SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Angiotensin-converting enzyme: mechanism of endogenous inhibition and the effectiveness of medicinal treatment

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The Examination takes place at the Institute of Laboratory Medicine, Faculty of Medicine, University of Debrecen. May 28, 2014. 11:00

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1. Introduction and literature review

Cardiovascular morbidity is the leading cause of death both in developed and developing countries, they are responsible for about 30% of the overall death rate. According to the World Health Organization, 17.3 million people died from cardiovascular disease in 2008 and this number will reach 23.3 million for 2030.

Inhibiting the renin-angiotensin-aldosterone system (RAAS) proves to be a successful treatment option in most cardiovascular diseases including hypertension, systolic heart failure, acute coronary syndrome and peripheral arterial disease. This suggests that RAAS plays an important role in the development and progression of cardiovascular diseases. The most commonly prescribed member of the RAAS family is the group of ACE-inhibitors. ACE-inhibitors may reduce cardiovascular mortality by almost 40% reflecting the distinguished role of ACE among RAAS components.

Beside the well-known treatment success of ACE-inhibitors, the endogenous regulation of ACE and the presence or absence of endogenous ACE-inhibitors are less revealed.

The renin-angiotensin-aldosterone system

Renin, which is secreted by the granular cells of the renal juxtaglomerular apparatus, gives rise to angiotensin I by cleaving a 10-aminoacide peptide from the N-terminal site of the α2-globulin angiotensinogen produced in the liver. The most important renin enhancers are decreased kidney perfusion, decreased sodium-chloride concentration in the tubules and increased sympathetic nervous activity of the juxtaglomerular apparatus. ACE cleaves 2 amino acids from the C-terminal site of the inactive angiotensin I thereby forming angiotensin II (consisting 8-amino acids), the most important effector molecule of the RAAS. Angiotensin II acts on AT1 and AT2 receptors. The AT1 receptor mediates induction of aldosterone and vasopressin secretion, vasoconstriction, provokes thirst, enhances fibrosis, cell growth and migration, etc. AT2 receptor effects are mainly controversial to AT1

mediated effects: angiotensin II induces vasodilation, triggers NO-production and inhibits cell growth. By means of aminopeptidase A, angiotensin II is catabolized into angiotensin III, which possesses similar effects but significantly shorter half-life. Angiotensin III is converted into angiotensin IV by aminopeptidase M, this molecule has less important biological effects.

The angiotensin converting enzyme

The zinc endopeptidase angiotensin converting enzyme (ACE, CD143) is a key molecule of the RAAS, which catabolizes angiotensin I to angiotensin II conversion by hydrolyzing a 2-amino-acid peptide from the C-terminal site of angiotensin I.

Two ACE isoforms are explored. The somatic ACE (the larger isoform, 150-180 kDa), which is present in several tissues including the surface of endothelial cells, the cardiovascular system, the kidneys and the liver, etc. Concerning its structure, it contains a short C-terminal cytoplasmic domain followed by a hydophobic transmembrane domain, which ensures appropriate anchoring of ACE to the cell surface. The huge glycolized globular domain containing catalytic centers is situated outside the cell. The somatic ACE has 2 active centers. Although the amino acid sequences of the two active centers are identical (HEMGH), they have partially different characteristics and substrate specificity. The C-terminal active center more considerably depends on the presence of Cl^- ions and is less heat stabile compared to the more glycosylated N-terminal active center. Both active centers hydrolyze bradykinin with similar effectiveness, while angiotensin cleavage is more effective in the C-terminal site. ACE secretase cleaves ACE on the extracellular part of the transmembrane region hereby ensuring its secretion into the circulation.

Testicular or germinal ACE is expressed only in the testicle (95-105 kDa) and possesses one active center. Significantly decreased fertility of ACE-knockout mice highlights to the importance of ACE in reproduction.

The amount of circulating ACE shows significant intersubject variability as a consequence of ACE insertion/deletion (I/D) polymorphism. This means the insertion or deletion of a 287 base pair non-coding repetitive sequence in intron 16 of the ACE gene. A higher concentration of circulating ACE can be detected in the serum of subjects expressing allele D in contrast to dose expressing allele I due to the I/D polymorphism.

ACE inhibitors

ACE inhibitors are widely used to treat several diseases including hypertension, cardiac insufficiency and diabetic nephropathy. This is the fifth most frequently ordered group of drugs in the United States, different type of ACE inhibitors were prescribed in 162,8 cases in 2009.

A substance with ACE inhibitor effect was first identified in 1965 by Ferreira in the toxin from vipera Bothrops jararca (teprotid). The first stable ACE inhibitor captopril was innovated by 1975 and was followed by several newer types in patient care. According to the chemical structure of their moiety they can be classified into 3 groups: (a) sulfhydril-containing drugs (captopril, zofenopril), (b) phosphinyl-containing drugs (enalapril, ramipril, perindopril, lisinopril, etc.), (c) carboxyl-containing drugs (fosinopril). Captopril and lisinopril are active agents, the other types are pro-drugs, which are metabolized into active agents by the liver. Effectiveness of ACE inhibitors has been proved in several clinical studies, which confirmed that ACE inhibitors decrease cardiovascular mortality and the risk for nonfatal myocardial infarct and sudden cardiac arrest in stable coronary artery disease. They improve the prognosis and the 5-week mortality after myocardial infarct, decrease left ventricular remodeling, decelerate development of hypertension, decrease left ventricular mass index in hypertrophy, decrease albuminuria and the risk for diabetic nephropathy in type 2 diabetes.

