 at 10 µg/ml (Rockland Immunochemicals), followed by incubation with goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (Invitrogen). The samples were mounted in FluoroshieldTM medium with DAPI (Sigma-Aldrich). Images were captured by LSM 710 confocal microscope (Zeiss, GmbH).

Production of SARS-CoV-2 Spike typed PVPs (PVPs)

PVPs were prepared in human embryonic kidney cells HEK-293T/17 according to the modified protocol of Millet *et al*. [20]. The modification included the establishment of a novel cell line, constitutively expressing Spike protein and its use for the production of PVPs. The stable cell line was transfected with HIV-1 NL4-3 ΔEnv Vpr Luciferase Reporter Vector [21-23]. PVPs were harvested 48-72 h after the transfection, aliquoted and stored at -80 °C. Each batch of PVPs was characterized by the determination of a specific infection

dose using HEK-ACE2 cells. To validate the responsiveness of our HEK-ACE2 cell line and final experimental data presented in this study we employed SARS-CoV-2 Pseudoviral Particles from MyBioSource (Cat. No. MBS434275), serving as experimental standard. The optimal dilution of PVPs was 1:100 for HEK-ACE2 cells and 1:4 for Vero E6 cell line. All the experiments were performed in a BSL2 laboratory.

Quantification of angiotensin peptides in cell culture

The Ang I and Ang II peptides were purchased from Sigma and stored at -20 °C until use (Cat. No. A9650, A9525). The cells were spiked with 500 µM Ang I or 100 µM Ang II and incubated with or without SARS-CoV-2 PVP for 2 h. These concentrations of Ang I and Ang II were chosen according to detection limit for determination of the small peptides in culture media. After incubation the culture medium was collected and kept frozen at -80 °C till analyzing. Angiotensin peptides Ang II, Ang 1-7, Ang 1-5, Ang III (Ang 2-8) and Ang IV (Ang 3-8) were determined in triplicates.

A combination of liquid chromatography and mass spectrometry (LC-MS/MS)-based approach was used to simultaneously quantify individual angiotensin metabolites by RAS equilibrium analysis as described previously [24,25]. The analysis was performed at Attoquant Diagnostics (Vienna, Austria). The activity of ACE2 arm of RAS was expressed as (Ang 1-7 + Ang 1-5)/Ang I and (Ang 1-7 + Ang 1-5)/Ang II ratio, respectively. The activity of aminopeptidase arm of RAS was expressed as (Ang II + Ang III + Ang IV)/Ang I and (Ang III + Ang IV)/Ang II ratio, respectively.

PVP and ATP assay

The testing procedure was adapted according to Nie *et al.* [26]. The recipient cells (HEK-ACE2) were seeded in density 4×10^4 cells per well of 96-well plate and cultured overnight in normal growth medium (DMEM, pH=7.6) supplemented with 10 % FBS. Next day, the cells were pre-incubated with peptides alone (in 50 µl of cultivation medium) for 2 h then the mixture of peptides and PVPs (in 50 µl of cultivation medium) was added to the cells. 24 h later, the peptides were added to the cells again (in 50 µl of cultivation medium). Peptides in 2 to 10-fold dilutions were always freshly prepared. The positive and negative controls (cells with or without PVPs) were included in all experiments. The cells were lysed with 70 µl of cell lysis buffer (ONE-Glo™ Luciferase Assay Buffer, Promega) 48 h post-infection and 30 µl of lysate was used to determine the luciferase activity (ONE-Glo™ Luciferase Assay, Promega). Cell viability was estimated by determination of ATP using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The luminescence was recorded using Fluoroskan Ascent® FL (Labsystems). Viability and infectivity are expressed as a mean percentage of the control (CTRL) including standard deviation (SD). Statistical analysis was performed by one-way ANOVA test using GraphPad Prism 7 Software. Results were evaluated using the Dunnett's multiple comparisons test considering multiplicity adjusted p values (*** $p<0.001$; ** $p<0.01$; * $p<0.05$) as statistically significant.

