SUMMARY OF DOCTORAL (Ph.D.) THESIS

Regulation of blood vessel diameter by the renin-angiotensin system and the vanilloid receptors

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Debrecen

2006

1. INTRODUCTION

1.1. The renin-angiotensin system and the in-stent restenosis

The renin-angiotensin system (RAS) plays a key role in the regulation of blood pressure and fluid and electrolyte balance in mammals. In the classical pathway of the RAS, renin, which is secreted from the juxtaglomerular apparatus of the kidney in response to a variety of stimuli, acts on the circulating precursor angiotensinogen (AGT) to generate a decapeptide, called angiotensin I (Ang I). Ang I has little, if any, effect on blood pressure but is converted by angiotensin-converting enzyme (ACE) to an octapeptide (angiotensin II, Ang II), which is one of the most potent vasopressor in the body. Ang II acts through two G-protein-coupled receptors, AT_1 and AT_2. In addition to the acut vasoconstrictor effect of Ang II it has long term effects on regulation of gene expression controling cell growth and proliferation, which can be resulted in myocardial and vascular hypertrophy and hyperplasia. These deleterious effects of Ang II are mediated by the AT_1 receptor whereas the AT_2 receptor generally mediates opposing effects.

There are various negative feed-back pathways which might have a role in fine-tuning of the rate at which Ang-derived peptides are formed and degraded. If disintegration of this balance leads to Ang II accumulation, consequently vasoconstriction, cellular hyperplasia and hypertrophy can be the initial steps of coronary artery disease, myocardial infarction and heart failure. Taken together, it is not surprising that among different elements of the RAS AGT, ACE and AT1R became the most investigated molecules. Cloning of the human genes of AGT, ACE and AT1R led to the discovery of numerous genetic polymorphisms – genetic variation of the gene with at least 1 % prevalence –, some of them have been implicated in the pathogenesis of different cardiovascular diseases.

AGT M235T polymorphism differs in the identity of the amino acid (methionine or threonine) in the 235^{th} amino acid position. The T235 allele was found to be associated with elevated plasma AGT levels. The gene encoding

ACE is characterized by an insertion/deletion (I/D) polymorphism. ACE activity was highest in DD homozygotes and intermediate in ID heterozygotes compared to the II genotype. Finally, in case of the AT1R gene, an adenine to citozine polymorphism was found in the 1166 nucleotide position. Several studies have shown that the C allele is accompanied with elevated cardiovascular risk.

The main causes of mortality are coronary artery disease and ischemic heart disease in Hungary. It is of primarily intrest to identify people carrying high risk of cardiovascular morbidity and mortality to improve their therapy.

Percutaneous transluminal coronary angioplasty (PTCA) is an established treatment for patients with occlusive coronary artery disease; however, it has the major drawback of restenosis of the mechanically dilated coronary arteries which occurs in approximately 20-30% of the patients. As well as coronary artery disease, restenosis is a complex phenomenon. Restenosis following coronary stenting depends mainly on neointimal proliferation and it is believed, that Ang II mediated smooth muscle cell overgrowth is predominantly responsible for in-stent restenosis (ISR).

In the last few decades a large number of studies tried to reveal the relationship between the appearance of ISR and genetic polymorphisms of the RAS. It is also important to consider, however, that the ethnic background of the study populations may differ, so the prevalence of gene mutations and/or polymorphisms in one population cannot always be extrapolated to others. Here we investigated the genetic association with ISR in a relatively large cohort of the Hungarian population.

1.2. Determination of AGT M235T polymorphism

Studies on the association of cardiovascular disorders with the AGT M235T polymorphism yielded inconsistant results. One reason of uncertainities could be that different methods were used to determine M235T genotype. Indeed, at least four methods were developed, such as (i) allele-specific oligonucleotide hybridization (PCR-ASO), (ii) mutagenically

separated PCR (MS-PCR) using three primers to amplify all possible alleles in PCR reaction, (iii) Single Strand Conformation Polymorphism analysis and (iv) PCR-RFLP.

During the optimalization of the PCR-RFLP method we encountered a lot of technical difficulties. To achieve our aims (to reveal the role of AGT M235T polymorphism in ISR) it became necessary to improve the published PCR-RFLP protocol to get more reliable genotyping.

1.3. The vanilloid receptor (TRPV1) and its role in the regulation of the arteriolar diameter

The vanilloid receptor (TRPV1) is a non-specific cation-channel with a relatively high selectivity for calcium. It is activated by noxious stimuli like heat, low pH, changes in extracellular osmolarity and pressure, by the pungent ingredient of hot chilli peppers, capsaicin, besides other vanilloids.

The TRPV1 is mainly expressed by nociceptive, primary afferent neurons. Additionally, functional receptors have been shown in different regions of the central nervous system, such as neurons in the cortex, hippocampus, corpus callosum and in the astrocytes and pericytes. Expression of TRPV1 was found in keratinocytes of epidermis, in the urothelium and smooth muscle of the urinary bladder in addition to the liver and macrophages in the periphery.