Beyond their advantages, ACE inhibitors have side effect of variable extent, which can be also serious in some cases. Their most common and

unfavourable side effects are coughing, angioedema (Quincke-edema) and renal impairment.

Natural ACE inhibitor substances

The first ACE inhibitor was isolated from the toxin of a viper, but this is not the only natural substance which influences angiotensin I to angiotensin II conversion. Numerous ACE inhibitory peptides derived from food proteins were identified in the last two decades but their clinical importance is still unrevealed.
The ACE inhibitory effect of casein from milk and of many whey proteins has been demonstrated. Lactokinin and casokinin have been proved to lower blood pressure in spontaneously hypertensive rats and similar effects were seen in human clinical trials.

Beside their antioxidant and antibacterial activity, ACE inhibitory effect of thyme honey and chestnut honey has been observed by Spanish researchers.

Oligopeptides and polipeptides possessing ACE inhibitory effect has been derived from several marine animals. These peptides may also originate from digested food but their absorption, and getting into the circulation still remains to be proved.

Endogenous ACE inhibition

The existence of endogenous ACE inhibitory mechanisms was already raised in 1979. At that time, researchers found an inhibitor with a molecular weight less than 10 kDa in the serum and urine of human, guinea pig and rat but they did not succeed in identifying this inhibitory molecule.

In the same year, Klauser et al described the ACE inhibitory effect of human serum albumin and acetil-tryptophane (conservation agent) in a commercially available plasma preparation. They established that the C-fragment of albumin, located between amino acids 124 and 298, exerts a significantly greater inhibitory effect compared to the whole protein.

Snyder et al defined the substrate analog des-Leu10-angiotensin I as endogenous ACE inhibitor in 1986. This peptide originates from angiotensin I in thrombocytes and mast cells by carboxipeptidase-like enzyme activity and acts as a competitive antagonist of angiotensin I. According to their theory, this peptide may play a role in the regulation of angiotensin I catalyzed local vascular effects.

In 1986, Lieberman et al found an inhibitor with a molecular weight higher than 50 kDa in the human serum, which hindered the measurement of ACE inhibition in people with sarcoidosis. Identification of the inhibitor was unsuccessful but the inhibitory effect proved to be reversible. Therefore, serum samples were suggested to be diluted 8 times to eliminate the confounding factor.

The N-terminal fragment of substance P was demonstrated to possess ACE inhibitory effect by Rogerson et al in 1989.

In 1989, Ikemoto et al examined rat hearts when found a sulfhydryl protein that competitively inhibited ACE activity. The molecular weight of the protein was less than 10 kDa and its effect could be inhibited by sulfhydryl group blocking agents. Altough the identification of the molecule was not successful, it was thought to play a role in the regulation of ACE catalyzed processes of the rat heart.

Researchers tried to prove the existence of endogenous ACE inhibitors still in 1990s. Brecher et al published data about two ACE inhibitors in rats, a smaller and a larger protein with reversible inhibitory effect. Still in 1996, Davidson et al identified the C-type natriuretic peptide as an endogenous ACE inhibitor peptide. Thevananther et al isolated an approximately 14 kDa protein showing ACE inhibitory effect from the human serum by affinity chromatography.

Although researchers have tried several times to identify endogenous ACE inhibitors in the last decades, there is still no consensus on their existence and significant biological effects. Moreover, endogenous inhibitory effect is considered to be of negligible importance beside the expressive effect of ACE inhibitors.

2. Aims

The aims of my scientific research were the followings:

- to validate our method for ACE-activity measuring against the physiological substrate of ACE,
- to detect and endogenous ACE inhibitor in the serum,
- to identify the endogenous ACE inhibitor,
- to describe endogenous ACE inhibition with biochemical methods,
- and to explore the vascular effects of the endogenous ACE inhibitor.

3. Materials and methods

3.1 Blood sample collection, serum isolation

Blood samples were collected from volunteers by using a standard aseptic technique. Native blood was incubated for 60 minutes at room temperature, serum fractions (separated by centrifugation at 1500 g for 15 min) were stored at -20 °C until further experiments.

3.2 ACE activity measurement using spectrophotometric assay

The basis of the ACE activity measurement was originally described by Beneteau et al and modified by us. This method is based on the change in optical density at 340 nm after the artificial substrate FAPGG (N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine) is cleaved by serum ACE. The activity measurements were performed in 96-well plates at 37°C, and the changes in optical density at 340 nm were measured at 5-min interval for at least 90 min with a NovoStar plate reader. Blank corrected optical density values were plotted as a function of reaction time and fitted by linear regression. The measurement and the goodness of fitting were accepted, when r^2 was >0.90. ACE activity was calculated by the equation:

activity = (S/k)*D,

where S is the rate of observed decrease in optical density (1/min), k is the change in optical density upon the complete cleavage of 1 μmol of FAPGG, and D is the dilution of the serum. ACE activity is given in units where 1 U is equivalent to the cleavage of 1 μmol of FAPGG in 1 min.

