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

Role of angiotensin converting enzyme (ACE) in cardiovascular diseases

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Péter Andréka MD, PhD

The Examination takes place at the library of Department of Laboratory Medicine, In Vitro Diagnostic Building, Faculty of Medicine, University of Debrecen

at 11:00 a.m., on 17th of June, 2015.

Head of the **Defense Committee**:   János Kappelmayer, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Auguszta Building, Faculty of Medicine, University of Debrecen

at 13:00 p.m., on 17th June, 2015.

INTRODUCTION

Amounting to a third of total mortality at present, cardiovascular mortality has been a leading cause of death worldwide. Compared to cancerous diseases, it is of approximately the same incidence in the developed countries. On launching the world's first prospective comprehensive study on the aetiology and risk factors of cardiovascular diseases in the USA, attention was focussed on the follow-up of the health state of the citizens of Framingham, a small provincial town. As a result, the role of blood lipids, the importance of diabetes, hypertension and smoking, an unequivocally harmful factor, in the development of atherosclerosis were confirmed. Current research into cardiovascular diseases is devoted to underlying genetic abnormalities.

Cardiovascular pathophysiology may manifest itself in several diseases of varying severity. Usually, risk factors appear first and the process may end up in myocardial infarction, stroke or the obstruction of arteries in the limbs. In the necrotized area, remodelling of the connective tissues is observed, which may lead to the failure of the affected organ, e.g. cardiac failure over time. In approximately 5-15 years on average, such diseases may cause the patient's death. Patients with ischaemic cardiomyopathy pose an increasingly great problem for society as, due to the spread of primary coronary intervention, there has been a spectacular decrease in mortality rates associated with acute myocardial infarction worldwide, but surviving patients make up the vast majority of the population with cardiac failure.

Currently, several methods are at our disposal to treat the ischaemic myocardium. The basic therapeutic arsenal includes treatment with medicines such as acetylsalicylic acid, beta-blockers, Ca^{+2} channel blockers and I_f – inhibitors. Symptomatic treatment is provided by giving nitrate therapy continuously or in unexpected attacks. Angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), statins to decrease cholesterol synthesis and ezetimibe to inhibit cholesterol absorption also form the base of prophylactic therapy. Trimetazidine is also used in symptomatic treatment to "strengthen" aerobic metabolism in the mitochondria. Having confirmed coronary sclerosis the physician can

supplement the aforementioned medicinal treatment with invasive therapeutic procedures (percutaneous coronary angioplasty and coronary bypass surgery) to treat ischaemia.

How does the renin-angiotensin-aldosterone system (RAAS) function?

As a result of efforts put into research activities, physicians have explored the renin-angiotensin-aldosterone system (RAAS) and studied its aspects in detail. Actually, RAAS functions as a hormonal system controlling the salt and water balance of the body and blood pressure.

In the first step of the process, renin, a protease produced by the juxtaglomerular cells of the kidney, splits off Angiotensin I (decapeptide) from the N-terminal end of angiotensinogen (alpha2-globulin) synthesized in the liver. Conversion of Angiotensin I into Angiotensin II is catalyzed by the angiotensin converting enzyme (ACE), which is also a protease. Splitting two amino acids off the Angiotensin I (Ang I) C terminal, ACE "creates" Angiotensin II (Ang II) octapeptide. Since ACE also plays a role in other processes, e.g. it inactivates bradykinine, it is also referred to as kininase. Alternatively, the conversion of Ang I into Ang II can take place differently from ACE, e.g. by chymase. Ang II binds with the cells' type 1 Angiotensin II receptors (AT1R) and type 2 Angiotensin II receptors (AT2R).

Ang II has opposite effects on the two receptors, although the activating effect of AT2R is negligible compared to that of AT1R. Ang II triggers vasoconstriction in the cardiovascular system in both the AT1 receptors of arteries and veins via the intracellular inositol and G-protein pathways. Ang II also has a prothrombolytic effect by interfering with the adhesion and aggregation of thrombocytes. Of the many effects of Ang II, the most important ones include the following: it causes hypertrophy in smooth muscle and myocardial cells, accelerates the progression of hypertension and atherosclerosis and starts off a thrombotic cascade in endothelial injuries through the activation of thrombocytes. Also affecting AT1R, Ang II enhances aldosterone release by the adrenal cortex contributing to increased renal Na^+ retention and K^+ secretion. It can cause weak constriction in the renal arteries and afferent arterioles but stronger vasoconstriction in the efferent ones. It can increase the sensitivity of tubuloglomerular feedback and decrease blood flow through the renal medulla. By contracting mesangial cells, it decreases the surface for secretion. Studies have confirmed that cellular migration, growth and connective tissue remodelling, i.e. the progression of fibrosis, increase on AT1R due to the effect of Ang II. Ang II has opposite effects on AT2R; it

increases the production of NO and induces vasodilation. Aminopeptidase A splits Ang II into angiotensin III, which is further split by aminopeptidase M into angiotensin IV, a biologically less effective substance. Angiotensin converting enzyme 2 (ACE 2) is one of the youngest members of RAAS; it can split both Ang I and Ang II thus decreasing the quantity of substrates. On Mas receptors, the resulting angiotensin 1-7 (Ang 1-7) usually generates opposite responses to the effect mediated by Ang II on AT1R. Ang 1-7 can improve inflammatory and fibrotic processes in several organs, e.g. lungs, kidneys and liver. It also has favourable effects on metabolism by improving glucose uptake and lipolysis, resulting in lowered insulin resistance and dyslipidaemia.

Interfering with RAAS by using medication

The complicated RAAS system outlined above is of physiologically enormous importance. Interfering at different points of the system has been a major preoccupation for researchers for some time now. As a result, populous families of ACE inhibitors and angiotensin receptor blockers were discovered. The latest development in this field was the discovery of direct renin inhibitors of which aliskiren was introduced into daily medical practice.