Activation of TRPV1 at the endings of sensory neurons leads to the elevation of intracellular potassium and calcium concentrations. Na^+-influx causes pain by the sensitization of the central nervous system through depolarization of nociceptors. At the same time elevation of intracellular Ca^{2+}-concentration leads to the local release of various neurotransmitters, like calcitonin-gene related peptide (CGRP) and substance P. These molecules cause the relaese of further mediators, so vanilloid receptors take part in an effectory mechanism beside sensory function.

Relatively little is known about endogenous ligands of the receptor. So far different lipoxygenase-products (12-HPETE, 15-HPETE, 20-HETE), *N*-

arachidonoyl-dopamin (NADA) and anandamide (*N*-arachidonoyl-ethanolamid) have been identified as endogenous agonists of TRPV1. Our studies focused on anandamide, which was originally identified as an endocannabinoid, activating the CB1 cannabinoid receptor.

Although anandamide had similar potency to activate CB1 and TRPV1 receptors, differences have been found in its efficacy: in some systems it was a full agonist while it had only a partial agonistic activity in others. It is noteworthy that the efficacy of anandamide is affected by its metabolism. First, the anandamide membrane transporter is thought to be responsible for the uptake of extracellular anandamide. Next, inside the cell, anandamide is rapidly metabolised by fatty acid amide hydrolase and various oxygenases.

It has been pointed out that the partial activation of TRPV1 may evoke partial inhibition for other endogenous ligands. Such ligands could be NADA or other as-yet-unidentified endogenous ethanolamides. It has also been suggested that stimulation of TRPV1 by anandamide leads do desensitization to subsequent agonist challenge (tachyfilaxis). The efficacy of the ligands acting on TRPV1 is largely influenced by the phosphorylation state of the receptor. Protein kinase A (PKA), protein kinase C (PKC) as well as Ca^{2+}/calmodulin dependent kinase II (CaMKII) are able to phosphorylate TRPV1. Phosphorylation of TRPV1 by PKC and PKA sensitizes TRPV1 to vanilloids, anandamide, heat and protons.

The physiological role of TRPV1 in several organs was investigated and the receptor was associated with the pathophysiology of various major diseases. Our interest turned to the effect of TRPV1 and its ligands on the diameters of microarterioles. These arterioles with a diameter less than 500 μm are the main regulators of local tissue circulation. Both anandamide and capsaicin cause vasodilatation of mesenteric, hepatic, basilar, and meningeal arterioles through the stimulation of cannabinoid and TRPV1 receptors. It has also been reported that activation of TRPV1 can cause vasoconstriction in rat and canine

mesenteric arteries or in coronary vessels. Moreover it was also found that *in vivo* anandamide treatment caused a transient vasoconstriction in renal, mesenteric, and hindquarters vessels in conscious rats. In these reports TRPV1-mediated substance P or endothelin release was suggested as possible mechanisms.

Here we investigated the physiological role of vanillod receptors in skeletal muscle arterioles which have essential role in the regulation of local tissue circulation. Our aim was to identify TRPV1 expressing cells and to investigate the stimulation of the receptor by anandamide in perfused arterioles isolated from the gracile muscle of rats. An exogenous TRPV1 expressing cell line (CHO-TRPV1) was used to specifically evaluate TRPV1 mediated effects of anadamide. Finally we characterized the role of phosphorylation state of the receptor.

2. **AIMS**

Our aims in this study were to:

1. determine ACE I/D, AT1R A1166C, and AGT M235T polymorphisms
2. investigate the possible associations between ACE I/D, AGT M235T, AT1R A1166C polymorphisms and in-stent restenosis following percutaneous coronary intervention
3. demonstrate functional vanilloid receptors in skeletal muscle arterioles
4. identify TRPV1 expressing cells
5. study the stimulation of the receptor with anandamide
6. specifically evaluate TRPV1 mediated cellular effects using an exogenous TRPV1 expressing cell line (CHO-TRPV1)
7. investigate the role of phosphorylation in the regulation of TRPV1 responsiveness to anandamide

3. METHODS

3.1. Study population of ISR

239 patients were involved in the study (all of them Caucasian and residents of North-East Hungary) who underwent coronary stent implantation and a follow-up angiography performed in the case of recurring complaints and/or positive stress tests. ISR was defined as an in-stent luminal narrowing exceeding 50% at the follow-up angiography as compared with the findings immediately after the intervention. Clinical data relating to conventional cardiovascular risk factors were obtained from the medical reports. The genotyping of the patients was performed before the follow-up angiography. The mean age of the patients was 58,9±9,4 years; 155 were men. The post-procedural pharmacological therapy consisted of aspirin (100 mg daily) and clopidogrel (75 mg daily) for all patients. Treatment with ACE inhibitors was prescribed at the discretion of the attending physician. The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All patients gave their informed consents to the diagnostic and interventional procedures.