3.3 Measurement of domain specific ACE activity

Domain specific ACE activity was measured as originally described by Carmona et al. Quenched fluorescent peptide substrates were used, Abz-SDK(Dnp)P-OH is highly specific for N domain active site, Abz-LFK(Dnp)-OH for C domain active site and Abz-FRK(Dnp)P-OH can be cleaved by both active sites. Measurements were performed in black, 96-well plates at 37 °C, λ_{ex} was 340

nm, λ_{em} was 405 nm. Changes in fluorescence intensities were measured at 4-min intervals in case of domain specific substrates for at least 90 min, and at 1.5-min intervals in case of Abz-FRK(Dnp)P-OH substrate for at least 30 min with a NovoStar plate reader. Fluorescence intensity values were plotted as a function of reaction time and fitted by a linear regression. The fit and the data were accepted when r^2 was > 0.95. ACE activity was calculated via the equation:

activity=(S/k)*D,

where S is the rate of observed increase in fluorescent intensity (1/min), k is the change in fluorescence intensity upon the complete cleavage of 1 µmol of fluorescent substrate, and D is the dilution of the sample. ACE activity is given in units where 1 U is equivalent to the cleavage of 1 µmol of fluorescent substrate in 1 min.

3.4 Direct measurement of ACE-catalyzed angiotensin I conversion

Serum samples containing angiotensin I in HEPES buffer were incubated at 37°C. 5 mM EDTA was added to stop the reaction. Angiotensin peptides were measured after filtering through a filter with a 10 kDa pore size. HPLC analysis was performed with a HPLC technique on a reverse-phase C18 column. Angiotensin peptides were separated by using an elution profile with a gradient from 22% acetonitrile to 55% acetonitrile. They were detected by a diode array detector at 230 nm and the area under the curve of each angiotensin peptide peek was compared with calibration curves recorded when the purified peptide was tested. The amounts of angiotensin peptides were plotted against the reaction time and fitted by linear regression. The kinetics of angiotensin I conversion was multiplied by the dilution of the sera and given in µmol angiotensin I cleavage in 1 L of serum in 1 min.

3.5 Measurement of serum ACE concentration

Serum ACE concentration was measured by a commercial human ACE ELISA development kit (R&D Systems) according to the manufacturer's instructions, with minor modifications. Enzyme-linked immunosorbent plates were coated with 80 ng/well capture antibody, and the remaining binding sites were then blocked with reagent diluent (10 mg/mL bovine serum albumin solution). Diluted sera were added to the wells, and the antibody-antigen complexes were labeled with a biotinylated detection antibody. Streptavidin-conjugated horseradish-peroxidase was added to the wells. Finally, the amounts of complexes were detected with a substrate solution containing tetramethylbenzidine. The reaction was terminated after 20 min by the addition of HCl, and the optical density was measured at 450 nm. ACE concentration was calculated by an ACE standard curve.

3.6 Fractionation of human sera

Serum samples from a healthy volunteer were ultrafiltered through ultrafiltration devices with a pore size of 50 or 100 kDa. One volume of initial serum sample was diluted to 250-fold, then diluted serum samples were ultrafiltered until the retained volumes reached the initial volumes of the serum samples (1 volume). ACE concentrations of an initial serum sample and the retained sample were compared to evaluate the potential loss (or enrichment) of the sample. No difference was found between the ACE concentration in the initial sample and in the retained fraction after filtration.

3.7 Measurement of human serum albumin concentration

Human serum albumin concentration was measured with bromocresol green diagnostic reagent according to the manufacturer's instructions. 10 μl serum sample was added to 1 mL bromocresol green reagent and the absorbance of the mixture was measured at 546 nm against reagent blank in a spectrophotometer using disposable cuvettes. HSA concentration was calculated via the equation:

HSA concentration= $(A_{sample}/A_{standard})*C_{standard}$,

where A is the absorbance of mixture at 546 nm, $C_{standard}$ is the HSA concentration of a diagnostic standard.

3.8 Properties of human serum albumin (HSA)

In some experiments, the ACE activity was measured in the presence of human serum albumin. The purity of the HSA preparation was tested by SDS-PAGE and mass spectrometry. Both assays showed a highly purified HSA. HSA was also tested for absorbed small molecular weight ACE inhibitors. HSA was diluted and filtered through 15 cycles with a membrane having a pore size of 5 kDa. The efficacy of remained the same compared to the initial sample.

3.9 Isometric contractile force measurement on human saphenous vein segments

Saphenous veins (remained from coronary artery bypass graft surgery) were cut into circular segments. Rings were then mounted on a dual wire myograph system. Vein segments were stretched at 15 mN and incubated under isometric conditions for 60 min at 37 °C. The viability of the mounted vascular rings was tested with KCl and norepinephrine. The mounted veins were then washed. The vascular contractile function was tested with 1 µM angiotensin I and II in the presence or absence of 20 mg/mL HSA. At the end of the measurement, the norepinephrine and KCl treatments were repeated in the presence of the angiotensin peptides to confirm the viability of the vascular rings. HSA was also applied together with captopril in some cases.

