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Molecular mechanism of intracellular signal transduction by the angiotensin-converting enzyme

Dissertation

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1. Introduction

Angiotensin I converting enzyme (ACE) is a zinc peptidyl dipeptidase that catalyses the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor and is also involved in the inactivation of bradykinin, a potent vasodilator (1). ACE plays an important role in blood pressure regulation by virtue of generating angiotensin II, which increases blood pressure by inducing aldosterone secretion, leading to sodium and fluid retention and the release of norepinephrine from the sympathetic nervous system (2). Apart from regulating blood pressure, angiotensin II, an important effector of the renin-angiotensin system (RAS), is a potent proinflammatory agent leading to expression of growth factors, cytokines, chemokines and adhesion molecules, in pathological conditions wherein RAS is activated (3-6). Increased local ACE activity leading to increased *de novo* production of angiotensin II coupled with degradation of bradykinin, impairs the balance between vasoconstriction and vasodilation, thrombogenesis and fibrinolysis, proinflammation and anti-inflammation thus promoting pathological effects in hypertension (7). ACE inhibitors are widely employed for the treatment of hypertension and various cardiovascular diseases (8). The observed beneficial cardiac, vascular and renal effects cannot be explained by the blood pressure lowering action of ACE inhibitors alone, and also the magnitude of risk reduction was far greater than that expected from the modest reduction in blood pressure. Therefore the study of ACE and mechanisms of ACE inhibitors is needed to fully explain the beneficial effects of ACE inhibitors. To explain the mode of action of ACE inhibitors, our group recently identified ACE as a signal transduction molecule because upon binding of ACE inhibitors, an outside-in signalling is initiated leading to a signalling cascade resulting in the phosphorylation of Ser^{1270} in the cytoplasmic tail of ACE, activation of JNK as well as the phosphorylation of c-Jun and its translocation to the nucleus affecting the increased expression of ACE and cyclooxygenase-2 (COX-2) in endothelial cells. This ACE inhibitor induced signalling cascade could potentially influence the expression of a spectrum of genes in endothelial cells and this could be an additional mechanism by which ACE inhibitors exert their protective effects on the cardiovascular system. This thesis describes the mechanism by which the ACE inhibitor initiates the signalling cascade and attempts to identify the downstream effectors of the ACE signalling cascade.

1.1. The Renin Angiotensin System

The RAS plays an important role in regulating blood volume, arterial pressure, cardiac and vascular function. Sympathetic stimulation (acting via β_1-adrenoceptors), renal artery hypotension, and decreased sodium delivery to the distal tubules stimulate the release of renin by the kidney. Renin is an enzyme that acts upon a circulating substrate, liver-derived angiotensinogen that undergoes proteolytic cleavage and forms the decapeptide angiotensin I. Angiotensin I, per se has only weak physiological activity and mainly acts as a precursor to angiotensin II. In addition to being a substrate for angiotensin II, angiotensin I can be converted to heptapeptide angiotensin 1-7 by neutral endopeptidase and also by the recently discovered angiotensin converting enzyme 2 (ACE2). ACE2 can also remove a single amino acid from the carboxyl terminus of angiotensin I to produce angiotensin 1-9 (Fig. 1).

ACE, located in the vascular endothelium, particularly in the lungs, cleaves two amino acids from the C-terminus to form the octapeptide, angiotensin II. Alternatively, angiotensin II can be converted to angiotensin 1-7 by ACE2. Angiotensin II can be converted to smaller peptides with biological activity by the action of aminopeptidase A, which removes a single amino acid from the amino terminus to generate angiotensin III or angiotensin 2-8. Additional action of aminopeptidases can generate angiotensin IV or angiotensin 3-8. Angiotensin III has 40% of the pressor activity of angiotensin II, but 100% of the aldosterone-producing activity. Angiotensin 1-7 has been reported to have vasodilatory effects (9).

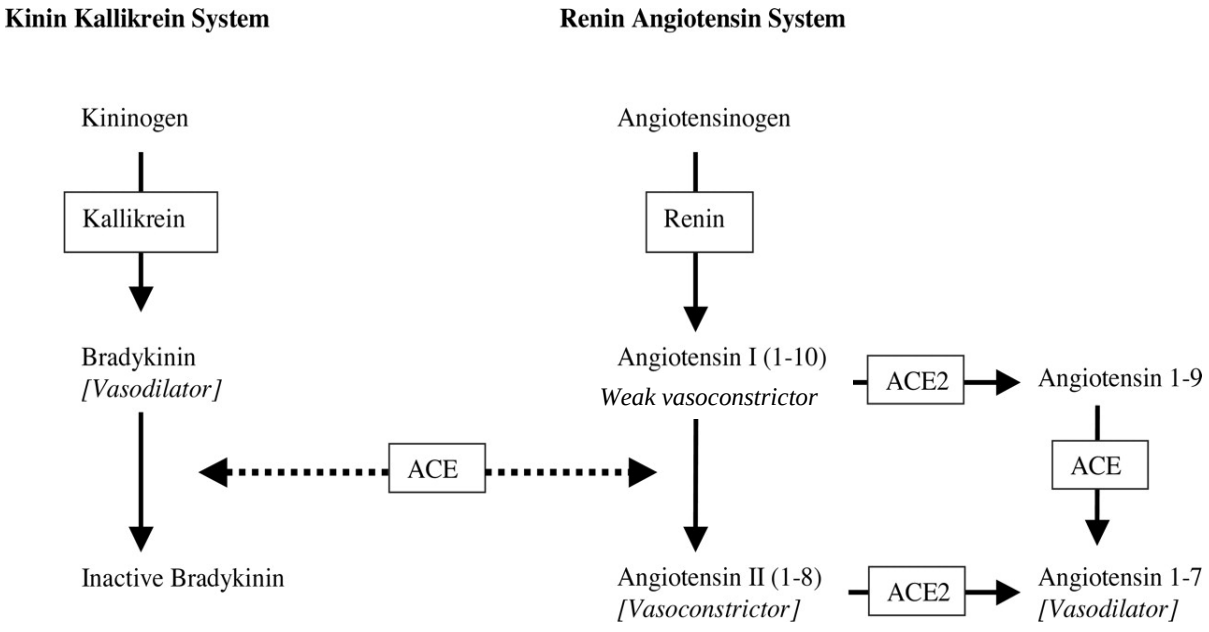

Fig 1. The components of the renin angiotensin system and the kinin kallikrein system**.**

Angiotensin II mediates its effects via binding to specific receptors on the cell surface. At least two types of angiotensin receptors which are G-protein coupled have been identified: angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors. Most of the effects of angiotensin II are mediated via AT_1 receptors to stimulate systemic vasoconstriction, vascular smooth muscle contraction, aldosterone secretion, dipsogenic responses, pressor and tachycardiac responses.

Angiotensin II acting via AT_1 receptors, can also cause cell growth, differentiation and proliferation directly by affecting several kinase pathways and indirectly by inducing several growth factors, including transforming growth factor β1 (TGF-β_1) and platelet-derived growth factor (PDGF) (10). The AT_2 receptor is highly expressed in fetal mesenchymal tissues from both rodents and man and diminishes rapidly to low levels a few days after birth. However AT_2 receptor protein is detectable in the adult kidney, heart and blood vessels. The expression of the AT_2 receptor is upregulated by sodium depletion and is inhibited by angiotensin II and growth factors such as platelet-derived growth factor, epidermal growth factor and also by insulin and insulin growth factor-I (11-13). Growing evidence shows that AT_2 receptors are also important in controlling the cardiovascular system (10). A physiological role for the AT_2 receptor was first suggested by the observations that mice lacking the AT_2 receptor have a slight but significant increase in baseline blood pressure (11;14). The AT_2 receptor subsequently mediates vasodilation by stimulating the production of bradykinin, nitric oxide (NO) and cyclic guanosine 3', 5'-monophosphate (cGMP) (15;16). In blood vessels, in addition to its vasodilatory actions, the AT_2 receptor exerts antiproliferative and apoptotic effects in vascular smooth muscle cells and decreases neointimal formation in response to injury by counteracting the effects of angiotensin II (17). It seems that most of the effects mediated by AT_2 receptors are the opposite of those mediated by AT_1 and it is also suggested that the AT_2 receptor exerts protective actions only when the AT_1 receptor is blocked (18;19). Apart from angiotensin II, angiotensin III also can bind to and signal through the AT_1 and AT_2 receptors.

The components of the RAS have been found in a number of tissues, which partly explains the increased concentration of circulating angiotensin II levels during long term ACE inhibitor therapy. Other peptidases are able to convert angiotensin I to angiotensin II and the serine protease chymase are thought to be responsible for more than 80% of the angiotensin II formation in the human heart and more than 60% of that in the arteries (20;21). However the

role of RAS in other tissues warrants further investigation. The physiological role of RAS is to maintain or increase extracellular fluid volume and to increase the total peripheral resistance. An effective RAS attenuates orthostatic hypotension and hypotension during low salt intake or during dehydration. Overactivity of RAS has been implicated in the development of various cardiovascular diseases, such as hypertension, congestive heart failure, coronary ischemia, atherosclerosis and renal insufficiency (22).

1.2. ACE inhibitors

ACE inhibitors are a preferred class of drugs used clinically to treat high blood pressure. ACE inhibitors are most effective in lowering blood pressure in experimental models of hypertension such as in genetically hypertensive rats (spontaneously hypertensive rats) and mice (23-25). Findings from large clinical trials have established that blocking the renin-angiotensin system with ACE inhibitors not only lower blood pressure but also positively influence a number of cardiovascular risk factors such as cardiac and vascular hypertrophy, endothelial dysfunction, atherosclerosis or insulin resistance.

ACE inhibitors are tight-binding competitive inhibitors of ACE. They interact with the constituents of the enzyme's active site, i.e. the hydrophobic pocket, positively charged groups of amino acids, the zinc ion and auxiliary binding sites. Captopril, the first clinically available ACE inhibitor, belongs to the group of sulfhydryl (SH)- containing ACE inhibitors (26). Based on the structure of captopril, other inhibitors were synthesized such as enalaprilat, lisinoprilat (27), ramiprilat (28;29) and perindoprilat (30). More than 15 inhibitors of the enzyme are now clinically available worldwide. ACE inhibitors can be divided into three chemical classes according to their zinc ligand (31;32). They mainly differ in their elimination half-life, potency, lipophilicity and the route of elimination.

ACE inhibitors significantly improved endothelial function by increasing the formation of NO and prostaglandin PGI_2 (33-36), vascular remodelling (37) inhibited the progression of arteriosclerosis (38) and delayed the onset of type-2 diabetes (39;40). ACE inhibitors results in significant reductions in mortality in heart failure and post-myocardial infarct patients (41). The effects of ACE inhibition in patients with high risk for coronary artery disease without left ventricular dysfunction or heart failure, patients at least 55 years old with a history of stroke, peripheral vascular disease and diabetic patients were examined in the heart outcomes prevention evaluation (HOPE) study. Treatment with ramipril significantly reduced the combined outcome of death, myocardial infarction and stroke by 22% and revascularizations

by 16%. In diabetic patients, ramipril lowered the risk of new onset of diabetes by 32%, diabetic complications by 16%. ACE inhibitors have been shown to decrease the clinical events in high risk patients with atherosclerosis with and without ventricular dysfunction, prior to and after myocardial infarction (42-44). The positive effects of ACE inhibitors observed in HOPE and other studies of ACE inhibition, cannot be explained by the blood pressure lowering action of ACE inhibitors alone, since the magnitude of risk reduction was greater than that expected from the modest reductions in blood pressure that occurred (45). Therefore the study of mechanism of action of ACE inhibitors apart from lowering blood pressure or independent of angiotensin II or bradykinin, is needed to explain the positive beneficial effects demonstrated by various large clinical trials.

1.3. The Angiotensin Converting Enzyme

Cells and tissues not implicated in the classical RAS are now known to possess the components of RAS and exert diverse actions in many organs. ACE is found in most mammalian tissues bound to the external surface of the plasma membrane of vascular endothelial cells. ACE is also present in epithelial, neural and neuroepithelial cell types (46). ACE is located in the parenchyma of the heart, kidneys, brain, and adrenal glands. Apart from the vasculature, ACE is also found in circulating leukocytes and monocytes. T lymphocytes have higher enzyme protein levels than monocytes (47). Various components of the renin-angiotensin system have been localized to human adipose tissue (48;49). Taken together, emerging picture of ACE expression in many other tissues suggests that the enzyme might play other roles apart from conversion of angiotensin I to angiotensin II, which needs further investigation. The structural aspects of the enzyme, isoforms and homologues of ACE, polymorphisms of ACE, ACE in other tissues apart from vasculature, wherein it might play a new role and its role as a signal transduction molecule, all of which will be discussed in the sections to follow.

1.3.1. Structure

ACE exists as a cell surface integral membrane glycoprotein (class I membrane protein) anchored to the plasma membrane with the N-terminus and active site facing the extra-cellular milieu, in particular at the luminal surface of vascular endothelial cells (Fig. 2) (50). ACE consists of a 28 residue carboxyl-terminal cytosolic domain and a 22 residue hydrophobic transmembrane domain and an extracellular domain consisting of 1227 residues.

The extracellular domain is further divided into two homologous domains, a 612 residue N-domain at the amino terminus linked by a 15 residue sequence to a 600 residue C domain. Each of the extracellular domains possess an active site with a HEXXH sequence in which the two histidine residues serve as zinc-binding ligands (51).

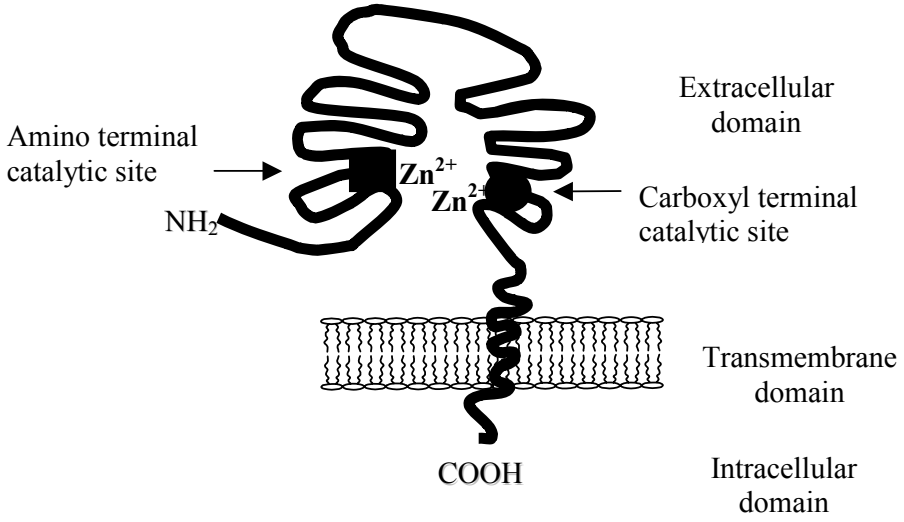

Fig. 2: Schematic representation showing the structure of angiotensin converting enzyme (ACE). Catalytic sites on each lobe binds a zinc (Zn^{2+}) atom. Modified from Johnston et al (52).