3.2. Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using FlexiGene DNA kit (Qiagen). The ACE gene I/D polymorphism was determined by the PCR-method of Rigat *et al.* (Nucleic Acids Res 1992; 20(6):1433.), with direct PCR amplification of the DNA-sequence containing the insertion.

To determine AGT M235T polymorphism a PCR-RFLP method developed by Russ *et al* (Hum Mol Genet 1993; 2(5):609-610.) was used at the beginning. Later the original protocol was improved, by adding 10% DMSO, using 64°C annealing temperature, and a new antisense primer: 5'– GCC AGG GTG CTG TCC ACA CTG ACT CCC – 3' (Sigma). These PCR-products were digested with Box I (Fermentas) restriction enzyme.

AT1R gene A1166C polymorphism was determined by PCR-RFLP method previuosly described by Steeds *et al* (Atherosclerosis 2001; 154(1):123-128.).

3.3. Statistical analysis of the patients data

Allele frequencies were counted and compared with Hardy-Weinberg predictions by χ^2 analysis. Data on quantitative clinical characteristics were expressed as means ± standard deviations, other characteristics were expressed as percentages or absolute numbers, as indicated. Comparisons between groups were made with the χ^2 test (nominal data) or Student's *t*-test for independent samples (continuous data). A value of $p<0,05$ was considered to be statistically significant. A logistic multivariate model was applied to assess the predictive value of factors on ISR. Statistical analyses were carried out with Analyse-it for Microsoft Excel (Analyse-it Software).

3.4. Isolation of skeletal muscle arterioles and measurement of the vascular diameter

Wistar rats were anesthetized. Using microsurgical instruments and an operating microscope, the gracile muscle arteriole running intramuscularly was isolated, cannulated and transferred into an organ chamber filled with physiological salt solution (Krebs) composed of (in mmol/L): 110 NaCl, 5,0 KCl, 2,5 $CaCl_2$, 1,0 $MgSO_4$, 1,0 KH_2PO_4, 5,0 glucose and 24,0 $NaHCO_3$ equilibrated with a gas mixture of 10% O_2 and 5% CO_2, balanced with nitrogen, at pH 7,4. After isolation, the vessels were cannulated, intraluminal pressure was set to 80 mmHg while temperature was set at 37^{o}C. The internal arteriolar diameter at the midpoint of the arteriolar segment was measured by videomicroscopy. During a 60-120 min regeneration period the arteries dveloped a spontaneous myogenic tone. At the beginning of every single experiment the endothelial function was checked by acethyl-choline (100 nM), and the smooth muscle function was tested by norepinephrine (100nM). Cumulative dose-response measurements were used to determinate capsaicin

and anandamide responsiveness. In addition to the TRPV1 ligands, vessels were also treated with the protein phosphatase 2B inhibitor Cyclosporin-A.

Animal experiments were in accordance with the standards established by the National Institutes of Health.

3.5. Demonstration of TRPV1 by immuno-histochemistry

Cryostat sections (thickness 10 μm) of frozen skeletal muscle (m. gracile) tissue samples were placed on adhesive slides and fixed in acetone for 10 min. The slices were blocked with normal goat sera (1,5% in PBS, Sigma) for 20 min and stained with an anti-capsaicin receptor antibody (raised in rabbit, Calbiochem) at 1:100 dilution in the blocking buffer. Then, the slices were incubated with a biotin-labeled anti-rabbit antibody (1:200, Vector Laboratories) and the immunocomplexes were visualized by a Vector VIP substrate (Vector Laboratories) according to the manufacturer's instructions. Finally, the slices were also stained with methyl green to visualize the nuclei.

We used the same method for immuno-fluorescence microscopy, with the following differences: the secondary antibody was a FITC-conjugated anti-rabbit antibody, raised in goat, at 1:100 dilution (Vector Laboratories). To visualize the smooth muscle cells a monoclonal mouse anti-smooth muscle-actin antibody (Novocastra) was used at 1:50 dilution. In this case the secondary antibody was a Texas red-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories), raised in goat, at 1:100 dilution. Finally the sections were covered with Vectashield (Vector Laboratories) cover-medium.

3.6. Confirming TRPV1 expression by RT-PCR

Messenger RNA samples of rat aortic tissue and cultured A7r5 smooth muscle cells were isolated by an RNeasy RNS extraction kit (Qiagen). Using these templates full length cDNA was synthetised by RevertAid H Minus kit (Fermentas). The TRPV1 encoding mRNA sequence was investigated with a sense primer: 5' – CTA CCT GGA ACA CCA ATG TGG G – 3' , and an antisense primer: 5' – GCT GGG TGG CAT GTC TAT CTC G – 3' (Sigma).

GAPDH was used as a constitutively expressing gene, and the sense primer: 5' – CTC CCT CAA GAT TGT CAG CAA – 3' and the antisense primer: 5' – CAG ATC CAC AAC GGA TAC ATT – 3' were. In this latter case a 269 bp PCR product appeared. During TRPV1 primer design we choosed a sequence which results a 596 bp product from genomic DNA (did not apperaed in our experiments) while a 149 bp product from cDNA.