The first ACE inhibitor, captopril, was introduced in the late 1970s. Since then, at least 16 ACE inhibitors have appeared in the market worldwide. They can be divided into three chemical groups, such as compounds containing sulfhydryl, dicarboxylate and phosphonate groups. Much of the developed drugs are so-called prodrugs. Captopril and lisinopril, however, are active agents. Various ACE inhibitors may differ from one another based on the duration of inhibiting circulating ACE and the degree to which they can inhibit ACE in the tissues. ACE inhibition in the tissues in equipotent doses is multifactorial, the binding strength to ACE's terminal C and dissociation kinetics of the inhibitory molecule as well as the extent of tissue penetration by the ACE inhibitor and its lipophilicity being some of the factors. Although extensive information is available about the function and elements of RAAS in blood circulation, many researchers think that the medical importance of RAAS modulation is due to the little-known renin-angiotensin-aldosterone system in tissues, which was described as early as the 1980s. While a decrease of 20% in primary end-point could be detected in studies on perindopril and ramipril, trials using trandolapril and quinapril,

substances of greater tissue activity given in secondary prevention, did not exhibit a significant effect on survival or the prevention of coronary events. In the latter cases, differences in the tissue affinity of ACE inhibitors may be detected in the background.

The efficiency of ACE inhibitors has been demonstrated in several clinical studies on large samples. These inhibitors decrease the incidence of cardiovascular death, non-fatal myocardial infarction or cardiac arrest in stable coronary disease; they improve the prognosis of myocardial infarction, inhibit the process of left ventricular remodelling, delay the development of hypertension and reduce left ventricular mass index in left ventricular hypertrophy. In addition, ACE inhibitors reduce the chances of nephropathy to develop, incidence of microalbuminuria and likelihood of newly diagnosed diabetes. The latest therapeutic guidelines take the above for granted; that is how ACE inhibitors have become medicines recommended as basic therapeutic agents in the primary and secondary prevention of diseases of the highest mortality.

Importance of the ACE genotype

The clinical efficiency of ACE inhibitors appears to be genetically determined as, comparing Afro-American patients and patients of the Caucasian race, ACE inhibiting treatment has been less efficient in the former group. Genetic studies have highlighted that ACE expression in the genetic substance of ACE is controlled by insertion/deletion (I/D) polymorphism, which in individuals of the DD genotype results in 50% higher ACE expression than in those of the II genotype. Accordingly, elevated serum ACE levels in the presence of the D allele are suggestive of the D allele acting as a major factor of risk for cardiovascular diseases. Although not to a significant extent, hypertension was ten percent more commonly detected among patients of the DD genotype. Together with genetic differences, environmental factors and lifestyle and ethnic dissimilarities may all play a decisive role in the development of hypertension. The presence of the D allele, for example, is an independent predictor of ischaemic stroke in the family of cardiovascular diseases. Diabetes sufferers carrying the DD allele are more likely to develop nephropathy. In 2003, Sayed-Tabatabaei published a meta-analysis based on the data of 9833 patients in 23 articles, in which the extent of atherosclerosis was established in view of the proportion between the tunica intima and tunica media in the carotid artery. This publication reported significantly higher levels of

atherosclerosis among patients of the DD genotype. Even more striking results were disclosed if cerebrovascular disease, diabetes and hypertension occurred simultaneously.

Endogenous ACE regulation

The issue of the potential of endogenous ACE inhibition was raised as early as the late 1970s. In 1979, two teams published their results independently of each other. Ryan et al detected a substance, capable of ACE inhibition, of approximately 10 kDA in molecular size in the serum and urine of guinea pigs and possums. Klauser and his team identified human albumin and acetyl tryptophan, a preservative, as endogenous ACE inhibitors in a commercially available plasma preparation. They justified that the C-terminal of albumin alone had a more significant endogenous inhibitory effect than the whole protein did. In the 1980s, four teams published their results about ACE inhibition. Among them, the results by Liebermann et al should be highlighted; they isolated a molecule from the sera of patients with sarcoidosis, exceeding 50 kD and capable of reversible inhibition, which made it difficult to measure ACE serum levels. After the dilution of the serum by eight times, the measurements could be performed. At the end of the 1990s, Thevananther et al separated a protein with ACE inhibiting effect of 14 kD in molecular mass using affinity chromatography and polyacrylamide gel electrophoresis.

In the past few years, our team also confirmed that human serum albumin (HSA) had endogenous ACE inhibiting properties. HSA inhibited serum ACE activity at 5.7 ± 0.7 mg/ml IC_{50} while the physiological concentration of human serum albumin fell in the range of 35-52 mg/ml. The above data show that human serum albumin has a significant inhibitory effect on angiotensin converting enzyme *in vivo*. We have found that human serum albumin can stabilize angiotensin converting enzyme (ACE) activity at a very low level, independently of the level of ACE expression.