ACE is a metallopeptidase with a zinc atom in its active center, its activity can therefore be inhibited by metal chelating agents. Despite their high degree of homogeneity, the N and C domains display distinct structural and functional differences. The C domain is primarily responsible for angiotensin I hydrolysis, while both N and C domains are responsible for the inactivation of bradykinin (53-55). The N domain preferentially hydrolyses the haemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) and angiotensin 1-7 (56). The C domain displays greater chloride dependence (55;57) and is more sensitive to proteolysis (58), while the N domain shows greater thermal stability (59). In addition, ACE inactivates the vasodilatory peptide brakykinin by the sequential removal of two C-terminal dipeptides (60). However bradykinin is the preferred substrate of both catalytic sites, as ACE hydrolyzes bradykinin 80 times faster than angiotensin I (61;62). Therefore, the net physiological effect of ACE is to increase the production of vasoconstrictor and decrease the availability of vasodilator.

Peptide	Sequence
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
	↑
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
	↑
Chemotactic Peptide	FMet-Leu-Phe
	↑
Enkephalins	
Pentapeptide	Tyr-Gly-Gly-Phe-Met
Heptapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Octapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
	↑
Neurotensin	<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
	↑
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	↑ ↑
LH-RH	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
	↑ ↑

Table 1: Arrows indicate the primary sites of cleavage by human ACE. Adapted from Skidgel et al (63)

The testicular ACE is O-glycosylated, whereas the somatic ACE is principally N-glycosylated. The role of glycosylation is not well understood; probably it may enhance the solubility of the enzyme. It has also been suggested that the extent of juxtamembrane glycosylation within somatic ACE regulates dimerization of the protein via the involvement of a carbohydrate-recognizing domain and subsequent formation of intermolecular disulphide bridges (64). On the other hand glycosylation per se does not seem to participate in the catalytic properties of ACE, although it can protect, in particular the N-terminal domain, against proteinases such as trypsin (65-67).

1.3.2. ACE isoforms

There are two ACE isoforms: a somatic form of about 150-180 kDa, and a smaller isoform (90-110 kDa) found exclusively in the testes (68). The human somatic form of ACE, a 170-kDa glycoprotein, has been shown by molecular cloning of its complementary deoxyribonucleic acid (cDNA) to consist of two homologous domains, each containing an active site (57;69). The somatic isoform of the enzyme consists of two highly homologous and functionally active domains, the so-called amino/N terminal and the carboxy/C terminal domain, resulting from gene duplication (57;70), while the testicular isoform has only one of these two domains (71-73).

1.3.3. ACE genetics

The human ACE gene is composed of 26 exons, but the mature endothelial transcript is 4.3 kb long and composed of only 25 exons as exon 13 is spliced off in this transcript. The testicular ACE transcript is 3 kb long and transcribed from exons 13 to 26. Therefore a single gene encodes both the somatic and testicular ACE transcripts by activation of alternate promoters (69;72;74). There are two ACE promoters, a somatic promoter which is located on the 5' side of the first exon of the gene and a germinal, promoter located on the 5' side of the specific testicular ACE mRNA (74). The two alternate promoters of the ACE gene exhibit highly contrasting cell specificities, as the somatic promoter is active in endothelial, epithelial and neuronal cell types, whereas the germinal promoter is only active in a stage specific manner in male germinal cells (75). Expression of both the somatic and testicular ACE is under different hormonal regulation i.e. the endothelial enzyme is induced by glucocorticoids, whereas the testicular form is stimulated by androgens (76;77).

1.3.4. ACE polymorphisms

It is generally thought that plasma ACE concentration mirrors the level of the synthesis of the somatic enzyme. The cloning of human ACE gene also enabled the detection of many polymorphisms. One of them consists of the presence (insertion allele I) or absence (deletion allele D) of a 287-base pair DNA fragment in the intron 16 of the ACE gene, corresponding to an Alu sequence. A strong association of these alleles and the level of serum ACE was detected (78;79). Individuals homozygous for the D allele (genotype DD) displayed twice as high serum ACE levels compared to individuals homozygous for the I allele (genotype II) (79-82). Also, carriers of the D allele have been found to have significantly higher risk of diabetic nephropathy (83).

1.4. ACE secretion

Despite the fact that ACE is expressed as a type I integral membrane protein, a soluble form of ACE is found in plasma, seminal fluids and other body fluids (84). This soluble form is derived from the membrane-bound form through the action of ACE secretase (85;86). Proteolytic cleavage of membrane-bound ACE occurs at different positions within the juxtamembrane stalk region (87;88). Of physiological relevance, a point mutation within the juxtamembrane region (Pro-1199 to Leu) has been shown to cause a dramatic increase in serum levels of ACE (89). The same mutation has been shown to enhance ACE shedding *in*

vitro (90). Intermolecular disulfide bridge formation and the extent of glycosylation within the ACE juxtamembrane region has been shown to modulate the degree of shedding of the enzyme (91) thereby also altering the cleavage site by secretases (92). Somatic ACE is shed much less efficiently than testicular ACE as the N-terminal domain of somatic ACE also plays an important regulatory role by occluding a recognition motif on C-domain required for ectodomain shedding (93).

Increased serum ACE levels has been reported in Sarcoidosis (94), diabetes, Gaucher's disease (95), leprosy and to a lesser extent in hyperthyroidism (96). Increased plasma ACE activity has been suggested to be a risk factor for patients with coronary artery disease and myocardial infarction (97;98). Elevated plasma ACE activity determined soon after the onset of MI has been suggested to be a significant predictor of development of left ventricular dilation (99). On the other hand, decreased soluble ACE has been reported in vascular pathologies involving an endothelial abnormality, for instance deep vein thrombosis (100), pulmonary injury (96;101) and in endothelial dysfunction related to the toxicity of chemo- and radiotherapy used in cancer, leukemia and hematopoietic or organ transplantation (96;102). Therefore soluble ACE can be of interest as a prognostic marker for monitoring arterial hypertension and diabetic patients treated with specific ACE inhibitors.

1.5. ACE in other tissues

1.5.1 Human monocytes

Both monocytes and macrophages are instrumental in maintaining the homeostasis of the immune system and also are involved in pathological events. Freshly isolated monocytes contain little or no detectable ACE activity (103). However, when cultured for 4 days and also when monocytes differentiate into macrophages (104), ACE levels increases substantially. Differentiation of monocytes into macrophages in culture is also accompanied by an increase in the expression of components of renin-angiotensin system (105;106). Dexamethasone also was able to induce ACE expression in monocytes in culture (107). The biological relevance of ACE induction in monocytes is not yet clear, however due to its carboxypeptidase activity, ACE enhances the presentation of certain endogenously synthesized peptides to MHC class I-restricted cytotoxic T-lymphocytes, by generation of optimally sized class I-binding peptides from larger protein fragments (108-110). The level of ACE expression in the mononuclear cells that synthesize ACE is genetically determined and dependent on ACE polymorphisms *in vivo* (47). Activation of monocytes occurs in pathological conditions and it was demonstrated that dialysis patients with manifest cardiovascular disease had significant ACE levels in

circulating monocytes thus suggesting a general activation of the renin-angiotensin system contributing to atherosclerosis (111). It was reported that there is an increased accumulation of ACE in atherosclerotic coronary artery disease (112). In the regions of plaques where macrophages and foam cells accumulate, there is also an elevated concentration of ACE which produces increased amounts of angiotensin II, which in turn is atherogenic and has proinflammatory properties, thereby promoting the risk of plaque rupture.

1.5.2. Heart and vasculature

The components of RAS have been found in the cardiomyocytes, vascular smooth muscle cells and endothelial cells. By means of immunohistochemistry, ACE expression has been localized to the cardiac blood vessels and the endocardium (113). ACE levels are increased in vascular and cardiac hypertrophy. Adminstration of ACE inhibitors led to efficient treatment of cardiac disease. Local ACE expression plays an important role in cardiac remodeling as reported that the in-vivo gene transfer of ACE into the uninjured rat carotid artery results in the development of vascular hypertrophy independent of its effect on blood pressure per se (114).

1.5.3. Adipose tissue

Local RAS components have also been detected in white and brown adipose tissue. ACE mRNA was found in both human subcutaneous and extraperitoneal adipose tissue, especially in adipocytes (115). ACE synthesis at protein levels was detected in human preadipocytes (116). The expression of RAS components seems to be related to body weight because obese women are reported to have higher circulating angiotensinogen, renin, aldosterone and ACE than lean women. Body weight reduction (approximately 5%) reduced angiotensinogen, renin and aldosterone levels and decreased ACE expression (117).

1.5.4. Pancreas

Renin mRNA was detected in the connective tissue surrounding the blood vessels and in reticular fibres within the islets of human pancreas. Renin protein was localized in the beta cells of the human islets of Langerhans and in endothelial cell of the pancreatic vasculature. AT_2 receptor was detected in the beta cells of the human islets of Langerhans and also in endothelial cells of the pancreatic vasculature (118). The pancreatic RAS appears to regulate the secretion of pancreatic hormones. This finding and the overall functional relevance of the tissue RAS in the pancrease awaits further study and might account for the beneficial effects of ACE inhibitors and angiotensin II recpetor blocker therapy on the onset of type 2 diabetes.

1.6. Homologues of ACE

Apart from the angiotensin receptors (type I and II), a new member of RAS has been unraveled by genomic-based strategies which led to the discovery of ACE2, a human ACE homologue (119). ACE2 expression has been found in the heart, kidneys, testes, gastrointestinal tract, brain and lung (120-123). Unlike ACE, ACE2 hydrolyzes angiotensin I to angiotensin (1-9), angiotensin II to angiotensin (1-7) and bradykinin to [des-Arg^9]-bradykinin (an inactive metabolite). The full length ACE2 cDNA encodes an 805 amino acid protein with a molecular weight of ~ 120 kDa. ACE2 is a zinc metallopeptidase sharing 42% sequence homology with ACE (119;121;124). The ACE2 protein sequence reveals a potential signal peptide at the N-terminus and a hydrophobic region near the C-terminus which likely serves as a cell membrane anchor. Like ACE, ACE2 is also an ectoenzyme capable of hydrolyzing circulating peptides and is susceptible to cleavage and secretion from the cell surface (121). However unlike ACE, ACE2 has only one isoform. The N-terminal domain of ACE2 has an active site while its C-terminal domain shows 48% sequence identity with collectrin. Collectrin is a renal specific type-1 transmembrane glycoprotein that lacks the metalloprotease catalytic domains of ACE and ACE2 (123) suggesting that collectrin has other physiological roles that are shared with ACE2. Gene targeting studies indicates that ACE2 is an important regulator of cardiac function. ACE2 knockout mice exhibit severe reduction in cardiac contractility, increased angiotensin II levels in plasma and heart and upregulation of hypoxia genes (120). Cardiac dysfunction observed in these mutant mice is completely reversed by a second mutation that causes ACE deficiency. In sharp contrast to ACE, ACE2 does not convert angiotensin I to angiotensin II, and its enzymatic activity is not inhibited by ACE inhibitors. ACE2 probably counterbalances the enzymatic actions of ACE and thus might be a new target for hypertension.

1.6. ACE as a signal transduction molecule

To explain the mode of action of ACE inhibitors it was suggested that apart from inhibiting the formation of angiotensin II, ACE inhibitors increase the accumulation of bradykinin and also potentiates the responses to bradykinin, by inhibiting bradykinin B_2 receptor desensitization (125;126). ACE inhibitors have been demonstrated to attenuate or partly reverse the bradykinin-induced translocation of the B_2 receptor to caveolin-rich membranes and reactivate signalling events such as increase in intracellular calcium ions, activation of extracellular-regulated kinases 1/2 in B_2 receptor-desensitized cells (125) and thus was

proposed that cross-talk might occur between ACE and the B_2 kinin receptor (125-127). Many of the effects of ACE inhibitors in both animals and humans, such as the increase in endothelial NO synthase expression and improved vasodilator responsiveness can be inhibited by blocking the B_2 kinin receptor (128). The question as to how this interaction between the two membrane proteins, ACE and B_2 receptor occur, largely remains unanswered. Moreover angiotensin II levels are not decreased during long-term ACE inhibition and also other enzymes such as chymase can also generate angiotensin II and therefore the beneficial effects of ACE inhibitors cannot solely be attributed to a decrease in the generation of angiotensin II or accumulation of bradykinin, thus hinting towards the existence of an alternative pathway whereby ACE inhibitors might exert their positive actions on vascular system apart from the inhibition of angiotensin II production.

For ACE per se to be a signal transduction molecule upon binding of an ACE inhibitor, it was hypothesized that ACE should be able to bind to soluble intracellular signal molecules or adaptor proteins. The ACE protein sequence revealed that somatic ACE has a short cytoplasmic tail which harbours five serine residues, one of which (Ser^{1270} of the human sequence) is located in a highly conserved 13-amino acid sequence at the C-terminal end of the protein. ACE is phosphorylated by protein casein kinase 2 (CK2) at the serine residue 1270 in the cytosolic tail of ACE. In endothelial cells, CK2 which phosphorylates the enzyme has also been demonstrated to physically interact with ACE and the phosphorylation of ACE by CK2 has been shown to stabilize its localization in the plasma membrane as either the mutation of this residue or the inhibition of CK2 both enhance the cleavage/secretion of the enzyme (129). Interestingly, bradykinin, albeit to a lesser extent than an ACE inhibitor, increased ACE Ser^{1270} phosphorylation and CK2 activity in endothelial cells lacking AT_1 receptors and bradykinin B_2 receptors suggesting a signal transduction pathway for ACE that does not require B_2 receptors. Angiotensin I did not increase the phosphorylation of the enzyme on Ser^{1270} and CK2 activity. Apart from CK2, mitogen-activated protein kinase kinase 7 and the c-Jun NH_2-terminal kinase (JNK) were also found to be associated with the cytoplasmic tail of ACE in endothelial cells. Following the CK2-dependent phosphorylation of Ser^{1270}, activity of ACE associated JNK is also increased in cells treated with ACE inhibitors and bradykinin. Activation of JNK results in the translocation of phosphorylated c-Jun to the nucleus, and enhanced binding of the activator protein-1 transcription factor to DNA, followed by the increased expression of ACE (130) and cyclooxygenase-2 (COX-2) (131). This signalling pathway has been demonstrated in primary cultures of human endothelial cells and also in mice. The identification of ACE as a signalling molecule that can be activated upon binding of ACE inhibitors may account for some of the beneficial

effects of these drugs on the cardiovascular system. The ACE/CK2/JNK pathway may not be the only ACE signalling pathway activated by ramiprilat since in endothelial cells ACE also has been found to interact physically with other intracellular molecules including CaM kinase II, annexin 2, the phosphatase PPI, β-actin and the non-muscle myosin heavy chain IIA (MYH9) and needs further investigation.

1.8. Aim

Clinical trials have established an increasingly important role for ACE inhibition in the treatment and prevention of cardiovascular disease, improvement in endothelial function and delaying the onset of type 2 diabetes. These relevant findings cannot be attributed only to the inhibition of angiotensin II formation or decreased bradykinin degradation as it is well known that chronic ACE inhibition nevertheless does not decrease angiotensin II levels. The mechanisms of ACE inhibitors underlying these effects are so far unclear and needs investigation and go beyond just the metabolism of the kinins.