3.7. CHO-TRPV1 cell culturing

The TRPV1 expression was induced by tetracyclin withdrawal in the selected, stable CHO cell clone expressing TRPV1 (Tet-Off induced CHO-TRPV1 cells).

3.8. $^{45}Ca^{2+}$-uptake experiments

For $^{45}Ca^{2+}$ uptake, CHO-TRPV1 cells were plated in 24-well plates to yield a cell density of 20-40% confluency. The next day, the media was changed to remove the tetracycline in order to induce TRPV1 expression. Experiments were performed approximately 48 h after induction. CHO-TRPV1 cells were used immediately upon removal from the CO_2 incubator or were preincubated with 100 nM PMA, 100 nM CY-A or with 100 nM PMA and 100 nM CY-A applied together. For the $^{45}Ca^{2+}$ uptake assay, cells were incubated for 5 min at 37 °C in a total volume of 500 µl of serum-free DMEM (Life Technologies) containing 1,8 mM $CaCl_2$ in the presence of 0,5 mg/ml bovine serum albumin (BSA, Sigma), 1 µCi/ml $^{45}Ca^{2+}$ (ICN), and increasing concentrations of anandamide. Maximal response was determined by the response caused by 300 nM capsaicin on the same plate. Immediately after the incubation, extracellular $^{45}Ca^{2+}$ was removed by washing the cells three times with ice cold DPBS (Life Technologies) containing 1,8 mM $CaCl_2$. In order to measure internalized $^{45}Ca^{2+}$ activity the cells were lysed. Then the cell lysate was transferred from each well into a scintillation vial and radioactivity was determined by scintillation counting. Data from these experiments were analyzed by computer fit to the Hill equation.

3.9. Ca^{2+} imaging

For intracellular Ca^{2+} concentration measurements, CHO-TRPV1 cells were plated on 25 mm round glass coverslips in maintaining media. The next day, the media was changed to inducing media. Experiments were done approximately 24 hours after induction. The CHO-TRPV1 cells were transferred to DPBS containing 1 mg/ml BSA and 5 µM fura2-AM (Molecular Probes) for 2 hours at room temperature. The cells were then kept in maintaining media at room temperature, in the dark, until the measurements, which were carried out in DPBS, containing 1,8 mM Ca^{2+}. The fluorescence of individual cells was measured with an InCyt Im2 fluorescence imaging system (Intracellular Imaging). The cells within a field were illuminated alternately at 340 and 380 nm. Emitted light >510 nm was measured. Data were analyzed with the InCyt 4.5 software and further processed with Excel (Microsoft) and GraphPad Prism 2.0 (GraphPad Software) software.

4. RESULTS

4.1. Determination of ACE I/D, AT1R A1166C, and AGT M235T polymorphisms

Previously described methods were successfully applied in the case of the ACE I/D and the AT1R A1166C polymorphisms. In contrast, we encountered problems in the determination of M235T polymorphism. With the application of the PCR-RFLP method of Russ *et al* we did not find patients carrying the TT genotype among the 123 DNA samples, although the calculated allele frequencies were 0,634 (M-allele) and 0,366 (T allele). If this population was in a Hardy-Weinberg equillibrium, 0,366 x 0,366 x 123 = 16,48 ≈ 16 people should have carried the TT genotype. This fact – considering previously described allele frequencies – suggested technical problems either in the PCR amplification or in the restriction digestion.

To eliminate these problems, a higher hybridization temperature – 64°C – was used to ensure about the specificity of PCR reaction. We found better efficiency of restriction digestion, but complete cleavage of AGT fragments were not achieved. The Psy 1 restriction enzyme was also tested instead of Tth 111I, to investigate the restriction enzyme dependent effects on the digestion, but complete digestion was not found.

Increasing amount (0,1-10 U) or increased reaction time (1-24 hours) were also tested. Although a clear tendency of more complete digestion was observed in parallel with longer time and with higher enzyme activities, complete digestion was still not achieved.

After these trials, we set out to develop a more reliable PCR-RFLP protocol. Since there is no known restriction enzyme specific for the AGT sequence in the region which is responsible for M235T polymorphism, we designed a protocol where only one mismatch would be enough to generate sites for restriction enzymes. Accordingly, a new antisense primer was applied, in which only one mismatch – instead of the two in the original primer – was introduced to generate a restriction site for the Box I enzyme. Following optimalization of the PCR reaction with the new primer, the digestion with the Box I resulted complete cleavage. The sequence of the PCR products used during the optimalization were determined by direct sequencing and confirmed the PCR-RFLP results.

Re-analyzing DNA-samples of the 123 patients we found 33 MM, 56 MT, and 34 TT genotypes in contrast to the original protocol. Using the new method, statistical analyses of the data revealed allele frequencies of M: 0,496 T: 0,504. These frequencies represent a population in Hardy-Weinberg equilibrium ($p>0,5$, with the Chi-square test, no significant difference from the expected values).

