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Authors:

De Hert Emilie[1], Bracke An[1], Lambeir Anne-Marie[1], Van der Veken Pieter[2], De Meester Ingrid[1,*]

[1]Laboratory of Medical Biochemistry, University of Antwerp, Antwerp, Belgium

[2]Laboratory of Medicinal Chemistry, University of Antwerp, Antwerp, Belgium

*Corresponding author:
De Meester Ingrid
Universiteitsplein 1, 2610 Antwerp, Belgium
+3232652741
ingrid.demeester@uantwerpen.be

Abstract

The renin-angiotensin system, with the octapeptide angiotensin II as key player, is important in the renal, cardiac and vascular physiology. Prolyl carboxypeptidase (PRCP), prolyl endopeptidase (PREP) and angiotensin converting enzyme 2 (ACE2) are reported to be involved in the conversion of angiotensin II to angiotensin (1-7). Previous investigations showed that the processing of angiotensin II is cell- and species-specific and little is known about its conversion in human endothelial cells. Therefore, we aimed to investigate the C-terminal processing of angiotensin II and III in comparison to the processing of des-Arg^9-bradykinin in human endothelial cells. To this end, human umbilical vein and aortic endothelial cells (HUVEC and HAoEC) were incubated with the peptides for different time periods. Mass spectrometry analysis was performed on the supernatants to check for cleavage products. Contribution of PRCP, ACE2 and PREP to the peptide cleavage was evaluated by use of the selective inhibitors compound 8o, DX600 and KYP-2047. The use of these selective inhibitors

revealed that the C-terminal cleavage of angiotensin II and III was PRCP-dependent in HUVEC and HAoEC. In contrast, the C-terminal cleavage of des-Arg^9-bradykinin was PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC. With this study, we contribute to a better understanding of the processing of peptides involved in the alternative renin-angiotensin system. We conclude that PRCP is the main enzyme for the C-terminal processing of angiotensin peptides in human umbilical vein and aortic endothelial cells. For the first time the contribution of PRCP was investigated by use of a selective PRCP-inhibitor.

Keywords

Angiotensin converting enzyme 2; des-Arg^9-bradykinin; human endothelial cells; prolyl carboxypeptidase; prolyl endopeptidase; renin-angiotensin system.

1. Introduction

The renin-angiotensin system (RAS) is important in the renal, cardiac and vascular physiology. The key player of this system is the octapeptide angiotensin (Ang) II, which executes its actions by binding on the angiotensin type 1 and 2 receptor (AT_1R, AT_2R). The AT_1R is ubiquitously expressed in adult tissues, while AT_2R expression is high in the fetus but low in adults. However, the AT_2R expression increases in cardiovascular tissue during injury or AT_1R blocking therapy [1]. Activation of the AT_1R leads to numerous physiological and pathophysiological effects like increase in blood pressure, vasoconstriction, inflammation, fibrosis and cardiac hypertrophy, while AT_2R activation leads to opposite effects. Not only Ang II, but also its metabolic products, which were first thought to be inactive, are important in the RAS. Ang 1-7, the result of C-terminal cleavage of Ang II, exerts opposite effects when binding to the Mas-receptor, like vasodilation and decrease in blood pressure, cardiac hypertrophy and fibrosis. Ang III, the result of N-terminal cleavage of Ang II, also acts on the AT_1R and plays a direct role in the secretion of aldosterone [2,3].

The conversion of Ang II to Ang (1-7) was investigated before and is attributed to prolyl carboxypeptidase (PRCP, EC 3.4.16.2), prolyl endopeptidase (PREP, EC 3.4.21.26) and angiotensin converting enzyme 2 (ACE2, EC 3.4.17.23) by biochemical *in vitro* assays with the pure enzymes [1–3]. Previous investigations using cellular and *in vivo* approaches, learn us that the processing of Ang II is very cell-, tissue- and species-specific. One of the largest limitations of previous studies, is the use of the non-selective PRCP/PREP-inhibitor, Z-Pro-Prolinal (ZPP). When ZPP is used in low concentrations, PREP is selectively inhibited (IC_{50} = 0.4 nM) [4,5]. Being a weak PRCP-inhibitor, PRCP-selective inhibition can never be reached using ZPP. IC_{50} values of ZPP for PRCP are dependent on the conditions and reported values range from 10 µM to 75 µM [6,7]. However, in the past, a lot of conclusions were based on the use of this non-selective inhibitor. Based on *ex vivo* studies with an ACE2-inhibitor and ZPP, Grobe *et al.* concluded that all three enzymes contribute to the C-terminal processing of Ang II in the mice renal cortex, but in fact ZPP was used in a concentration (10 µM) that makes interpretation of results extremely difficult [8]. In human glomerular endothelial cells, the involvement of PRCP/PREP and ACE2 was confirmed by use of inhibitors, but ZPP was used in a concentration (100 nM) that only inhibited PREP to a significant extent [9]. Kovarik *et al.* attributed the *ex vivo* C-terminal Ang II conversion in human cardiac tissue to ACE2 and PRCP, but ZPP (20 µM) was used, so involvement of PREP cannot be ruled out [10]. Using ACE2 and PREP knockout (KO) mice and PRCP gene trap mice, it was observed that ACE2 is responsible for the C-terminal conversion of Ang II in the kidney at neutral and basic pH, while PRCP catalyzes the same reaction at acidic pH. PREP did not contribute to this cleavage in the kidney [11]. Serfozo *et al.* observed that the conversion of Ang II in the circulation was PREP-dependent and ACE2- and PRCP-independent by use of a PREP KO mice model and a combined ACE2/PRCP KO mice model. From *ex vivo* studies, they concluded that the conversion of Ang II was PREP-dependent in lung lysates, by use of ZPP in a concentration

(10 µM) that gives mixed PREP/PRCP inhibition, and they showed ACE2-dependent conversion in mice kidney lysates, by use of a selective ACE2-inhibitor [12]. A recent investigation into human myocardial tissue RAS regulation concluded that Ang II processing to Ang (1-7) was mediated by PRCP and not by ACE2. However, ZPP was used in a concentration (20 mM) that inhibited both PREP and PRCP, so contribution of PREP cannot be ruled out [13,14]. Given the limitations of previous research, and the fact that the C-terminal cleavage of Ang II is only rarely investigated in human cells, we aimed to study the Ang II processing in human endothelial cells.

In this study, we investigate the processing of Ang II in two types of human endothelial cells, with a focus on the contribution of PRCP, ACE2 and PREP by use of selective inhibitors (Figure 1). We are the first to introduce the selective PRCP-inhibitor compound 8o [15] for the study of peptide conversion in human cells. To include endothelial cells of veins as well as arteries, we effected the experiments in human umbilical vein and aortic endothelial cells (HUVEC and HAoEC). We also investigated the processing of Ang III and des-Arg^9-bradykinin. Ang III is an important player in the RAS as pressor agent and is a reported substrate of PRCP [16]. As a comparator, we included des-Arg^9-bradykinin, an important mediator in the kinin-kallikrein system, which interacts with the RAS and is a reported substrate of PRCP, ACE2 and PREP [3,17–19].

2. Materials and Methods

2.1 Inhibitors

The PRCP-inhibitor compound 8o [15] was custom made in the Department of Medicinal Chemistry of the Latvian Institute of Organic Synthesis (purity > 95%). The ACE2-inhibitor DX600 [20] was obtained from BioVision (Milpitas, CA, USA). The PREP-inhibitor KYP-2047 [21,22] was synthesized in the Laboratory of Medicinal Chemistry of the University of Antwerp (UAMC).

2.2 Substrates

Ang II (Ang 1-8, DRVYIHPF), Ang III (Ang 2-8, RVYIHPF) and des-Arg^9-bradykinin (bradykinin 1-8, RPPGFSPF) were purchased from Bachem (Bubendorf, Switzerland).

2.3 Cell culture

HUVEC and HAoEC were purchased from PromoCell (Heidelberg, Germany) and Cell Applications (San Diego, CA, USA), respectively. The cells were cultured in endothelial cell growth medium (R&D Systems, Minneapolis, MN, USA) supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco (Waltham, MA,USA)) at 37 °C in 5% CO_2. Medium was replaced every 2-3 days. After reaching 70-80% confluence, cells were detached using TrypLE^{TM} Express (Life Technologies, Carlsbad, CA, USA) and counted using the Scepter^{TM} 2.0 Cell Counter with a 60 µM Scepter sensor (Merck-Millipore, Burlington, MA, USA). Passage numbers 2-8 were used for HUVEC and 2-5 for HAoEC.

2.4 Substrate cleavage in endothelial cells

Endothelial cells were seeded at a density of 10 000 cells/well in 96-well plates in 100 µL full medium. After 24 h, full medium was removed and cells were incubated with vehicle control (1% DMSO), 1 µM compound 8o, 1 µM DX600, 1 µM KYP-2047, all combinations of two inhibitors or the three inhibitors in assay medium (5% full medium in Hank's Balanced salt solution (Gibco (Waltham, MA,USA)), all 1% final DMSO concentration) for 15 min at 37 °C in 5% CO_2. This resulted in eight treatment options (control, PRCP-inhibited, ACE2-inhibited, PREP-inhibited, PRCP/ACE2-inhibited, PRCP/PREP-inhibited, ACE2/PREP-inhibited and PRCP/ACE2/PREP-inhibited cells). Then, substrate (Ang II, Ang III or des-Arg^9-bradykinin) or vehicle control (PBS) was added to the wells at a final concentration of 100 µM. The reaction was stopped by acidification (pH<3) with 0.1% trifluoroacetic acid (TFA, Alfa Aesar, Haverhill, MA, USA) after five different time periods (0 h, 2 h, 4 h, 8 h or 24 h) and samples were stored at -80 °C until further analysis. The experiment was independently conducted four

times. Concentrations of compound 8o and DX600 were based on prior experiments in HUVEC and HAoEC [23]. Viability of HUVEC and HAoEC in the presence of compound 8o or DX600 was assessed and inhibitor potency was analyzed [23,24]. The used concentration of the PREP-inhibitor KYP-2047 was based on prior publications, a concentration of 1 µM KYP-2047 was chosen to ensure that PRCP was not inhibited [22,25,26].

2.5 MALDI-TOF/TOF analysis

To investigate the cleavage of Ang II (Ang (1-8), m/z 1046.5), Ang III (Ang (2-8), m/z 931.5) and des-Arg^9-bradykinin (bradykinin (1-8), m/z 904.4), the samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight/TOF (MALDI-TOF/TOF). Hydrolysis of the substrates at the C-terminus leads to additional product peaks in the mass spectra of respectively Ang (1-7) (m/z 899.4), Ang 2-7 (m/z 784.4) and bradykinin (1-7) (m/z 757.4). Samples were desalted and concentrated using C_{18} ZipTips (Merck-Millipore, Burlington, MA, USA) and eluted directly on the MALDI target in 70% acetonitrile/0.1% TFA. Next, a MALDI matrix solution of 2.5 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Saint-Louis, MO, USA) in 70% acetonitrile/0.1% TFA was applied on each spot and air-dried at room temperature. The target was introduced into a 4800 plus MALDI TOF/TOF™ Analyzer. MS spectra (mass range: 600-4000 Da) were acquired in positive reflector mode with a laser intensity of 5000. To confirm the identity of the peptides, MS/MS spectra were generated by use of collision induced dissociation with a laser intensity of 5500. Calibration was done during analysis using the 6-peptide mixture (AB Sciex, Framingham, MA, USA). Mass spectra were generated by use of the 4000 Series Explorer (Applied Biosystems, Waltham, MA, USA) and analyzed via mMas [27]. The ratio of the peak intensity of the cleaved substrate to the peak intensity of the intact substrate is an indication for the C-terminal cleavage of the substrates. To check for additional cleavage sites, a search for other product peaks was conducted.

2.6 Cleavage by recombinant PREP in a cell-independent assay

Recombinant human PREP [28] was pre-incubated for 15 min with 1 µM KYP-2047 or vehicle control at pH 7.4 (0.1 M potassium phosphate (Jena Bioscience, Jena, Germany), 1 mM EDTA (Carl Roth, Karlsruhe, Germany) and 5 mM dithiothreitol (Fisher Scientific, Waltham, MA, USA). Substrates (Ang II, Ang III or des-Arg^9-bradykinin) or vehicle control (PBS) were added at a final concentration of 100 µM. The reaction was stopped by acidification (pH < 3) with 0.1% TFA after different time periods (0 s, 30 s, 1 min, 5 min, 15 min, 30 min or 1 h) and samples were stored at -80 °C until further processing. The samples were analyzed by MALDI-TOF/TOF as described above.

2.7 Incubation of peptides with 24 h cellular supernatant

HUVEC were seeded at a density of 10 000 cells per well in 96-well plates in 100 µL full medium. After 24 h, medium was replaced by assay medium and the cells were incubated for another 24 h at 37 °C in 5% CO_2. Subsequently, conditioned media were harvested and incubated with vehicle control (1% DMSO) or 1 µM compound 8o for 15 min at 37 °C. Then, Ang II, Ang III, des-Arg^9-bradykinin or vehicle control (PBS) was added in a final concentration of 100 µM. The reaction was stopped by acidification (pH < 3) with 0.1% TFA after 1 h and the samples were stored at -80 °C until further processing. The experiment was independently conducted four times. To detect the cleavage of Ang II, Ang III or des-Arg^9-bradykinin the samples were analyzed by MALDI-TOF/TOF as described above.

2.8 Statistical analysis

Statistical analysis was performed using SPSS software version 27 (IBM, Endicot, NY, USA). Two-way ANOVA analysis was conducted to examine the effects of the two independent variables (time point and treatment option) on the ratio of the peak intensities. For results that showed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities, simple main effects analysis was executed. $P<0.05$ was considered

as statistical significance and significant results are indicated on the graphs (Graphpad Prism 9).

3. Results

3.1 Cleavage of Ang II to Ang (1-7) is PRCP-dependent in HUVEC and HAoEC

C-terminal cleavage of Ang II to Ang (1-7) is shown in Figure 2 and is expressed as the ratio of the peak intensity of Ang (1-7) to the peak intensity of Ang II measured in the supernatant. After 24 h, the cleavage of Ang II to Ang (1-7) is significant in both types of endothelial cells, except for PRCP- and PRCP/ACE2/PREP-inhibited HUVEC and HAoEC and PRCP/ACE2-inhibited HAoEC. In PREP- and PRCP/PREP-inhibited HUVEC, the cleavage is significant after 2 h, 4 h and 24 h of incubation. Contrary to the cell supernatants, none of the peptide forms could be detected in the cell lysate under our experimental conditions.

Unexpectedly, significantly more Ang (1-7) was detected in the supernatant of HUVEC after PREP- and ACE2/PREP-inhibition in comparison with all the other treatment groups after 24 h. In comparison with the 0 h time point, significantly more Ang (1-7) was detected in the supernatant of control HUVEC after 24 h, but not in the supernatant of PRCP- or PRCP/ACE2/PREP-inhibited HUVEC. Therefore, we can assume that PRCP contributes to the cleavage of Ang II.