The aim of this study was to investigate the intracellular events implicated in ACE signalling in endothelial cells and monocytes/macrophages and the physiological relevance of ACE-mediated signalling in these cells. Furthermore we set out to identify the downstream effectors of ACE signalling which might be responsible for the beneficial effects by ACE inhibitors in various clinical conditions. As ACE inhibitors have been reported to delay the onset of type 2 diabetes, investigation also assessed whether ACE inhibitors activate peroxisome proliferator-activated receptor gamma (PPARγ), which influences multiple genes involved in carbohydrate and lipid metabolism, via the ACE signalling cascade.

2. Materials and Methods

2.1. Materials

All media, enzymes and buffer as well as antibiotics, which were used for the cell and organ culture, were from the company GIBCO Life Technology (Karlsruhe), fetal calf serum (FCS) from Life Technologies Inc., human serum AB from PAA laboratories, GmbH and the cell culture dishes by Falcon (Becton Dickinson).

The monoclonal anti-body (MAB 4051) against the extracellular, N-terminal segment of the angiotensin-converting enzyme (ACE), related for the immunoprecipitation and the immunohistochemical experiments, was from Chemicon international (Temecula, APPROX). The monoclonal antibody (C78) used for Western blotting also recognized the N-terminal domain and was provided by Dr. Peter Bünning (Sanofi-Aventis, Frankfurt, Germany). The ACE monoclonal antibodies (clone 9B9, 3A5, and 5F1) recognizing different epitopes of the N-terminal domain of the enzyme were from Chemicon International (Temecula, CA). The phosphospecific p-Ser1270 antibody was generated from the peptide sequence HGPQFGpSEVELR (position 1263 to 1275 in human somatic ACE protein) by Eurogentec (Seraing, Belgium). The monoclonal (MAB575) as well as the polyclonal (PAB568) anti-body against the cytoplasmic, C-terminal segment of ACE were generated in mice and rabbits immunized with a synthetic peptide corresponding to to amino acids 1250 to 1277 in the ACE C-terminus. The serum from these immunized animals was further purified by affinity chromatography. The antibodies against CD31 and JNK-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The beads for immunoprecipitation, protein A sepharose was from Amersham (Freiburg) and protein G Sepharose was from Zymed (Berlin).

The radioactive nucleotide ([^{32}P γ- adenosine triphosphate was from Hartmann analytics (Braunschweig). Bradykinin as well as angiotensin I and II were from Bachem Biochemica (Heidelberg).

The ACE inhibitor ramiprilat was provided by Aventis (Frankfurt/Main). The oligonucleotide for the polymerase chain reaction (PCR) was synthesized by, Biospring GmbH (Frankfurt/Main. The transfection reagent Superfect was from Qiagen (Hilden). Oligonucleotide chips were from Affymetrix. The plasmids related to split-ubiquitin assay were generous gifts from Dr. Nils Johnsson (Westfälische Wilhelms-Universität Münster, Münster, Germany). All other substances were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords, using dispase as described (132) and cultured in endothelium cell medium MCDB 131 (Invitrogen, Karlsruhe, Germany) with FCS (8%), L-glutamine (10 mmol/L), fibroblast growth factor (1 ng/mL), epidermal growth factor (0,1 ng/mL), endothelial growth-stimulating factor from cattle brain (0.4%), penicillin (50 U/mL) and streptomycin (50 µg/mL) on cell culture dishes coated with fibronectin. HUVEC's were used from passages between 0-2 as ACE expression goes down with passaging of the cells(133). Porcine endothelial cells (PAEC) were isolated from fresh porcine aorta using dispase and cultured in growth medium containing a 1:1 mixture. MCDB 131 (Invitrogen, Karlsruhe, Germany) and M199 (PAA laboratory, Pasching, Austria) containing 14% FCS, EGF (0.05 ng/mL), bFGF (0.5 ng/mL), endothelial cell growth supplement (1.5 mg/mL), heparin (22.5 mg/mL), penicillin (50 U/mL) and streptomycin (50 µg/mL). Although the porcine endothelial cells no longer endogenously expressed ACE or functional AT_1 and B_2 receptors, they expressed a number of characteristic endothelial cell proteins (von Willebrand factor, CD31, the endothelial nitric-oxide synthase, and VE-cadherin). These cells were transfected stably either with human wild type ACE or with non-phosphorlatable ACE mutant, and thus developed into a cell line, in order to examine the effects of the ACE mutation on the intracellular signal transduction pathways and the posttranslational modification of ACE in endothelial cells.

2.2.1. Monocyte isolation

Peripheral blood was obtained from healthy donors (Blood bank, Frankfurt am Main) and mononuclear cells were isolated by Ficoll gradient centrifugation. CD14 positive monocytes were separated from the enriched pelleted cells by Easysep method (Stem cell technologies) according to manufacturer's instructions. 95% of the monocytes thus obtained were CD14 positive by FACS analysis. Resulting monocytes were cultured in Primaria cell culture dishes (Becton Dickinson), in RPMI medium supplemented with L-glutamine, penicillin-streptomycin, 5% human AB serum from PAA, 1X non essential amino acids, 1X MEM vitamins (GIBCO,BRL), 1 mM sodium pyruvate (PAA) and 10 mM HEPES (GIBCO, BRL).

2.3. Stable transfection of endothelial cells

High-passaged ACE deficient porcine aortic endothelial cells (PAEC) were stably transfected with wild-type ACE, S1253A, S1263A, or S1270A using calcium phosphate precipitation or electroporation. Cells stably overexpressing wild type ACE and mutant ACE, were cultured in presence of selection antibiotic Geneticin G418 (500µg/mL), so that only the positive clones, which carry the geneticin resistance cloned in transfection plasmid were selected. Individual clones were selected and ACE expression and enzyme activity was assessed by means of western blot and spectrophotometric enzyme assay (129).

2.4. Adenoviral infection of human endothelial cells

For the infection of endothelial cells, the cells were serum-starved (medium containing 0.1% BSA) for 4-6 hours. Cells were infected with recombinant adenoviruses expressing human wild-type ACE or nonphosphorylatable S1270A ACE for 4 hours in the same medium. Virus containing medium was removed and the cell monolayer was washed three times with PBS in order to remove any traces of virus and/or virus particles and full medium was added and cultured for 24 hrs and then used for experiments.

2.5. Protein isolation and Western blot analysis

Cultivated or native endothelial cells, monocytes were lysed in Nonidet lysis buffer (20 mmol/L TRIS/HCl, pH 8.0, 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 10% (v/v) glycerol, 2 mmol/L sodium pyrophosphate, 10 nmol/L okadaic acid, 2 mmol/L sodium-orthovanadate, 2 µg/mL leupeptin, 2 µg/mL pepstatin A, 2 µg/mL antipain, 2 µg/mL aprotinin, 10 µg/mL trypsin inhibitor, 44 µg/mL PMSF and 1% (v/v) nonidet P40 and placed on ice for 10 minutes and subjected to centrifugation (10000 g, 4°C, 10 minutes). The protein concentration of the supernatant was quantified by Bradford's method (134) or by Amido black staining (135;136). Protein extracts (20-50 µg) were denatured by heating in Laemmli buffer (2% sodiumdodecylsulfate (SDS), 5% β-mercaptoethanol, 8.5 % glycerin, 0,002% bromine-phenol-blue and 63 mmol/L TRIS/HCl, pH 6.8) at 95°C for 5 minutes and separated by SDS-PAGE. The proteins were then subjected to electrophoresis (running buffer: 190 mmol/L glycine, 0.1% SDS and 25 mmol/L Tris-HCl) and resolved for about 2 hours with approximately 35 mA/gel. Western blotting was performed using the tank blot technique onto

nitrocellulose membranes (Biorad, Munich, Germany) for 90 minutes, in transfer buffer (190 mmol/L glycine, 25 mmol/L Tris-HCl and 20% methanol) with 250 mA per apparatus for 90 minutes. The nitrocellulose membranes were stained with Ponceau S (0.2% w/v) to determine the quality of transfer and equal loading of the protein. After destaining with distilled water, the membranes were blocked in Tris buffered saline with tween (TBST, 200 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 0.3% Tween-20) containing 3% BSA for 1 hour at room temperature. The membranes were incubated with the primary antibody (routinely diluted 1:1000) in TBST containing 3% BSA overnight at 4°C. The membranes were then washed in TBST for 30 minutes at an interval of 10 minutes for 30-45 minutes. The membranes were blocked again for 1 hour. The secondary antibody coupled to horse-radish-peroxidase (HRP, 1:10000 in TBST) was incubated with the membranes for 1-2 hours at room temperature and washed again. The proteins were visualized on X-ray films (Fuji RX-film), by enhanced chemiluminescence solution (ECL, Amersham Pharmacia, Freiberg, Germany). For semi quantitative determination of protein expression, the films were densitometrically evaluated. In order to probe the membranes with alternative antibodies, the nitrocellulose membranes were incubated in Stripping buffer (67.5 mmol/L TRIS/HCl, pH 6.8, 100 mmol/L β-mercaptoethanol, 2% (w/v) SDS) at 50°C for 30 minutes. After decanting the stripping buffer, membranes were thoroughly washed several times with TBST and then incubated with specific antibody and processed as described above.

2.6. Immunoprecipitation

Immunoprecipitation (IP) is employed to isolate protein of interest using a specific antibody which is subsequently pulled down using protein A/G Sepharose beads. Cells were lysed in 1% Nonidet lysis buffer, and incubated on ice for 10 minutes. The lysate was centrifuged at 10000 g (4°C for 10 min) and the supernatant was transferred into a fresh tube. All subsequent steps were performed at 4°C. Cell extract (30-40 µg) was kept aside to check for total expression of the protein of interest and the rest volume was used for immunoprecipitation. The cell lysate (300 µg) was subjected to preclearing with 30 µl protein A/G Sepharose beads (Santa Cruz Biotechnology, Heidelberg, Germany) and incubated for 30-60 minutes at 4°C on a rotating wheel. The preclearing step, allows the clearing of the lysate of non-specific protein binding to the sepharose. The supernatant of the preclearing was used for immunoprecipitation with the specific antibodies. For this, 5 µg of the antibody of interest was added to the lysate. The mixture was incubated on the rotating wheel at 4°C, for 2

hours. Then 50 µl of protein A/G Sepharose beads were added and incubated with the sample for 1 hour on the rotating wheel at 4°C. The complex of antigen, antibody and sepharose was then pulled down by centrifugation at 10000 g (4°C for, 10 minutes) and washed three times with Nonidet lysis buffer. Finally, 30 µl of Laemmli buffer was added and the protein denatured at 95°C for 5 minutes. Briefly, the samples were centrifuged for 1 minute in a microcentrifuge at 10000g and the supernatants were separated by SDS-PAGE and Western blotting.

2.8. JNK activity Assay

To assess JNK activity, cells were serum starved for 24 hours prior to stimulation, and rinsed twice with ice-cold phosphate-buffered saline, and then lysed in Nonidet lysis buffer (20 mmol/L Tris-HCl, pH 8.0, containing 1% Nonidet, 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L $Na_2H_2P_2O_7$, 2 mmol/L EDTA, pH 8.0, 10% glycerol, and protease inhibitors (100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml leupeptin). The cell lysate was cleared by centrifugation at 10000 g at 4^0C for 10 minutes. The supernatant was precleared by incubating with 30 µl of Protein A/G sepharose beads for 30 minutes and centrifuged again for 10 minutes. The supernatant was incubated with 5 µl of rabbit anti-JNK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C and then with 50 µl of protein A/G-Sepharose for another 1 hour. The immunoprecipitates were washed twice with Nonidet lysis buffer and once with kinase buffer (25 mmol/L HEPES, pH 7.6, 20 mmol/L $MgCl_2$, 20 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 2 mmol/L dithiothreitol). The kinase assays were performed at 30 °C for 30 min using 2 µg of GST-c-Jun (Cell Signalling, Beverly, MA) as substrate as described earlier(137), 20 µmol/L ATP, and 5 µCi of [γ-^{32}P]dATP (Hartmann Analytic, Braunschweig, Germany) in 30 µl of kinase buffer. The reactions were stopped by the addition of 10µL of 3X Laemmli sample buffer, and the products were resolved by SDS-PAGE (10%). The incorporation of [^{32}P] phosphate was visualized by autoradiography and quantified by scanning densitometry.

2.9. RNA isolation

Endothelial cells were grown to 80-90% confluence and infected with either human somatic ACE or S1270A mutant ACE. Cells were washed with PBS to remove traces of serum. Cells were then lysed with TRI reagent (Sigma, Germany) and incubated at room temperature for

5-10 minutes to permit the dissociation of nucleoprotein complexes. Chloroform (0.2 mL per mL of TRI reagent) was added and the homogenate was shaken vigorously. The cell homogenates were further incubated at room temperature for 5 minutes and were centrifuged at 13000 rpm for 10 minutes at 2 to 8^0C. Following centrifugation, RNA is then precipitated from the aqueous phase by mixing with isopropanol (0.5 mL per mL of TRI reagent) and incubated at room temperature for 10 minutes followed by centrifugation at 13000 rpm for 10 minutes at 2 to 8^0C. After the centrifugation, the RNA precipitate is then washed once with 75% ethanol and again with 95% alcohol, and centrifuged each time at 10000 rpm for 5 minutes at 2 to 8^0C. The resulting RNA pellet is air dried briefly in a fume hood and reconstituted with RNase-free water. The quantity of the purified RNA samples was determined by measuring the absorbance at 260 nm (A_{260}) using a spectrophotometer and the purity by measuring the A_{260}/A_{280} ratio. A ratio of ≥1.7 (260/280) indicates that the RNA is free from protein and deoxy ribonucleic acid. The overall quality of RNA preparation was assessed further by electrophoresis on a denaturing agarose gel and visualized by ethidium bromide staining.

2.10. Gene array (Affymetrix) for expression analysis

Gene arrays have become a powerful approach for many applications to name a few, for the determination of the expression profiles of genes and identification of sequence (gene mutation). We used the gene array to compare the profile of expression of the genes in endothelial cells overexpressing somatic ACE or non-phosphorylatable ACE S1270 mutant and treated with or without ACE inhibitor. Affymetrix's gene chips are glass slide arrays manufactured using special photolithographic methods and combinatorial chemistry, which allow the oligonucleotide spots to be synthesized directly onto the array substrate. For the Affymetrix gene array, endothelial cells were stimulated and RNA was extracted. One cycle eukaryotic target labeling assay was used. Total RNA (10 μg) was first reverse transcribed using a T7-Oligo (dT) promoter primer in the first strand cDNA synthesis reaction. Following Rnase H-mediated second strand cDNA synthesis, the double-stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription reaction. The *in vitro* transcription was carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets are then cleaned up, fragmented and hybridized to GeneChip expression arrays. The hybridized cRNA was then stained with a

Streptavidin-phycoerythrin conjugates and visualized with an array scanner. The experiment was performed three times with different batches of cells to limit experimental variations.