Importance of coronary bypass surgery

Coronary artery bypass grafting (CABG) is the most commonly performed cardiovascular surgical intervention, in which new pathways are created to replace narrowed or obstructed vessels in an attempt to improve the oxygen and nutriment supply to tissues with poor perfusion. Surgeons have used saphenous vein grafts to bypass coronary arteries since the 1960s. They are commonly used owing to their elasticity, easy accessibility and low dissection rate. Unfortunately, practice has soon established that the life expectancy of

venous grafts is relatively short; various degenerative processes may cause early degeneration in the first year after insertion. The ratio of graft obstruction is 15-30% and 70% in the first postoperative year and within ten years, respectively. Atherosclerotic degeneration of the grafts plays an important role, and it may differ from processes characteristic of the patient's own vessels as far as duration and certain aetiological factors are considered. Two periods are distinguished in the literature, the first is the postoperative one-year period while the second is the late graft degeneration period, following the first postoperative year. Insufficient adaptation of the venous endothelium to increased pressure in the arterial system is thought to be the most important degenerative factor, as it puts mechanical stress on the venous system. Early graft degeneration is mainly attributed to technical reasons, such as perioperative damage to the venous endothelium (e.g. due to tension forces caused by inflation), winding course of the venous graft (it encourages the deposition or aggregation of thrombocytes) and disproportion between the output and input surfaces of venous grafts and arteries, respectively, which results in difficulty of unification and an increase in the shearing effect of local turbulence. Due to all of the above, thrombotic processes play a leading role in the first month, which is taken over by hyperplasia of the tunica intima in the 2nd to 12th months. After the first postoperative year, it is atherosclerosis that becomes the most harmful process.

Although patients undergoing CABG operation have an increasingly higher risk, perioperative mortality rates are decreasing. On the other hand, it is reported that the patients' long term survival after bypass surgery has not actually improved over the past 20 years. Several studies have been devoted to the exploration of optimal medication to slow down the progression of atherosclerosis in patients after bypass surgery, secondary prevention being in the focus of attention. Acetyl salicylic acid, beta blockers, statins and the family of ACE inhibitors or angiotensin receptor blockers (ARB) are baseline medicines in the postoperative period.

 Whether the different types of grafts stay open or not or if they survive, depends on the graft's tissue composition. The internal mammary artery exhibits the longest, occasionally lifelong survival. Prepared radial arteries stay permeable for 10-15 years on average. Saphenous veins have the poorest chance to stay open due to the aforementioned degenerative processes (mean: 7-9 years). According to a Canadian medical team, the

chances for arterial and venous grafts to stay open is predictable. Enhanced graft degeneration is expected in diabetes sufferers, and the levels of certain biomarkers, such as plasma fibrinogen, creatinine and high density lipoprotein (HDL) as well as those of glutation-S-transferase $\alpha 3$, forecast increased graft degeneration. Comparing the smooth, short-term survival period among patients having received an internal mammary artery graft or a saphenous vein graft, it seems necessary to individually treat saphenous vein grafts after surgery in order to improve graft viability.

GOALS

- Checking endogenous ACE inhibition triggered by serum albumin in previous clinical studies.

- Exploring the possible role of ACE inhibiting serum albumin at different ACE levels.

- Studying the cardiovascular role of the ACE I/D polymorphism in a Hungarian sample of patients.

- Identifying relationships between ACE expression and the life expectancy of vessels used in coronary bypass grafting in hope for finding individually tailored therapies.

MATERIALS AND METHODS

Ethical approval

All of the investigations were done after approval issued by the Ethical Committee of the Medical and Health Science Centre at the University of Debrecen (UDMHSC REC/IEC, 2894-2008) and the Hungarian Medical Research Council. Every subject included in the study has signed an informed consent.

Collecting blood samples, serum and DNA isolation

Blood samples were collected in two ways; peripheral venous blood was drawn from 151 volunteers at the outpatient unit of Department of Cardiology, UDMHSC, using the traditional aseptic method and collecting 1x10 ml of untreated blood and 1x5 ml blood treated with an anticoagulant, per person. Further samples (n=76) were obtained from patients undergoing bypass surgery at the Department of Cardiac Surgery, UDMHSC (1x10 ml of untreated blood and 1x5 ml of blood treated with an anticoagulant). Serum and cellular fractions of the untreated samples were separated in a centrifuge and stored at -20 °C. Genomic DNA was prepared from anticoagulated venous blood using a DNA separation kit (Qiagen).

Measuring ACE activity

ACE activity was measured according to the methods by Beneteau (Beneteau et. al. 1986) and Murray (Murray et. al. 2004). Briefly, ACE activity was determined at pH 8.2, using an artificial substrate and a reagent consisting of 25 mM HEPES, 0.5mM FAPGG, 300 mM NaCl and the serum in the required dilution. The examination was performed in a 96-well plate (Greiner-Bio One) at 37 °C. Changes in optical density (340 nm) were detected at 5-minute intervals for at least 90 minutes (NovoStar plate reader, BMG Labtech). Values of optical density were plotted against the time of reaction and straight linear regression line was fitted to it. Fitting and results were accepted at r^2 >0.90. ACE activity was calculated as follows:

ACE activity = *-(S/k)xD*

in which S is the rate of decrease in optical density (1 per minute), k is the change in optical density until the splitting of 1 nmol of FAPGG is complete and D is serum dilution. ACE activity was given in units (U); 1 U equals 1 nmol FAPGG splitting per minute. The inhibiting effect of captopril (ACE inhibitor) on human serum ACE was examined in the 0.1 nM-1.0 mM concentration range.

Measuring serum ACE concentration

Serum ACE concentration was measured using a commercially available ACE ELISA kit (R&D Systems). The plates had been coated with 80 ng/well anti-ACE antibodies and the remaining binding sites were blocked using inert proteins in Dulbecco's phosphate-buffered saline solution. Using diluted reagent, we added 100-times-diluted serum to the surface and introduced antibody-antigen complexes labelled with biotinylated detection antibodies into the wells. Eventually, the quantity of complexes was detected using a substrate solution containing 0.3 mg/ml of tetramethylbenzidine, $0.1 \mu M$ of H_2O_2 and 50 mM of acetic acid. The reaction was terminated in 20 minutes, by the addition of 0.5 M of hydrochloric acid. Optical density was measured at 450 nm. The result of serum ACE expression was given as the quantity of ng ACE/ml serum.