3.10 Expression and purification of recombinant ACE

Recombinant ACE was produced with a Bac-to-Bac TOPO expression system according to the manufacturer's instructions. A chemically competent E. coli strain was transformed with an ACE gene containing cDNA plasmid. After antibiotic selection and plasmid isolation, the pFastBac construct containing the

ACE coding sequence was transformed into DH10Bac competent E. coli to generate recombinant bacmid. The bacmid DNA was transfected into the SF9 insect cell line, in which baculovirus was generated. Further SF9 insect cells were infected with these bacoluviruses. On day 4, the insect cells were centrifuged and the pellets were washed in PBS to remove the cell culture medium. The pellet was then homogenized in radioimmunoprecipitation assay buffer by sonication (Bandelin Electronic). The supernatant was collected by centrifugation and injected onto an anion-exchange column. The ACE was eluted with a gradually increasing concentration of NaCl (from 168 mM to 540 mM, hatched). ACE activity was measured in each collected fractions and fractions with at least 50 U/L activity (determined by FAPGG hydrolysis, hatched) were combined. No differences were noted in ACE inhibition by captopril (activity was determined by FAPGG hydrolysis) when endogenous and recombinant ACE were used.

3.11 Detection of the molecular interactions of human ACE

Molecular interactions were stabilized by heterobifunctional crosslinkers and the ACE and the crosslinked proteins were then immunoprecipitated (with biotinylated goat anti-human ACE antibody) The immunoprecipitated complexes were then prepared for SDS-PAGE and ACE was detected with the same goat anti-human ACE antibody. The molecular interaction between purified human serum ACE (>100 kDa serum fraction) and HSA was also tested. The interaction between ACE and HSA was further tested on ELISA plates. HSA, gelatin or ACE antibody were added to the wells and the wells were incubated overnight with crosslinkers. After the addition of recombinant ACE, we detected the immobilized HSA-linked ACE.

3.12 Direct measurement of ACE-catalyzed bradykinin cleavage

Recombinant ACE was incubated with bradykinin in the absence or presence of HSA at 37°C. EDTA was added to stop the reaction. Bradykinin

peptides were measured after filtering through a filter with a 10 kDa pore size. Analysis was performed with a HPLC technique on a reverse-phase C18 column. Bradykinin peptides were separated by using an elution profile with a gradient from 18% acetonitrile to 44.2% acetonitrile. The area under the curve of each bradykinin peptide peek was compared with calibration curves recorded when the purified peptide was tested. The amounts of bradykinin peptides were plotted against the reaction time and fitted by linear regression. The kinetics of bradykinin cleavage was normalized to the background (recombinant ACE plus 1 μM captopril), and compared to vehicle (recombinant ACE without HSA).

3.13 Ethical approval

All of the studies were approved by the Regional and Institutional Ethics Committee, Medical and Health Science Center, University of Debrecen, (UDMHSC REC/IEC number: 2894-2008) and by the Medical Research Council of Hungary. All of the individuals involved gave their written informed consent.

3.14 Statistical analysis

Statistical analysis was performed with Graphpad Prism 5.0 (GraphPad Software) by paired and unpaired t-tests. Differences were considered to be significant when $p<0.05$.

4. Results

4.1 Detection of the endogenous ACE inhibitor

Serum ACE activity was significantly affected by dilution, i.e. specific ACE activity increases by the degree of dilution. In contrast, no such effects of dilution were found on the purified tissue ACE activity. These data suggest that ACE activity is modulated by additional factors besides the ACE itself in the human serum. We hypothesized that an endogenous reversible inhibitor is present in the human sera. According to this hypothesis the endogenous reversible inhibitor dissociates from the ACE at high dilutions, resulting in an apparent increase in specific activity.

4.2 Validation of the ACE activity measurement

The cleavage of the artificial substrate FAPGG by human sera was compared with the conversion of the physiological substrate angiotensin I *in vitro,* in separate sets of experiments. ACE activity determined by FAPGG cleavage was directly proportional to angiotensin I to angiotensin II, albeit FAPGG cleavage was about 30-fold faster and easier to measure.

The specificity of FAPGG hydrolysis was also tested with a set of protease inhibitors. Protease inhibitors did not affect FAPGG conversion by human serum. On the other hand, 1 µM captopril (an ACE inhibitor) successfully inhibited FAPGG conversion.

The increase in specific ACE activity upon dilution was tested under different assay conditions. Increase in specific activity was present at physiological pH and Cl-concentrations and specific ACE activity was not affected by the buffer concentration.

4.3 Description of the endogenous ACE inhibition

Efforts were made to prove the presence of the endogenous inhibiting factor. Serum samples were filtered through ultrafilter devices with different pore

sizes. No effect was observed when proteins with <50 kDa molecular masses were removed, while depletion of proteins with molecular masses <100 kDa from the human serum resulted in significantly elevated ACE activities, without the apparent gradual increase in ACE activity seen upon dilution in the original serum samples.

A Lineweaver-Burk plot was designed showing competitive ACE inhibition by captopril, while the inhibition was found to be non-competitive in the presence of the serum fraction.