2.12. Nuclear isolation of proteins

Cells were stimulated for the indicated times and harvested in ice cold PBS with protease inhibitors. All the procedures was handled on ice. Cells collected in PBS with protease inhibitors were centrifuged briefly for 30 sec at 14000 g at 4^0 C. The cell pellets were resuspended in 100 µL of the buffer A (Hypotonic buffer: 10 mmol/L HEPES 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA), 1mmol/L DTT, 0.5mmol/L PMSF, and mixed gently with pipetting. The cell pellets were incubated on ice for 10 minutes and 7.5 µL of 10% nonidet was added to lyse the cell membrane and incubated on ice for further 1 min, vortexed and centrifuged briefly at 14000 g at 4^0 C for 1 minute. The supernatant (post nuclear fraction) was transferred to fresh eppendorf tubes. The resulting pellet was briefly washed with PBS with protease inhibitors by vortexing and centrifugation at 14 000 g at 4^0 C for 1 minute in order to remove the traces of the post nuclear fraction. The pelleted nuclei were resuspended in 30-50 µL of the buffer C (Hypertonic buffer: 20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1mmol/L EDTA, 1mmol/L EGTA, 10% glycerol), and incubated on ice for 30 minutes, vortexing for 10 seconds every 10 minutes for 30 minutes. The insoluble fraction was removed by centrifugation at 14 000 g for 10 minutes at 4^0 C. The nuclear extracts were stored at -80^0C until analysed further.

2.13. Electrophoretic Mobility Shift Assay (EMSA)

This technique is used to study gene regulation and determining protein-DNA interactions. The EMSA technique is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (138). Monocytes were cultured for 4 days and treated thereafter with/without ramiprilat for 24 hours. Nuclear extracts were prepared as described above. Protein concentration was measured by the method of Bradford. The NF-κB binding protein was used according to the manufacturer's instructions. Briefly, NF-κB oligonucleotide was end labeled using γ^{32}P-ATP using Ready-to-go T4 Polynucleotide kinase (Amersham Biosciences, Germany) kit. Unincorporated nucleotide was removed from the DNA probe by using the G-25 spin columns (Amersham Pharmacia biotech, Germany). Briefly the column

containing the resin is vortexed gently. The cap is loosened and the bottom closure is snapped off and placed in a 1.5 mL eppendorf tube for support. The column is centrifuged for 1 minute at 3000 rpm. The column is placed in a fresh eppendorf tube and the labeled sample is applied on to the center of the resin bed and centrifuged again for 2 minutes at 3000 rpm. The purified sample collects at the bottom of the eppendorf tube. 1 μL of the sample is counted. Nuclear extracts of 5 to 10 μg were incubated with 20000 counts per minute of the labeled oligonucleotide in binding buffer (20% glycerol, 5mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl -pH 7.5) on ice for 30 minutes. Poly (dI-dC) was used as a non-specific competitor. The samples were then separated on a 6% denaturing gel. The gel was dried and subjected to autoradiography.

2.14. Statistical analysis

Data are expressed as mean ± SEM, and statistical evaluation was performed by using the Student's *t* test for paired or unpaired data, one way ANOVA followed by a Bonferroni *t* test or ANOVA for repeated measures, where appropriate. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. ACE dimerization in endothelial cells

ACE has been defined as a signal transduction molecule which upon binding to ACE inhibitor turns on a signalling cascade culminating in changes in the gene expression in endothelial cells, however the mechanism by which ACE inhibitor initiates this signalling cascade was not clear. It was hypothesized that ACE which is anchored to the membrane with a single transmembrane domain should dimerize preceding the downstream signalling events. Therefore, we sought to determine whether or not ACE dimerizes in endothelial cells and whether ACE dimerization is involved in ACE inhibitor-induced ACE signalling. We used porcine aortic endothelial cells stably overexpressing human somatic ACE which were subjected to native gel electrophoresis. Two forms of ACE were detected by native gel electrophoresis: a dominant form of approximately 270 kDa (monomer) and a minor form of approximately 520 kDa, the putative dimer (Fig. 3). To confirm ACE dimerization in endothelial cells, experiments were also performed under nonreducing conditions. The ACE dimer (~ 520 kDa) was only detected in SDS- and DTT- free conditions suggesting the involvement of disulphide bonds in ACE dimerization. In the presence of SDS and at high temperature of 95^0C, only the lower monomeric ACE was detected, the reason being, large protein aggregates are destroyed by detergent-SDS and high temperature. Endothelial cells treated with trypsin which cleaves ACE at the C-terminal stalk region (139) resulted in the loss of both the 520- and the 270-kDa forms of ACE.

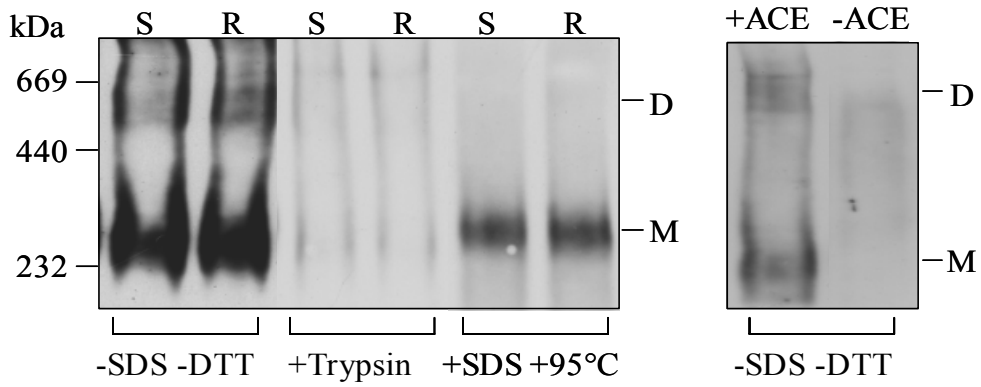

Fig. 3: Dimerization of ACE under basal conditions and in the presence of ramiprilat.
Representative Western blots showing somatic ACE monomers (M) and dimers (D) in lysates of ACE-overexpressing porcine aortic endothelial cells treated with either solvent (S) or ramiprilat (R; 100 nM; 7 min) (left side) or ACE overexpressing (+ACE) and ACE-deficient (-ACE) porcine aortic endothelial cells (right side). Experiments were performed under nonreducing conditions (-SDS-DTT). As a control, lysates were treated with trypsin (10 U/ml; 1 h) before adding nonreducing sample buffer or were heated for 10 min in Laemmli SDS sample buffer (+SDS +95°C) before analysis.

ACE inhibitors are reported to increase the expression of ACE in endothelial cells in a time-dependent manner (130). However, stimulation of endothelial cells with ramiprilat (100μM; 7 min) significantly increased the relative amount of 520-kDa ACE by 137± 11% (n=5; $p < 0.05$) as compared to the controls treated only with the solvent (left panel, Fig. 3) suggesting that ramiprilat increases the dimerization of ACE in endothelial cells.

3.2. Existence of ACE dimerization in living cells system.

ACE dimers were reported to exist in reverse micelles (64), so to determine homodimer formation of ACE in living cells, split-ubiquitin system was used. For the split ubiquitin system, ubiquitin is split into an N-terminal domain (N_{ub}) and into a C-terminal domain (C_{ub}), and neither is recognized by the ubiquitin-specific proteases (Ubps). This assay is based on the association of the N_{ub} and C_{ub} of ubiquitin that are fused to the cytosolic domain of full-length human ACE generating ACE-N_{ub} and ACE-C_{ub}. The C-terminal of ACE-C_{ub} is fused to an Ura3p reporter protein whose first amino acid has been replaced by arginine (RUra3p) thus generating ACE-C_{ub}-RUra3p (Fig. 4). Dimerization-induced association of N_{ub} and C_{ub} in cotransformants creates a native-like ubiquitin which is then recognized by ubiquitin-specific proteases cleaving RUra3p.

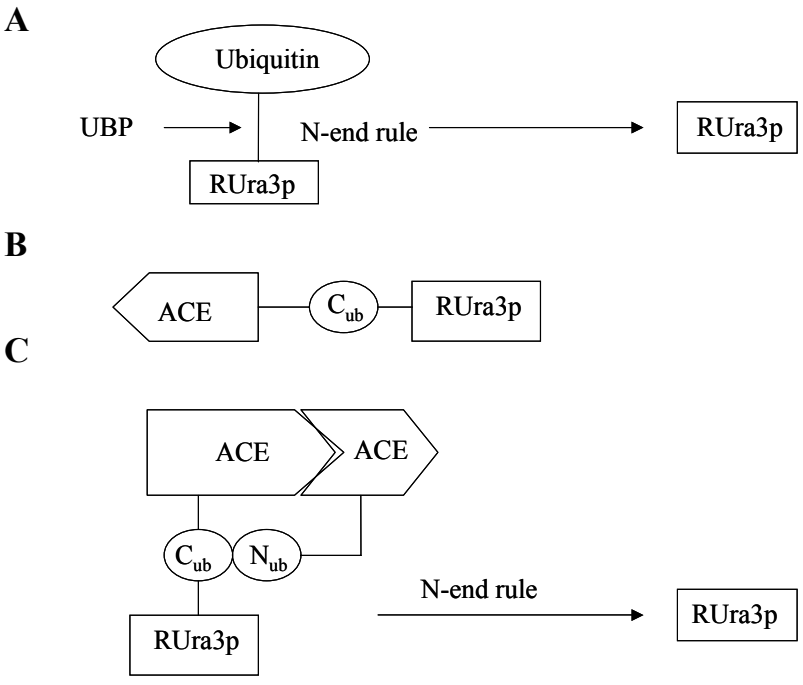

Fig. 4: Schematic representation of conditional degradation design. **A**, The first amino acid of Ura3p enzyme is replaced by arginine (Rura3p) and fused to ubiquitin. The fusion protein is cleaved by the Ubps and the free Ura3p is degraded rapidly by the enzymes of N-end rule that the cells are uracil auxotrophic and resistant to the drug 5-fluro-orotic acid (FOA). **B,** A fusion containing ACE, C_{ub} and the Rura3p is not cleaved by the Ubps. The fusion is active and cells are uracil prototropic and sensitive to FOA. **C,** Co-expression of N_{ub} and C_{ub} fused to ACE leads to increased interaction and a native-like ubiquitin is formed which is cleaved. RUra3p is rapidly degraded. The protein-protein interaction between ACE monomers can be detected by the absence of growth on plates lacing uracil and by growth on plates containing FOA.

In this way, the split-Ub system provides a direct readout of the N_{ub}-C_{ub} association state indicating existence of dimer forms.

To check for the expression of both ACE-N_{ub} and ACE-C_{ub} proteins, transformed JD53 cells were further analyzed by Western blotting. Cells coexpressing ACE-N_{ub} and ACE-C_{ub} were unable to grow on ura$^-$ plates but expanded on 5-FOA plates, indicating that ACE forms stable dimers (and/or higher oligomers) in intact cells (Fig. 6). While cells expressing ACE-C_{ub} and an unrelated construct in which the ubiquitin conjugating enzyme Ubc6 was fused to the N-terminal portion of N_{ub} grew on ura$^-$ plates but not on ura$^+$ and 5-FOA$^+$ plates, indicating that ACE and Ubc6 do not interact under these indications. To enhance the discriminating power of the assay system, two N_{ub} mutants containing alanine (N_{ua}) or glycine (N_{ug}) in position 13, which have a lower affinity for C_{ub} than wild-type $N_{ub,}$ were also used. Using these modified probes, a strong interaction between ACE-N_{ua} or ACE-N_{ug} and ACE-$C_{ub,}$ was observed indicating the existence of ACE dimers/oligomers in living cells (Fig. 5).

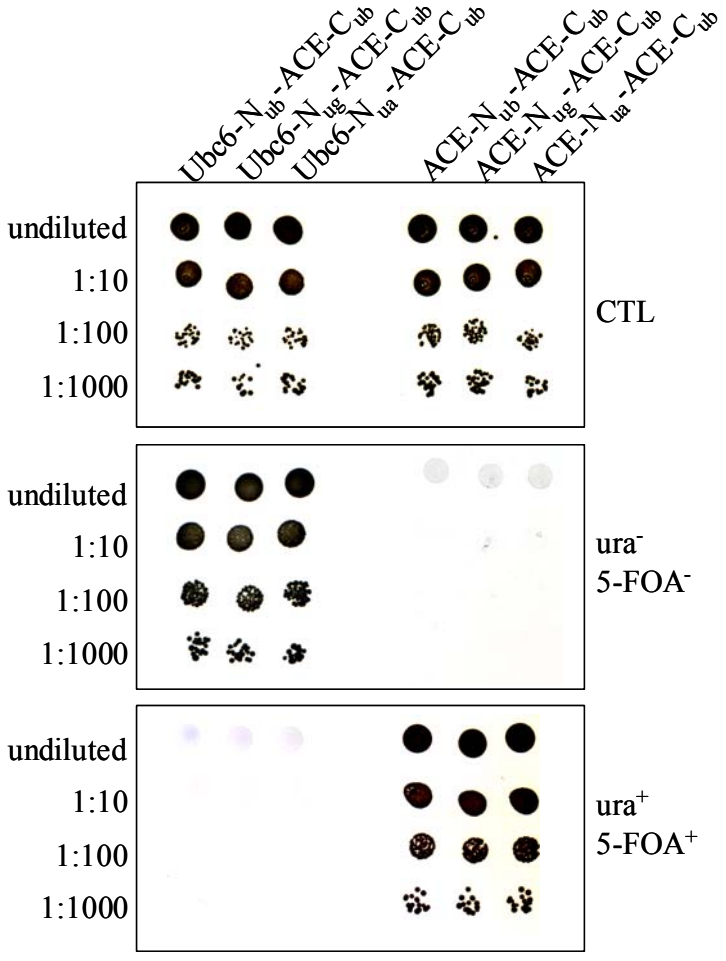

Fig. 5: Serial dilutions of JD53 cells coexpressing ACE-N_{ub}, ACE-N_{ug}, or ACE-N_{ua} and ACE-C_{ub} were spotted on plates lacking Trp/His (CTL), or uracil and 5-FOA (ura$^-$ 5-FAO$^-$), or on plates containing uracil and 5-FOA (ura$^+$ 5-FOA$^+$). Interaction between ACE monomers is indicated by cell growth on ura$^+$ 5-FOA$^+$ plates and growth inhibition on ura$^-$ 5-FAO$^-$ plates and vice versa. Similar results were obtained in three additional experiments.

3.3 ACE inhibitors induce dimerization of ACE in endothelial cells

To investigate the effects of ACE inhibitor ramiprilat on ACE dimerization, endothelial cells were treated with the ACE inhibitor and then exposed to chemical cross-linkers DSS or BS_3 to stabilize the dimers formed. The chemical cross-linker BS_3 (which is membrane impermeable and has a spacer arm of 11.4 Å) reacts with lysine residues. Cross-linking experiments were then followed by Western blotting to assess dimerization of ACE. Ramiprilat induced a rapid (within 2 min) 2-fold increase in the dimerization of ACE, which was maintained for up to 60 min (Fig. 6A).