Calculating ACE activity in undiluted human serum

ACE activity in undiluted serum was calculated using the formula below:

K_i=([Active ACE]x[HSA])/[Inactive ACE]

K_i is the constant of HSA inhibition (83 μM). [Active ACE] means the concentration of active forms of ACE. [Inactive ACE] stands for the concentration of the inhibited forms of ACE, while [HSA] represents human serum albumin concentration determined using colorimetry.
In the calculations the following were regarded:
[Full ACE quantity] = [Active ACE] + [Inactive ACE], in which [Full ACE quantity] was measured using ELISA,
[Active ACE] = (K_i x [Full ACE quantity]) / ([HSA] + K_i).
In order to assess activity in undiluted human serum, ACE activity was measured in the samples of the same patient, the dilution being 20 times, which was related to [Active Ace] and in that case ACE activity was regarded uninhibited by HSA.

Collecting human tissue samples during bypass surgery

A prospective study was conducted in order to examine angiotensin conversion in human vascular tissue. Blood samples and distal segments of the saphenous vein and mammary artery were collected from patients having undergone coronary bypass surgery. The study involved 76 patients. The medical team consisted of four cardiac surgeons who harvested vascular grafts using the same technique. Still in the operating theatre, extra grafts left over from operations were immersed into ice-cold physiological saline solution (KREBS solution) and transferred to our laboratory. These tissue samples were used in functional measurements (to measure isometric contractile force) as well as to determine ACE expression in the tissues. Vessel samples were cut into circular, ring-like preparations of 4 mm in width and put into ice-cold oxygenated physiological saline solution (KREBS) for further functional examinations. Some were dried and frozen for biochemical tests. Blood samples were collected in order to determine ACE genotype and serum ACE.

Measuring vasoconstriction in bypass grafts

The aforementioned vessel rings were fixed and their contractility was measured in an isometric contractility measuring system (DMT 510A, Danish Myotechnology). Smooth muscle functions and endothelial integrity were tested after the addition of 30 μM norepinephrine and 10 μM methacholine, respectively. Vascular contractility was tested using angiotensin I and II (both manufactured by Sigma-Aldrich) in increasing concentrations. In many cases, tests were also performed by adding type 1 angiotensin II blocker (AT1R) – telmisartan 1.0-10 nM – while in other cases, type 2 angiotensin II receptor blocker (AT2R) – 10 μM of PD123319 – as well as ACE inhibitor, captopril (0.1-100 μM), and chymase inhibitor, chimostatin (300 μM), were added. (All of the chemicals were produced by Sigma-Aldrich). Contractility response was either compared to the maximum effect caused by the medicine or the maximum effect of norepinephrine achieved prior to treatment with angiotensin.

Measuring tissue ACE expression in patients after bypass surgery

The frozen vessel samples were homogenized and separated in a centrifuge (at 16,000 g, 4°C, for 5 minutes). The supernatant had been collected and stored frozen (-20 °C) before

the measurements. ACE concentration was determined using the Human ACE ELISA system (described above), following the manufacturer's protocol (R&D) closely. At least two independent measurements were used on average in order to assess ACE concentration, while protein determination was based on three parallel measurements. The expressed quantity of ACE was given in ng ACE/mg protein units.

Determining the I/D polymorphism of ACE

Genomic DNA was isolated from leukocytes of peripheral blood using FlexiGene[®] DNA Kit (Qiagen). The insertion/deletion (I/D) polymorphism of ACE was determined by the PCR amplification of alleles I and D, according to the standard protocol elaborated by Rigat et. al. (Rigat et. al. 1992). The products obtained by amplification were separated by polyacrylamide gel electrophoresis and visualized using ethidium bromide staining. The presence of I or D alleles was detectable in products of 490 bp or 190 bp obtained by PCR.

Statistical analysis

We carried out one-way ANOVA using Graphpad Prism software (GraphPad Software) supported by Dunnett multiple comparison test and t-test, the differences of $p < 0.05$ being regarded significant. On examining the relationship of continuous clinical parameters, we used regression analysis.

RESULTS

ACE genotypes of blood samples obtained for human serum albumin investigations in view of morphometric and laboratory parameters, and the drugs used

Blood samples from 151 hypertensives were collected. Based on the I/D genotype of ACE, they were divided into three groups (DD, ID, II). The most important morphometric parameters, such as distribution according to gender, age, body mass index, smoking habits, left ventricular systolic ejection fraction, systolic and diastolic pressure, and heart rate were included in a table. Presence of diabetes and hyperlipidaemia, current total cholesterol level and the most important parameters of renal function (serum urea, creatinine and glomerular filtration rate) were also recorded. We divided antihypertensive medicines commonly taken by the patients into three groups but found no significant statistical differences among the groups.

Relationship between serum ACE concentration and activity

First, we checked the endogenous ACE inhibiting potential of human serum albumin (HSA). We mapped ACE activity as the functioning of the serum angiotensin converting enzyme, which showed linear correlation in both individual patients and groups of patients with different endogenous ACE expression. But the five-fold rise in serum ACE concentration resulted in only 2.3-fold elevation in serum ACE activity. Accordingly, the rise of serum ACE concentration from 62 ± 11 ng/ml to 252 ± 32 ng/ml (elevation in expression by 4.1 times) was accompanied with partial elevation in ACE activity (by 2.1 times) from 27 ± 7 U/ml to 56 ± 2 U/ml.