In HAoEC, significantly less Ang (1-7) was observed after PRCP-, PRCP/ACE2- and PRCP/ACE2/PREP-inhibition in comparison with the control after 24 h. Moreover, in these three groups, no significant cleavage could be detected. As we did not observe a significant decrease in Ang (1-7) levels after ACE2- or PREP-inhibition alone and we did not observe a more pronounced decrease after PRCP/ACE2-inhibition or inhibition with the three inhibitors in comparison with the PRCP-inhibited HAoEC, we can assume that only PRCP contributes to the C-terminal cleavage of Ang II.

The check for additional cleavage sites showed Ang (2-8) (=Ang III) as cleavage product of Ang II in both HUVEC and HAoEC, no effects of the inhibitors were seen. Figure 3A shows the MS spectrum of intact Ang II in the HUVEC supernatant at the 0 h time point. Figure 3B shows the MS spectrum of Ang II and its cleavage products Ang (1-7) and Ang (2-8) in the HUVEC supernatant after 24 h of incubation.

3.2 Cleavage of Ang III to Ang (2-7) is PRCP-dependent in HUVEC and HAoEC

C-terminal cleavage of Ang III to Ang (2-7) is shown in Figure 4 and is expressed as the ratio of the peak intensity of Ang (2-7) to the peak intensity of Ang III measured in the supernatant. The C-terminal cleavage of Ang III is significant from 2 h, 4 h or 8 h onwards, depending on the treatment, in both types of endothelial cells. In PRCP- and PRCP/ACE2-inhibited HAoEC, no significant cleavage could be detected. After 24 h, the peak of intact Ang III or fragments of the intact peptide could not be observed, probably due to full degradation of Ang III.

After 8 h, significantly less Ang (2-7) was observed in the supernatant of PRCP-, PRCP/ACE2-, PRCP/PREP- and PRCP/ACE2/PREP-inhibited HUVEC in comparison with the control. As there was no significant difference in Ang (2-7) levels between the PRCP-inhibited HUVEC and the groups where PRCP-inhibition is combined with other inhibitors, only PRCP contributes to the C-terminal cleavage of Ang III in HUVEC. Unexpectedly, from 4 h onwards significantly more Ang (2-7) was observed in the supernatant of PREP- and ACE2/PREP-inhibited HUVEC in comparison with all the other treatment groups.

After 4 h, significantly less Ang (2-7) was observed after PRCP- and PRCP/ACE2-inhibition in HAoEC in comparison with the control. After 8 h, the same was observed in all treatment groups where PRCP was inhibited. Only PRCP contributes to the C-terminal cleavage of Ang III, as there was no significant difference in Ang (2-7) levels between the PRCP-inhibited HAoEC and the groups in which PRCP-inhibition was combined with other inhibitors. As no significant cleavage was observed after 8 h in PRCP- and PRCP/ACE2-inhibited HAoEC,

PRCP is probably the only enzyme responsible for this cleavage in HAoEC. In PRCP/PREP- and PRCP/ACE2/PREP-inhibited HAoEC, there was still formation of Ang (2-7) after 8 h, this is probably due to the effect caused by PREP-inhibition. Also in these PREP-inhibited cells, significantly more Ang (2-7) was observed from 4 h onwards compared to the levels at 0 h.

The check for additional cleavage sites revealed Ang (3-8) and Ang (4-8) as cleavage products of Ang III in both HUVEC and HAoEC. After PREP-inhibition, these peptide forms were more prominently present. Figure 3C shows the MS spectrum of intact Ang III in the HUVEC supernatant at the 0 h time point. Figure 3D shows the MS spectrum of Ang III and its cleavage products Ang (2-7), Ang (3-8) and Ang (4-8) in the HUVEC supernatant after 8 h of incubation.

3.3 Cleavage of bradykinin (1-8) to bradykinin (1-7) is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC

C-terminal cleavage of bradykinin (1-8) is shown in Figure 5 and is expressed as the ratio of the peak intensity of bradykinin (1-7) to the peak intensity of bradykinin (1-8) measured in the supernatant. The C-terminal cleavage of bradykinin (1-8) was significant from 2 h, 4 h or 8 h onwards, depending on the treatment in both types of endothelial cells. In control and ACE2-inhibited HUVEC and PRCP-inhibited HAoEC, the cleavage was only significant after 24 h. In PRCP- and PRCP/ACE2-inhibited HUVEC and PRCP/ACE2- and PRCP/ACE2/PREP-inhibited HAoEC, no significant cleavage could be observed.

In HUVEC, after 24 h a significant decrease in bradykinin (1-7) was observed after PRCP- and PRCP/ACE2-inhibition in comparison with the control. Only PRCP contributes to the C-terminal cleavage of bradykinin (1-8), as we did not observe a significant decrease in bradykinin (1-7) after ACE2-inhibition alone or a more pronounced decrease after PRCP/ACE2-inhibition in comparison with PRCP-inhibition alone. Moreover, after PRCP-inhibition, no significant cleavage could be detected. We observed more bradykinin (1-7) in the supernatant of PREP-inhibited HUVEC from 2 h onwards in comparison with all the other groups. The same was

seen in the supernatant of PRCP/PREP- and ACE2/PREP-inhibited HUVEC, but less pronounced than after PREP-inhibition alone. After PRCP/PREP-inhibition or PRCP/ACE2/PREP-inhibition, we did not see a decrease in bradykinin (1-7), probably due to the interference caused by PREP-inhibition. This fact is strengthened by the observation that PRCP/PREP-inhibition caused a smaller increase in bradykinin (1-7) levels than PREP-inhibition alone.

In PRCP/ACE2-inhibited and PRCP/ACE2/PREP-inhibited HAoEC, a significant lower level of bradykinin (1-7) was observed in comparison with the control after 24 h. So we can conclude that both PRCP and ACE2 are responsible for the cleavage of bradykinin (1-8) in HAoEC and a significant decrease in bradykinin (1-7) was only observed when PRCP and ACE2 were inhibited at the same time. There was a significant difference between the ACE2-inhibited HAoEC and the PRCP/ACE2-inhibited HAoEC, but not between the PRCP-inhibited HAoEC and the PRCP/ACE2-inhibited HAoEC. Thus, PRCP contributed more to the cleavage than ACE2. Unexpectedly, PREP-inhibition caused an increase in bradykinin (1-7) in the supernatant of HAoEC at the 4 h and 8 h time points compared to the levels at 0 h.

Figure 3E shows the MS spectrum of intact bradykinin (1-8) in the HUVEC supernatant at the 0 h time point. Figure 3F shows the MS spectrum of bradykinin (1-8) and its cleavage product bradykinin (1-7) in the HUVEC supernatant after 24 h of incubation.

3.4 Ang II and des-Arg^9-bradykinin are cleaved at their C-terminus by recombinant PREP in a cell-independent assay

A cell-independent assay showed that Ang II and des-Arg^9-bradykinin were cleaved by recombinant PREP at the C-terminus in function of time. The cleavage of these substrates was abolished by 1 µM KYP-2047 (Figure 6). No other fragments could be observed. Ang III was not cleaved by recombinant PREP (Figure 6). Based on the amino acid sequence of Ang III,

one could expect cleavage by PREP. However, this finding is line with the fact that, to the best of our knowledge, PREP is never reported as an Ang III-processing enzyme.

3.5 C-terminal cleavage of Ang II, Ang III and des-Arg^9-bradykinin after incubation with the 24 h cellular supernatant

To verify whether secreted PRCP is responsible for the observed cleavages, we incubated the 24 h cellular supernatant of HUVEC with the different peptides during 1 h in the absence of cells. If secreted PRCP is responsible for the cleavage of these peptides, we should observe the formation of significant levels of the cleaved forms in this cell-free experiment. After incubation of Ang II with the 24 h cellular supernatant, Ang II is cleaved to form Ang (1-7). The ratio Ang (1-7)/Ang II is comparable with the ratio detected in the supernatant of control HUVEC at the 24 h time point in the cellular experiment. But in contrast, no effect of PRCP-inhibition was seen (Figure 7A). While in the cellular experiment all the added Ang III was degraded after 24 h, in this experiment only a part of the Ang III was converted to Ang (2-7). Contrary to Ang II, Ang III levels were influenced by PRCP-inhibition (Figure 7B). Also bradykinin (1-8) was converted to bradykinin (1-7) in this assay and an effect of PRCP-inhibition was seen (Figure 7C).

4. Discussion

The present study is, to the best of our knowledge, the first investigation into the contribution of PRCP to angiotensin cleavage in human cells by use of a selective PRCP-inhibitor. We observed that in both human umbilical vein and aortic endothelial cells, PRCP is responsible for the C-terminal cleavage of Ang II. We could not observe contribution of the two other enzymes studied, PREP and ACE2, to this cleavage in endothelial cells, although Ang II is a reported substrate of these proteases [1–3,8–10,12].

The C-terminal cleavage of Ang III is only attributed to PRCP [16,29], which can be confirmed by our experiments. We observed that cleavage by PRCP is evident after 8 h in HUVEC and after 4 h in HAoEC. In HAoEC, no significant cleavage could be observed after PRCP-inhibition, while a certain degree of Ang (2-7) formation could still be detected in HUVEC. So we assume that in HUVEC, there is another enzyme besides PRCP, contributing to the C-terminal cleavage of Ang III, while in HAoEC, PRCP is the only enzyme involved. After 24 h, the peak of intact Ang III or fragments of the intact peptide could not be observed, probably due to full degradation of Ang III. This indicates that Ang III is more rapidly converted than Ang II. The N-terminus of Ang II probably protects the peptide from rapid degradation. In the past, it was already shown that PRCP cleaved Ang III at a faster rate than Ang II *in vitro* [16,29]. The results of our study suggest that this also applies in human endothelial cells.

Our experiments suggest that the C-terminal cleavage of des-Arg^9-bradykinin is different in both types of endothelial cells. In HUVEC, we see that PRCP is responsible for this cleavage, while in HAoEC, both PRCP and ACE2 are contributing. Moreover, in HAoEC, a significant decrease in bradykinin (1-7) was only observed when both PRCP and ACE2 were inhibited at the same time. As in PRCP-inhibited HUVEC and PRCP/ACE2-inhibited HAoEC, no significant levels of bradykinin (1-7) could be observed, we assume that no other enzymes are involved in this cleavage. Both enzymes were described as bradykinin-processing enzymes before [3,30,31]. Although PREP is also described as a bradykinin-cleaving enzyme, we could not observe any contribution of PREP in these endothelial cells [19]. On the contrary, we see an increase in bradykinin (1-7) levels after PREP-inhibition in both types of endothelial cells.

The search for other cleavage sites showed that Ang II is N-terminally cleaved to form Ang (2-8) (= Ang III). In the past, this cleavage was attributed to aminopeptidase A [32], an enzyme that was not studied here. We also observed the formation of Ang (3-8) and Ang (4-8) from Ang III. These peptide forms were more prominently present after PREP-inhibition. Ang (3-8)

is a known angiotensin form, also named Ang IV. In human glomerular endothelial cells, this cleavage was also observed and shown to be inhibited by aminopeptidase N-inhibitor bestatin [9].

To our surprise, PREP-inhibition caused an increase in levels of many cleaved peptide forms. The mechanism for this phenomenon remains to be clarified. As we found the results after PREP-inhibition with KYP-2047 rather intriguing, we set-up a cell-independent assay. As expected, we detected C-terminal cleavage of Ang II and des-Arg^9-bradykinin and the hydrolysis of these peptides was abolished by KYP-2047. Apart from Ang (1-7) and bradykinin (1-7), no other fragments were found. This cell-independent assay learns us that the increase in cleaved peptide forms upon PREP-inhibition with KYP-2047 is a cellular phenomenon observed in endothelial cells. There is evidence that PREP is involved in the regulation of autophagy and that KYP-2047 acts as an autophagy inducer [33], thus indirectly affecting peptide and protein degradation in general. The increase in cleaved peptide forms seen after PREP-inhibition, can be a consequence of this effect. Our results offer the perspective to study PREP during autophagy induction in endothelial cells. Furthermore, off-target effects of KYP-2047 cannot be ruled out, although extensive selectivity testing was carried out in the past [21,22]. We tested the ability of KYP-2047 to inhibit angiotensin converting enzyme (ACE, EC 3.4.15.1), an enzyme that is involved in angiotensin peptide turnover, as this could possibly clarify the intriguing results after inhibition with KYP-2047. However, incubation of recombinant human ACE with 1 µM KYP-2047 did not alter its activity (data not shown).

Our findings clearly show that the enzymes involved in the processing of peptides differ between HUVEC and HAoEC. Angiotensin peptide processing is very cell- and tissue-specific and even differs between very related cell types. Previous investigations already pointed in that direction [8–10,12]. For example, Grobe *et al.* detected that the conversion of Ang II to Ang (1-7) was favored over the formation of Ang III in the mice renal cortex, while the opposite

was observed in the medulla. When low concentrations of Ang II (up to 100 µM) were incubated with the kidney tissue for short incubation times (up to 5 min), Ang (1-7) formation was inhibited by the ACE2-inhibitor MLN-4760. When larger concentrations of Ang II (up to 1 mM) were incubated with the kidney tissue for longer incubation times (up to 15 min), Ang (1-7) formation was inhibited by ZPP [8]. By use of ACE2 KO mice, it was later confirmed that the *in situ* generation of renal Ang 1-7 is dependent on Ang II concentrations and incubation time [11]. Moreover, it was observed that the Ang II processing was dependent on pH. Generation of Ang (1-7) was detected from pH 4-9 in kidney homogenates of WT and ACE2 KO after *ex vivo* incubation with Ang II. At pH 4 and 5, no significant differences in Ang (1-7) formation were observed between kidney homogenates of WT and ACE2 KO mice, while at pH 6-9, Ang (1-7) formation was significantly decreased in ACE2 KO mice. At high Ang II concentrations or at pH 5, the formation of Ang (1-7) from Ang II was significantly inhibited by ZPP in WT and ACE2 KO mice. Further studies in PREP KO and PRCP gene trap mice showed that PRCP was responsible for the Ang (1-7) formation in the kidney at low pH, while PREP did not contribute to this cleavage [11]. Serfozo *et al.* observed that the conversion of Ang II in the circulation was PREP-dependent and ACE2- and PRCP-independent by use of KO mice models. Moreover, they showed PREP-dependent conversion of Ang II in lung lysates and ACE2-dependent conversion in kidney lysates [12]. Also in human cells differences in Ang II processing were already observed. In human glomerular endothelial and mesangial cells the formation of Ang (1-7) was favored, while in human podocytes Ang III formation was preferred [9]. In human myocardial tissue of end-stage heart failure patients, ACE2-inhibition with MLN-4760 did not affect C-terminal Ang II conversion, while PREP/PRCP-inhibition with ZPP did [13]. Apart from differences in enzyme expression and/or activity, mechanisms of peptide uptake may vary between cell types and, in certain cases, the peptide and peptidase may simply

not encounter each other because they are in different cellular compartments or the ideal circumstances for cleavage (e.g. pH) are not met.