Results

The ACE2-expressing cell line was established according to the procedure described above. The important feature of HEK-ACE2 is shown in Figure 1. It is the expression of recombinant ACE2 and its localization on the cell surface as seen by the green fluorescence signal detected with laser scanning confocal microscopy (Fig. 1a). The biochemical proof of the ACE2 expression is presented by Western blot showing the molecular size of ACE2 extracted from the cells and comparison of its size with the recombinant ACE2 protein (Fig. 1b). The cells appeared to be a convenient system for the assessment of cell membrane-bound ACE2 receptor function in the context of SARS-CoV-2 cell entry. Figure 1c shows the results of the dose-response experiment, representing the infectivity of well-characterized PVPs from commercial source (MyBioSource).

The infectivity of PVPs prepared using the "in house" established HEK cell lines (clone S/3 and S/7), was comparable to those from commercial source (Fig. 1d), therefore, in all subsequent experiments we used PVPs of our own production although the final results were double checked with commercial PVPs.

To test the effect of angiotensin peptides and their potential influence on SARS-CoV-2 infectivity, we pre-incubated the cells expressing human angiotensin-converting enzyme ACE2 (HEK-ACE2 and Vero E6) with the peptides and then added PVPs to the cells.

In parallel with PVP infectivity, we monitored the viability of cells by determining the intracellular ATP level. In HEK-ACE2 model, we did not see any effect of Ang I on the PVPs cell entry up to the concentration of 100 µM. However, at 200 µM, the

increased luciferase signal was observed, which indicates the increase of PVP infectivity. As shown in the Figure 2a, the PVP infectivity was equal to 115.63±8.81 % (mean ± SD) of the control. Although mild, it is a statistically significant increase ($p<0.001$). The cell viability as determined by ATP level was slightly, however, non-significantly decreased (88.23±6.00 %). At the higher concentration of the peptide, the cell viability was strongly reduced (not shown; at 500 μM we observed a reduction up to 74.73±3.77 % of control). Ang II induced a slight however significant increase of PVPs infectivity already at the 20 μM; at 100 μM the increase (129.74±3.99 %; Fig. 2b, red line) was highly significant ($p<0.001$), while there was no cytotoxic effect observed at this concentration (Fig. 2b, blue line).

In the Vero E6 cells, naturally expressing angiotensin-converting enzyme and the Ang I receptor for angiotensin peptide processing, we showed a significantly increased infectivity of PVPs at 500 μM of Ang I (131.85±13.09 %; Fig. 2c, red line) and 100 μM of Ang II (118.62±5.35 %; Fig. 2d, red line); the increase was statistically significant ($p<0.001$) for both peptides. However, these cells appeared to be more sensitive to peptides, which resulted in a statistically significant decrease in the level of ATP as shown by the blue line in Figure 2c and 2d (Ang I: 87.35±2.50 %; Ang II: 87.89±4.91 %).

Next, we have evaluated the potential effect of PVPs on Ang I and Ang II metabolism. In HEK-ACE2 cells PVP significantly inhibited ACE2 arm (Fig. 3a) activity of RAS expressed as (Ang 1-7+Ang 1-5)/Ang II ratio (Fig. 3b). The same effect of PVP was noticed in Vero E6 cells as well (Fig. 3b). The aminopeptidase arm of RAS behaved differently. PVP slightly but significantly increased (Ang III + Ang IV)/Ang II ratio in HEK-ACE2 cells with an opposite effect seen in Vero E6 cells (Fig. 3c). When Ang I was used as a substrate for RAS the ACE2 arm activity of the RAS was inhibited by PVP in HEK-ACE2 cells (Fig. 3d). In Vero E6 cells the production of Ang 1-7 and Ang 1-5 was negligible (the ratio (Ang 1-7 + Ang 1-5)/Ang I was below 0.027). The aminopeptidase arm of RAS was upregulated in Vero E6 cells by PVP (Fig. 3e) mainly due to an increase in Ang II production (control: 22.56±2.90 vs. PVP: 36.40±3.70 μM, $p<0.007$). In HEK-ACE2 cells the production of Ang II, Ang III and Ang IV was negligible (the ratio (Ang II + Ang III + Ang IV)/Ang I was below 0.009).