Decisive role of ACE genotype in serum ACE concentration and activity

In patients homozygous to the D (deletion) allele of the ACE gen, higher serum ACE concentration could be detected compared to those who were homozygous to the I (insertion) allele (Insertion, II=47-194 ng/ml; mean: 94.5 ng/ml, n=28, ID=36-202 ng/ml, mean: 112.9 ng/ml, n=70, DD=74-188 ng/ml, mean: 154.9 ng/ml n=53). ACE activity was also elevated in DD homozygous patients (II=15.6-55.4 U/ml, mean: 32.74 U/ml, n=28, ID =15.2-

59.3 U/ml, mean: 35.94 U/ml, n=70, DD=27.3-59.8 U/ml, mean: 42,95 U/ml, n=53). Overall, elevation of serum ACE concentration by 64% was accompanied with only 31% rise in ACE activity compared to patients of genotype II.

Role of serum albumin concentration in controlling serum ACE activity

These data suggest that, in addition to human serum ACE concentration, ACE activity is controlled by further factors. Based on our earlier results we have presumed that human serum albumin acts as a certain kind of ACE inhibitor which has a concentration-dependent effect on serum ACE activity. In *in vitro* trials, we tried to find correlation between serum albumin concentration and serum ACE activity in human serum. Specific ACE activity was higher in case serum albumin concentration was decreased owing to the dilution of the serum. ACE activity decreased from 56.13±1.17 U/ml to 38.55±0.78 U/ml if HSA concentration rose from 2.42±0.02 mg/ml to 12.12±0.12 mg/ml. In relation to human serum ACE-HSA, the constant of stability was (K_i) 5.7±0.7 mg/ml, which was suggestive of ACE activity of 8.47±0.18 U/ml (calculated values) in the presence of 48.46±0.46 mg/ml HSA.

Examining the relationship between morphometric parameters and serum ACE concentration

We have tested if the endogenous inhibitory effect of HSA on serum ACE is suitable to compensate for the differences in ACE expression levels in patients suffering from hypertension in whom therapeutic ACE inhibition has been partly effective. A sample of patients (n=151) on ACE inhibition were included in the study. Serum ACE concentration showed no relationship with amorphometric parameters, e.g. age, body weight and height or body mass index.

Examining the relationship between serum lipid levels and serum ACE concentration

Total cholesterol and triglyceride levels were checked and plotted against the function of serum ACE concentration (n=151 patients). A straight linear regression line was fitted to the points in order to find the possible correlation among the parameters in the graph. The normal values of total cholesterol and triglyceride were 5.2 mmol/l and below 1.7 mmol/l,

respectively. Serum ACE concentration showed no relationship with total cholesterol or triglyceride levels.

Examining the relationship between serum ACE concentration and cardiovascular parameters

Systolic and diastolic parameters (ejection fraction, heart rate, systolic and diastolic pressure) were plotted against serum ACE concentration (n=151). Ejection fraction, heart rate, and systolic and diastolic pressure were regarded normal above 50%, between 60-100/min, and in the range of 90-140 mmHg and 60-100 mmHg, respectively. Cardiovascular parameters (ejection fraction, heart rate, systolic and diastolic pressure) were also independent of serum ACE concentration, due to inaccurate fitting (based on low r^2 values).

Relationship between serum ACE concentration and renal function parameters

Renal functions were established on the basis of the mean serum concentrations of creatinine and uric acid and using the glomerular filtration rate (GFR). Values were given in the function of serum ACE concentration (n=151). Normal values were as follows: urea 3.6-7.2 mmol/l, creatinine 44-97 µmol/l (female) and 62-106 µmol/l (male), GFR below 90 ml/min/1.73m^2, uric acid 140-340 µmol/l (female) and 220-420 µmol/l (male). Renal function parameters (serum urea, creatinine and glomerular filtration rate) were widely scattered (low r^2 values), denying correlation with serum ACE concentration.

Relationship between the patients' parameters and I/D genotype of ACE

Different parameters (age, weight, body mass index, total cholesterol, triglyceride, ejection fraction, heart rate, systolic and diastolic pressure, urea, creatinine, glomerular filtration rate and uric acid) were correlated with the patients' genotypes (insertion: II, n=28, deletion: DD, n=53, and heterozygous: ID, n=70). Like above, no relationship was found between these clinical parameters and genotype of ACE or the individual ACE expression levels.

Analysis of the distribution of samples from the saphenous vein and radial artery in view of morphometric and laboratory parameters and the drugs used

Further investigations were targeted at patients having undergone bypass surgery. Samples of the *radial artery* (120 segments from 31 patients) and *saphenous vein* (148 segments from

45 patients) were harvested from a total of 76 patients. The most important morphometric parameters such as distribution according to gender, age, body mass index, systolic and diastolic pressure and heart rate were included in a table. In addition to checking cholesterol and triglyceride levels, as well as fasting blood glucose concentration, we analyzed the presence of diabetes and the NYHA status at the time of surgery. Common, preoperatively administered primary and secondary prophylactic medicines were also included in the table. No significant statistical differences were found between the two groups.

Contractile responses to angiotensin I and angiotensin II

Our investigations were aimed at characterizing ACE in the tissues via checking the effect of vasoconstriction, ACE expression and ACE activity with regard to tissue specificity (radial artery and saphenous vein grafts) and genotype. Treatment with angiotensin II (Ang II) caused significant vasoconstriction in both the samples from the radial artery and segments of the saphenous vein. Vasoconstriction in samples from the radial artery (82±6%, n=30) was significantly higher (p<0.01) compared to the relevant parameters for the saphenous vein (61±5%, n=39). Maximum vasoconstriction was assessed after the addition of 30 μM norepinephrine. Although the differences in maximum vasoconstriction responses were quite significant to Ang II, Ang II sensitivity was also much higher in samples from radial arteries (p<0.01) compared to those from saphenous veins (3.0±0.5 nM, p=31 and 6.6±0.9 nM, p=36, respectively). Preliminary treatment with the type 2 angiotensin receptor II (AT2R) inhibitor (PD123319 10 μM) caused no change in Ang II sensitivity in radial arteries (EC_{50}=4.4±0.7 nM, n=27) or saphenous veins (EC_{50}=8.2±2.0 nM, n=25), therefore it was concluded that AT2R played a limited role in triggering vascular responses in such experimental systems.