Captopril had higher efficacy on recombinant ACE catalyzing angiotensin I to angiotensin II conversion than human serum containing the endogenous inhibitor measured by HPLC, while no inhibitory effect was detected in the case of serum proteins below 50 kDa. The same was noted in FAPGG hydrolysis. Moreover, inhibitory effect of captopril was not affected by <50 kDa or 50-100 kDa serum fractions.

Serum ACE has two catalytically active domains. Application of specific fluorescent substrates for these active sites revealed that captopril have the same potency and efficacy on both active, while the endogenous serum inhibitor had higher potency on the C-terminal active site than that is for the N-terminal active site. Interestingly, the hydrolysis of the fluorescent substrate, which is non-specific for the catalytic sites, was identical than that for the N-terminal site specific substrate in this latter case. Recombinant ACE was also inhibited by the endogenous serum inhibitor and captopril, when measured by the fluorescent substrate, similarly to the angiotensin I and FAPGG hydrolysis, suggesting that the ACE inhibition by the 50-100 kDa endogenous serum inhibitor is not a substrate specific feature. On the other hand, the serum fraction containing all of the components below 50 kDa (fraction <50 kDa) was again without effects on recombinant ACE activity on the fluorescent substrate hydrolysis, similarly to angiotensin I and FAPGG hydrolysis.

These data suggested that ACE is inhibited by a protein with an apparent molecular mass of 50-100 kDa in human serum. It was tested whether this inhibition is species dependent or an evolutionary conserved general feature. Normal serum samples from mouse, bovine, goat and donkey were tested and compared to human. Serum ACE activities were significantly different in these species. Nonetheless, specific ACE activities significantly increased upon dilution in each species.

4.4 Identification of the endogenous ACE inhibitor and its effects on serum ACE

The interaction of ACE with its suspected endogenous inhibitor was stabilized by crosslinkers. ACE-containing complexes were then identified by immunoprecipitation and then by using an ACE-specific antibody in Western blotting. Besides the 180 kDa band indicative of free ACE, an extra band appeared in the crosslinked samples, with an apparent molecular mass of about 250 kDa. The size of the crosslinked product (about 250 kDa) suggested that the interaction partner is about 70 kDa. The most abundant plausible protein with that molecular mass is the human serum albumin (HSA, 66 kDa). This hypothesized interaction (between ACE and HSA) was directly tested with purified ACE and HSA by the same crosslinking technique. The 250 kDa adduct was again observed and positively stained by both anti-ACE and anti-HSA antibodies in Western blot. The interaction between HSA and ACE was further confirmed by ELISA.

Potential inhibitory effect of HSA was tested on recombinant and partially purified ACE from the human sera. Separation of the ACE from the HSA in human sera resulted in an increase in ACE activity.

HSA also inhibited recombinant human ACE and partially purified serum ACE activities directly, with half maximal inhibitory concentrations (IC_{50}) of 9.5 and 5.7mg/mL, respectively.

A common clinical side effect of ACE inhibitory therapy is coughing as a result of elevated bradykinin levels. Effects of 40 mg/mL HSA were tested on bradykinin hydrolysis by recombinant ACE. HSA only partially inhibited bradykinin breakdown.

ACE has two catalytically active sites, which have slightly different substrate specificities. Effects of HSA were tested on these sites by specific fluorescent substrates. It was found that HSA has a higher affinity for the C-terminal active site than for the N-terminal active site.

4.5 The effect of HSA on the angiotensin-catalyzed vascular responses

ACE inhibition by HSA was tested on tissue-bound endogenous ACE in human vascular preparations (saphenous vein). Angiotensin I or angiotensin II was applied in the absence or in the presence of 20 mg/mL HSA. There were no significant differences in constrictions to angiotensin I and II in the absence of HAS. Angiotensin I mediated contractions decreased in the presence of HSA while angiotensin II evoked contractions were not affected. Addition of captopril to 20 mg/mL HSA did not affect vascular constriction to angiotensin I compared to HSA alone.

These transient angiotensin responses were investigated in detail. Several parameters of the contractile response were determined in each and every individual trace, including the kinetics of the constriction, duration of the half maximal contraction, kinetics of desensitization and the level of desensitization besides to the maximal constriction described before. The kinetics of constriction was about 3-fold slower in the presence of HSA than in its absence. In contrast, the presence of HSA had no significant effect on the kinetics of desensitization, the duration of the half-maximal contraction or the level of steady-state desensitization in response to angiotensin I treatment. HSA displayed no significant effect on the angiotensin II-evoked responses.