A remarkable observation of this research is the fact that the effect of PRCP- and ACE2-inhibitors only became significant after a longer incubation time of 8 h or 24 h. Therefore, it can be hypothesized that the angiotensin and bradykinin peptides are first taken up into the cell via their respective receptors, processed in the cell and secreted subsequently. PRCP is reported to be present in lysosomes and on the cell membrane of HUVEC. However, the PRCP activity was mainly associated with the lysosomal fraction and PRCP was enriched from this fraction in HUVEC [34]. PRCP's lysosomal location was confirmed by our research group using immunofluorescence and PRCP activity levels were measured in both types of endothelial cells [23]. Therefore, we assume that PRCP-mediated cleavage takes place in the cells rather than on the cell membrane. Furthermore, lysosomes are a perfect environment for PRCP-mediated processing given the acidic pH optimum of PRCP. It is also possible that PRCP is only secreted after longer incubation times and that the cleavage of the peptides occurs in the extracellular environment after PRCP secretion. Incubation of the peptides with the 24 h cellular supernatant showed that the peptides are also cleaved outside the cells. Ang III and des-Arg^9-bradykinin cleavage was partially inhibited by compound 8o, while Ang II cleavage was not. These findings show that secreted PRCP and other secreted enzymes are capable of cleaving these peptides, but this does not exclude that the peptides are cleaved inside the cell as well. As ACE2 is a transmembrane enzyme, it is difficult to explain why ACE2-mediated cleavage is not observed earlier. Maybe the interaction of the peptides with their receptor is more favored than the interaction with ACE2. Moreover, ACE2 activity is low in HUVEC and HAoEC [23].

A limitation of this study is that a cell culture environment may not reflect an *in vivo* setting. Moreover, we had to add the peptides exogenously to the cells, as their endogenous concentrations were too low to detect cleavage patterns. As we use a semi-quantitative method,

we can conclude that PRCP is involved in the cleavage of the different peptides, but the extent to which should be further evaluated in the future. Lastly, there are some inherent limitations to the pharmacological approach to study the involvement of peptidases in peptide metabolism. No matter how extensive selectivity testing is carried out, off-target effects can never be excluded. We therefore have tried to silence the enzymes in endothelial cells, but despite great effort, we did not succeed to produce silenced cells in a reproducible manner. Nevertheless, we are convinced that this pharmacological approach contributes to a better understanding of peptide processing in human endothelial cells, especially because this is the first study using the most potent and selective PRCP-inhibitor available to investigate the involvement of PRCP in the cleavage of these peptides.

In conclusion, this study contributes to a better understanding of the processing of peptides involved in the alternative renin-angiotensin system. We report that the C-terminal cleavage of Ang II and Ang III is PRCP-dependent in HUVEC and HAoEC. The C-terminal cleavage of des-Arg^9-bradykinin is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC.

Author contributions

Emilie De Hert: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization

An Bracke: Formal analysis, Investigation, Writing – Review & Editing

Anne-Marie Lambeir: Writing – Review & Editing

Pieter Van der Veken: Resources, Writing – Review & Editing

Ingrid De Meester: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision

Acknowledgements

This work was supported by the Research Foundation Flanders (grant numbers FWO-SB 1S22417N and FWO-SBO S001017N) and by a GOA BOF 2015 grant of the University of Antwerp (No. 30729; www.uantwerp.be). We thank the Centre for Proteomics (University of Antwerp) for the use of their mass spectrometry equipment.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Cleavage pathways of angiotensin II, angiotensin III and des-arg^9-bradykinin. The contribution of PRCP, ACE2 and PREP in the cleavage of these peptides was studied in human endothelial cells by use of their respective selective inhibitors compound 8o, DX600 and KYP-2047. PRCP: prolyl carboxypeptidase; ACE2: angiotensin converting enzyme 2; PREP: prolyl endopeptidase; APA: aminopeptidase A.

Figure 2: Cleavage of Ang II to Ang (1-7) is PRCP-dependent in HUVEC and HAoEC. C-terminal cleavage of Ang II to Ang (1-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of Ang (1-7) to the peak intensity of Ang II. Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of Ang (1-7) versus Ang II ($p<0.001$ for HUVEC and $p=0.002$ for HAoEC) in both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 8 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

Figure 3: MS spectra for Ang II, Ang III and bradykinin (1-8) and their cleavage products in the HUVEC supernatant at the 0 h timepoint and after different incubation times. A) MS spectrum for intact Ang II (m/z = 1046.5) at the 0 h time point; B) MS spectrum for Ang II and its cleavage products Ang (1-7) (m/z = 899.4) and Ang (2-8) (m/z = 931.5) after 24 h of incubation; C) MS spectrum for intact Ang III (m/z = 931.5) at the 0 h time point; D) MS spectrum for Ang III and its cleavage products Ang (4-8) (m/z = 676.3), Ang (3-8) (m/z = 775.4) and Ang (2-7) (m/z = 784.4) after 8 h of incubation; E) MS spectrum for intact bradykinin (1-8) (m/z = 904.4) at the 0 h time point; F) MS spectrum for bradykinin (1-8) and its cleavage product bradykinin (1-7) (m/z = 757.4) after 24 h of incubation.

Figure 4: Cleavage of Ang III to Ang (2-7) is PRCP-dependent in HUVEC and HAoEC. C-terminal cleavage of Ang III to Ang (2-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of Ang (2-7) to the peak intensity of Ang III. Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of Ang (2-7) versus Ang III ($p<0.001$) in

both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 24 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

Figure 5: Cleavage of bradykinin (1-8) to bradykinin (1-7) is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC. C-terminal cleavage of bradykinin (1-8) to bradykinin (1-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of bradykinin (1-7) to the peak intensity of bradykinin (1-8). Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of bradykinin (1-7) versus bradykinin (1-8) ($p<0.001$) in both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 24 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from PRCP/PREP-inhibited group; e: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

Figure 6: MS spectra for Ang II, Ang III and bradykinin (1-8) and their cleavage products after incubation with recombinant PREP in presence or absence of the PREP-inhibitor KYP-2047. A) Ang II (m/z = 1046.5) is cleaved by PREP to form Ang (1-7) (m/z = 899.4); B) The cleavage of Ang II (m/z = 1046.5) to Ang (1-7) by PREP is abolished by KYP-2047; C) Ang III (m/z = 931.5) is not cleaved by PREP to form Ang (2-7) (m/z = 784.4); D) MS spectrum of Ang III in presence of PREP and KYP-2047; E) Bradykinin (1-8) (m/z = 904.4) is cleaved by PREP to form bradykinin (1-7) (m/z = 757.4); F) The cleavage of bradykinin (1-8) (m/z = 904.4) to bradykinin (1-7) (m/z = 757.4) by PREP is abolished by KYP-2047.

Figure 7: Ang II, Ang III and bradykinin are cleaved in the extracellular environment of endothelial cells. C-terminal cleavage of Ang II (A), Ang III (B) and bradykinin (1-8) (C) after incubation with the 24 h cellular supernatant of HUVEC (n=4). (A) Ang II was converted to Ang (1-7). No significant effect of PRCP-inhibition was seen (p=0.730). (B) Ang III was converted to Ang (2-7). PRCP-inhibition caused a significant decrease in Ang (2-7) formation. (C) Bradykinin (1-8) was converted to bradykinin (1-7). PRCP-inhibition caused a significant decrease in bradykinin (1-7) formation. Values are represented as mean with SD. (Two-Way ANOVA, *p<0.05, ***p<0.001).

The C-terminal cleavage of angiotensin II and III is mediated by prolyl carboxypeptidase in human umbilical vein and aortic endothelial cells

Authors:

De Hert Emilie[1], Bracke An[1], Lambeir Anne-Marie[1], Van der Veken Pieter[2], De Meester Ingrid[1,*]

[1]Laboratory of Medical Biochemistry, University of Antwerp, Antwerp, Belgium

[2]Laboratory of Medicinal Chemistry, University of Antwerp, Antwerp, Belgium

*Corresponding author:
De Meester Ingrid
Universiteitsplein 1, 2610 Antwerp, Belgium
+3232652741
ingrid.demeester@uantwerpen.be

Abstract

The renin-angiotensin system, with the octapeptide angiotensin II as key player, is important in the renal, cardiac and vascular physiology. Prolyl carboxypeptidase (PRCP), prolyl endopeptidase (PREP) and angiotensin converting enzyme 2 (ACE2) are reported to be involved in the conversion of angiotensin II to angiotensin (1-7). Previous investigations showed that the processing of angiotensin II is cell- and species-specific and little is known about its conversion in human endothelial cells. Therefore, we aimed to investigate the C-terminal processing of angiotensin II and III in comparison to the processing of des-Arg^9-bradykinin in human endothelial cells. To this end, human umbilical vein and aortic endothelial cells (HUVEC and HAoEC) were incubated with the peptides for different time periods. Mass spectrometry analysis was performed on the supernatants to check for cleavage products. Contribution of PRCP, ACE2 and PREP to the peptide cleavage was evaluated by use of the selective inhibitors compound 8o, DX600 and KYP-2047. The use of these selective inhibitors

revealed that the C-terminal cleavage of angiotensin II and III was PRCP-dependent in HUVEC and HAoEC. In contrast, the C-terminal cleavage of des-Arg^9-bradykinin was PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC. With this study, we contribute to a better understanding of the processing of peptides involved in the alternative renin-angiotensin system. We conclude that PRCP is the main enzyme for the C-terminal processing of angiotensin peptides in human umbilical vein and aortic endothelial cells. For the first time the contribution of PRCP was investigated by use of a selective PRCP-inhibitor.

Keywords

Angiotensin converting enzyme 2; des-Arg^9-bradykinin; human endothelial cells; prolyl carboxypeptidase; prolyl endopeptidase; renin-angiotensin system.

1. Introduction

The renin-angiotensin system (RAS) is important in the renal, cardiac and vascular physiology. The key player of this system is the octapeptide angiotensin (Ang) II, which executes its actions by binding on the angiotensin type 1 and 2 receptor (AT_1R, AT_2R). The AT_1R is ubiquitously expressed in adult tissues, while AT_2R expression is high in the fetus but low in adults. However, the AT_2R expression increases in cardiovascular tissue during injury or AT_1R blocking therapy [1]. Activation of the AT_1R leads to numerous physiological and pathophysiological effects like increase in blood pressure, vasoconstriction, inflammation, fibrosis and cardiac hypertrophy, while AT_2R activation leads to opposite effects. Not only Ang II, but also its metabolic products, which were first thought to be inactive, are important in the RAS. Ang 1-7, the result of C-terminal cleavage of Ang II, exerts opposite effects when binding to the Mas-receptor, like vasodilation and decrease in blood pressure, cardiac hypertrophy and fibrosis. Ang III, the result of N-terminal cleavage of Ang II, also acts on the AT_1R and plays a direct role in the secretion of aldosterone [2,3].

The conversion of Ang II to Ang (1-7) was investigated before and is attributed to prolyl carboxypeptidase (PRCP, EC 3.4.16.2), prolyl endopeptidase (PREP, EC 3.4.21.26) and angiotensin converting enzyme 2 (ACE2, EC 3.4.17.23) by biochemical *in vitro* assays with the pure enzymes [1–3]. Previous investigations using cellular and *in vivo* approaches, learn us that the processing of Ang II is very cell-, tissue- and species-specific. One of the largest limitations of previous studies, is the use of the non-selective PRCP/PREP-inhibitor, Z-Pro-Prolinal (ZPP). When ZPP is used in low concentrations, PREP is selectively inhibited (IC_{50} = 0.4 nM) [4,5]. Being a weak PRCP-inhibitor, PRCP-selective inhibition can never be reached using ZPP. IC_{50} values of ZPP for PRCP are dependent on the conditions and reported values range from 10 µM to 75 µM [6,7]. However, in the past, a lot of conclusions were based on the use of this non-selective inhibitor. Based on *ex vivo* studies with an ACE2-inhibitor and ZPP, Grobe *et al.* concluded that all three enzymes contribute to the C-terminal processing of Ang II in the mice renal cortex, but in fact ZPP was used in a concentration (10 µM) that makes interpretation of results extremely difficult [8]. In human glomerular endothelial cells, the involvement of PRCP/PREP and ACE2 was confirmed by use of inhibitors, but ZPP was used in a concentration (100 nM) that only inhibited PREP to a significant extent [9]. Kovarik *et al.* attributed the *ex vivo* C-terminal Ang II conversion in human cardiac tissue to ACE2 and PRCP, but ZPP (20 µM) was used, so involvement of PREP cannot be ruled out [10]. Using ACE2 and PREP knockout (KO) mice and PRCP gene trap mice, it was observed that ACE2 is responsible for the C-terminal conversion of Ang II in the kidney at neutral and basic pH, while PRCP catalyzes the same reaction at acidic pH. PREP did not contribute to this cleavage in the kidney [11]. Serfozo *et al.* observed that the conversion of Ang II in the circulation was PREP-dependent and ACE2- and PRCP-independent by use of a PREP KO mice model and a combined ACE2/PRCP KO mice model. From *ex vivo* studies, they concluded that the conversion of Ang II was PREP-dependent in lung lysates, by use of ZPP in a concentration

(10 µM) that gives mixed PREP/PRCP inhibition, and they showed ACE2-dependent conversion in mice kidney lysates, by use of a selective ACE2-inhibitor [12]. A recent investigation into human myocardial tissue RAS regulation concluded that Ang II processing to Ang (1-7) was mediated by PRCP and not by ACE2. However, ZPP was used in a concentration (20 mM) that inhibited both PREP and PRCP, so contribution of PREP cannot be ruled out [13,14]. Given the limitations of previous research, and the fact that the C-terminal cleavage of Ang II is only rarely investigated in human cells, we aimed to study the Ang II processing in human endothelial cells.

In this study, we investigate the processing of Ang II in two types of human endothelial cells, with a focus on the contribution of PRCP, ACE2 and PREP by use of selective inhibitors (Figure 1). We are the first to introduce the selective PRCP-inhibitor compound 8o [15] for the study of peptide conversion in human cells. To include endothelial cells of veins as well as arteries, we effected the experiments in human umbilical vein and aortic endothelial cells (HUVEC and HAoEC). We also investigated the processing of Ang III and des-Arg^9-bradykinin. Ang III is an important player in the RAS as pressor agent and is a reported substrate of PRCP [16]. As a comparator, we included des-Arg^9-bradykinin, an important mediator in the kinin-kallikrein system, which interacts with the RAS and is a reported substrate of PRCP, ACE2 and PREP [3,17–19].

2. Materials and Methods

2.1 Inhibitors

The PRCP-inhibitor compound 8o [15] was custom made in the Department of Medicinal Chemistry of the Latvian Institute of Organic Synthesis (purity > 95%). The ACE2-inhibitor DX600 [20] was obtained from BioVision (Milpitas, CA, USA). The PREP-inhibitor KYP-2047 [21,22] was synthesized in the Laboratory of Medicinal Chemistry of the University of Antwerp (UAMC).