The above results confirm that HEK-ACE2 cells are producing a negligible quantity of angiotensin-converting enzyme (ACE) and aminopeptidase-originated angiotensin peptides as well as a low concentration of Ang 1-5. For example, Ang II substrate is converted to approximately 97 % of Ang 1-7 and 3 % of Ang 1-5 (142.04±10.72 vs. 3.80±0.27 μM) suggesting a low level of ACE activity in HEK-ACE2 cells. On the other hand, Vero E6 cells are producing high portion of Ang II from Ang I (96 % from sum of Ang II, Ang III and Ang IV, (22.56±2.90 vs. 0.70±0.11 vs. 0.15±0.02 μM, respectively) indicating the presence of substantial amount of ACE activity. The predominant production of Ang 1-7 in HEK-ACE2 cells and Ang II in Vero E6 cells was not changed significantly by SARS-CoV-2 pseudovirus action.

Discussion

In the presented study angiotensin peptides Ang I and Ang II stimulated SARS-CoV-2 Spike typed PVPs cell entry at micromolar concentrations. The effect was moderate, however visible in both HEK-ACE2 and Vero E6 cells. Ang II was more effective than Ang I, since the effect was attained at lower concentrations of 20 μM (Ang II) vs. 200 μM (Ang I) in HEK-ACE2 cells and at 100 μM (Ang II) vs. 50 μM (Ang I) in Vero E6 cells, respectively. Difference in Ang I and Ang II effectivity roughly corresponds to binding affinity of these angiotensin peptides to ACE2 [8]. Similarly, the effect of peptides was more noticeable in HEK-ACE2 cells probably due to overexpressed ACE2 in comparison to Vero E6 cells. Since the Vero E6 cells do express endogenous AT 1 angiotensin II receptors [27], one might consider their involvement in SARS-CoV-2 pseudoviral invasion. Indeed, blockage of the AT 1 angiotensin II receptors in Vero E6 cells in angiotensin II-free system upregulated the ACE2 expression and increased SARS-CoV-2 production in infected cells [27]. On the other hand, the plasma Ang II levels are elevated in SARS-CoV-2 infected patients [12], suggesting that this peptide might have a role in viral infection *in vivo*. With regard to "*in vivo*" blockage of Ang II receptors by angiotensin receptor blockers (ARBs) the recent general conclusion is that the ARBs have either neutral or positive impact on hypertensive SARS-CoV-2 infected patients [28].

SARS-CoV-2 can enter the target cell by two mechanisms: *via* endocytosis mediated by ADAM17 where ACE2 ectodomain shedding and fusion is activated by cathepsins B/L or by membrane fusion mediated by