4.6 Dilution of serum is an appropriate technique for the judgement of ACE inhibitor therapy

Upon dilution of the serum, the endogenous inhibitor HSA dissociates from ACE, which retrieves its activity. Both the effect of the endogenous inhibitor and the drug can be observed in patients on ACE inhibitor therapy. In these patients, a high level of dilution results in the dissociation of both the HSA and the drug molecules, herewith ACE regains its activity. Specific activity of the highly diluted serum sample indicates individually the maximal ACE activity, while the activity of the concentrated serum indicates the inhibited ACE activity. The difference between the maximal and the inhibited activity values reflects the degree of ACE inhibition. Our method was also tested in the clinical practice. 502 subjects with hypertension were enrolled in the study, 384 received ACE inhibitor therapy. Blood pressures were under the target value 140/90 mmHg in the study population. The degree of ACE inhibition was measured individually. The inhibited activity values of some of the patients on ACE inhibitor therapy was similar to that of the control group (n=66). ACE inhibition was insufficient in these patients and had significantly higher blood pressure values. These 66 patients were recalled for a second visit and asked to take their medicine according to the instruction of their treating physician. Of the 66 patients, 30 patients did not present on the second visit, in 11 patients we found an adequate level of ACE inhibition, and in 6 patients the treating physician ordered therapy intensification on account of the persisting low level of inhibition. Dose adjustment in these 6 patients resulted in adequate therapy effect. 17 patients did not take their medicine because of coughing as a side effect (they changed for another type of blood pressure lowering drugs), 2 patients refused to take any kind of medicine. A significant decrease was observed in the blood pressure values of those 17 patients, in whose serum samples we detected an adequate level of inhibition on the second visit after therapy intensification.

5. Discussion

The degree of ACE activity remarkably depends on the level of serum dilution applied for the measurement. The higher the level of dilution is, the higher the value of specific activity is. This phenomenon is not observed concerning purified ACE, which can be explained by suboptimal experimental circumstances or the inhibited state of ACE in concentrated serum samples owing to the endogenous reversible inhibitor (inhibition ceases only for higher degrees of dilution).

The speed of FAPGG hydrolysis (artificial ACE substrate) is directly proportional to that of the natural ACE substrate (angiotensin I) at different levels of dilution. Our method for enzyme activity measuring is specific for ACE, when tested with different kind of peptidase inhibitors, only captopril inhibited the hydrolysis of ACE. For enzyme activity measuring, in vitro circumstances are different from in vivo, therefore we modelled the effect of natural circumstances for ACE in our experiments. Physiological pH and Cl^- concentration did not have an influence on serum dilution induced specific ACE activity increase. It can be established that the experimental conditions were optimal, therefore increase of enzyme activity refers to the presence of an endogenous inhibitor.

Only the ultrafiltered serum fraction containing proteins between 50 to 100 kDa exerted ACE inhibitory effect both on the recombinant and the purified enzyme. Lineweaver-Burk plot indicated that this is a non-competitive inhibitory effect.

Adding C- and N-terminal active center specific ACE substrates to the reaction mix demonstrated that the inhibition by the endogenous inhibitor is more effective in the C-terminal site of ACE.

Dilution induced ACE activity increase and the presence of an endogenous ACE inhibitor in the serum is not specific for human, they were demonstrated in several animal species (mouse, bovine, goat and donkey) as well.

It testifies that endogenous ACE inhibition is an evolutionary conserved general feature.

To identify the endogenous inhibitor, molecular interactions between ACE and the inhibitor were stabilized by heterobifunctional crosslinkers.
The size of the crosslinked product suggested that the interaction partner may be the HSA. This crosslinked product was also observed when purified ACE and HSA was were crosslinked and could be detected both with anti-ACE and anti-HSA antibodies.

We tested the inhibitory effect of HSA on both purified and recombinant ACE. The inhibition was similar in both cases (IC_{50} values are 5.7 and 9.5 mg/ml, respectively).

Circulating angiotensin converting enzyme activity is significantly controlled by the human serum albumin. We also tested the effect of HSA on endothelial cell surface bound ACE. On saphenous vein circular segments (remained from coronary artery bypass graft surgery), we experienced that angiotensin I mediated contractions decreased in the presence of HSA while angiotensin II evoked contractions were not affected. These all conclude that HSA inhibits both circulating and tissue bound ACE activity.

Endogenous ACE inhibition is a particularly interesting phenomenon in the context of the successfulness of ACE-inhibitor therapy. ACE inhibitors are effective in the treatment of several cardiovascular diseases demonstrated by numerous clinical studies on large patient populations and are recommended by cardiovascular guidelines. We demonstrated that HSA significantly inhibits ACE activity, which is a possible protective mechanism against the development and progression of cardiovascular diseases. Acting as a buffering system, stabilizes the activity of ACE on a low level independently from ACE concentration.

Serum ACE concentration is genetically determined. The insertion/deletion polymorphism of the ACE gene accounts for the 20-50% interindividual variability of ACE concentration suggesting that I/D polymorphism

may play a special role in the pathomechanism of several cardiovascular diseases including myocardial infarct, coronary heart disease, heart failure and hypertension. In spite of this, most clinical studies failed to confirm the connection between cardiovascular diseases and ACE polymorphism. It can be explained by the fact that HSA buffers ACE activity by holding it on a low level independently from ACE concentration.

Ignoring the contribution of ACE genotypes to ACE activity, a metaanalysis on more than 30 000 patients concluded that ACE polymorphism does not influence blood pressure and not increases the risk for myocardial infarct, ischaemic heart disease and ischaemic celebrovascular diseases. We suppose that the buffering effect of HSA on ACE activity may be its explanation. ACE is suggested to play an important role in the pathomechanism of these diseases, because ACE inhibitors are especially important in their therapy.