2.2 Substrates

Ang II (Ang 1-8, DRVYIHPF), Ang III (Ang 2-8, RVYIHPF) and des-Arg^9-bradykinin (bradykinin 1-8, RPPGFSPF) were purchased from Bachem (Bubendorf, Switzerland).

2.3 Cell culture

HUVEC and HAoEC were purchased from PromoCell (Heidelberg, Germany) and Cell Applications (San Diego, CA, USA), respectively. The cells were cultured in endothelial cell growth medium (R&D Systems, Minneapolis, MN, USA) supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco (Waltham, MA,USA)) at 37 °C in 5% CO_2. Medium was replaced every 2-3 days. After reaching 70-80% confluence, cells were detached using TrypLE™ Express (Life Technologies, Carlsbad, CA, USA) and counted using the Scepter™ 2.0 Cell Counter with a 60 µM Scepter sensor (Merck-Millipore, Burlington, MA, USA). Passage numbers 2-8 were used for HUVEC and 2-5 for HAoEC.

2.4 Substrate cleavage in endothelial cells

Endothelial cells were seeded at a density of 10 000 cells/well in 96-well plates in 100 µL full medium. After 24 h, full medium was removed and cells were incubated with vehicle control (1% DMSO), 1 µM compound 8o, 1 µM DX600, 1 µM KYP-2047, all combinations of two inhibitors or the three inhibitors in assay medium (5% full medium in Hank's Balanced salt solution (Gibco (Waltham, MA,USA)), all 1% final DMSO concentration) for 15 min at 37 °C in 5% CO_2. This resulted in eight treatment options (control, PRCP-inhibited, ACE2-inhibited, PREP-inhibited, PRCP/ACE2-inhibited, PRCP/PREP-inhibited, ACE2/PREP-inhibited and PRCP/ACE2/PREP-inhibited cells). Then, substrate (Ang II, Ang III or des-Arg^9-bradykinin) or vehicle control (PBS) was added to the wells at a final concentration of 100 µM. The reaction was stopped by acidification (pH<3) with 0.1% trifluoroacetic acid (TFA, Alfa Aesar, Haverhill, MA, USA) after five different time periods (0 h, 2 h, 4 h, 8 h or 24 h) and samples were stored at -80 °C until further analysis. The experiment was independently conducted four

times. Concentrations of compound 8o and DX600 were based on prior experiments in HUVEC and HAoEC [23]. Viability of HUVEC and HAoEC in the presence of compound 8o or DX600 was assessed and inhibitor potency was analyzed [23,24]. The used concentration of the PREP-inhibitor KYP-2047 was based on prior publications, a concentration of 1 µM KYP-2047 was chosen to ensure that PRCP was not inhibited [22,25,26].

2.5 MALDI-TOF/TOF analysis

To investigate the cleavage of Ang II (Ang (1-8), m/z 1046.5), Ang III (Ang (2-8), m/z 931.5) and des-Arg^9-bradykinin (bradykinin (1-8), m/z 904.4), the samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight/TOF (MALDI-TOF/TOF). Hydrolysis of the substrates at the C-terminus leads to additional product peaks in the mass spectra of respectively Ang (1-7) (m/z 899.4), Ang 2-7 (m/z 784.4) and bradykinin (1-7) (m/z 757.4). Samples were desalted and concentrated using C_{18} ZipTips (Merck-Millipore, Burlington, MA, USA) and eluted directly on the MALDI target in 70% acetonitrile/0.1% TFA. Next, a MALDI matrix solution of 2.5 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Saint-Louis, MO, USA) in 70% acetonitrile/0.1% TFA was applied on each spot and air-dried at room temperature. The target was introduced into a 4800 plus MALDI TOF/TOF™ Analyzer. MS spectra (mass range: 600-4000 Da) were acquired in positive reflector mode with a laser intensity of 5000. To confirm the identity of the peptides, MS/MS spectra were generated by use of collision induced dissociation with a laser intensity of 5500. Calibration was done during analysis using the 6-peptide mixture (AB Sciex, Framingham, MA, USA). Mass spectra were generated by use of the 4000 Series Explorer (Applied Biosystems, Waltham, MA, USA) and analyzed via mMas [27]. The ratio of the peak intensity of the cleaved substrate to the peak intensity of the intact substrate is an indication for the C-terminal cleavage of the substrates. To check for additional cleavage sites, a search for other product peaks was conducted.

2.6 Cleavage by recombinant PREP in a cell-independent assay

Recombinant human PREP [28] was pre-incubated for 15 min with 1 µM KYP-2047 or vehicle control at pH 7.4 (0.1 M potassium phosphate (Jena Bioscience, Jena, Germany), 1 mM EDTA (Carl Roth, Karlsruhe, Germany) and 5 mM dithiothreitol (Fisher Scientific, Waltham, MA, USA). Substrates (Ang II, Ang III or des-Arg^9-bradykinin) or vehicle control (PBS) were added at a final concentration of 100 µM. The reaction was stopped by acidification (pH < 3) with 0.1% TFA after different time periods (0 s, 30 s, 1 min, 5 min, 15 min, 30 min or 1 h) and samples were stored at -80 °C until further processing. The samples were analyzed by MALDI-TOF/TOF as described above.

2.7 Incubation of peptides with 24 h cellular supernatant

HUVEC were seeded at a density of 10 000 cells per well in 96-well plates in 100 µL full medium. After 24 h, medium was replaced by assay medium and the cells were incubated for another 24 h at 37 °C in 5% CO_2. Subsequently, conditioned media were harvested and incubated with vehicle control (1% DMSO) or 1 µM compound 8o for 15 min at 37 °C. Then, Ang II, Ang III, des-Arg^9-bradykinin or vehicle control (PBS) was added in a final concentration of 100 µM. The reaction was stopped by acidification (pH < 3) with 0.1% TFA after 1 h and the samples were stored at -80 °C until further processing. The experiment was independently conducted four times. To detect the cleavage of Ang II, Ang III or des-Arg^9-bradykinin the samples were analyzed by MALDI-TOF/TOF as described above.

2.8 Statistical analysis

Statistical analysis was performed using SPSS software version 27 (IBM, Endicot, NY, USA). Two-way ANOVA analysis was conducted to examine the effects of the two independent variables (time point and treatment option) on the ratio of the peak intensities. For results that showed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities, simple main effects analysis was executed. $P<0.05$ was considered

as statistical significance and significant results are indicated on the graphs (Graphpad Prism 9).

3. Results

3.1 Cleavage of Ang II to Ang (1-7) is PRCP-dependent in HUVEC and HAoEC

C-terminal cleavage of Ang II to Ang (1-7) is shown in Figure 2 and is expressed as the ratio of the peak intensity of Ang (1-7) to the peak intensity of Ang II measured in the supernatant. After 24 h, the cleavage of Ang II to Ang (1-7) is significant in both types of endothelial cells, except for PRCP- and PRCP/ACE2/PREP-inhibited HUVEC and HAoEC and PRCP/ACE2-inhibited HAoEC. In PREP- and PRCP/PREP-inhibited HUVEC, the cleavage is significant after 2 h, 4 h and 24 h of incubation. Contrary to the cell supernatants, none of the peptide forms could be detected in the cell lysate under our experimental conditions.

Unexpectedly, significantly more Ang (1-7) was detected in the supernatant of HUVEC after PREP- and ACE2/PREP-inhibition in comparison with all the other treatment groups after 24 h. In comparison with the 0 h time point, significantly more Ang (1-7) was detected in the supernatant of control HUVEC after 24 h, but not in the supernatant of PRCP- or PRCP/ACE2/PREP-inhibited HUVEC. Therefore, we can assume that PRCP contributes to the cleavage of Ang II.

In HAoEC, significantly less Ang (1-7) was observed after PRCP-, PRCP/ACE2- and PRCP/ACE2/PREP-inhibition in comparison with the control after 24 h. Moreover, in these three groups, no significant cleavage could be detected. As we did not observe a significant decrease in Ang (1-7) levels after ACE2- or PREP-inhibition alone and we did not observe a more pronounced decrease after PRCP/ACE2-inhibition or inhibition with the three inhibitors in comparison with the PRCP-inhibited HAoEC, we can assume that only PRCP contributes to the C-terminal cleavage of Ang II.

The check for additional cleavage sites showed Ang (2-8) (=Ang III) as cleavage product of Ang II in both HUVEC and HAoEC, no effects of the inhibitors were seen. Figure 3A shows the MS spectrum of intact Ang II in the HUVEC supernatant at the 0 h time point. Figure 3B shows the MS spectrum of Ang II and its cleavage products Ang (1-7) and Ang (2-8) in the HUVEC supernatant after 24 h of incubation.

3.2 Cleavage of Ang III to Ang (2-7) is PRCP-dependent in HUVEC and HAoEC

C-terminal cleavage of Ang III to Ang (2-7) is shown in Figure 4 and is expressed as the ratio of the peak intensity of Ang (2-7) to the peak intensity of Ang III measured in the supernatant. The C-terminal cleavage of Ang III is significant from 2 h, 4 h or 8 h onwards, depending on the treatment, in both types of endothelial cells. In PRCP- and PRCP/ACE2-inhibited HAoEC, no significant cleavage could be detected. After 24 h, the peak of intact Ang III or fragments of the intact peptide could not be observed, probably due to full degradation of Ang III.

After 8 h, significantly less Ang (2-7) was observed in the supernatant of PRCP-, PRCP/ACE2-, PRCP/PREP- and PRCP/ACE2/PREP-inhibited HUVEC in comparison with the control. As there was no significant difference in Ang (2-7) levels between the PRCP-inhibited HUVEC and the groups where PRCP-inhibition is combined with other inhibitors, only PRCP contributes to the C-terminal cleavage of Ang III in HUVEC. Unexpectedly, from 4 h onwards significantly more Ang (2-7) was observed in the supernatant of PREP- and ACE2/PREP-inhibited HUVEC in comparison with all the other treatment groups.

After 4 h, significantly less Ang (2-7) was observed after PRCP- and PRCP/ACE2-inhibition in HAoEC in comparison with the control. After 8 h, the same was observed in all treatment groups where PRCP was inhibited. Only PRCP contributes to the C-terminal cleavage of Ang III, as there was no significant difference in Ang (2-7) levels between the PRCP-inhibited HAoEC and the groups in which PRCP-inhibition was combined with other inhibitors. As no significant cleavage was observed after 8 h in PRCP- and PRCP/ACE2-inhibited HAoEC,

PRCP is probably the only enzyme responsible for this cleavage in HAoEC. In PRCP/PREP- and PRCP/ACE2/PREP-inhibited HAoEC, there was still formation of Ang (2-7) after 8 h, this is probably due to the effect caused by PREP-inhibition. Also in these PREP-inhibited cells, significantly more Ang (2-7) was observed from 4 h onwards compared to the levels at 0 h.

The check for additional cleavage sites revealed Ang (3-8) and Ang (4-8) as cleavage products of Ang III in both HUVEC and HAoEC. After PREP-inhibition, these peptide forms were more prominently present. Figure 3C shows the MS spectrum of intact Ang III in the HUVEC supernatant at the 0 h time point. Figure 3D shows the MS spectrum of Ang III and its cleavage products Ang (2-7), Ang (3-8) and Ang (4-8) in the HUVEC supernatant after 8 h of incubation.

3.3 Cleavage of bradykinin (1-8) to bradykinin (1-7) is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC

C-terminal cleavage of bradykinin (1-8) is shown in Figure 5 and is expressed as the ratio of the peak intensity of bradykinin (1-7) to the peak intensity of bradykinin (1-8) measured in the supernatant. The C-terminal cleavage of bradykinin (1-8) was significant from 2 h, 4 h or 8 h onwards, depending on the treatment in both types of endothelial cells. In control and ACE2-inhibited HUVEC and PRCP-inhibited HAoEC, the cleavage was only significant after 24 h. In PRCP- and PRCP/ACE2-inhibited HUVEC and PRCP/ACE2- and PRCP/ACE2/PREP-inhibited HAoEC, no significant cleavage could be observed.

In HUVEC, after 24 h a significant decrease in bradykinin (1-7) was observed after PRCP- and PRCP/ACE2-inhibition in comparison with the control. Only PRCP contributes to the C-terminal cleavage of bradykinin (1-8), as we did not observe a significant decrease in bradykinin (1-7) after ACE2-inhibition alone or a more pronounced decrease after PRCP/ACE2-inhibition in comparison with PRCP-inhibition alone. Moreover, after PRCP-inhibition, no significant cleavage could be detected. We observed more bradykinin (1-7) in the supernatant of PREP-inhibited HUVEC from 2 h onwards in comparison with all the other groups. The same was

seen in the supernatant of PRCP/PREP- and ACE2/PREP-inhibited HUVEC, but less pronounced than after PREP-inhibition alone. After PRCP/PREP-inhibition or PRCP/ACE2/PREP-inhibition, we did not see a decrease in bradykinin (1-7), probably due to the interference caused by PREP-inhibition. This fact is strengthened by the observation that PRCP/PREP-inhibition caused a smaller increase in bradykinin (1-7) levels than PREP-inhibition alone.

In PRCP/ACE2-inhibited and PRCP/ACE2/PREP-inhibited HAoEC, a significant lower level of bradykinin (1-7) was observed in comparison with the control after 24 h. So we can conclude that both PRCP and ACE2 are responsible for the cleavage of bradykinin (1-8) in HAoEC and a significant decrease in bradykinin (1-7) was only observed when PRCP and ACE2 were inhibited at the same time. There was a significant difference between the ACE2-inhibited HAoEC and the PRCP/ACE2-inhibited HAoEC, but not between the PRCP-inhibited HAoEC and the PRCP/ACE2-inhibited HAoEC. Thus, PRCP contributed more to the cleavage than ACE2. Unexpectedly, PREP-inhibition caused an increase in bradykinin (1-7) in the supernatant of HAoEC at the 4 h and 8 h time points compared to the levels at 0 h.

Figure 3E shows the MS spectrum of intact bradykinin (1-8) in the HUVEC supernatant at the 0 h time point. Figure 3F shows the MS spectrum of bradykinin (1-8) and its cleavage product bradykinin (1-7) in the HUVEC supernatant after 24 h of incubation.

3.4 Ang II and des-Arg^9-bradykinin are cleaved at their C-terminus by recombinant PREP in a cell-independent assay

A cell-independent assay showed that Ang II and des-Arg^9-bradykinin were cleaved by recombinant PREP at the C-terminus in function of time. The cleavage of these substrates was abolished by 1 µM KYP-2047 (Figure 6). No other fragments could be observed. Ang III was not cleaved by recombinant PREP (Figure 6). Based on the amino acid sequence of Ang III,

one could expect cleavage by PREP. However, this finding is line with the fact that, to the best of our knowledge, PREP is never reported as an Ang III-processing enzyme.