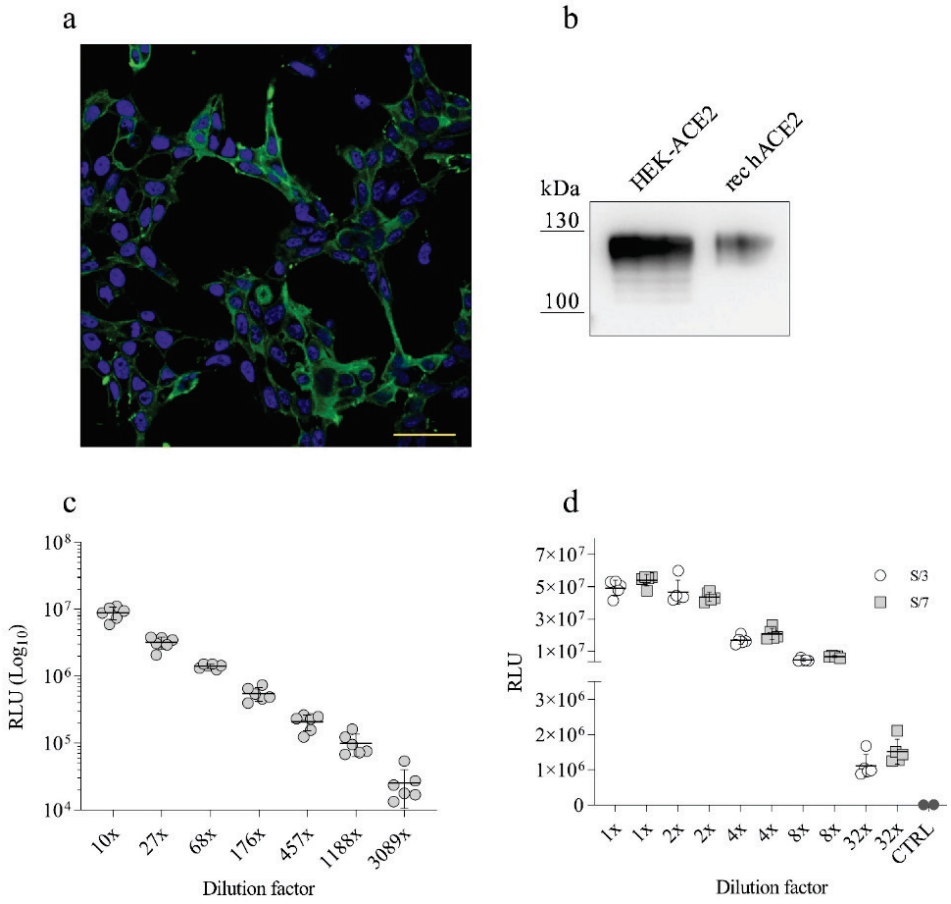

Fig. 1. Characterization and use of HEK-ACE2 stable cell line. (**a, b**) Cytochemical and biochemical features of the model. The fluorescent photomicrographs of cells demonstrate the expression of ACE2 receptor on the cell surface (green: anti-ACE2 antibody, blue: nuclei stained with DAPI, scale bar: 20 μm) and Western blot analysis confirming the expression of ACE2. (**c**) Aa relative luminescence (RLU) recorded after incubation of HEK-ACE2 cells with PVPs for 48 h (MyBioSource) in serial dilutions (n=6). (**d**) The infectivity of PVPs produced in newly established cell line clones S/3 and S/7 in the cell-entry assay (n=5).

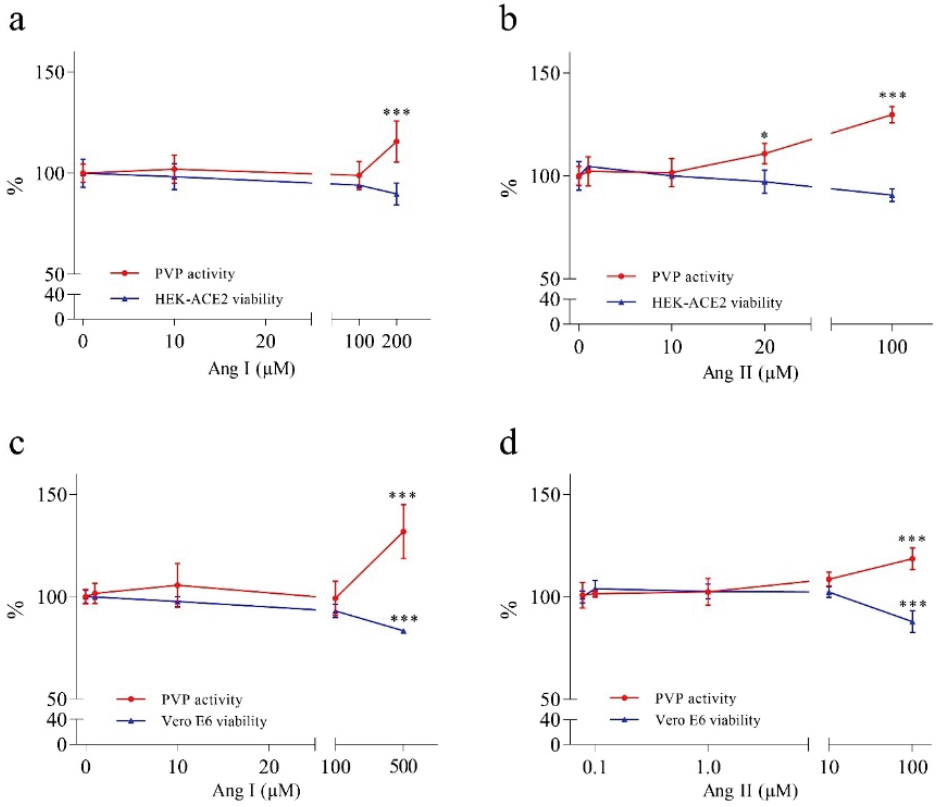

Fig. 2. The effect of Ang I and Ang II on PVP cell entry. The PVP infectivity (red line) was assessed using two cell lines (HEK-ACE2: **a**, **b** and Vero E6: **c**, **d**) at the indicated concentrations of peptides. The cell viability (blue line) was determined at the same concentrations of peptides in parallel cultures. Ang I and Ang II peptides stimulated the PVP entrance in HEK-ACE2 cells (red line, panel a, and b) at the concentration of 100 μM or higher (not shown), while the cell viability was not significantly decreased at the indicated concentrations (blue line). The increased infectivity of PVPs after the pretreatment with Ang I (c) and Ang II (d) peptides in Vero E6 cells was observed; however, these cells showed decreased viability at the corresponding concentrations (blue line) (n=6, *** $p<0.001$; ** $p<0.01$; * $p<0.05$).