Responses to Ang I were measured in order to examine the conversion of Ang I to Ang II in the tissues. The effect of Ang I at half maximum was EC_{50}=31±6 nM, (n=26) on the radial artery while it was found 30±4 nM (n=40) in the saphenous vein. As the effect of Ang I on tissues develops after it is converted into Ang II, the above experimental results suggest significant conversion of Ang I into Ang II in the tested vessels. We have calculated the proportion of Ang I and Ang II effect (EC_{50}), to quantify the extent of the conversion of Ang I into Ang II. This effect was significantly lower (p=0.02) in the samples of the radial artery (0.17±0.03, n=23) compared to that of the saphenous vein samples (0.51±0.14, n=24). These

results show three times more effective Ang I conversion in the saphenous veins than in the radial arteries.

Mechanisms of angiotensin-induced vasoconstriction

The results have also confirmed that the Ang I effect is triggered by the local conversion of Ang I into Ang II in the saphenous veins. Responses to the dose-dependent inhibition of Ang II (1.0 nM-10 nM) by telmisartan suggest that the Ang II effect involves type 1 angiotensin II receptors (AT1R). Similarly, by administering telmisartan we could inhibit vasoconstriction induced by Ang I and concluded that the effect of Ang I was also communicated by means of the AT1R receptors. We also examined the identity of angiotensin I converting enzyme. After the addition of captopril (100 μM), widely known for its effect to inhibit the angiotensin converting enzyme (ACE), there was substantial decrease in the response to Ang I, which supports the role of captopril in ACE conversion. Moreover, Ang I was also inhibited by simultaneously adding 100 μM of captopril and 300 μM of chymastatin, a chymase inhibitor. Results suggest, that chymastatin can be successfully used if Ang I is present in higher concentrations.

Examining the inhibitory effects of captopril on circulatory and tissue ACE

The inhibitory effects of captopril on both circulatory and tissue ACE were examined in detail. First, we could confirm circulatory ACE inhibition in circulatory ACE. Significantly lower ($p < 0.01$) serum ACE activities (7.0±1.0 U/l, n=15) were measured in patients treated with ACE inhibitors compared to ACE activities in untreated patients (22.0±2.0 U/l, n=15). Next, the effect of captopril on human tissue ACE was tested in vitro. Based on the results it was evident, that captopril was capable of completely inhibiting human serum ACE at 15 nM (IC_{50}). We also tested the converting effect of captopril on tissue angiotensin I in order to assess the inhibitory potential of this drug on tissue ACE. Captopril was ineffective at a concentration of 1 μM, but concentrations of 10 μM and 100 μM had non-competitive inhibitory effects.

Expression of angiotensin converting enzyme in blood circulation (serum) and tissues (saphenous vein)

ACE expression was measured in both tissue samples. Twice as high ACE expression was detected in saphenous veins as in tissues from the radial artery (5.3 ± 0.7 ng/mg tissue protein, n=14 in the radial artery and 9.7 ± 1.0 ng/mg, n=35 tissue protein in samples from the saphenous vein, p<0.01). These results are in harmony with the threefold Ang I conversion efficiency. In contrast, no difference in ACE concentration was detected in the specific patients' blood samples (p=0.12, serum ACE concentration in samples from the radial artery: 164 ± 21 ng/ml n=14, whereas in samples from the saphenous vein: 134 ± 10 ng/ml, n=34).

ACE expression in tissues and circulation depending on the ACE genotype

Other research teams have also suggested serum ACE expression is controlled by genetic polymorphism. In our study, we have also managed to support this hypothesis about ACE expression. In patients of the DD genotype, serum ACE expression has been significantly higher than in those of the II genotype (171 ± 15 ng/ml, n=13, and 97 ± 15 ng/ml, n=11, respectively, p<0.01). However, in the samples from the saphenous vein from the same patients, no relationships determined by the same genetic polymorphism in the background of tissue ACE expression has been detected (9 ± 1 ng/mg, n=11 in genotype II and 9 ± 1 ng/mg in genotype DD, n=13). Further detailed research has demonstrated that tissue (from the saphenous vein) and serum ACE concentrations are regulated independently of each other (the results cannot be fitted with a straight line of linear regression; in the best fit: $r^2=0.014$, n=35).

Accordingly, human serum ACE expression has been detected in a very wide range (47-288 ng/ml) and it was found 64% higher in patients of the DD genotype than of the II genotype. The obviously missing correlation between cardiovascular parameters and ACE expression (genotype) is surprising, especially because the cardiovascular efficiency of ACE inhibitors is supported by several clinical studies on large samples, and approved guidelines. In view of previous data we have presumed that the differences in ACE expression, associated with the I/D polymorphism of ACE, may play a role in the development of myocardial infarction, coronary diseases, sclerosis of the coronary arteries, cardiac failure and hypertension. We could not detect a similar relationship, which was in agreement with the results of a meta-analysis including more than 30 000 subjects.

In the present study we have directly tested the relationship between ACE expression and ACE activity in the serum of hypertensives. We have found that the serum ACE concentration of patients of the DD genotype was 64% higher than those of the II genotype; the difference in ACE activities was only 31%, suggesting that higher ACE expression is buffered under physiological conditions. Our observations are in agreement with the ones according to which the local conversion of angiotensin I into angiotensin II and the II/I proportion of angiotensin are independent of the genotype in human samples.