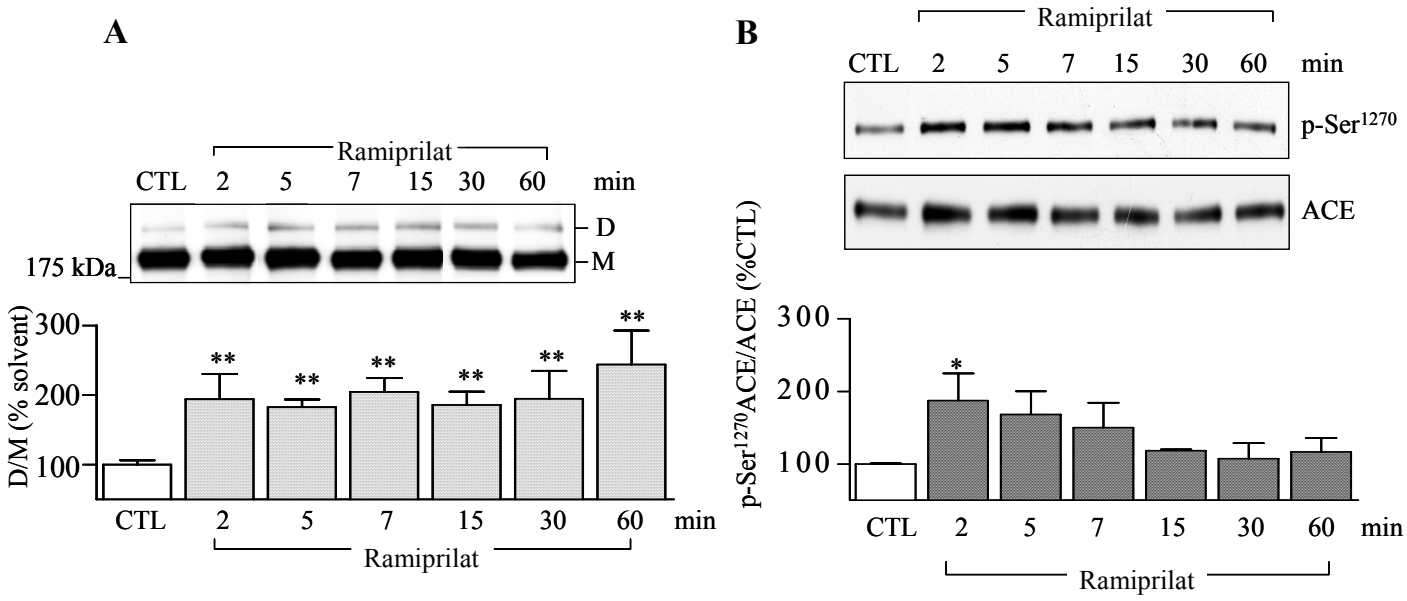

Fig. 6: Effect of inhibitors on ACE dimerization and phosphorylation in endothelial cells. Porcine aortic endothelial cells stably overexpressing human somatic ACE were treated with ACE inhibitors. **A**, Time course (2-60 min) of the ramiprilat (100 nM) induced dimerization assessed after chemical cross-linking. The cross-linking of ACE on the surface of confluent cells was performed at room temperature for 30 min using 1 to 2 mM DSS or BS_3. After quenching the reaction with 20 mM Tris-HCl, pH 7.4, for 15 min at room temperature, cells were lysed and immunoblotting performed. **B**, Phosphorylation of ACE on Ser^{1270}. Results were quantified by calculating the D/M ratio and comparing changes relative to the ratio detected in unstimulated endothelial cells (CTL) or by calculating the p-Ser1270ACE/ACE signal ratio by densitometry and comparing changes relative to the signal obtained in CTL cells. The bar graphs represent the results of four to six independent experiments; * $p<0.05$;** $p<0.01$ versus CTL.

On binding of an ACE inhibitor to ACE, CK2 phosphorylates the Ser^{1270} residue in the cytoplasmic tail, a process that initiates a cascade of signalling events (130). Therefore phosphorylation of ACE was also assessed in parallel experiments.

We observed the ramiprilat-induced phosphorylation of ACE was maximal at 2 min after treating the cells with inhibitor but decreased back to the baseline in the next 10 to 15 min (Fig. 6B).

To exclude an artifact arising from ACE-overexpressing endothelial cells we performed experiments in primary cultures of human umbilical vein endothelial cells which endogenously express ACE, and we observed ACE inhibitor-induced dimerization of ACE (Fig. 7A). The mutation of Ser^{1270} to alanine prevents the ACE inhibitor-induced phosphorylation of ACE and the subsequent ACE signalling (130). To assess whether ACE dimerization is dependent on phosphorylation of the cytoplasmic tail of ACE, we performed experiments in endothelial cells overexpressing the nonphosphorylatable ACE. We however observed that even in endothelial cells overexpressing the nonphosphorylatable S1270A ACE mutant, ramiprilat was able to induce the dimerization of ACE (Fig. 7B) suggesting that ACE dimerization is not dependent on the phosphorylation status of the cytoplasmic tail.

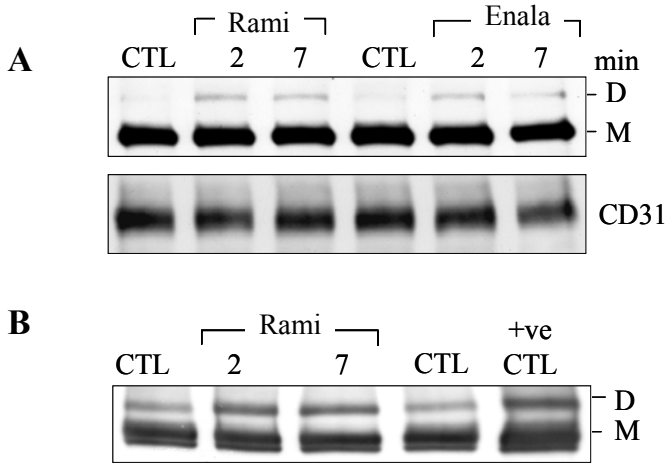

Fig. 7. Effect of ACE inhibitors on dimerisation after cross-linking. Human umbilical vein endothelial cells were treated with ACE inhibitors and assessed for ACE dimerization after cross-linking with BS_3. **A**, Effect of ramiprilat (Rami) and enalaprilat (Enala; each 100 nM; 2 and 7 min) on ACE dimer formation. Equivalent loading of protein in each sample was verified by reprobing the blots for CD31. This blot is representative of four to six additional experiments. **B**, Effect of Rami (100 nM; 2 and 7 min) on ACE dimerization in porcine aortic endothelial cells stably overexpressing the nonphosphorylatable ACE mutant S1270A. Porcine aortic endothelial cells stably overexpressing human somatic ACE were used as a positive control (+ve CTL).

A similar dimerization of ACE was observed with other ACE inhibitors (i.e., enalaprilat, quinaprilat, captopril) (Fig. 8) but with varying efficacies. The effect of ramiprilat and other ACE inhibitors on the dimerization of ACE in ACE-overexpressing porcine endothelial cells was concentration-dependent with significant effects being observed using concentrations of 10 nM or greater (Fig. 8), thus suggesting that the ACE inhibitor-induced dimerization of ACE is a specific effect of this class of compounds, rather than a unique property of ramiprilat and also that the increase in dimerization of ACE was dependent on the binding of a compound (ACE inhibitors).

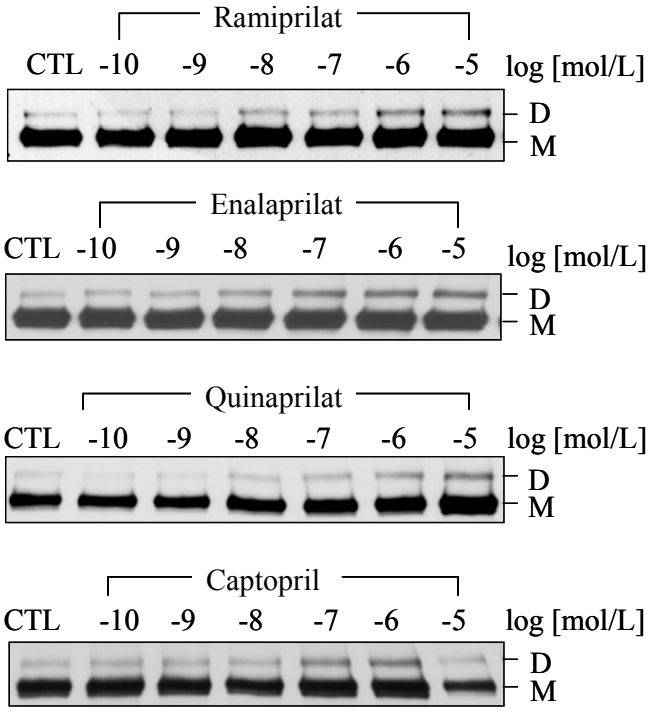

Fig. 8: Concentration-dependent effect of different ACE inhibitors on ACE dimerization. Confluent endothelial cells were treated with different ACE inhibitors and assessed for ACE dimer formation after chemical cross-linking. Representative western blots showing the concentration-dependent effects of ramiprilat, quinaprilat, captopril, and enalaprilat (0.1 nM-10 µM; 2 min) on ACE dimerization. Similar results were obtained in three additional experiments.

3.4. Effect of carbohydrates on ACE dimerization

ACE is an extensively glycosylated protein. Numerous studies have demonstrated that glycosylation plays an important role in the folding of ACE and also affects the transport and enzyme release (140-142). Human somatic ACE is reported to form carbohydrate-mediated

dimers and ACE contains a specific carbohydrate-recognizing domain (143). To assess the role of carbohydrates in enzyme dimerization, we used the monosaccharides galactose or glucose. ACE-overexpressing porcine aortic endothelial cells were incubated with 10 µM galactose, 10 µM glucose, or 10 µM mannitol (as an osmotic control) for 30 min, prior to the addition of 100 nM ramiprilat for 2 min as we had observed robust rapid dimerization with ACE inhibitors after 2 minutes. The basal or ramiprilat-induced dimerization of ACE in endothelial cells was not influenced by galactose, glucose, or mannitol (Fig. 9). Furthermore the pretreatment of cells with the monosaccharides did not influence either the basal or the ramiprilat-induced phosphorylation of ACE on Ser^{1270}.

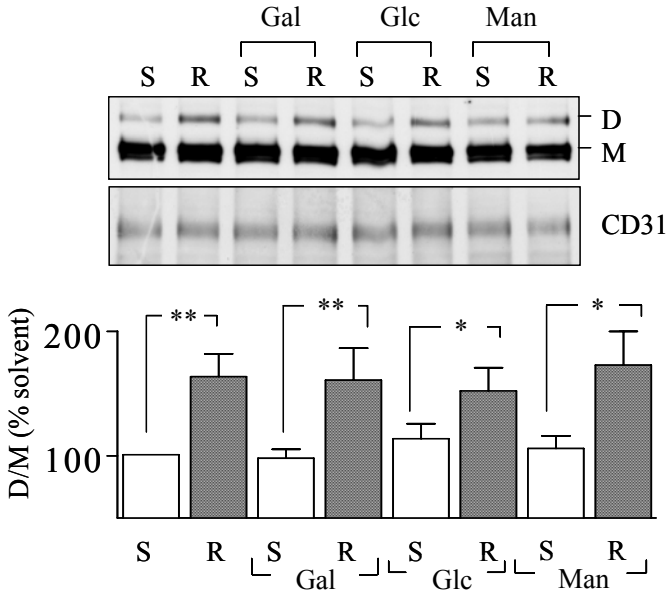

Fig. 9: Effect of carbohydrates on the dimerization of ACE in ACE-overexpressing endothelial cells. Endothelial cells overexpressing human somatic ACE were pre-incubated with galactose (Gal), glucose (Glc), or mannitol (Man) (each 10 µM; 30 min). Representative western blot depicts the effect of pretreatment with monosaccharides on ramiprilat (R; 100 nM; 2 min)-induced dimerization of ACE. The results were quantified by calculating the cross-linked D/M ratio and comparing changes relative to the ratio detected in unstimulated endothelial cells (S, solvent). The bar graphs represents results obtained in four to six different experiments; *, $p < 0.05$; **, $p < 0.01$ versus CTL.

3.5. Effect of monoclonal antibodies on ACE dimerization and shedding

The carbohydrate recognition domain that was suggested to be important for ACE dimerization is reported to be recognized and thus shielded by the monoclonal antibody 9B9 (143). To determine the effect of different monoclonal ACE antibodies (9B9, 3A5, 5F1, and C78) directed to different epitopes within the N-domain on ACE dimer formation, ACE-overexpressing porcine aortic endothelial cells were incubated with the above mentioned antibodies and assessed for dimer formation (143).

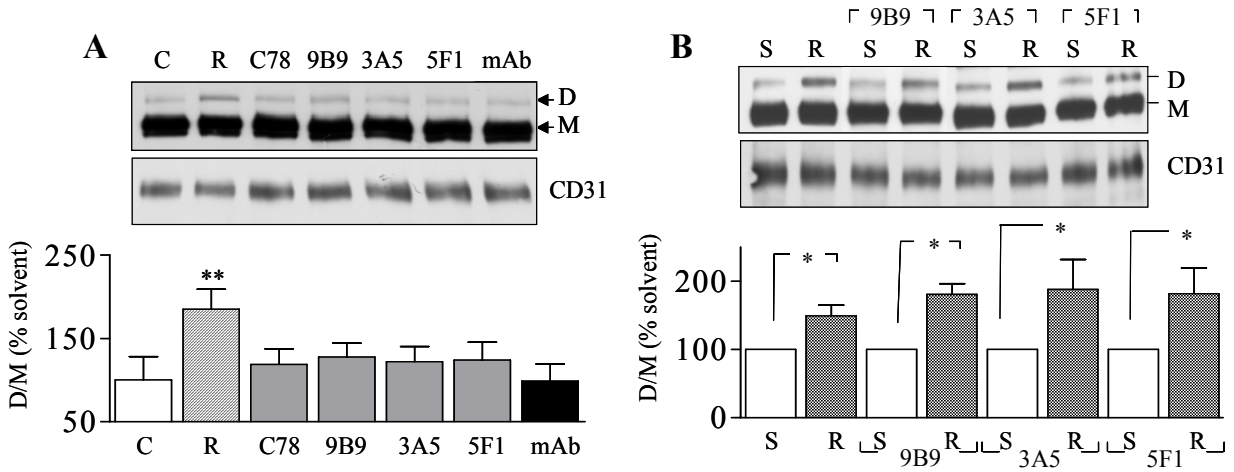

Fig. 10: Effect of anti-ACE monoclonal antibodies on ACE dimerization. A, Effect of solvent (S), ramiprilat (R; 100 nM; 7 min), monoclonal antibodies to the ACE N domain C78, 9B9, 3A5, and 5F1 (each 10 µg/ml; 7 min) and an unrelated monoclonal antibody (mAb; anti-caveolin-1), on ACE dimerization in ACE-overexpressing porcine aortic endothelial cells. **B**, Effect of pretreatment with solvent and antibodies 9B9, 3A5, and 5F1 (each 10 µg/ml; 4 h) on the ramiprilat (R; 100 nM; 2 min)–induced dimerization of ACE assessed after chemical cross-linking. The bar graphs summarize the results of three to six independent experiments; * $p < 0.05$; ** $p < 0.01$ versus the respective buffer alone.