3.5 C-terminal cleavage of Ang II, Ang III and des-Arg^9-bradykinin after incubation with the 24 h cellular supernatant

To verify whether secreted PRCP is responsible for the observed cleavages, we incubated the 24 h cellular supernatant of HUVEC with the different peptides during 1 h in the absence of cells. If secreted PRCP is responsible for the cleavage of these peptides, we should observe the formation of significant levels of the cleaved forms in this cell-free experiment. After incubation of Ang II with the 24 h cellular supernatant, Ang II is cleaved to form Ang (1-7). The ratio Ang (1-7)/Ang II is comparable with the ratio detected in the supernatant of control HUVEC at the 24 h time point in the cellular experiment. But in contrast, no effect of PRCP-inhibition was seen (Figure 7A). While in the cellular experiment all the added Ang III was degraded after 24 h, in this experiment only a part of the Ang III was converted to Ang (2-7). Contrary to Ang II, Ang III levels were influenced by PRCP-inhibition (Figure 7B). Also bradykinin (1-8) was converted to bradykinin (1-7) in this assay and an effect of PRCP-inhibition was seen (Figure 7C).

4. Discussion

The present study is, to the best of our knowledge, the first investigation into the contribution of PRCP to angiotensin cleavage in human cells by use of a selective PRCP-inhibitor. We observed that in both human umbilical vein and aortic endothelial cells, PRCP is responsible for the C-terminal cleavage of Ang II. We could not observe contribution of the two other enzymes studied, PREP and ACE2, to this cleavage in endothelial cells, although Ang II is a reported substrate of these proteases [1–3,8–10,12].

The C-terminal cleavage of Ang III is only attributed to PRCP [16,29], which can be confirmed by our experiments. We observed that cleavage by PRCP is evident after 8 h in HUVEC and after 4 h in HAoEC. In HAoEC, no significant cleavage could be observed after PRCP-inhibition, while a certain degree of Ang (2-7) formation could still be detected in HUVEC. So we assume that in HUVEC, there is another enzyme besides PRCP, contributing to the C-terminal cleavage of Ang III, while in HAoEC, PRCP is the only enzyme involved. After 24 h, the peak of intact Ang III or fragments of the intact peptide could not be observed, probably due to full degradation of Ang III. This indicates that Ang III is more rapidly converted than Ang II. The N-terminus of Ang II probably protects the peptide from rapid degradation. In the past, it was already shown that PRCP cleaved Ang III at a faster rate than Ang II *in vitro* [16,29]. The results of our study suggest that this also applies in human endothelial cells.

Our experiments suggest that the C-terminal cleavage of des-Arg^9-bradykinin is different in both types of endothelial cells. In HUVEC, we see that PRCP is responsible for this cleavage, while in HAoEC, both PRCP and ACE2 are contributing. Moreover, in HAoEC, a significant decrease in bradykinin (1-7) was only observed when both PRCP and ACE2 were inhibited at the same time. As in PRCP-inhibited HUVEC and PRCP/ACE2-inhibited HAoEC, no significant levels of bradykinin (1-7) could be observed, we assume that no other enzymes are involved in this cleavage. Both enzymes were described as bradykinin-processing enzymes before [3,30,31]. Although PREP is also described as a bradykinin-cleaving enzyme, we could not observe any contribution of PREP in these endothelial cells [19]. On the contrary, we see an increase in bradykinin (1-7) levels after PREP-inhibition in both types of endothelial cells.

The search for other cleavage sites showed that Ang II is N-terminally cleaved to form Ang (2-8) (= Ang III). In the past, this cleavage was attributed to aminopeptidase A [32], an enzyme that was not studied here. We also observed the formation of Ang (3-8) and Ang (4-8) from Ang III. These peptide forms were more prominently present after PREP-inhibition. Ang (3-8)

is a known angiotensin form, also named Ang IV. In human glomerular endothelial cells, this cleavage was also observed and shown to be inhibited by aminopeptidase N-inhibitor bestatin [9].

To our surprise, PREP-inhibition caused an increase in levels of many cleaved peptide forms. The mechanism for this phenomenon remains to be clarified. As we found the results after PREP-inhibition with KYP-2047 rather intriguing, we set-up a cell-independent assay. As expected, we detected C-terminal cleavage of Ang II and des-Arg^9-bradykinin and the hydrolysis of these peptides was abolished by KYP-2047. Apart from Ang (1-7) and bradykinin (1-7), no other fragments were found. This cell-independent assay learns us that the increase in cleaved peptide forms upon PREP-inhibition with KYP-2047 is a cellular phenomenon observed in endothelial cells. There is evidence that PREP is involved in the regulation of autophagy and that KYP-2047 acts as an autophagy inducer [33], thus indirectly affecting peptide and protein degradation in general. The increase in cleaved peptide forms seen after PREP-inhibition, can be a consequence of this effect. Our results offer the perspective to study PREP during autophagy induction in endothelial cells. Furthermore, off-target effects of KYP-2047 cannot be ruled out, although extensive selectivity testing was carried out in the past [21,22]. We tested the ability of KYP-2047 to inhibit angiotensin converting enzyme (ACE, EC 3.4.15.1), an enzyme that is involved in angiotensin peptide turnover, as this could possibly clarify the intriguing results after inhibition with KYP-2047. However, incubation of recombinant human ACE with 1 µM KYP-2047 did not alter its activity (data not shown).

Our findings clearly show that the enzymes involved in the processing of peptides differ between HUVEC and HAoEC. Angiotensin peptide processing is very cell- and tissue-specific and even differs between very related cell types. Previous investigations already pointed in that direction [8–10,12]. For example, Grobe *et al.* detected that the conversion of Ang II to Ang (1-7) was favored over the formation of Ang III in the mice renal cortex, while the opposite

was observed in the medulla. When low concentrations of Ang II (up to 100 µM) were incubated with the kidney tissue for short incubation times (up to 5 min), Ang (1-7) formation was inhibited by the ACE2-inhibitor MLN-4760. When larger concentrations of Ang II (up to 1 mM) were incubated with the kidney tissue for longer incubation times (up to 15 min), Ang (1-7) formation was inhibited by ZPP [8]. By use of ACE2 KO mice, it was later confirmed that the *in situ* generation of renal Ang 1-7 is dependent on Ang II concentrations and incubation time [11]. Moreover, it was observed that the Ang II processing was dependent on pH. Generation of Ang (1-7) was detected from pH 4-9 in kidney homogenates of WT and ACE2 KO after *ex vivo* incubation with Ang II. At pH 4 and 5, no significant differences in Ang (1-7) formation were observed between kidney homogenates of WT and ACE2 KO mice, while at pH 6-9, Ang (1-7) formation was significantly decreased in ACE2 KO mice. At high Ang II concentrations or at pH 5, the formation of Ang (1-7) from Ang II was significantly inhibited by ZPP in WT and ACE2 KO mice. Further studies in PREP KO and PRCP gene trap mice showed that PRCP was responsible for the Ang (1-7) formation in the kidney at low pH, while PREP did not contribute to this cleavage [11]. Serfozo *et al.* observed that the conversion of Ang II in the circulation was PREP-dependent and ACE2- and PRCP-independent by use of KO mice models. Moreover, they showed PREP-dependent conversion of Ang II in lung lysates and ACE2-dependent conversion in kidney lysates [12]. Also in human cells differences in Ang II processing were already observed. In human glomerular endothelial and mesangial cells the formation of Ang (1-7) was favored, while in human podocytes Ang III formation was preferred [9]. In human myocardial tissue of end-stage heart failure patients, ACE2-inhibition with MLN-4760 did not affect C-terminal Ang II conversion, while PREP/PRCP-inhibition with ZPP did [13]. Apart from differences in enzyme expression and/or activity, mechanisms of peptide uptake may vary between cell types and, in certain cases, the peptide and peptidase may simply

not encounter each other because they are in different cellular compartments or the ideal circumstances for cleavage (e.g. pH) are not met.

A remarkable observation of this research is the fact that the effect of PRCP- and ACE2-inhibitors only became significant after a longer incubation time of 8 h or 24 h. Therefore, it can be hypothesized that the angiotensin and bradykinin peptides are first taken up into the cell via their respective receptors, processed in the cell and secreted subsequently. PRCP is reported to be present in lysosomes and on the cell membrane of HUVEC. However, the PRCP activity was mainly associated with the lysosomal fraction and PRCP was enriched from this fraction in HUVEC [34]. PRCP's lysosomal location was confirmed by our research group using immunofluorescence and PRCP activity levels were measured in both types of endothelial cells [23]. Therefore, we assume that PRCP-mediated cleavage takes place in the cells rather than on the cell membrane. Furthermore, lysosomes are a perfect environment for PRCP-mediated processing given the acidic pH optimum of PRCP. It is also possible that PRCP is only secreted after longer incubation times and that the cleavage of the peptides occurs in the extracellular environment after PRCP secretion. Incubation of the peptides with the 24 h cellular supernatant showed that the peptides are also cleaved outside the cells. Ang III and des-Arg^9-bradykinin cleavage was partially inhibited by compound 8o, while Ang II cleavage was not. These findings show that secreted PRCP and other secreted enzymes are capable of cleaving these peptides, but this does not exclude that the peptides are cleaved inside the cell as well. As ACE2 is a transmembrane enzyme, it is difficult to explain why ACE2-mediated cleavage is not observed earlier. Maybe the interaction of the peptides with their receptor is more favored than the interaction with ACE2. Moreover, ACE2 activity is low in HUVEC and HAoEC [23].

A limitation of this study is that a cell culture environment may not reflect an *in vivo* setting. Moreover, we had to add the peptides exogenously to the cells, as their endogenous concentrations were too low to detect cleavage patterns. As we use a semi-quantitative method,

we can conclude that PRCP is involved in the cleavage of the different peptides, but the extent to which should be further evaluated in the future. Lastly, there are some inherent limitations to the pharmacological approach to study the involvement of peptidases in peptide metabolism. No matter how extensive selectivity testing is carried out, off-target effects can never be excluded. We therefore have tried to silence the enzymes in endothelial cells, but despite great effort, we did not succeed to produce silenced cells in a reproducible manner. Nevertheless, we are convinced that this pharmacological approach contributes to a better understanding of peptide processing in human endothelial cells, especially because this is the first study using the most potent and selective PRCP-inhibitor available to investigate the involvement of PRCP in the cleavage of these peptides.

In conclusion, this study contributes to a better understanding of the processing of peptides involved in the alternative renin-angiotensin system. We report that the C-terminal cleavage of Ang II and Ang III is PRCP-dependent in HUVEC and HAoEC. The C-terminal cleavage of des-Arg^9-bradykinin is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC.

Author contributions

Emilie De Hert: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization

An Bracke: Formal analysis, Investigation, Writing – Review & Editing

Anne-Marie Lambeir: Writing – Review & Editing

Pieter Van der Veken: Resources, Writing – Review & Editing

Ingrid De Meester: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision

Acknowledgements

This work was supported by the Research Foundation Flanders (grant numbers FWO-SB 1S22417N and FWO-SBO S001017N) and by a GOA BOF 2015 grant of the University of Antwerp (No. 30729; www.uantwerp.be). We thank the Centre for Proteomics (University of Antwerp) for the use of their mass spectrometry equipment.

Conflict of interest

The authors declare no conflict of interest.

References

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Figure Legends

Figure 1: Cleavage pathways of angiotensin II, angiotensin III and des-arg^9-bradykinin. The contribution of PRCP, ACE2 and PREP in the cleavage of these peptides was studied in human endothelial cells by use of their respective selective inhibitors compound 8o, DX600 and KYP-2047. PRCP: prolyl carboxypeptidase; ACE2: angiotensin converting enzyme 2; PREP: prolyl endopeptidase; APA: aminopeptidase A.

Figure 2: Cleavage of Ang II to Ang (1-7) is PRCP-dependent in HUVEC and HAoEC. C-terminal cleavage of Ang II to Ang (1-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of Ang (1-7) to the peak intensity of Ang II. Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of Ang (1-7) versus Ang II ($p<0.001$ for HUVEC and $p=0.002$ for HAoEC) in both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 8 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

Figure 3: MS spectra for Ang II, Ang III and bradykinin (1-8) and their cleavage products in the HUVEC supernatant at the 0 h timepoint and after different incubation times. A) MS spectrum for intact Ang II (m/z = 1046.5) at the 0 h time point; B) MS spectrum for Ang II and its cleavage products Ang (1-7) (m/z = 899.4) and Ang (2-8) (m/z = 931.5) after 24 h of incubation; C) MS spectrum for intact Ang III (m/z = 931.5) at the 0 h time point; D) MS spectrum for Ang III and its cleavage products Ang (4-8) (m/z = 676.3), Ang (3-8) (m/z = 775.4) and Ang (2-7) (m/z = 784.4) after 8 h of incubation; E) MS spectrum for intact bradykinin (1-8) (m/z = 904.4) at the 0 h time point; F) MS spectrum for bradykinin (1-8) and its cleavage product bradykinin (1-7) (m/z = 757.4) after 24 h of incubation.

Figure 4: Cleavage of Ang III to Ang (2-7) is PRCP-dependent in HUVEC and HAoEC. C-terminal cleavage of Ang III to Ang (2-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of Ang (2-7) to the peak intensity of Ang III. Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of Ang (2-7) versus Ang III ($p<0.001$) in

both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 24 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

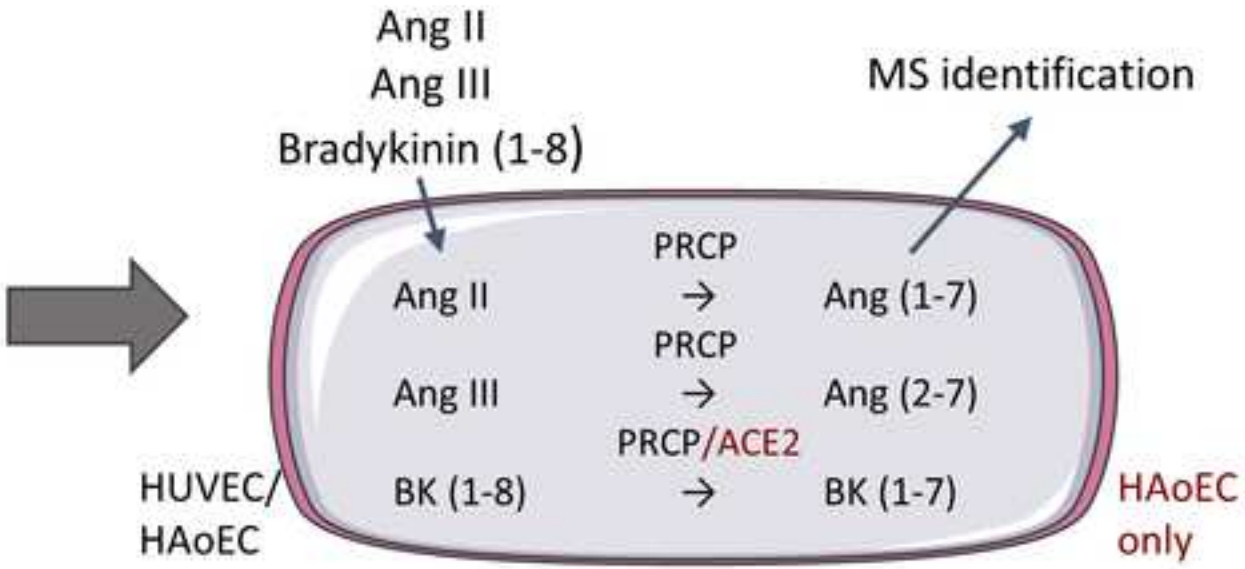
Figure 5: Cleavage of bradykinin (1-8) to bradykinin (1-7) is PRCP-dependent in HUVEC and PRCP- and ACE2-dependent in HAoEC. C-terminal cleavage of bradykinin (1-8) to bradykinin (1-7) in HUVEC (A) and HAoEC (B) (n=4 per group), expressed as the ratio of the peak intensity of bradykinin (1-7) to the peak intensity of bradykinin (1-8). Two-way ANOVA analysis revealed a significant interaction between the effects of time point and treatment option on the ratio of the peak intensities of bradykinin (1-7) versus bradykinin (1-8) ($p<0.001$) in both types of endothelial cells. Values are represented as mean with SD. Significant p-values (at least $p<0.05$) are indicated on the graph (α: different from 0 h; β: different from 2 h; γ: different from 4 h; δ: different from 24 h; a: different from control; b: different from ACE2-inhibited group; c: different from PREP-inhibited group; d: different from PRCP/PREP-inhibited group; e: different from ACE2/PREP-inhibited group; within treatment group and time point group respectively).