It is important to remark that the issue is more complicated than just determining serum ACE activity accurately. It is partly due to the fact that previous investigations informed about the significant effect of diluting the serum on the values of ACE activity. In order to reduce such effect the use of a high dilution factor is recommended. This is consistent with the results by our team as we have identified serum albumin as an endogenous ACE inhibitor at K_i = 5.7±0.7 mg/ml. According to our experience, if serum albumin is removed from the human serum, the actual serum ACE activity is higher and there is no dilution effect.

The novelty of our study is that it demonstrates the inhibitory effect of serum albumin on ACE in clinical circumstances. In addition to the 4.1-fold elevation in the ACE concentration in the patients' sera, there was a 2.1-fold rise in ACE activity, which suggests that serum ACE activity is under control. In addition to enzyme concentration, other factors also contribute to ACE activity. These factors may suppress ACE activity at different levels of ACE expression if the presence of a mechanism to stabilize endogenous ACE activity is presumed. Suppression may be clinically significant even if circulatory ACE levels are elevated due to genetic factors (e.g. I/D polymorphism of ACE) or disease. Point mutation arising from the *saar* region of the ACE gene, can cause five-fold increase in circulatory ACE concentration. Several other mutations of the ACE gene are accompanied by mild elevation in ACE concentration without causing increase in the incidence of cardiovascular diseases. All these observations suggest that the significant differences in ACE concentration can be well tolerated. This is explained by the fact that ACE activity is stabilized by endogenous factors at a lower level. Our group's earlier results justified HSA being such an endogenous ACE inhibitor. The present study was aimed to examine to what extent human serum albumin in human serum samples was responsible for the inhibition of the ACE gene. ACE activity can be determined in the presence of a specific amount of HSA (e.g. 56±14 U/ml 2.4±0.3 mg/ml

of HSA) but it decreases, in correlation with the ACE-inhibiting potential of HSA, if the concentration of HSA is increased.

Unfortunately, direct measurements of ACE activity were impossible in undiluted serum. ACE activities were measured after four-fold dilution as the serum has substantial power to absorb light at 340 nm, which does not allow us to measure the conversion of the specific substrate. ACE activity was assessed in undiluted serum by measuring serum albumin and ACE concentrations. In the assessment we also used the results of ACE activity measured at 20-fold dilution and using the HSA constant (K_i= 5.7±0.7 mg/ml), determined in human serum earlier. Considering these measurements and calculations – based on our results – the ACE activity in human serum can be approximately 8.5±2.2 U/ml.

ACE (127 ng/ml) and serum albumin (48 mg/ml) concentrations measured in human serum by us suggest that the differences in ACE concentrations are caused by the buffer effect of HSA. Serum albumin is present in a concentration ten times higher than the ACE-inhibiting K_i quantity of HSA. The results of these measurements have been suggestive of ACE being present in circulation in a significantly inhibited form, dependent on HSA.

Suppression of serum activity can serve as a basis for important conclusions in practice. In case of in vivo endogenous ACE inhibition to a great extent, physiological angiotensin II concentration can be profoundly affected by the elimination of angiotensin II. In that case, RAAS activation, serving as the basis for cardiovascular diseases (hypertension, cardiac failure), can be caused by decreasing elimination of angiotensin II. ACE2 is one of the best characterized enzymes which plays a role in the elimination of angiotensin II; and, as the latest findings suggest, its activation provides protection against hypertension, myocardial fibrosis, remodelling and renal damage. In accordance with the above, our research team has also confirmed the role of ACE2 between hypertension and the systolic function of the heart.

Based on another conclusion, serum albumin concentration can also have an effect RAAS function via the modulation of ACE activity. The results support that ACE is well suppressed until serum albumin concentration is found to be at least 30 mg/ml. Nonetheless, human serum albumin concentration may decrease below the aforementioned level, e.g. in both protein malnutrition and renal failure. Also based on our findings, it can be proposed that

administering serum albumin intravenously we can increase ACE inhibition. This has been supported by the observation that postoperative human serum albumin given intravenously has often caused hypotension in patients receiving ACE inhibiting therapy.

Further research was carried out in human vascular samples, such as the *saphenous vein* and *radial artery* to examine tissue ACE in samples for patients having undergone coronary bypass graft surgery (CABG).

Long-term survival in patients after bypass surgery is greatly influenced by the conductivity of the graft, which, on the other hand, is dependent on vascular tissue type. Arterial grafts survive longer than those from the saphenous vein. Due to the short survival of saphenous vein grafts there has been growing demand for a graft-specific treatment by which the uneventful postoperative period of patients after CABG-surgery could be extended. In order to devise a graft-specific treatment, one has to understand factors affecting the much greater vulnerability of saphenous grafts compared to the radial artery. In the first postoperative month, early degeneration of the saphenous grafts is caused by thrombolytic occlusion. Later, processes involving atherosclerotic obstruction associated with neointimal hyperplasia play a dominant role. Angiotensin converting enzyme (ACE) can play a role in these processes because ACE inhibition deceases thrombocyte aggregation, improves endothelial function and moderates neointimal growth. Several studies in the past (QUO VADIS, APRES, HOPE) also reported significant improvement after ACE inhibiting treatment. The study entitled IMAGINE, however, challenged the useful effects of ACE inhibition in the treatment of patients after bypass surgery. All of these contradicting results have urged us to precisely devise and execute our examinations.

We put a lot of effort into trying to confirm the importance of ACE effect on saphenous grafts. We compared tissue samples from the saphenous vein and radial artery. Based on the results, angiotensin converting enzyme expression was double and the conversion of angiotensin I into angiotensin II (Ang I into Ang II) was two and a half time more effective in samples from the saphenous veins than in the ones from the radial arteries.