Several point mutations on the ACE gene are known to influence circulating ACE concentration more than the I/D polymorphism does. Kramers et al published that the level of the circulating ACE concentration elevated 5-times owing to a point mutation on the ACE gene. At least eight families were affected, but nobody suffered from any kind of disease in connection with ACE or hypertension. Nesterovich et al published that a certain mutation elevated the serum ACE-level 13-times, but they failed to connect any kind of disease to this elevated ACE amount. A proline to leucin mutation at position 1199 on the ACE gene is called asymptomatic autosomal-dominant hyper-ACE-emia. These observations demonstrate that the change in ACE concentration is well tolerated in a wide range.

Interestingly, we did not find evidence of small molecular weight ACE inhibitors in human sera, although they have been described previously. We did not detect any effect of <50 kDa serum filtrates on ACE activity. One of the most probable reasons for interpersonal variances can be the diet of the individuals. It has been reported that dietary factors, such as bovine alphaS2-casein or

components of the honey can have ACE inhibitory activities. These data suggests that diet rich in milk products or honey may result in the appearance of small molecular weight inhibitors. This is an important issue (regulation of ACE activity by dietary factors) which needs to be tested in future studies. An alternative explanation is that the observed small molecular weight inhibitors described in previous studies are the products of the degradation of large molecular weight ACE inhibitors, such as HSA and that this degradation did not occur in our samples. Alternatively, the small molecular weight inhibitors were further metabolized and therefore inactivated in our case.

It is an important question whether HSA or some HSA absorbed molecules are responsible for ACE inhibition. The effect of purified HSA on ACE activity suggested that this inhibitory effect is rather linked to the HSA than to any other absorbed molecules. This was further supported by two data of this present manuscript. First, the applied HSA was found to be remarkably pure by mass spectrometry. Second, HSA ability to inhibit recombinant ACE was not affected by up to 15 filtration cycles, suggesting that HSA inhibition is not the result of some dissociative absorbed molecules.

Interestingly, some studies designed to identify dietary components with ACE inhibitory activity lead to the recognition of Acein-1 and albutensin A as tryptic fragments of serum albumin with ACE inhibitory properties. These peptides were synthesized and inhibited ACE, pinpointing HSA regions potentially important in ACE inhibition. Acein-1 was identified as a heptapeptide spanning the region 138-144 in HSA, while albutensin A is a nonapeptide spanning the region 210-218 in HSA. The existence of these peptides suggest that HSA may have multiple ACE inhibitory sites. Moreover, both peptides have IC_{50}values similar to that determined for HSA here (16 µM for acein-1 and 1.2 µM for albutensin A). Interestingly, the synthetic peptide, which is a single residue longer than acein-1 had a dramatically lower IC_{50} (500 µM versus 16 µM for acein-1) suggesting that

position and exposition of these segments of HSA on the surface may have a dramatic effect on their effectiveness.

Physiological HSA concentrations are several times higher (35-52 mg/mL) than the determined IC_{50}values for HSA (5.7 and 9.75 mg/mL), suggesting complete suppression of ACE activity by HSA, *in vivo*. Moreover, ACE inhibition by HSA were tested on human sera and human blood vessels. Our data suggest that ACE activity is significantly suppressed as long as the HSA concentration is at least ~30 mg/mL. Nonetheless, the HSA concentration may be lower in conditions associated with protein malnutrition and liver failure, among others. Our findings tend to indicate that under these conditions the infusion of HSA may increase the inhibition of ACE. In accordance with this, it has been found, that the postoperative infusion of HSA frequently evokes hypotension in patients receiving ACE inhibitor therapy.

ACE inhibitor drugs are particularly effective in cardiovascular diseases, pinpointing ACE as a major angiotensin I converting enzyme, *in vivo*. HSA was identified here as a major inhibitor of circulating ACE, suggesting that ACE inhibitor drugs are probably not acting on the circulating ACE, since it has already been suppressed by the physiological concentration of the endogenous HSA. ACE inhibitor drugs should therefore target a tissue-bound ACE population. HSA inhibited angiotensin I conversion in the vascular tissue, resulting in a slower and lower maximal angiotensin I mediated constriction in human vascular bed. This effect of HSA was independent of angiotensin II evoked constrictions, suggesting a direct effect on tissue bound ACE, but the efficacy of HSA at 20 mg/mL appeared to be lower in the case of tissue-bound ACE than that is for serum ACE. In addition, captopril (an ACE inhibitory drug) was not more effective than HSA in the same assay (even applied at high concentrations), suggesting that either captopril is not a complete antagonist at vascular ACE or alternatively, angiotensin I responses were not exclusively mediated by ACE in this location.

We hypothesize that HSA does not inhibit all forms of ACE similarly. According to this hypothesis ACE in some tissues has lower affinity for HSA or has higher local concentration (expression level) than that is in the serum. HSA mediated inhibition is therefore only limited in these locations, while ACE inhibitory drugs can potently inhibit these enzymes. Under these conditions HSA may inhibit somewhat local ACE activity, but it can be further inhibited by these ACE inhibitor drugs. This is in accordance with a recent consensus report stating that “the plasma ACE represents only a small proportion of the body’s total ACE activity, therefore its role is thought to be minimal”. As a matter of fact we have provided a mechanism for this “minimal contribution” by showing HSA mediated suppression of plasma ACE.