4.2. Investigation of the associations between ACE I/D, AGT M235T, and AT1R A1166C polymorphisms and in-stent restenosis following percutaneous coronary intervention

ACE I/D, AGT M235T, AT1R A1166C genotypes of 239 patients with coronary artery disease were determined in the study. In general, 347 stents were implanted in 330 stenoses. Angiographic ISR was present in 116 patients (48,5%). There were no significant differences between the patients with or without ISR with regard to age, blood pressure status, plasma lipid profile, prior myocardial infarction, diabetes mellitus or pharmaceutical treatments. The two groups exhibited no deviation with respect to the extent of the stenoses, the distribution of the lesions among the three main coronary vessels or other procedural parameters, such as the stent length, the stent type, the vessel diameter or the application of predilatation.

The observed allele frequencies did not deviate significantly from the Hardy-Weinberg equilibrium in the overall study group (ACE I: 0,44, D: 0,56 p>0,1; AGT M: 0,53, T: 0,47 p>0,1; AT1R A: 0,72, C: 0,28 p>0,9), which is an indicator of genetic balance. There were no significant differences in genotype repartitions between the two groups. The distributions of the ACE II, ID and DD genotypes were 24,2 %, 40,5 % and 35,3 % in the ISR group and 20,3 %, 45,5 % and 34,2 % in the non-ISR group, respectively (p=0,80). In the case of the AGT M235T polymorphism, the frequencies of the genotypes were MM 26,7% vs 31,7%, MT 50,9% vs 43,1%, and TT 22,4% vs 25,2% in patients with and without ISR, respectively (p=0,96). Moreover, no significant difference was found in the prevalence of the different AT1R genotypes: AA 50,9% vs 52%, AC 38,8% vs 41,5%, and CC 10,3% vs 6,5% in the ISR and non-ISR groups, respectively (p=0,65).

Likewise, logistic regression analysis with adjustment for several covariates, such as age, gender, blood pressure, lipid profile, the presence of diabetes mellitus and procedural characteristics, failed to demonstrate any

correlation between the genotype and the degree of lumen loss at the follow-up examination. Among the conventional risk factors, only a previous myocardial infarction had statistically significant effect on the lumen narrowing.

To elucidate the synergistic effect of the gene polymorphisms, patients with at least one D, T and C allele were selected and compared to the other patients. However, again no significant difference was observed in the prevalence of ISR between the two groups.

4.3. Demonstration of functional vanilloid receptors in skeletal muscle arterioles

The vasoactive effects of anandamide and capsaicin were measured in resistance arterioles (132-223 μm in diameter) of skeletal muscle (m. gracile of the rat. The isolated arteries were mounted in a perfusion myograph system and developed spontaneous myogenic tone without the use of any vasoactive agent (myogenic constriction: 25 ± 4%, diameter in the presence of Ca^{2+} is 179 ± 33 μm and in the absence of Ca^{2+} is 234 ± 20 μm at 80 mmHg, n = 15, p<0,01). The functionality of the endothelium was tested at the beginning of each experiment by administration of acetylcholine or 1 min (10^{-7} M, 95 ± 13% dilation, n = 15), while smooth muscle functions were checked by addition of norepinephrine for 1 min (10^{-7} M, 31 ± 14% constriction, n = 15).

First, the effects of TRPV1 stimulation were tested on the isolated vessels. In this series of experiments 1 μM capsaicin caused 58 ± 8 % constriction (n=5) which was blocked by the competitive TRPV1 antagonist capsazepine (10 μM, constriction is 2 ± 5 %, n=5, p<0,01). To investigate the desensitization of TRPV1 in this system, isolated vessels were incubated with 1 μM capsaicin for 20 min, followed by 40 min regeneretion and the vessels were tested again with 1 μM capsaicin. According to the results, the maximal constriction was 51 ± 12 % and it did not decreased significantly after the 20 min incubation and 40 min regenaration (response is 35 ± 7 %, n=5, p=0,29).

4.4. Identifying TRPV1 expressing cells

Immuno-histochemistry revealed a strong TRPV1-like staining in skeletal muscle arterioles. Since the picture was characteristic to smooth muscle cells we further investigated the possibility of TRPV1 expression in smooth muscle cells. RT-PCR method revelaed the presence of mRNA of the receptor in rat aortic tissue and in cultured vascular smooth muscle cells (A7r5 cells). Immuno-fluorescent staining was also performed on the m. gracile tissue samples. Smooth muscle actin staining and TRPV1 staining showed remarkable overlap, and this fact supported the expression of TRPV1 in smooth muscle cells.