Figure 6: MS spectra for Ang II, Ang III and bradykinin (1-8) and their cleavage products after incubation with recombinant PREP in presence or absence of the PREP-inhibitor KYP-2047. A) Ang II (m/z = 1046.5) is cleaved by PREP to form Ang (1-7) (m/z = 899.4); B) The cleavage of Ang II (m/z = 1046.5) to Ang (1-7) by PREP is abolished by KYP-2047; C) Ang III (m/z = 931.5) is not cleaved by PREP to form Ang (2-7) (m/z = 784.4); D) MS spectrum of Ang III in presence of PREP and KYP-2047; E) Bradykinin (1-8) (m/z = 904.4) is cleaved by PREP to form bradykinin (1-7) (m/z = 757.4); F) The cleavage of bradykinin (1-8) (m/z = 904.4) to bradykinin (1-7) (m/z = 757.4) by PREP is abolished by KYP-2047.

Figure 7: Ang II, Ang III and bradykinin are cleaved in the extracellular environment of endothelial cells. C-terminal cleavage of Ang II (A), Ang III (B) and bradykinin (1-8) (C) after incubation with the 24 h cellular supernatant of HUVEC (n=4). (A) Ang II was converted to Ang (1-7). No significant effect of PRCP-inhibition was seen (p=0.730). (B) Ang III was converted to Ang (2-7). PRCP-inhibition caused a significant decrease in Ang (2-7) formation. (C) Bradykinin (1-8) was converted to bradykinin (1-7). PRCP-inhibition caused a significant decrease in bradykinin (1-7) formation. Values are represented as mean with SD. (Two-Way ANOVA, *p<0.05, ***p<0.001).

Use of selective inhibitors to investigate the C-terminal processing of angiotensins and kinin in endothelial cells:

Compound 8o ⊣ PRCP
DX600 ⊣ ACE2
KYP-2047 ⊣ PREP



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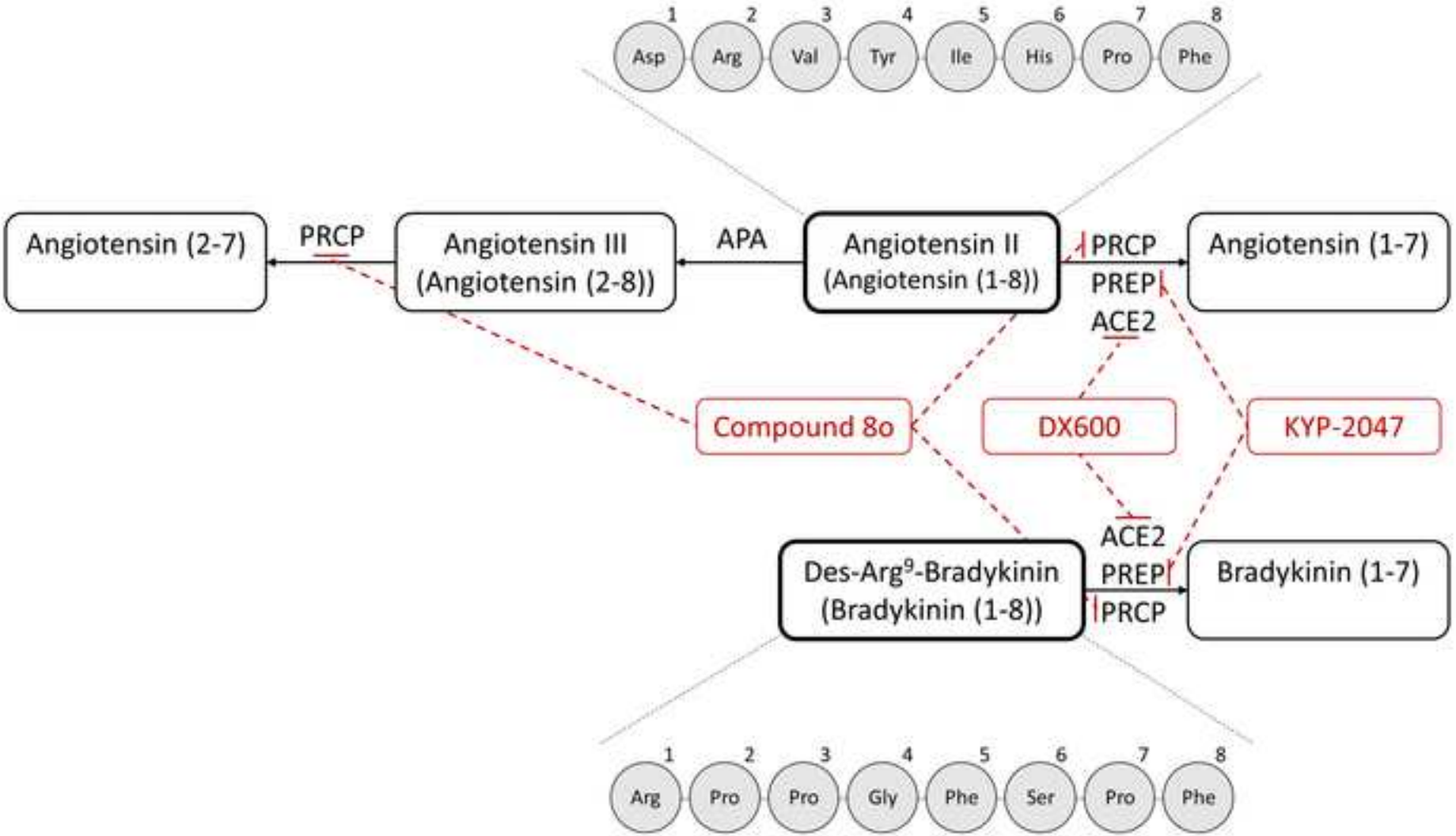

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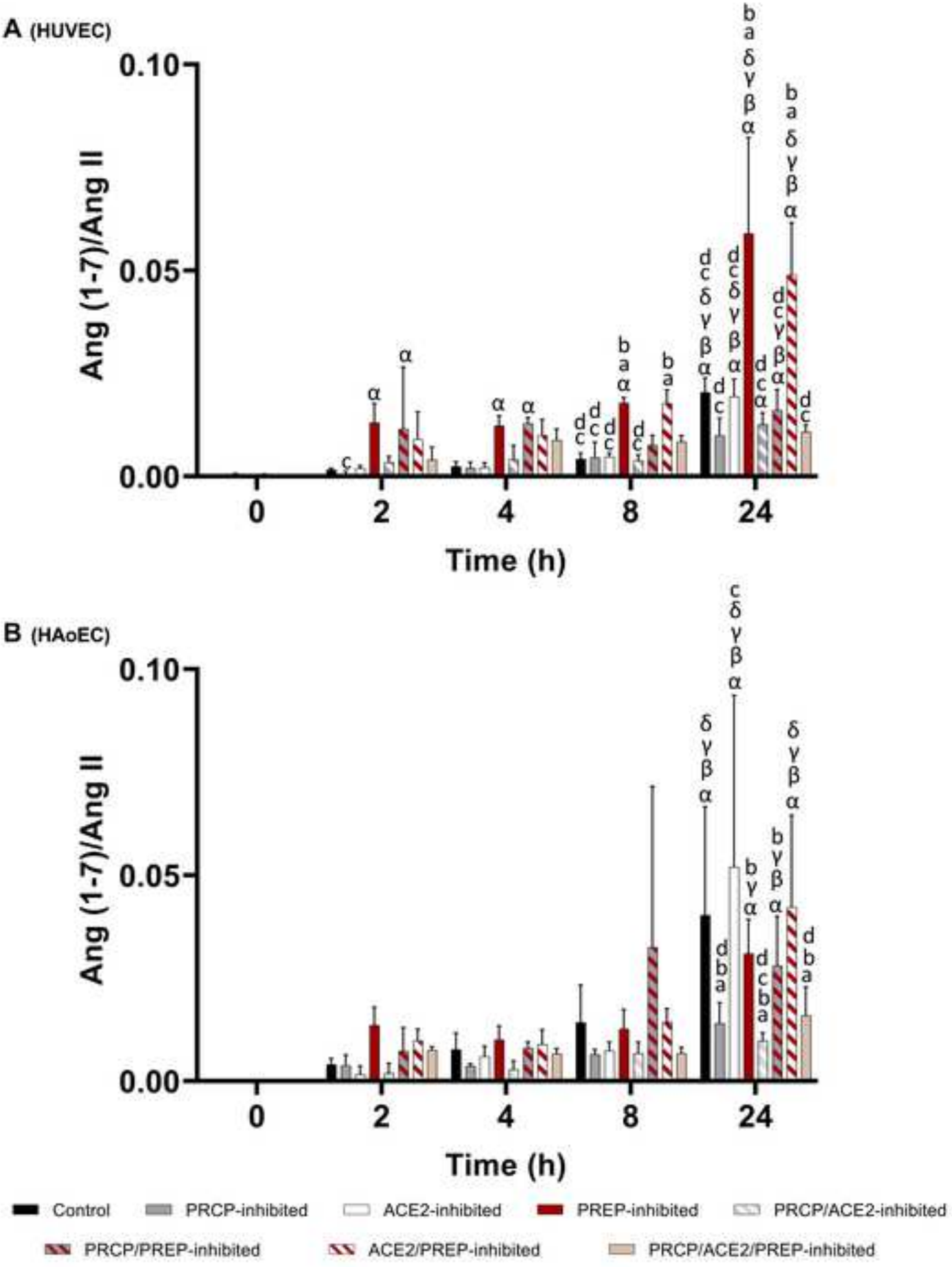

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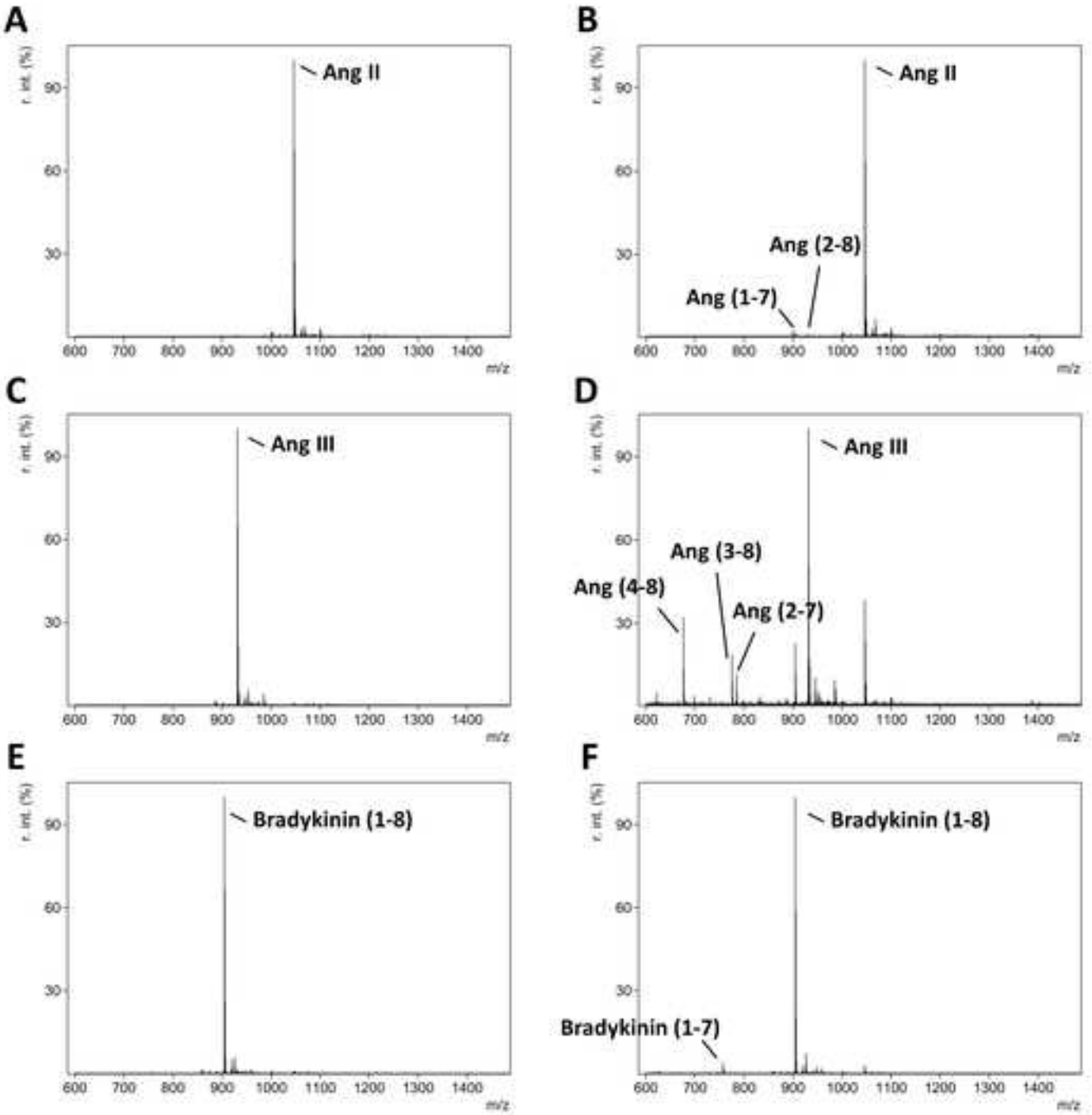