We have made further efforts to compare angiotensin converting enzyme in circulation and bound in tissues. We have presumed that determining circulatory ACE activity can help us to assess individual risks and tailor the treatment to the individual. Interestingly enough, we

could not find a relationship between the expression of circulatory ACE or tissue ACE. This striking observation can be explained as follows: circulatory ACE expression is determined by a genetic polymorphism but no such background could be detected in the case of tissue ACE expression. This observation can also explain that ACE levels (ACE polymorphism) are not of great significance in different cardiovascular diseases, while, in the same group of patients, ACE inhibitors represent a wide therapeutic arsenal of agents. But beyond that, we have found substantial differences in the quantities of ACE in circulation (serum) and in the tissues (saphenous vein) after inhibition using captopril. Captopril was capable of inhibiting serum ACE at 15 nM, while tissue ACE inhibition could be achieved by administering the drug in doses a thousand times higher (10 μM).

Values of tissue ACE activity were given on the basis of functional measurements. The conversion of Ang I into Ang II was examined in graft tissues obtained prior to bypass surgery. It should be remarked that local ACE levels and ACE activity were determined from venous blood drawn theoretically at the same time with graft implantation.

Finally, mention must be made of the limitations of our study. Putting experimental results into clinical practice appeared to be the greatest challenge for us. While captopril could effectively inhibit serum ACE activity in an order corresponding to the physiological range, it was less effective on tissue ACE inhibition. These observations suggest that certain ACE inhibitors are ineffective in inhibiting ACE activity in grafts from the saphenous vein. Therefore they mask or veil the results of clinical tests performed to prevent the degeneration of saphenous vein grafts. Moreover, graft permeability can be beneficially influenced by higher ACE activity: increased tonicity of the vessels mediated by angiotensin II contributes to the rate of blood flow through them causing decrease in local haemostasis. On the other hand, increased angiotensin II levels can activate neurohormonal mechanisms, making the picture even more complicated. Last but not least, the permeability and rheological characteristics of saphenous veins implanted into coronary circulation change significantly. It would probably highlight the issue of graft permeability, improving due to the effect of ACE inhibitors, if we could carry out further tests to compare two different ACE inhibitors, one of which has a selective effect on ACE found in the tissue of the saphenous vein. In a constellation like that, complications due to the beneficial effects of systemic ACE

inhibition (e.g. consequences of the effect on volume homeostasis and renal metabolism) could be avoided.

SUMMARY

Angiotensin converting enzyme (ACE) inhibitors rank fifth among the most commonly prescribed groups of drugs; they serve as baseline medicines in the treatment of cardiovascular diseases.

In a previous study we identified serum albumin as an endogenous ACE inhibitor at K_i = 5.7±0.7 mg/ml. If serum albumin was removed from the human serum, specific serum ACE activity was higher. The novelty of our study is that it demonstrates the inhibitory effect of serum albumin on ACE in clinical circumstances. Serum ACE activity showed incomplete correlation with ACE concentration. In addition to a 4.1-fold elevation in the ACE concentration in the patients' serum, there was a 2.1-fold rise in ACE activity, which suggested that serum ACE activity was under control. In addition to enzyme concentration, other factors also contribute to ACE activity. A factor like that may suppress ACE activity at different levels of ACE expression if the presence of a mechanism to stabilize endogenous ACE activity is presumed. We also investigated the importance of the effect of angiotensin converting enzyme on grafts from the saphenous vein. We compared samples from the saphenous vein and radial artery. Twice as high angiotensin converting enzyme (ACE) expression and 2.5 times more effective tissue conversion from angiotensin I into angiotensin II were found in the more vulnerable saphenous veins, compared to the findings in the radial arteries. In saphenous veins, tissue ACE expression was independent of the ID gene polymorphism of ACE, which was in contrast with the serum ACE concentration in the same patients, due to the regulatory role of gene polymorphism detected in the background. The expression of ACE in the serum and tissues represents independent processes. The inhibitory potentials of captopril on tissue and serum ACE differ significantly.

In summary it can be concluded that the inhibition of physiological ACE activity using HSA can contribute to tolerating different ACE expression levels determined by the I/D polymorphism of ACE, and, consequently, a decrease in the probable incidence of various cardiovascular diseases. The importance of ACE inhibiting treatment is given special attention in case patients receive saphenous vein grafts: the use of a tissue-specific ACE inhibitor can improve the permeability of saphenous vein grafts.

Key words: saphenous vein graft, angiotenzin converting enzyme, human serum albumin

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2. **Daragó, A.**, Fagyas, M., Siket, M.I., Facskó, A., Megyesi, Z., Kalász, J., Galajda, Z., Szerafin, T.,
   Hársfalvi, J., Édes, I., Papp, Z., Tóth, A., Szentmiklósi, J.: Differences in Angiotensin
   Convertase Enzyme (ACE) Activity and Expression May Contribute to Shorter Event Free
   Period After Coronary Artery Bypass Graft Surgery.
   Cardiovasc. Ther. 30 (3), 136-144, 2012.
   DOI: http://dx.doi.org/10.1111/j.1755-5922.2010.00252.x
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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) I: Endogenous angiotensin converting enzyme (ACE) inhibition.
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4. 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:3.534 (2013)

5. Czikora, Á., Lizanecz, E., Boczán, J., **Daragó, A.**, Papp, Z., Édes, I., Tóth, A.: Vascular metabolism of anandamide to arachidonic acid affects myogenic constriction in response to intraluminal pressure elevation.
   Life Sci. 90 (11-12), 407-415, 2012.
   DOI: http://dx.doi.org/10.1016/j.lfs.2011.12.016
   IF:2.555

6. Domokos É., Kardos L., Bednárszky I., Bene O., Bódi A., **Daragó A.**, Fülöp T., Homoródi N., Péter A., Rácz I., Hegedűs I., Kertész A.B.: Az interventrikuláris szeptum mérése echokardiográfiával: Mérési különbségek és klinikai következményeik.
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