4.5. Investigation of the TRPV1 stimulation by anandamide

The TRPV1 mediated vasoconstriction in skeletal muscle arteries was in marked contrast to the vasodilatation observed on mesenteric vessels earlier. Therefore, mesenteric vessels were also tested to support the experimental conditions and design of this study, to measure vascular effects of TRPV1 stimulation and to confirm earlier data. Indeed, we found a concentration dependent dilatation of mesenteric vessels evoked by both capsaicin and anandamide although the efficacy of capsaicin was significantly higher (maximal dilatation is 96,3 ± 2,1 % for capsaicin and 23,6 ± 3,3 % for anandamide, $p<0,01$, $n=5$). This capsaicin and anandamide mediated dilatation (without apparent vasoconstriction) is in accordance with earlier reports.

Effects of anandamide on the skeletal muscle vessel diameter were tested by cumulative dose-response measurements. 1 min incubation with anandamide (1 nM to 100 μM) evoked no significant changes in vessel diameter (dilatation: 3 ± 5 %) compared to the control.

It is suggested that anandamide may desensitize the TRPV1. We therefore tested the effects of 30 μM anandamide (for 20 min followed by a 40 min regeneration period) on the responsiveness of TRPV1 in this system. 1 μM capsaicin alone caused a significant constriction of these vessels (constriction is 51 ± 12 % $n = 5$, $p = 0,018$). Capsaicin-induced constriction was completely

abolished by anandamide (30 μM) pretreatment (constriction decreased to 1 ± 0,6 %, n = 5). The inhibitory effect of anandamide on TRPV1 was reversed by the calcineurin (protein phosphatase 2B) inhibitor cyclosporin-A (CY-A, 100 nM) (response to 1 μM capsaicin was 31 ± 1 %, n = 4, p = 0,014 for constriction, but no significant difference from capsaicin alone, p = 0,12). Furthermore, anandamide caused a significant vasoconstriction (constriction is 7 ± 2 %, n=4, p=0,01) when applied after CY-A (100 nM, 5 min).

4.6. Specific investigation of cellular effects of TRPV1 activation using a TRPV1 overexpressing cell line

To explore the possible mechanisms leading to the phosphorylation-dependent anandamide-mediated desensitization of TRPV1 in skeletal muscle arterioles we used an exogenous TRPV1 expressing cell line (CHO-TRPV1). $^{45}Ca^{2+}$ uptake experiments indicated that anandamide is a partial agonist in this system (with a maximal effect of 65 ± 8 %, n = 7 than that induced by 300 nM capsaicin). This partial agonism was converted to full agonism by the activation of PKC with 4α-phorbol-myristate-acetate (PMA) (100 nM, 98 ± 14 %, n = 3, p = 0,059), by inhibition of phosphatase 2B (calcineurin) using CY-A (100 nM, 145 ± 14 % n = 5, p<0,001), or by the combination of PKC activation and calcineurin inhibition (137 ± 13 %, n = 3, p = 0,001). Changes in the apparent K_d values were also observed in parallel with the maximal effects (K_d values: anandamide alone: 30 ± 6 μM, n = 7; PMA: 9 ± 4 μM, n = 5, p = 0,027; CY-A: 11 ± 5 μM, n = 5, p = 0,047; CY-A and PMA applied together: 11 ± 3 μM, n = 3, p = 0,117). Finally, we also tested the effect of the fatty acid amid hydrolase inhibitor phenil-methane-sulfonil-fluoride PMSF (0,5 mM) and found a moderate sensitization of TRPV1 to anandamide in the presence of PMSF (81,0 ± 4,8 % efficacy and 10,5 ± 2,8 μM potency, n=4).

To test the anandamide mediated desensitization specifically on TRPV1 receptors, CHO-TRPV1 cells, which do not express cannabinoid receptors, were

preincubated with anandamide (1-100 μM for 15 min at 37 °C), then $^{45}Ca^{2+}$ uptake was initiated by 50 nM capsaicin (the half maximally effective dose of capsaicin in this system). Anandamide inhibited the capsaicin induced $^{45}Ca^{2+}$ uptake in a dose dependent manner, with an apparent IC_{50} value of 21 ± 2 μM (n = 3). This IC_{50} is close to the K_d determined in the previous assays (K_d = 30 ± 6 μM, n = 7).

4.7. Investigation of the role of phosphorylation in the regulation of anandamide responsiveness of TRPV1

Finally, we characterized the anandamide mediated cellular effects on TRPV1 receptors at the level of the intracellular Ca^{2+} concentrations of CHO-TRPV1 cells. Anandamide alone (30 μM) caused a transient elevation of intracellular Ca^{2+} concentration, but this elevation was lower than that induced by capsaicin. Preincubation of the cells with PMA (100 nM, 15 min) sensitized the cells to anandamide (30 μM) and desensitization was not prominent (n = 3). Similar effects were detected in case of CY-A (100 nM).

In addition to receptor sensitization, the resensitization characteristics of TRPV1 were also tested, using a similar experimental protocol to that used with the isolated rat vessels. Both PMA and CY-A were able to resensitize TRPV1 which had been desensitized by anandamide (n = 3).