The antibodies tested had no acute effect on ACE dimerization per se (Fig. 10A). The antibodies tested were not able to prevent the ramiprilat-induced dimerization of ACE in ACE-overexpressing porcine aortic endothelial cells (Fig. 10B).

Anti-ACE monoclonal antibodies which are epitope specific, have been reported to enhace the shedding of ACE (144). To determine the shedding of ACE, endothelial cells were incubated with anti-ACE monoclonal antibodies. Among the anti-ACE antibodies used only the 9B9 and 3A5 antibodies (but not 5F1) significantly enhanced the amount of ACE precipitated from the endothelial cell supernatant (Fig. 11), reflecting the enhanced cleavage/secretion of the enzyme.

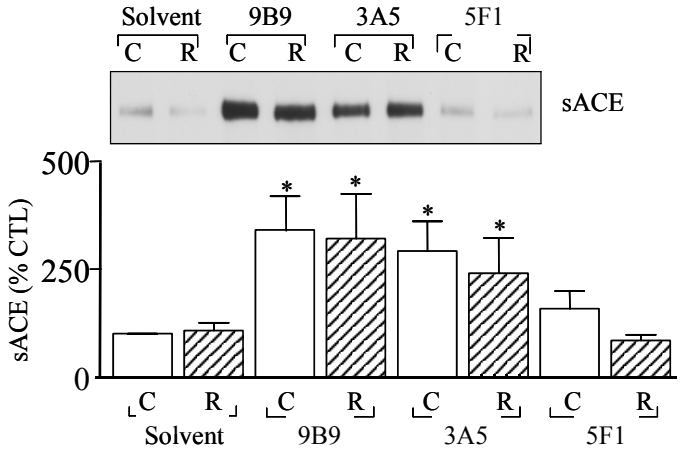

Fig. 11: **Effect of anti-ACE monoclonal antibodies on ACE shedding.** Effect of the 9B9, 3A5, 5F1 antibodies (each 10 µg/ml; 4 h) on the amount of soluble ACE (sACE) recovered from the supernatant of endothelial cells. The culture medium was collected and centrifuged (for the precipitation of the detached cells) and subjected to immunoprecipitation. The bar graph represents data from three to six independent experiments; * $p < 0:05$.

3.6. Ramiprilat-induced dimerization initiates ACE signalling in CHO cells

Glu^{362} in the N-domain and Glu^{960} in the C-domain are essential catalytic residues, and the His doublets 361/365 and 959/963 are most likely involved in the binding of Zn^{2+} (57). To determine whether the active sites are necessary for the dimer formation, CHO cells were stably transfected with either wild-type ACE or different ACE mutants; two with an inactivated N-domain (N_{His} and N_{Glu}), two with an inactivated C-domain (C_{His} and C_{Glu}), and one mutant with interfering mutations in both the N and C domains ($N+C_{Glu}$). Mutation of the glutamyl residues within the C and/or N domains (N_{Glu}, C_{Glu}, and $N+C_{Glu}$) did not have any effect on the ramiprilat-induced dimerization of ACE or on its phosphorylation on Ser^{1270} (Fig. 12A and B). Replacement of the two histidyl residues within the C domain of ACE (C_{His}) however attenuated the basal formation of ACE dimers as well as Ser^{1270} phosphorylation of the ACE mutant, whereas the N_{His} mutant did not show any change in dimerization and increased basal Ser^{1270} phosphorylation in CHO cells overexpressing these mutants (Fig. 12 A and B).

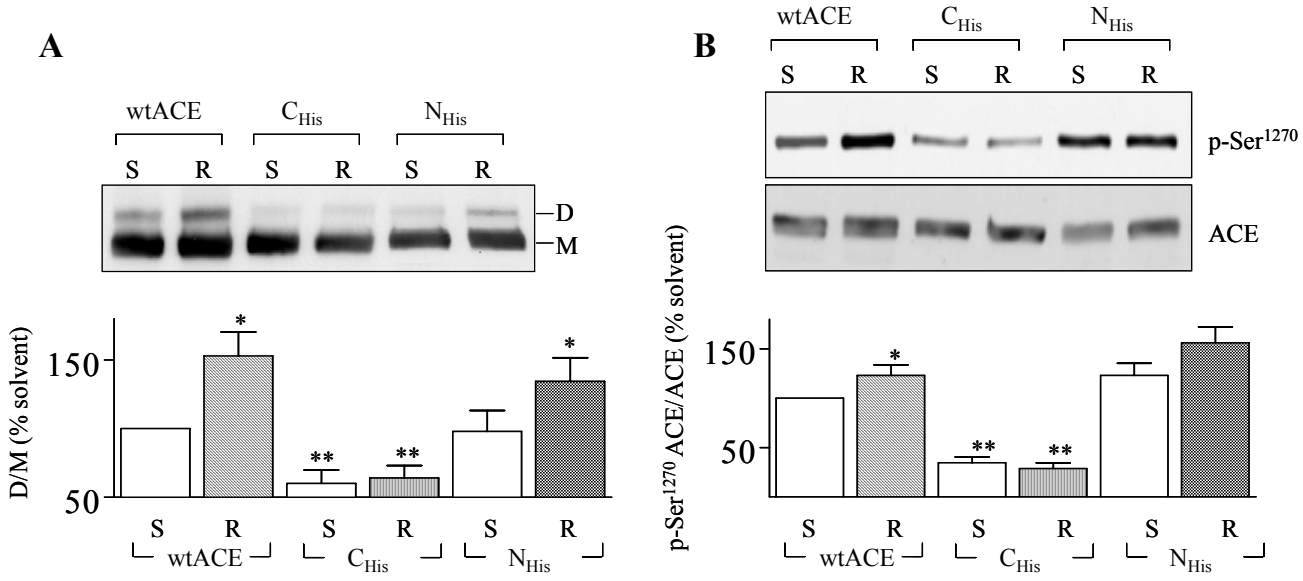

Fig. 12: Effect of ramiprilat on dimerization and phosphorylation of N_{His} amd C_{His} mutants. A, Representative Western blot showing the effect of solvent (S) and ramiprilat (R; 100 nM; 2 min) on ACE dimerization. **B,** Effect of ramiprilat (R; 100nM) on phosphorylation at Ser^{1270} in CHO cells overexpressing human somatic ACE (wtACE) or N_{His} and C_{His} mutants. The monomeric (M) and the dimeric (D) forms of ACE are indicated. The bar graphs summarize results obtained in four to six independent experiments; **, $p < 0.01$ versus basal wild-type ACE dimerization or phosphorylation.

ACE signalling involves the phosphorylation of Ser^{1270} residue, leading to the activation of JNK (130). The effects of ramiprilat treatment on CHO cells expressing wild-type ACE were similar to those observed in cells expressing N_{His}, or any of the Glu mutants (N_{Glu}, C_{Glu}, and $N+C_{Glu}$; Fig. 13). However ramiprilat was unable to activate JNK in C_{His} expressing cells (Fig. 13), indicating that ramiprilat was able to elicit signalling when the two His residues mediating Zn^{2+} binding of the active site in the C-domain were present.

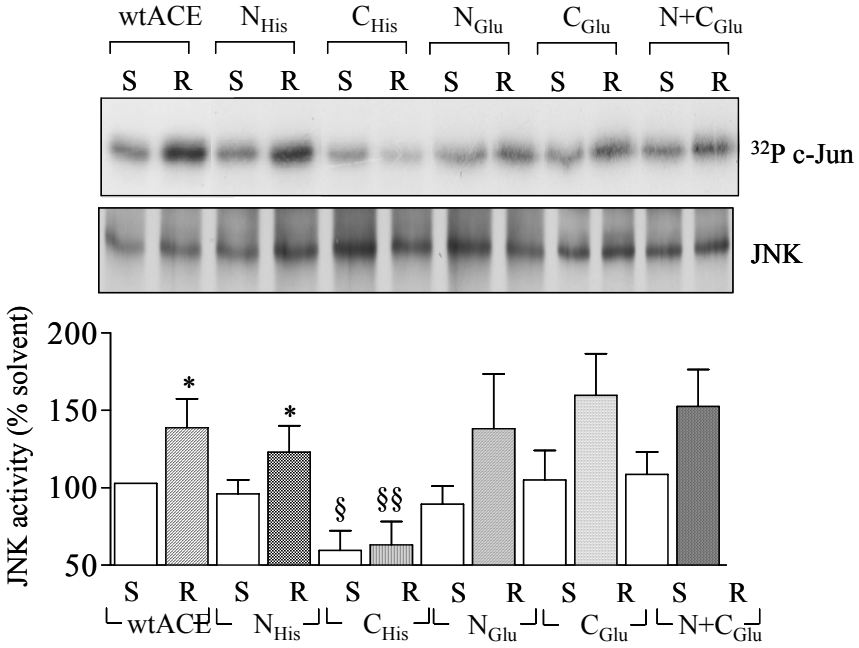

Fig. 13: Effect of ramiprilat on signalling by ACE mutants. Autoradiography of ^{32}P-labeled c-Jun and statistical analysis showing the effect of solvent (S) and ramiprilat (R; 100 nM; 7 min) on the activity of JNK immunoprecipitated from CHO cells overexpressing human somatic ACE (wt-ACE) or one each of the N_{His}, C_{His}, N_{Glu}, C_{Glu}, or $N+C_{Glu}$ mutants. To ensure equal immunoprecipitation of JNK, blots were reprobed with an antibody against JNK. The bar graph summarizes the results obtained from four independent experiments; * $p < 0.05$ versus respective solvent; §, $p < 0.05$; §§, $p < 0.01$ versus basal JNK activity in cells expressing wild-type ACE.

3.6. Divergent gene expression induced by ramiprilat in primary endothelial cells

ACE signalling leads to the activation of JNK/c-Jun pathway and the expression of ACE and COX-2 (131). To identify the genes affected by ramiprilat-induced dimerization and consequent ACE signalling, DNA microarray was used. Human umbilical vein endothelial cells were multi-passaged to ensure that these cells did not posses B_2 receptor and had very little endogenous ACE as both ACE and B_2 receptor levels diminish with passaging the cells. Wild type ACE or the non-phosphorylatable S1270 ACE mutant was then overexpressed in these endothelial cells. Briefly, human umbilical vein endothelial cells overexpressing either human somatic ACE and/or the non-phosphorylatable S1270 ACE mutant were treated with ramiprilat for 24 h and 48 h (Fig. 14). Total RNA was extracted from cultured cells and the integrity of RNA was assessed by visualization of ethidium bromide-stained gels. After prehybridisation, the labeled cDNA probes were hybridized onto Affymetrix oligonucleotide microarray. The relative expression level of each gene was determined by comparing the signal intensity of each gene in the array after background subtraction and normalization to the housekeeping genes. Genes were grouped depending on their expression levels in the control and treated samples in the cells expressing the wild-type ACE and not in the cells overexpressing the nonphosphorylatable mutant ACE which was the criteria for ACE signalling. Upregulation or downregulation of genes was considered if the magnitude of change was at least 4-fold in three independent experiments.

Twenty-one genes were identified out of which seven genes were upregulated and fourteen were downregulated (Table. 2) by ramiprilat in endothelial cells expressing human somatic ACE but not in endothelial cells expressing the non-phosphorylatable S1270 ACE mutant. The genes which were regulated belong to different classes of molecular functions as tabulated (Table. 2). All of the genes, whose induction was four fold changed, were selected for further analysis.

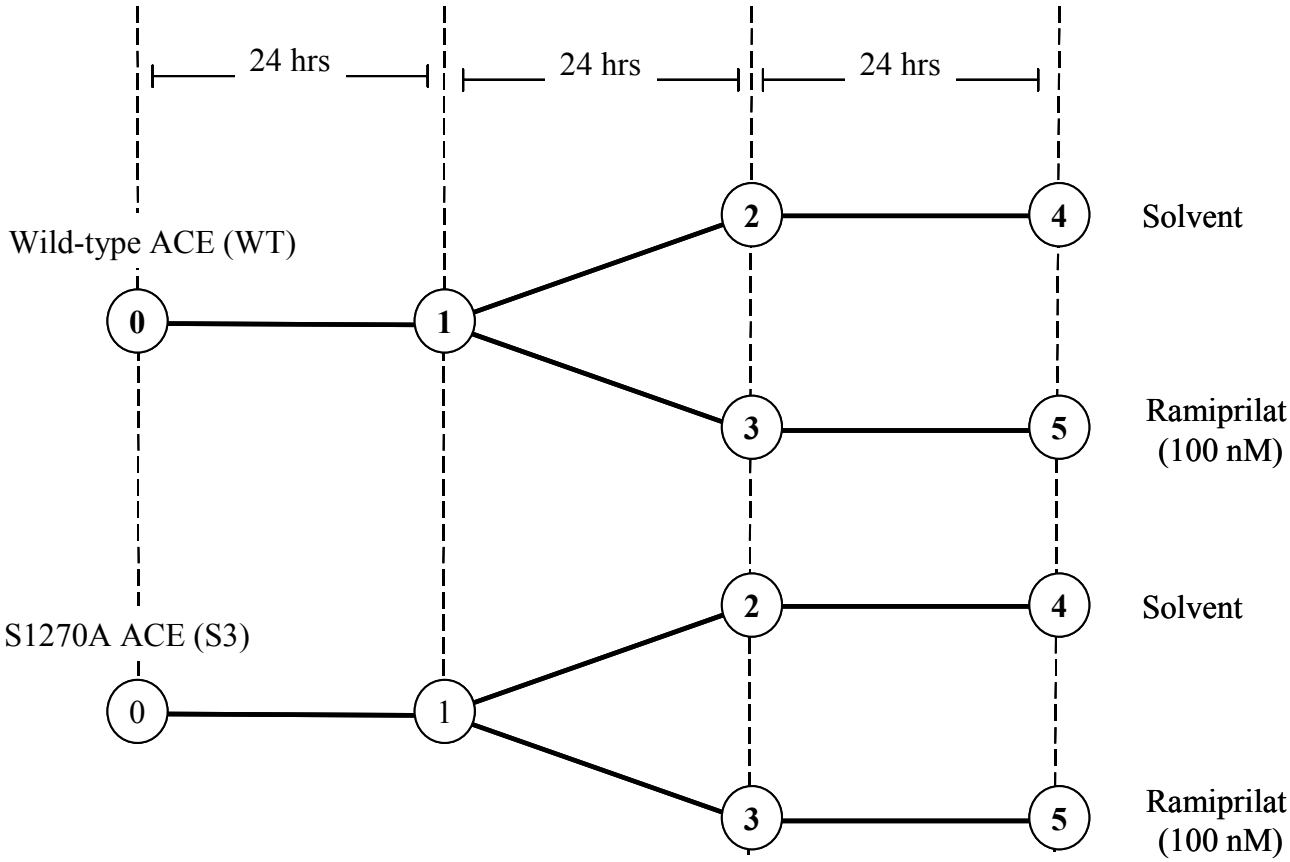

Fig. 14: Schematic representation of the protocol followed for the DNA microarray. Human umbilical vein endothelial cells expressing human somatic ACE or non-phosphorylatable S1270 mutant ACE were treated with ramiprilat for 24 h and 48 h. Total RNA was isolated and hybridized to Affymetrix oligo chips. All microarray analysis were performed with three completely different cell batches to ensure the reproducibility of results.