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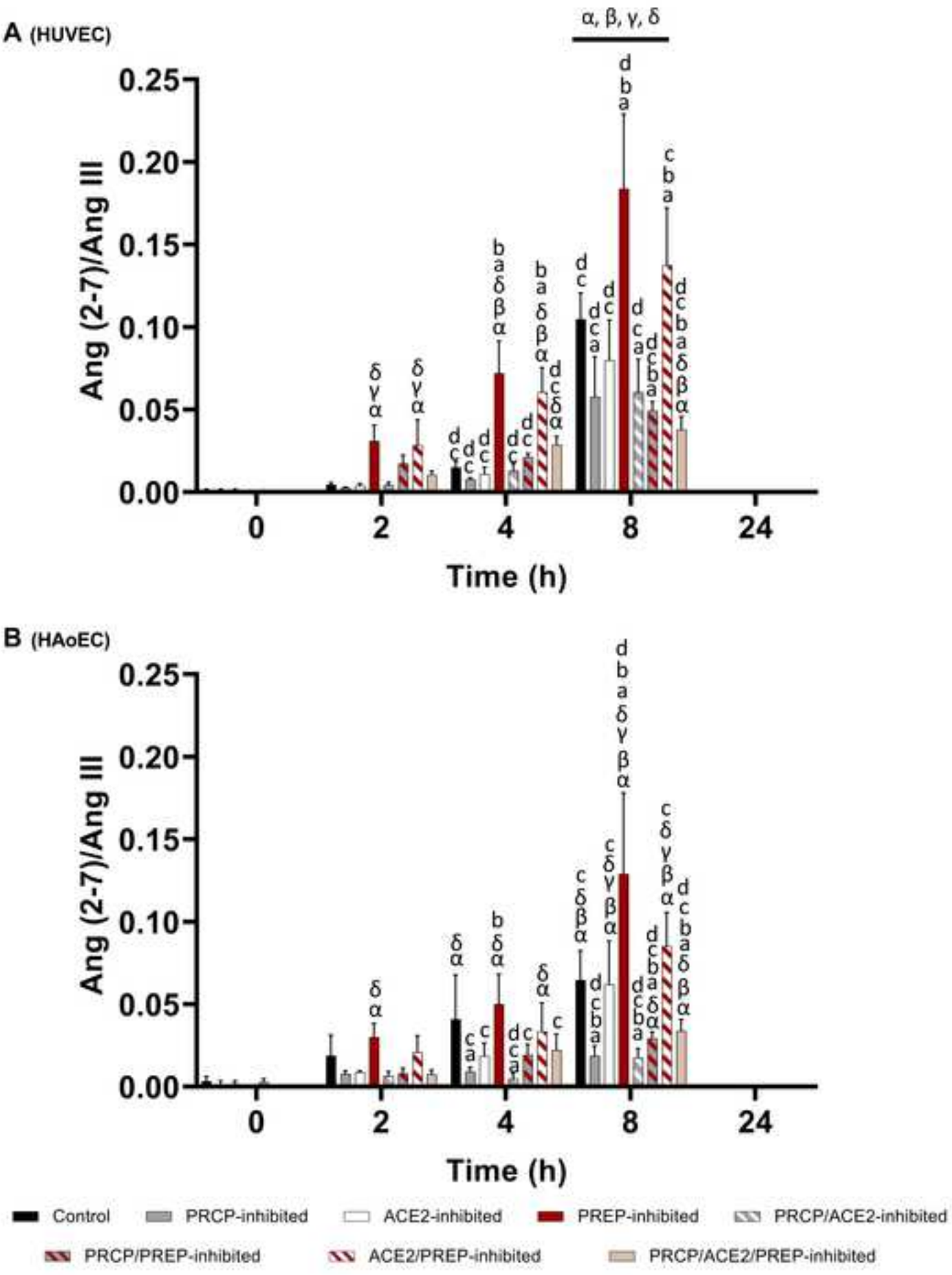

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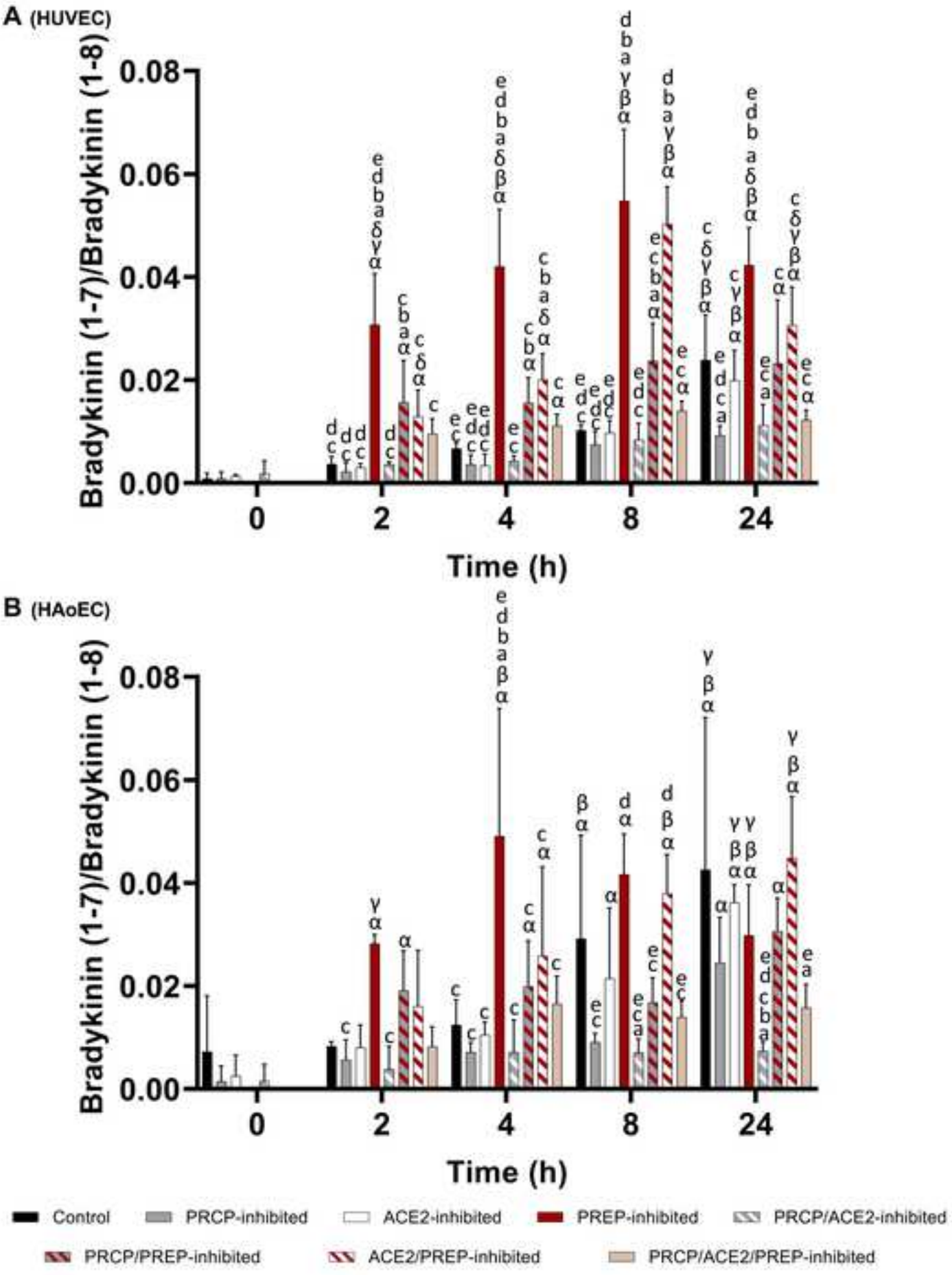

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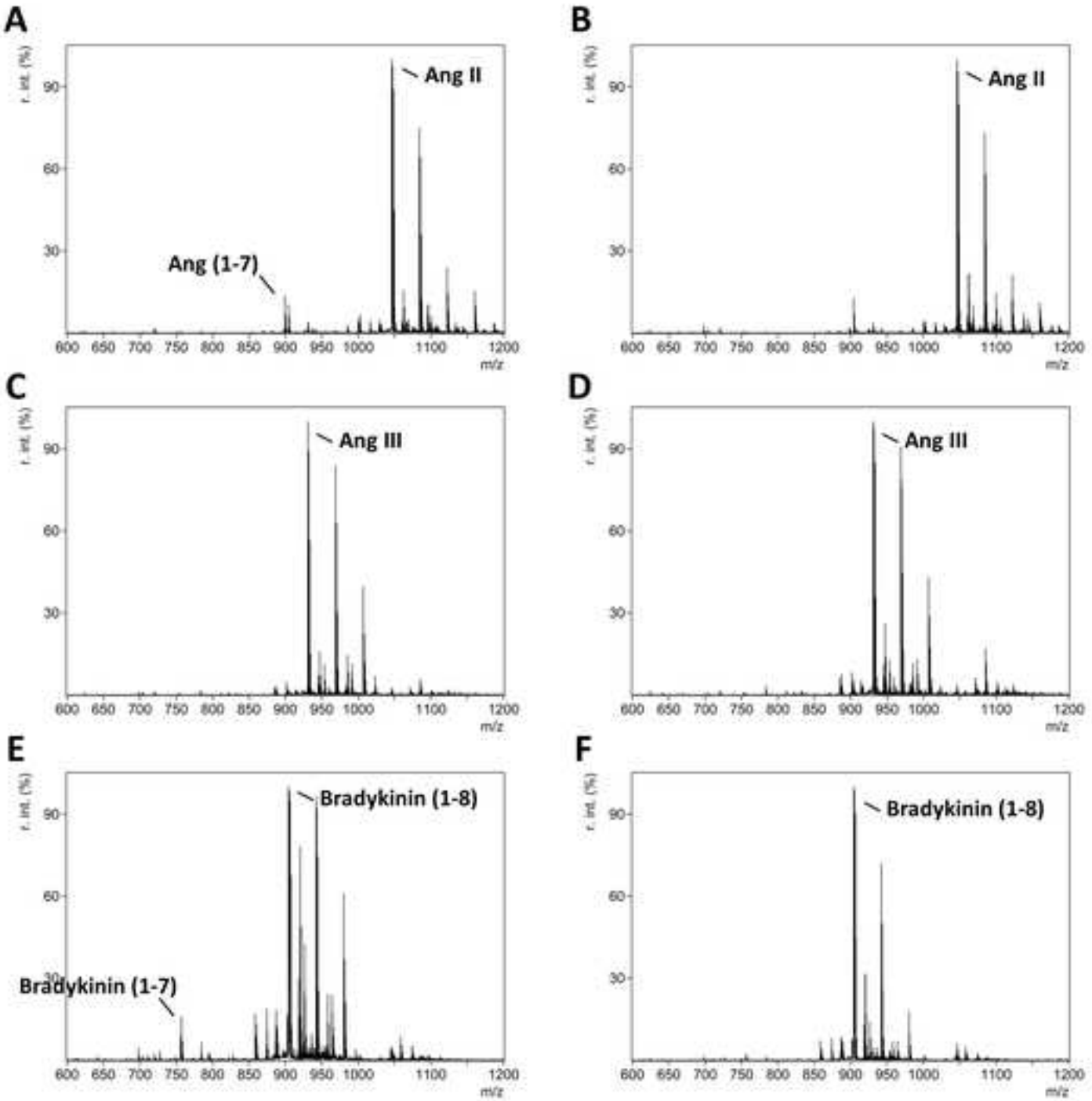