5. DISCUSSION

5.1. Associations of gene polymorphismsm of the RAS and development of in-stent restenosis (ISR)

Treatments with antihypertensive drugs developed to date have shown satisfactory therapeutic effects, but not in all of the patients. These facts fueled the efforts to characterize the molecular genetics of hypertension. The very first gene found to be associated with human hypertension was the AGT M235T polymorphism. To date that work has received more than 1000 citations indicating the interest and debates of this original finding.

One of the reasons of these controversial data could be the uncertainities in the methods applied to determine the genotype. During the optimalization of the reaction described by Russ *et al* we were not able to reach satisfactory identification using our equipment similarly to others . We also tried to optimalize both the PCR and restriction digestion, but still we encountered uncertainities. These uncertainities could lead to the overestimation of M235 allele, since the MT and TT genotypes could not be differentiated unambiguously. Therefore we designed a new antisense primer and consequently a new restriction enzyme to be able to identify AGT M235T polymorphism.

Conflicting results are reported on the relationship between RAS-related polymorphisms and ISR in the literature. The I/D polymorphism of the ACE gene is one of the most extensively studied polymorphisms, but the association between the ACE DD genotype and ISR is very controversial. The initial studies indicated a strong association between the ACE D allele and ISR. Later larger cohorts revealed that the ACE I/D polymorphism did not have an effect on ISR. A recently published meta-analysis demonstrated that the combined odds ratio for ISR in individuals with the DD genotype was 1,22 (95% CI 1,04-1,44), and accordingly an association between theI/D polymorphism and restenosis seemed to be weak (if any). Another meta-analysis drew attention to the need for larger epidemiological investigations, because studies with more than 200 cases showed no association between the ACE genotype and ISR. Ethnic differences might also be taken into the consideration: in Japan studies, the presence of the D allele promoted the progress of ISR and correlated with the progression of the inward remodeling within the stented lesion. Our results suggest no association between ISR and ACE I/D polymorphism.

In humans, a number of studies have been made to reveal the relationships between cardiovascular disorders and the M235T variant of the AGT gene. Some of them demonstrated that there were significantly more carriers of the

angiotensinogen 235T allele in the restenosis group, suggesting that the T allele affects the development of ISR and may be an independent predictor of recurrent restenosis after repeated angioplasty. In contrast, a study on a Japanese population found no association between the 235T variant and restenosis after coronary angioplasty. According to meta-analyses the AGT 235T allele was associated with an elevation in the level of plasma angiotensinogen, and TT homozygotes had a 6% higher risk of hypertension as compared with MM homozygotes. However, no association was found between the M235T polymorphism and the risk of ischemic heart disease or myocardial infarction, thus the genotype presumably does not predict the risk of ISR in either ethnic group. Our results, similarly to those on the Japanese population failed to demonstrate any genetic relationship between the T allele and restenosis after stent implantation.

Far fewer data are available that relate to the A1166C polymorphism. Our study did not confirm a substantive genetic relationship between the 1166C-allele and ISR.

These conflicting data might be due to the variable contributions that a single mutation can have on populations with different genetic backgrounds, and the difficulty in assigning predictive values to separate genetic risk factors in multifactorial diseases. For this reason, we performed a multiple regression analysis and investigated the synergistic effect of the presence of D, T and C alleles. We found that these molecular variants were not risk factors of binary restenosis, either directly or via interactions with other risk factors, and no synergistic effect between them and ISR was apparent.

In conclusion, the polymorphisms under discussion cannot identify a subset of patients at increased risk of restenosis following stent implantation.

5.2. Investigation of the physiological role of TRPV1

It was found that anandamide itself has no effect on the vasotone of skeletal muscle arterioles in contrast to the dilatation which has been demonstrated by us and previously by others. This result could arise from at least three different mechanisms. First, anandamide induced stimulation of cannabinoid or TRPV1 receptors might have low efficacy to achieve vasoactive responses in skeletal muscle arterioles. Second, the simultaneous stimulation of cannabinoid and TRPV1 receptors may cause opposite effects in this system. Third, there are no functional receptors present in the preparations to be activated. This latter explanation can be excluded since capsaicin caused a significant vasoconstriction, indicating the presence of functional TRPV1 receptors in our preparations. Moreover, pretreatment of the arteioles with the proteion phosphatase 2B (calcineurin) inhibitor Cyclosporin-A revealed an anandamide mediated vasoconstriction. These data suggest that anandamide induces dephosphorylation mediated desensitization of the receptor, and in this process calcineurin plays key role.

Since some of the kinases influencing TRPV1 activity (PKC isoenzymes, CaM kinase II) and the calcineurin are calcium-dependent enzymes, it is possible that the actual activity of kinases and phosphatases depends on the intracellular Ca^{2+}-concentration and the Ca^{2+} sensitivity of the TRPV1 receptors.