Another implication of the high degree of inhibition of serum ACE by HSA is that ACE mediated angiotensin I to angiotensin II conversion can be a rate limiting step in the renin-angiotensin-aldosterone system (RAAS). It is in accordance with the co-existence of both angiotensin I and angiotensin II in the circulation in a comparable level, suggesting that the rate of angiotensin I generation (by renin) and conversion (by ACE) are not much different, albeit angiotensin I cannot be present at such concentrations without the activation of the renin. Nonetheless, this high degree of ACE inhibition by HSA suggests that the processes responsible for the elimination of the angiotensin II can play a significant role in the determination of local angiotensin II levels.

The protective role of HSA is hypothetical, while the advantageous effects of the ACE-inhibitors in the development and progression of cardiovascular diseases are well established. The beneficial effects of a drug highly depend on patient adherence and adequate dosing. Treating physicians may have a great influence on adherence, but it is the patient who finally decides for taking his medicine. Adherence could be easily assessed by a blood pressure measurement, but in most cases the beneficial effects of ACE inhibitors, other that their blood pressure lowering effect, are desired.

Our method is suitable for the objective assessment of ACE inhibitor therapy. Only one tube of blood is required for the determination of the degree of ACE inhibition in each patient. Our study including 502 patients concluded that the ACE inhibitor therapy is ineffective in about one fifth of the patients and it can mostly be explained by inadequate patient adherence to the therapy. When physicians know which patients do not take their medication, they can improve adherence and thereby increase therapy efficiency, decrease cardiovascular mortality and the incidence of hospitalization. In a smaller proportion of patients who had ineffective ACE inhibition the applied dose of the ACE inhibitor was low and dose adjustment was necessary. The beneficial effect of dose adjustment could be observed both in the blood pressure values and *in vitro* in the measurement of efficiency of the ACE inhibitor therapy.

It was also observed in our study that in some patients the degree of ACE inhibition is over 99%. In these cases dose reduction should be considered to minimize the number and intensity of side effects, which ultimately improves patient adherence. Finally, our method may initiate a new chapter in personalized therapy.

6. Conclusion

Angiotensin converting enzyme inhibitors are one of the most commonly prescribed drugs in cardiovascular diseases. The presence of endogenous ACE inhibitors was first published in 1979 but their effects remained unrevealed.

Our aim was to detect and identify the endogenous ACE inhibitor in the serum and investigate its influence on circulating and vascular ACE.

Our results confirmed that human serum albumin significantly inhibits circulating ACE activity. The novelty of our research is the demonstration of the followings: (1) HSA mediated ACE inhibitory effect is also present under physiological conditions (pH, Cl^- concentration) *in vitro*, (2) HSA inhibits the C-terminal active site of ACE with higher affinity than the N-terminal active site, (3) HSA mediated inhibitory effect is evolutionary conserved, (4) HSA inhibits not only circulating but also endothelial cell membrane bound ACE, (5) specific enzyme activity measuring in serum samples at different dilution levels is a suitable method for the objective assessment of ACE inhibitor therapy efficacy (is the therapy effective?) and enables personalized treatment.

The most relevant conclusion of our work is that HSA is an effective endogenous inhibitor of ACE, which almost completely inhibits circulating ACE under physiological conditions, in vivo. Translation of our findings into practice opens the door to individualized ACE-inhibitor therapy.

7. List of publications

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PUBLICATIONS

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Register number: DEENKÉTK/79/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Miklós Fagyas

Neptun ID: NMHRCK

Doctoral School: Kálmán Laki Doctoral School

Mtmt ID: 10037028

List of publications related to the dissertation

1. **Fagyas, M.**, Úri, K., Siket, M.I., Daragó, A., Boczán, J., Bányai, E., Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) I: Endogenous angiotensin converting enzyme (ACE) inhibition.
PLoS One. 9 (4), 29 p., 2014.
DOI: http://dx.doi.org/10.1371/journal.pone.0087843
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2. **Fagyas, M.**, Úri, K., Siket, M.I., Fülöp, G.Á., Csató, V., Daragó, A., Boczán, J., Bányai, E., Szentkirályi, I.E., Maros, T.M., Szerafin, T., Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) II: Albumin suppresses angiotensin converting enzyme (ACE) activity in human.
PLoS One. 9 (4), 28 p., 2014.
DOI: http://dx.doi.org/10.1371/journal.pone.0087844
IF:3.73 (2012)

List of other publications

3. **Fagyas, M.**, Úri, K., Siket, M.I., Daragó, A., Boczán, J., Bányai, E., Édes, I., Papp, Z., Tóth, A.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) III: Endogenous inhibition of angiotensin converting enzyme (ACE) provides protection against cardiovascular diseases.
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4. Úri, K., **Fagyas, M.**, Mányiné Siket, I., Kertész, A., Csanádi, Z., Sándorfi, G., Clemens, M., Fedor, R., Papp, Z., Édes, I., Tóth, A., Lizanecz, E.: New perspectives in the renin-angiotensin-aldosterone system (RAAS) IV: Circulating ACE2 as a biomarker of systolic dysfunction in human hypertension and heart failure.
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