Activity	Gene	Accession number	Function
Upregulated	Cellular retinol binding protein 1	NM_002899	Intracellular transport of retinol
	P57KIP2	D64137	Negative regulator of cell proliferation
	CIS2 (STAT induced STAT inhibitor-2)	AB004903	Negative regulator of IGF1/ growth hormone signalling pathway
	CD26 (Dipeptidylpeptidase IV)	M74777	Peptidase, T-cell activation
	SOX-4	AI989477	Transcriptional activator
	CC-1(small inducible cytokine subfamily A)	NM_004166	Cytokine-cytokine receptor interaction
	G8	NM_016947	No known function
Downregulated	Interleukin 8	NM_000584	Chemotactic factor for neutrophils, basophils and T-cells
	Mitofusin 2	NM_014874	Transmembrane mitochondrial GTPase
	Cyclin F	NM_001761	Regulation of cell cycle
	Cyclin E2	AF112857	Regulation of cell cycle
	BRCA2 and CDKN1A interacting protein	NM_016567	Involved in double-strand break repair and/or homologous recombination
	Metallothionein 1H	NM_005951	Binds to various heavy metals
	Kinesin-like 6 (mitotic centromere associated kinesin	U63743	Associates with centromeres at early prophase
	BRCA1 (Breast cancer 1, early onset)	NM_007295	DNA repair
	Kinesin-like 7	NM_020242	Associates with microtubules
	Survivin (baculoviral IAP repeat-containing 5)	NM_001168	Inhibitor of caspases 3 & 7
	Forkhead box M1	NM_021953	Transcriptional activatory factor
	RacGAP 1	AU153848	Electron transporter activity
	Cell division cycle 25 C	AF277724	Tyrosine protein phosphatase required for progression of the cell cycle
	PRO2000 protein	NM_014109	Nucleoside-triphosphatase

Table. 2: List of genes screened through microarray analysis.

3.7. Expression patterns of selected genes in human endothelial cells

Post transcriptional mechanisms could contribute to regulation of specific gene products. Therefore, a subset of genes detected by the gene array was analyzed by Western blot to check for their expression at the protein level. Fig. 15A shows the effect of ramiprilat treatment on the expression profiles of upregulated genes in the microarray data (SOX4, CRBP1, CCL14, p57KIP2). Fig. 15B shows the effect of ramiprilat on the downregulated genes-RacGap1, Survivin, FOXM1, BRCA1 and CDC25 at the protein level in endothelial cells overexpressing wild-type ACE. Surprisingly, in contrast to gene array results, there was no significant differences between the untreated control and ramiprilat (Rami; 100 nM; 24-48 h) treated cells. Although the expression of these genes was substantial enough to be detected by Western blot analysis, we were not able to reproduce similar effects seen in the gene array.

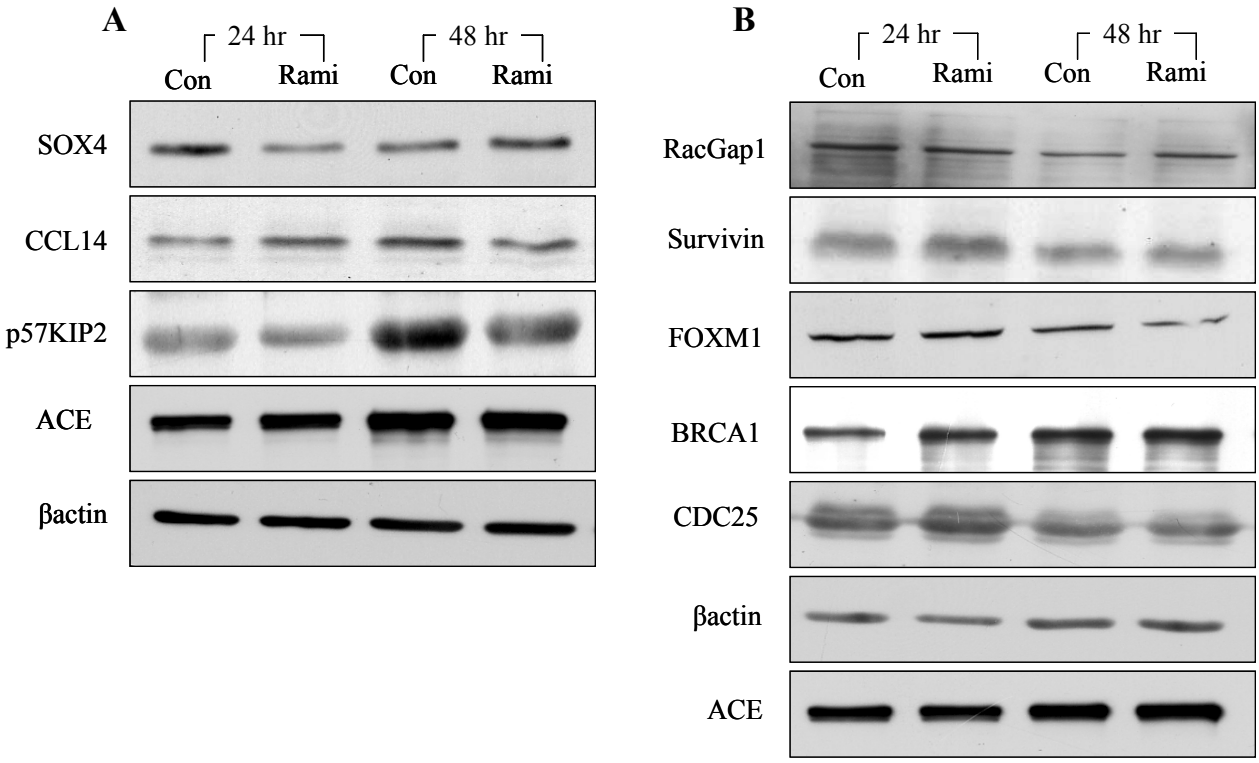

Fig. 15: Effect of ramiprilat on the expression profiles of modulated genes. Briefly, human umbilical vein endothelial cells overexpressing wild type ACE were treated with ramiprilat (Rami; 100 nM) for 24 h and 48 h. The cells were harvested and the whole cell lysate was analysed by Western blot. **A,** representative blots showing the expression of upregulated genes SOX4, CCL14, p57KIP2 and **B,** downregulated genes RacGap1, Survivin, FOXM1, BRCA1 and CDC25 in three to five independent experiments.

3.8. Ramiprilat enhances cellular retinol binding protein-1 levels in human Plasma

As several other cells and tissues also possess fully functional RAS, we screened plasma samples from healthy volunteers as well as from patients manifest with coronary artery disease and also from diabetic patients for the genes identified in the microarray. We observed that cellular retinol binding protein-1 (CRBP-1) was detectable albeit at low levels in plasma from patients (Fig. 16A).

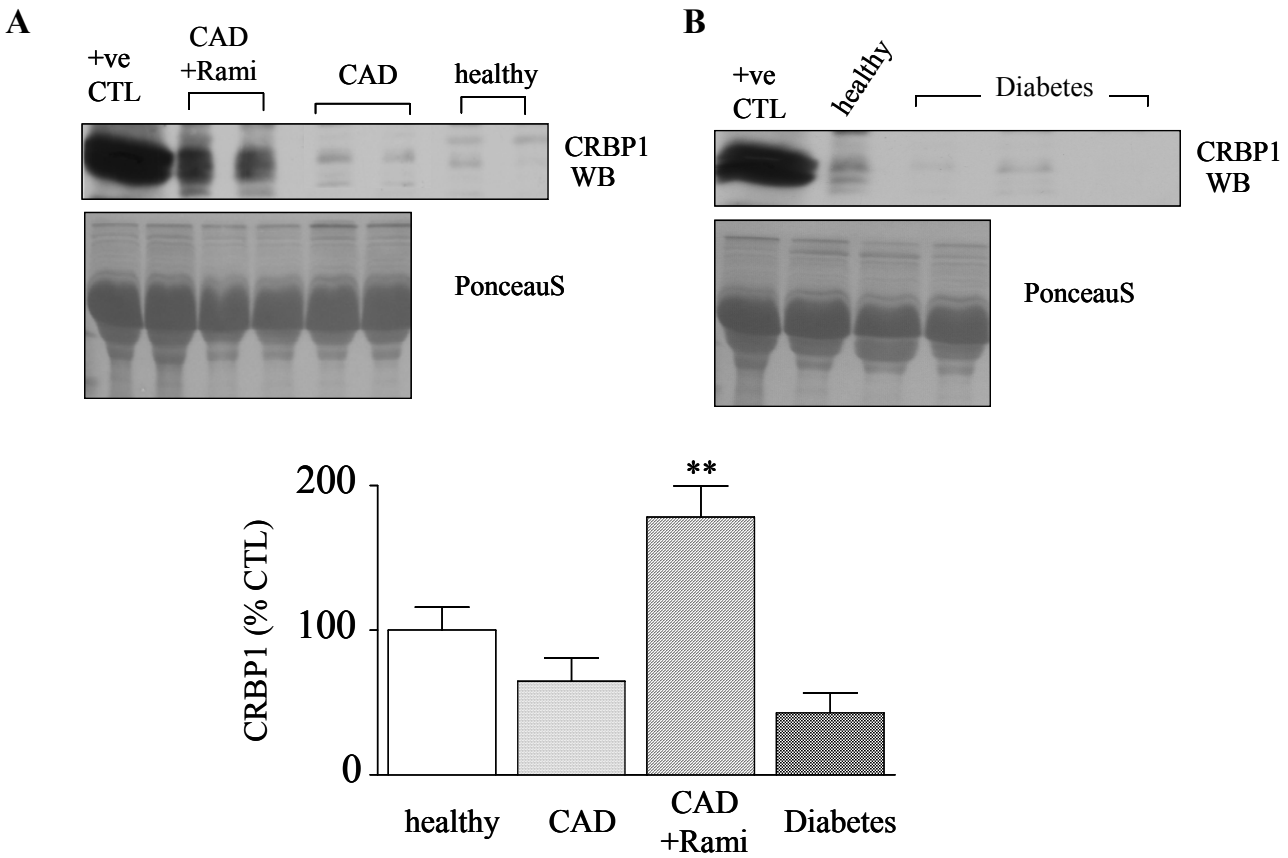

Fig. 16: Effect of ramiprilat on the CRBP-1 levels in human plasma. Plasma from the patients with coronary artery disease treated with/without ramipril, diabetic patients and healthy subjects were analyzed for CRBP-1 by Western blot. **A,** Representative Western blot showing the levels of CRBP-1 in treated coronary artery disease patients as opposed to untreated control patients and healthy subjects. Human testis protein lysate was used as a positive control. **B,** Blot showing CRBP-1 levels in diabetic patients compared to healthy subjects. The bar graph represents the percent increase in CRBP1 levels in plasma. ** $p < 0.01$.

However ramipril markedly enhanced the levels of CRBP-1 in the plasma of coronary artery disease patients as compared to the untreated patients as analyzed by Western blotting (Fig. 16A). CRBP-1 levels were also decreased in the plasma of type II diabetic patients compared

to healthy subjects (Fig. 16B). It is worth noting that the treatment of coronary artery disease patients with ramipril, enhanced the plasma CRBP-1 levels by 150% compared to healthy control subjects.

On the basis of gene array results, we analyzed the effects of ramiprilat on CRBP-1 expression. In contrast to the gene array results, wherein ramiprilat increased the gene expression of CRBP-1 by four fold, ramiprilat had no significant modulatory effect on the CRBP-1 expression at the protein level in primary human endothelial cells expressing the wild-type ACE. Next we investigated differences in the expression pattern of CRBP-1 in endothelial cells overexpressing the wild-type ACE or the non-phosphorylatable S1270 mutant ACE and the effect of ramiprilat on CRBP-1. There was no difference in the expression pattern of CRBP-1 between endothelial cells overexpressing wild-type ACE (Fig. 17A) and those overexpressing non-phosphorylatable S1270A mutant ACE (Fig. 17B) and also ramiprilat had no modulatory effect in CRBP-1 expression in either these endothelial cells overexpressing wild- type ACE and the mutant form of ACE.

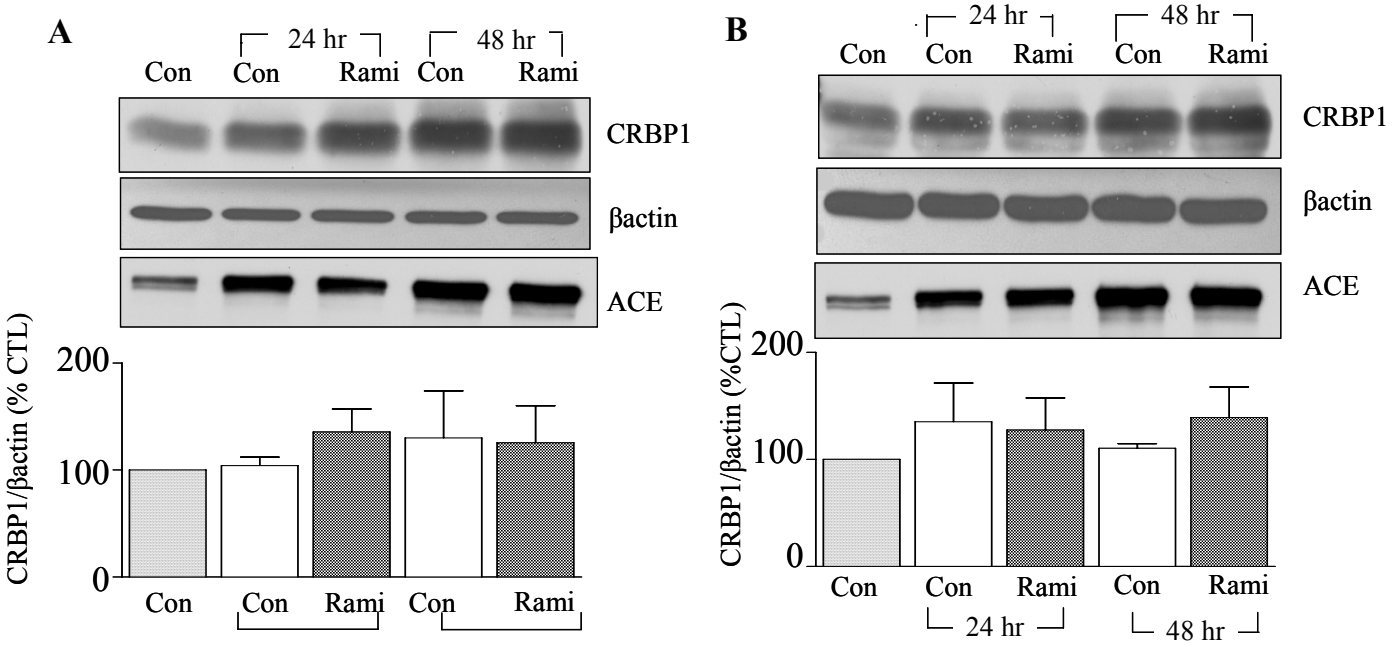

Fig. 17: Effect of ramiprilat on the expression of CRBP-1 in endothelial cells. Human umbilical vein endothelial cells expressing ACE were treated with ramiprilat for 24 h and 48 h and CRBP-1 expression was assessed in whole cell lysate by Western blot **A,** representative western blot showing the effect of solvent (CTL) and ramiprilat (R; 100 nM, 24 h and 48 h) on CRBP-1 expression in endothelial cells overexpressing the wild-type ACE and **B,** in endothelial cells overexpressing the non-phosphorylatable S1270 ACE mutant.