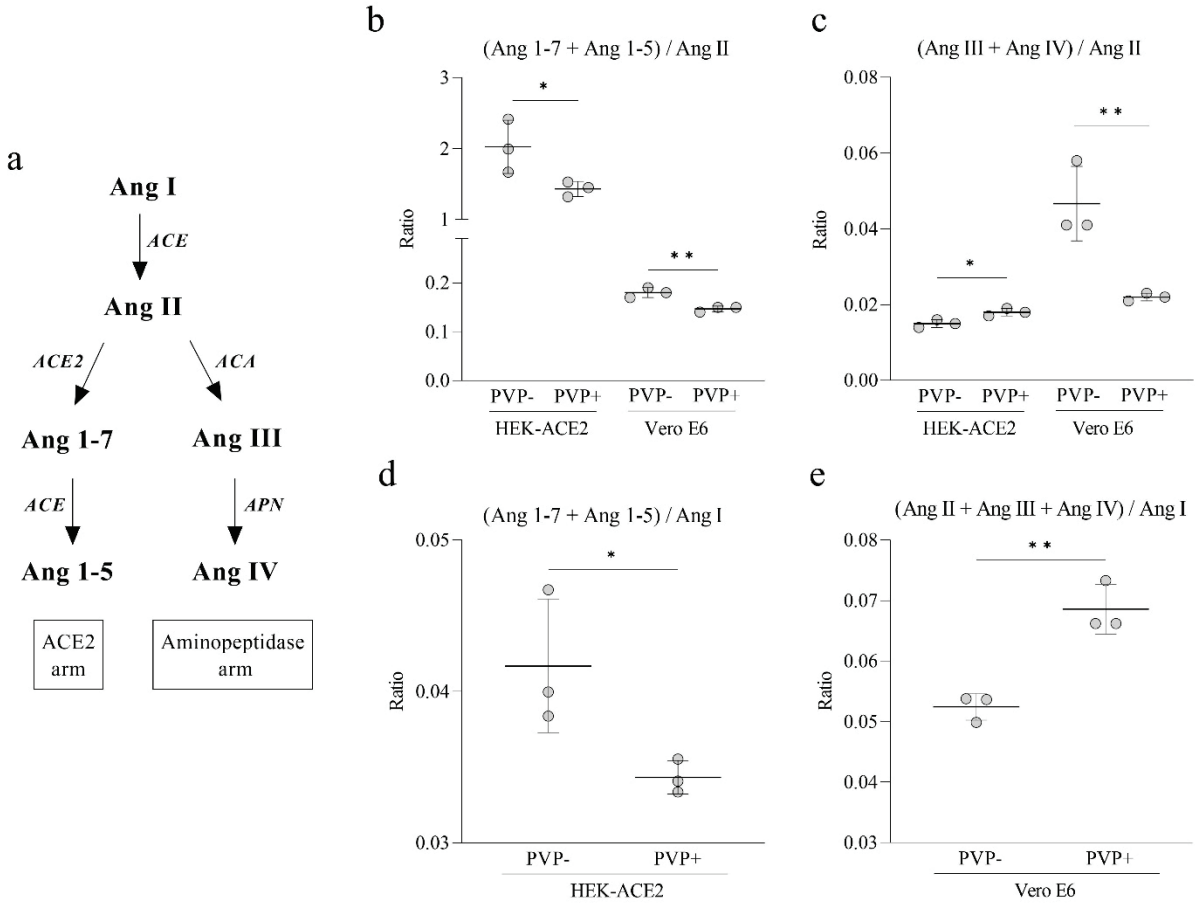

Fig. 3. *In vitro* metabolism of Ang I and Ang II in HEK-ACE2 and Vero E6 cells in absence and presence of PVP. (**a**) The expected products after the Ang I and Ang II cleavage by RAS enzymes. (**b**-**e**) Quantification of angiotensin peptides in cell culture (n=3, ** p<0.01; * p<0.05) as determined by liquid chromatography and mass spectrometry (Details are in Material and Methods).