To further investigate the possible cellular mechanisms, we used a heterologous TRPV1 expression system, in which TRPV1 of the rat was expressed in CHO cells and the effects of phosphorylation on the acute desensitization of TRPV1 were tested. The advantage of this system is that TRPV1 mediated effects could be specifically observed, since there are no endogenous expression of cannabinoid or TRPV1 receptors in the CHO cells. The observed efficacy (65 ± 8 % than that of capsaicin) and potency (30 ± 6 μM) of anandamide is somewhat lower than that of reported by others. One explanation of these controversies can be that fatty acid amid hydrolase

activities are different in the systems. Further possibilities may also explain the differences in the potency of anandamide, including different levels of TRPV1 expression, differences in protein phosphorylation, anandamide transporters, and other factors Some of them (PKC activation and protein phosphatase 2B inhibition) were also confirmed to be effective in this work.

The partial efficacy of anandamide on TRPV1 receptors allowed the investigation of phosphorylation in regulating the responsiveness of TRPV1 to anandamide. Our data supports the idea that both the potency and efficacy of anandamide are controlled by phosphorylation. The acute desensitization of TRPV1 is strong enough to cause reduced capsaicin mediated responses. The anandamide-mediated desensitization was blocked and reversed by PKC activation and calcineurin inhibition.

Based on our observations and of others, here we propose a mechanism for the regulation of TRPV1 responsiveness upon anandamide stimulation. According to this model, we suggest three different phases: resting, activated and desensitized. In the resting phase there are no ligands and the TRPV1 activity is suppressed. The steady state level of phosphorylation determines the sensitivity of TRPV1 to anandamide. The phosphorylation of TRPV1 is a function of the apparent phosphatase and kinase activities. If TRPV1 phosphorylation is enhanced, eg. by activation of PKC or inhibition of calcineurin, TRPV1 sensitivity to anandamide is higher. The second phase is the activation of the TRPV1 by anandamide. At the appearance of anandamide the channel activity increases and the intracellular Ca^{2+} concentration rises. This activation is, however, followed by a rapid desensitization, which is the third phase. During this acute desensitization period (in the continuous presence of the ligand) the activity of the receptor and the intracellular Ca^{2+} concentrations are apparently regulated by reversible phosphorylation: dephosphorylation keeps the TRPV1 desensitized, and the intracellular Ca^{2+} concentration low, while phosphorylation sensitizes the TRPV1. In this desensitized period, the TRPV1

behaves as a phosphorylation gated channel until anandamide is present in sufficient concentration. However, when anandamide concentration decreases, the channel will be closed independently from its phosphorylation state.

These observations could be of physiological importance if anandamide or anandamide like substances (eg. drugs with similar properties) are continously present and bound to the TRPV1. In this case TRPV1 is silenced by acute desensitization but can be activated by phosphorylation alone. Such anandamide like substances might be able to silence TRPV1 activity without the irritative effects of the full agonists (like capsaicin) providing an improved therapeutic strategy to control disorders caused by TRPV1 hypersensitivity.

6. Summary

Our clinical investigations focused on the association of the genetic polymorphisms (ACE I/D; AGT M235T; AT1R A1166C) of the renin-angiotensin system (RAS) with the in-stent restenosis (ISR) of coronary vessels. Our aim was to determine whether these polymorphisms are predictive markers of ISR. We set up the procedures to determine the genotypes of the patients and we improved the published PCR-RFLP based genotyping of AGT M235T polymorphism (by designing a new primer and the application of a new restriction enzyme). As a matter of the association of the gentical background of the patients with ISR, our data did not support a significant role of the determined polymorphisms in the development of ISR.

Our basic research efforts were directed to the vascular functions of the vanilloid receptor (TRPV1), which is a non-specific cation-channel, characterized mainly in primary sensory neurons. Surprisingly, TRPV1 stimulation with capsaicin caused a vasoconstriction, which was inhibited by the specific antagonist capsazepine in isolated, perfused arterioles (vessel diameter 132-223 μm at 80 mmHg) of rat skeletal muscle (m. gracile). RT-PCR experiments and immunohistochemistry confirmed TRPV1 expression in the smooth muscle cells of blood vessels, which observation provides an explanation for the constriction. Next, effects of the endogenous TRPV1 agonist anandamide were studied. Anandamide caused a phosphorylation-dependent desensitization of TRPV1 in skeletal muscle arterioles and in TRPV1 overexpressing CHO cells. This suggests that anandamide may be an endogenous desensitizer of TRPV1 instead of being an agonist on this receptor. Finally, we demonstrated that TRPV1 can function as a phosphorylation-gated (metabotrop) receptor in the continous presence of anandamide.

PUBLICATIONS

In extenso publications related to this the thesis:

1. **Lizanecz E.,** Bagi Z., Pásztor E.T., Papp Z., Édes I., Kedei N., Blumberg P.M., Tóth A. (2006): Phosphorylation-dependent desensitization by anandamide of vanilloid receptor-1 function in rat skeletal muscle arterioles and in Chinese hamster ovary cells expressing TRPV1 *Mol Pharmacol* **69**(3), 1015-1023. IF: 5,080
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Submitted:

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