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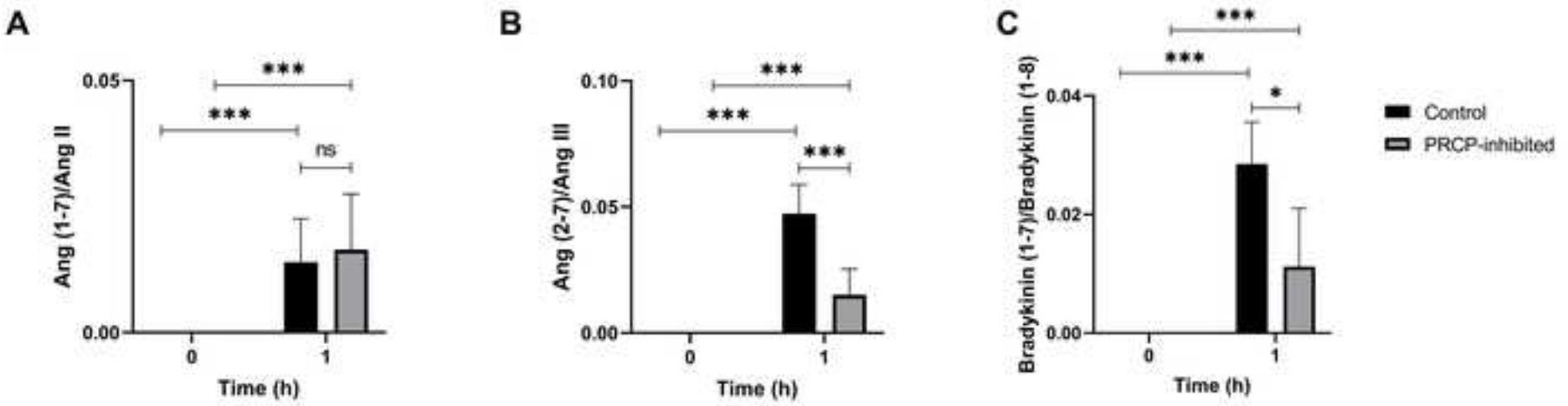
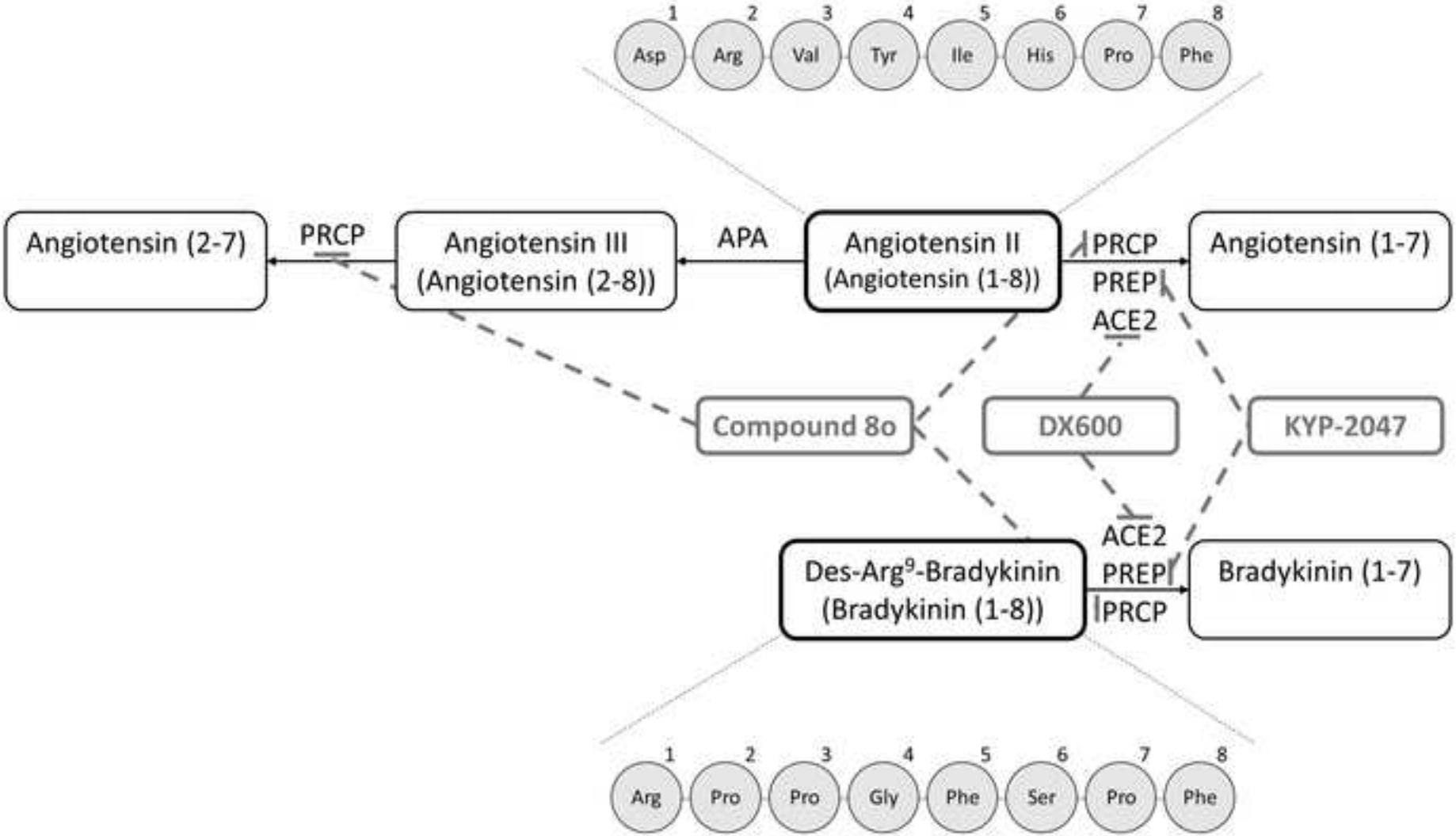
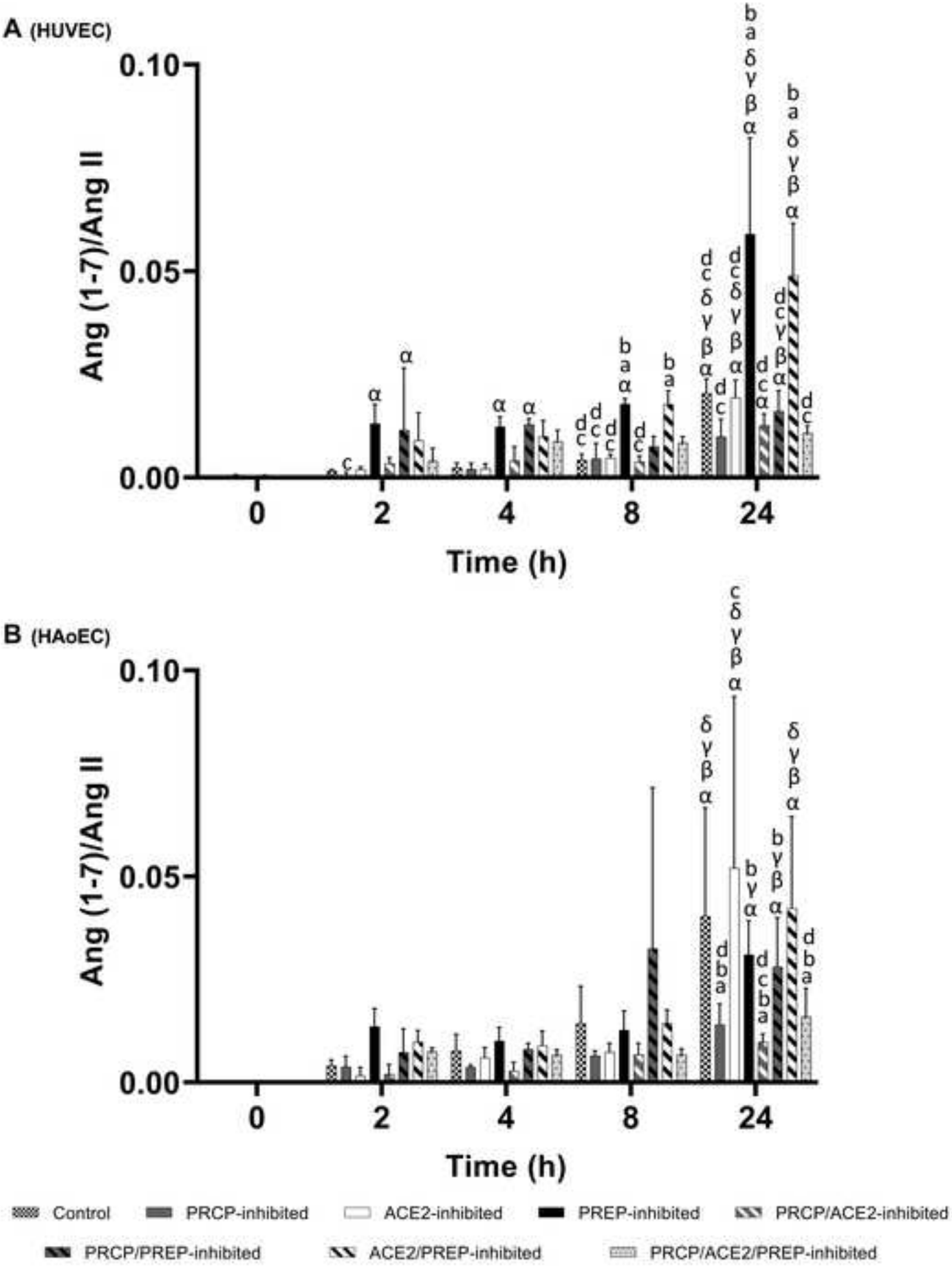
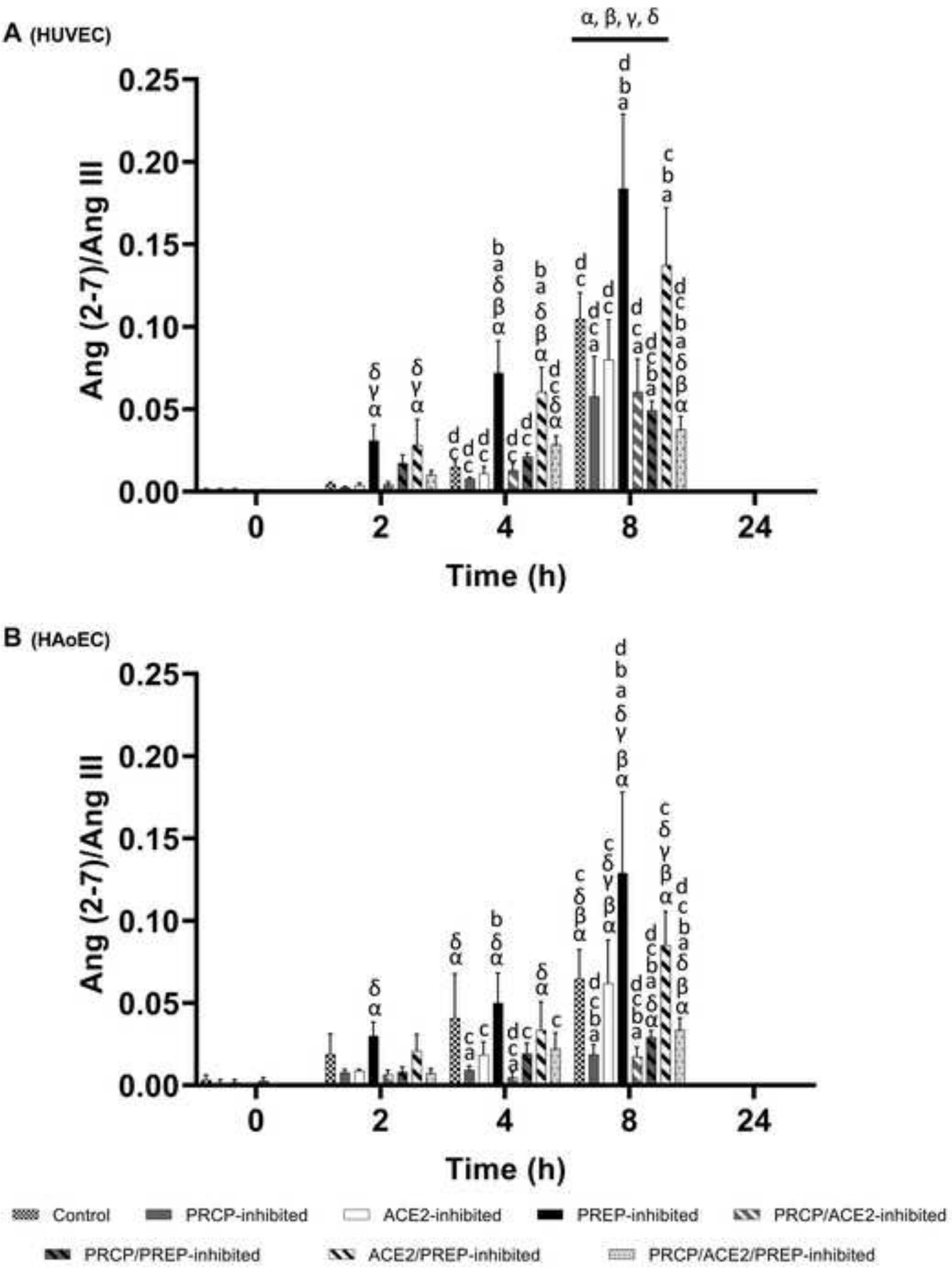
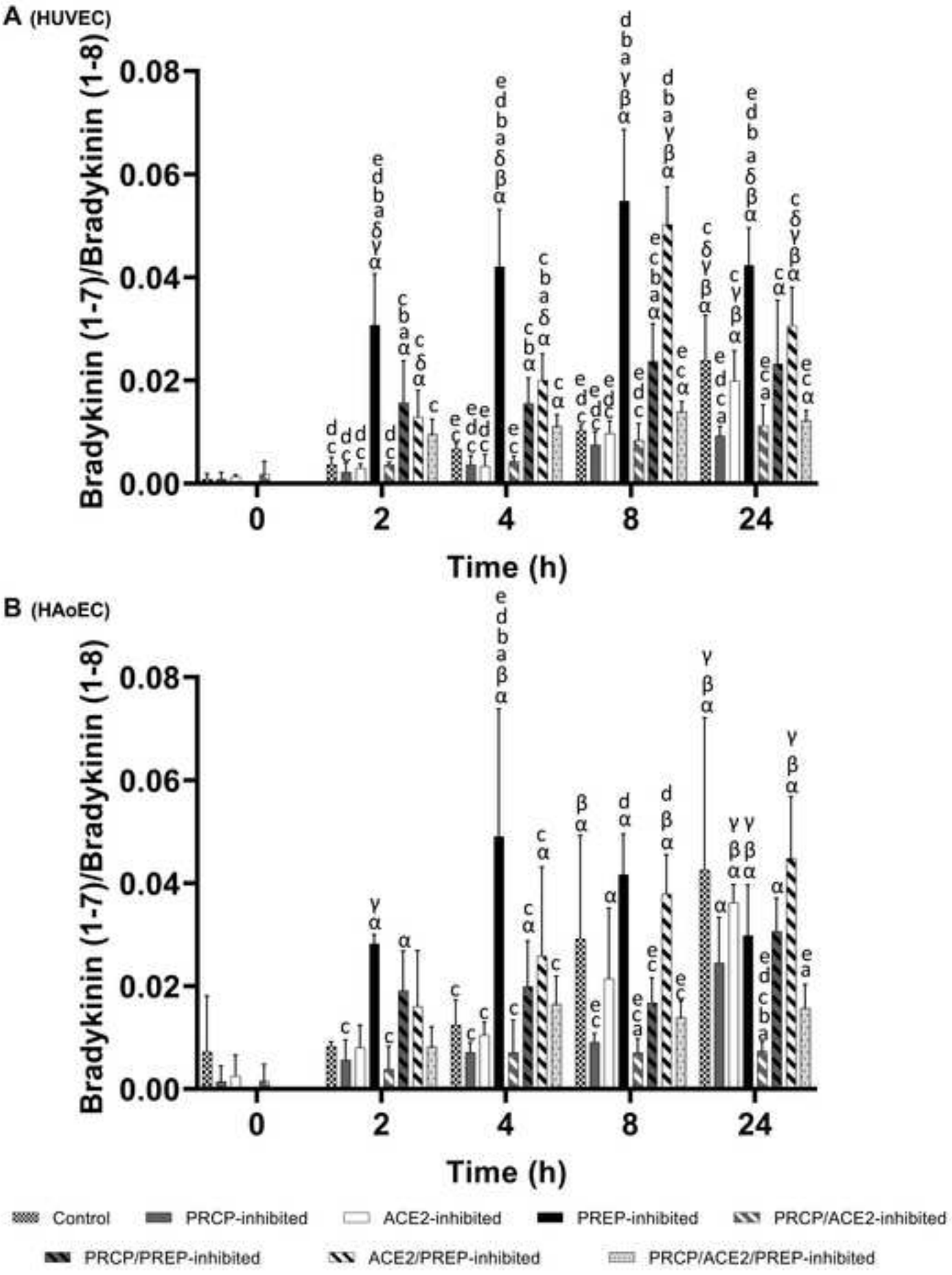





A (HUVEC)
Ang (1-7)/Ang II
0.10
0.05
0.00
0
2
4
8
24
Time (h)
B (HAoEC)
Ang (1-7)/Ang II
0.10
0.05
0.00
0
2
4
8
24
Time (h)
Control
PRCP-inhibited
ACE2-inhibited
PREP-inhibited
PRCP/ACE2-inhibited
PRCP/PREP-inhibited
ACE2/PREP-inhibited
PRCP/ACE2/PREP-inhibited


A (HUVEC)
α, β, γ, δ
Ang (2-7)/Ang III
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
8
24
Time (h)
B (HAoEC)
Ang (2-7)/Ang III
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
8
24
Time (h)
Control
PRCP-inhibited
ACE2-inhibited
PREP-inhibited
PRCP/ACE2-inhibited
PRCP/PREP-inhibited
ACE2/PREP-inhibited
PRCP/ACE2/PREP-inhibited


A (HUVEC)
Bradykinin (1-7)/Bradykinin (1-8)
0.00
0.02
0.04
0.06
0.08
0
2
4
8
24
Time (h)
B (HAoEC)
Bradykinin (1-7)/Bradykinin (1-8)
0.00
0.02
0.04
0.06
0.08
0
2
4
8
24
Time (h)
Control
PRCP-inhibited
ACE2-inhibited
PREP-inhibited
PRCP/ACE2-inhibited
PRCP/PREP-inhibited
ACE2/PREP-inhibited
PRCP/ACE2/PREP-inhibited

Credit Author Statement

Emilie De Hert: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization

An Bracke: Formal analysis, Investigation, Writing – Review & Editing

Anne-Marie Lambeir: Writing – Review & Editing

Pieter Van der Veken: Resources, Writing – Review & Editing

Ingrid De Meester: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision

Declaration of Interest

Declarations of interest: none