TMPRSS2 cleavage of Spike protein [28]. Ang II stimulates ADAM17 protein expression and activity *in vitro* [29,30]. The TMPRSS2 seems to be also upregulated with RAS activity at least in the lungs [31]. The above results are in favor of Ang II stimulation of SARS-CoV-2 entry. Indeed, experiments using human kidney organoids showed that Ang II (at 1 μM) stimulated the SARS-CoV-2 infection which was blocked by ARB losartan [32].

In our experiments, the PVP cell entry was stimulated by Ang II in HEK-ACE2 cells at low micromolar concentration and in Vero E6 cells at high micromolar concentration. The PVP infectivity in Vero E6 cells was concurrent with a decrease in cell viability. It seems that the toxic effect of high micromolar concentrations of Ang II in Vero E6 cells is mediated *via* overstimulated Ang II AT1 receptors. This effect was not observed in HEK-ACE2 cells, probably due to the absence of AT1 receptor expression in these cells [33].

We suppose that angiotensin-mediated increase of PVP infectivity in HEK-ACE2 cells is due to the steric interaction between the catalytic domain of ACE2 and the virus RBD binding site. The effect of Ang II on this interaction seems to be specific, since Ang I acted approximately at 10 times higher concentration.

Regarding the effect of PVP on angiotensin peptides metabolism the obtained data from HEK-ACE2 cells clearly show that the binding of SARS-CoV-2 to ACE2 is causing an inhibition of its enzymatic activity (Fig. 3b, d). This is in accordance with previously published data on downregulation of ACE2 by SARS-CoV-2 Spike protein in different tissues [34], as well as in HEK293A, Calu-3 and Vero cells [35]. On the other hand, *in vitro* experiments with the extracellular catalytic domain of recombinant human ACE2 and SARS-CoV-2 Spike full-length and Spike RBD show activation of ACE2 activity [12,36]. It is worth noting that in above experiments the ACE2 activity was measured fluorometrically using artificial ACE2 substrates. In our experiment we used natural ACE2 substrates Ang I and Ang II and measured Ang 1-7 and Ang 1-5 as their products. In addition, the ACE2 activity was estimated in

both HEK-ACE2 and Vero E6 cells expressing different levels of RAS components. Our results confirm the predominant expression of ACE2 in HEK-ACE2 cells and ACE in Vero E6 cells. In addition, Vero E6 cells are expressing angiotensin II AT1 receptor [27] which might play a role in ACE2 internalization by forming AT1/ACE2 complex [37]. Despite of above differences in RAS component expression, SARS-CoV-2 pseudovirus downregulated ACE2 activity. In our study we do not provide direct evidence for ACE2 activity inhibition or the enzyme internalization which may happen upon SARS-CoV-2 pseudovirus action. However, the indirect evidences such as fast action of PVP and the fact that ACE2 internalization by S1 spike protein requires the presence of AT1 expression [37], which is absent in HEK cells, are in favor of enzymatic activity change.

Regarding the aminopeptidase arm of RAS in HEK-ACE2 cells we noticed a small but significant increase in (Ang III + Ang IV)/Ang II ratio upon PVP action. However, it needs to be emphasized that the measured ratios were below 0.018 (Fig. 3c) suggesting negligible contribution of aminopeptidases to metabolism of Ang II in HEK-ACE2 cells. In Vero E6 cells the aminopeptidase arm of RAS was downregulated by SARS-CoV-2 pseudovirus (Fig. 3c).

When Ang I was used as a substrate the aminopeptidase arm showed upregulation in Vero E6 cells under PVP action (Fig. 3e). This is, however, due to predominant changes in the conversion of Ang I to Ang II by ACE. These results support the stimulation of ACE activity by SARS-CoV-2 infection.

In conclusion, our experiments showed that angiotensin peptides increase the entry of PVP into the recipient cells. Moreover, we show that SARS-CoV-2 downregulates ACE2 activity and increases ACE activity. Thus, we hypothesize that there is a bidirectional interaction between the SARS-CoV-2 RBD binding site and the catalytic pocket of ACE2 as well as the ACE enzyme, leading to an enhancement of the deleterious ACE/Ang II/AT1 RAS arm and a reduction of the protective ACE2 arm.

Conflict of Interest

There is no conflict of interest.

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