3.9. CRBP-1 overexpression increases the activity of retinoid X receptor (RXR) and PPAR response element (PPRE) promoter in endothelial cells

As we failed to observe any regulation of CRBP-1 protein expression by ramiprilat in endothelial cells, we next sought to investigate the promoter activation of retinoid X receptor element (RXRE) in endothelial cells overexpressing CRBP-1. Since physiologic retinoic acid receptor (RAR/RXR) activation is dependent on the retinoic acid transported by CRBP-1, we next sought to determine whether CRBP-1 overexpression influenced retinoid X receptor element (RXRE) activity in endothelial cells. Porcine endothelial cells overexpressing ACE were transfected with histidine tagged CRBP-1 (His-CRBP1). Control cells were transfected with green fluorescent protein (GFP).

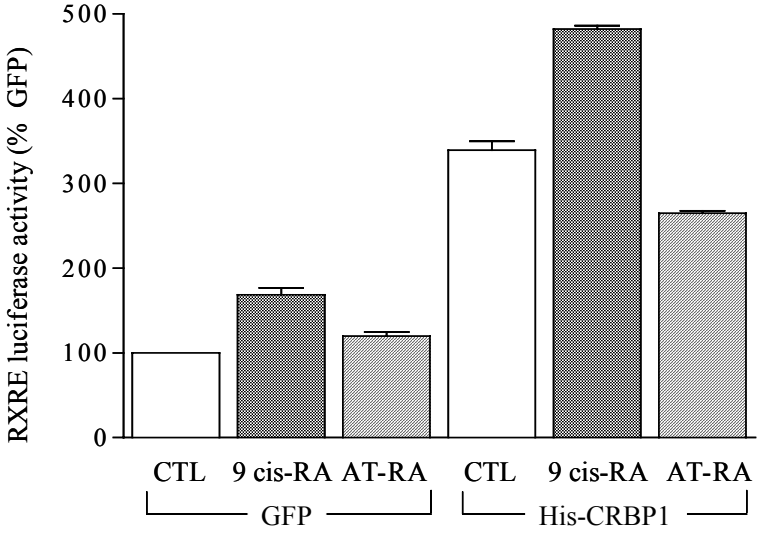

Fig. 18. Effect of overexpression of CRBP1 on the activity of RXRE in endothelial cells. Endothelial cells overexpressing his-CRBP1 were transfected with RXR promotor constructs and stimulated with 9-cis-retinoic acid (9 cis-RA; 1μM), all-trans-retinoic acid (AT-RA; 1μM) for 24 hours. Histogram showing the relative RXRE luciferase activity.

Overexpression of CRBP-1 in endothelial cells was associated in increased activity of RXR in response to stimulation by 9-cis- retinoic acid (1μM) treated for 24 hours. 9-cis-retinoic acid which activates both RXR and RAR family of receptors increased the RXRE activity by 1.9 fold in cells overexpressing CRBP-1 when compared to the cells transfected with GFP alone. In cells overexpressing CRBP-1, 9-cis-retinoic acid increased the RXRE activity by 1.7 fold compared to the solvent control (Fig. 18).

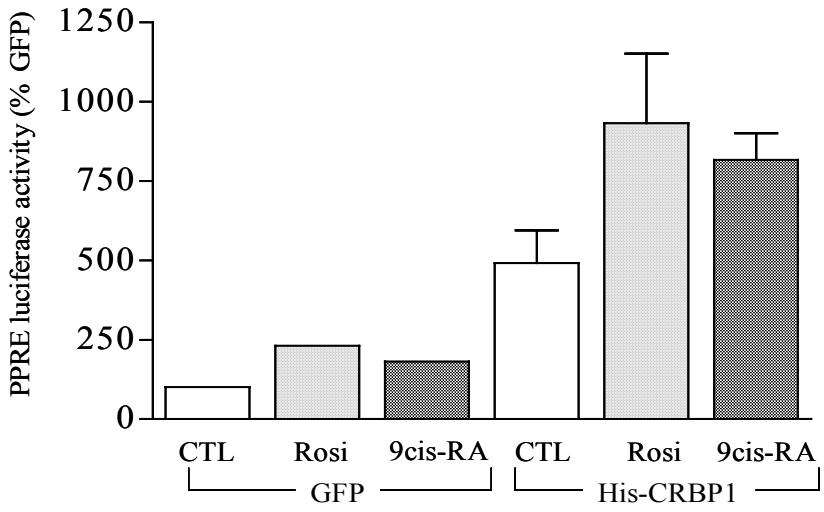

Fig. 19. Effect of overexpression of CRBP1 on the activity of PPRE in endothelial cells. Endothelial cells overexpressing CRBP-1 were transfected with PPRE promotor constructs and stimulated with 9-cis-retinoic acid (9 cis-RA; 1μM), rosiglitazone (Rosi: 100 nM) for 24 hours. Histogram showing the relative PPRE luciferase activity. Activity of PPRE is normalized to the relative luciferase activity of the controls.

PPARγ after binding to a ligand, forms heterodimers with the retinoid X receptor (RXR) and binds to a peroxisome proliferator response element (PPRE) in a gene promoter, leading to the regulation of the gene transcription (145). We next sought to determine if there is promoter activation of PPRE in endothelial cells overexpressing CRBP-1. We observed an increase in PPRE luciferase activity in response to its ligand rosiglitazone (Rosi; 100 nM) by 1.9 fold compared to solvent control in cells overexpressing GFP (Fig. 19), while rosiglitazone increased the PPRE activity by 4 fold in cells overexpressing CRBP-1 compared to cells not expressing CRBP-1 at all. Together these results suggest that overexpression of CRBP-1 influences the RXR and PPRE activity probably by increased availability and/or transport of retinol derivatives which serve as ligands for RXRE/PPRE.

3.10. ACE signalling in human monocytes

Although the ACE signalling pathway (ACE/CK2/JNK) was first demonstrated in endothelial cells, ACE signalling pathway has not been addressed in monocytes/macrophages which

possess functional local RAS. Circulating monocytes, which adhere to endothelial cell lesions, differentiate within the vascular wall to ACE-containing macrophages or foam cells with increased local synthesis of ACE and angiotensin II promoting atherosclerosis. No clear data is available on the mechanistic action of ACE inhibitors on monocytes/macrophages in reducing the events of atherosclerosis.

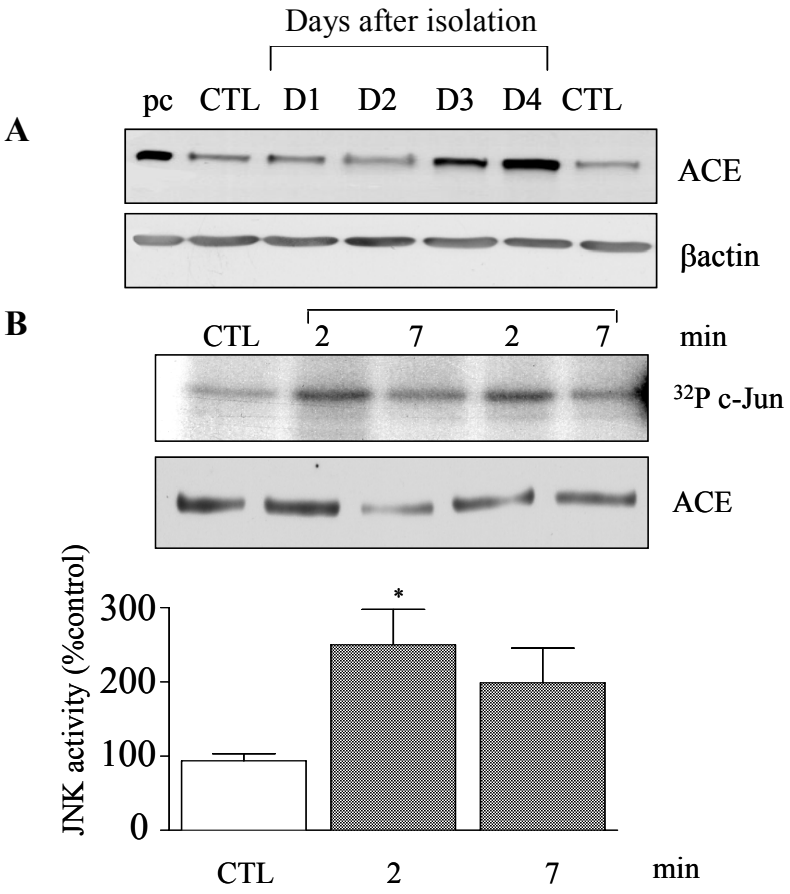

Fig. 20: ACE expression in monocytes in culture. Peripheral blood monocytes were isolated from whole blood and CD14+ monocytes were cultured with 5% human serum. **A,** Representative Western blot showing increase in ACE expression with time in culture. Endothelial cells overexpressing human somatic ACE was used as positive control. This blot represents results from 3 different experiments. **B,** Autoradiography of ^{32}P labeled c-Jun showing the effect of solvent (CTL) and ramiprilat (Rami; 100 nM; 2 and 7 min) in four days old ACE expressing monocytes. Bar graph summarizes data from 3 independent experiments; *, $p < 0.05$.

We first sought to study the expression levels of ACE in monocytes in culture. Substantial ACE expression increases with increase in time of culture, as seen from four days old monocytes cultured with 5% human serum (Fig. 20A). To investigate ACE signalling in monocytes, we assessed the ability of ramiprilat to activate JNK in ACE expressing

monocytes. Ramiprilat (Rami; 100 nM, 2 and 7 min) was able to increase the phosphorylation of c-Jun thus indicating the activation of JNK in ACE expressing human monocytes (Fig. 20B). Thus these results suggest the existence of ACE signalling in monocytes.

3.11. Effect of ramiprilat on nuclear peroxisome proliferator-activated receptor gamma (PPARγ) levels in human monocytes

One of the most intriguing and clinically relevant findings related to RAS inhibition using either ACE inhibitors or AT_1 receptor antagonists is their ability to delay the onset of type 2 diabetes. The mechanisms underlying this effect are not clear but may be related to the activation of PPARγ, a nuclear receptor that influences the expression of genes involved in carbohydrate and lipid metabolism (40). Enalapril activated PPARγ in aortae from apolipoprotein E-deficient mice (146) and also it was reported that certain angiotensin receptor blockers, like telmisartan induces PPARγ activity (147;148). Therefore we next sought to investigate if ACE inhibitors activate PPARγ in human monocytes cultured with human serum. Ramiprilat at 100 nM elicited a 1.7 fold increase in PPARγ levels in the nuclear extracts (Fig. 21A) which was comparable to the increase in PPARγ levels by potent agonist of PPARγ, rosiglitazone (100 nM; Fig. 21B). Ramiprilat (100 nM) was able to increase the PPARγ levels similar to rosiglitazone (100 nM) which is a potent agonist of PPARγ (Fig. 21B). The increased PPARγ levels were seen in the nuclear extracts of monocytes treated with ramiprilat but not in whole cell lysates suggesting that ramiprilat might increase the concentrations of PPARγ into the nucleus or diminish its degradation to increase the availability of PPARγ to result in expression of genes related to anti-inflammation in these cells.

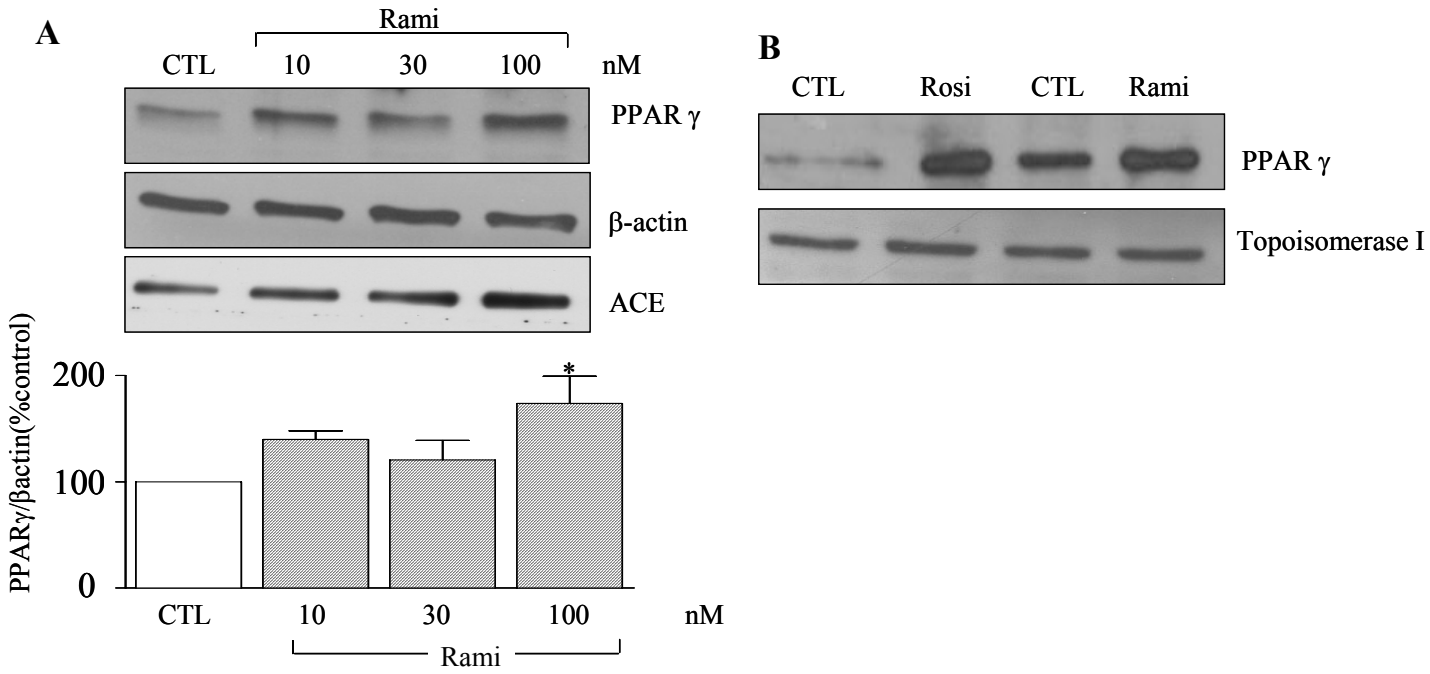

Fig. 21: Effect of ramiprilat on PPARγ levels in human monocytes. Human monocytes were cultured in 5% human serum for four days and treated thereafter with ramiprilat for 24 h. **A,** Representative blot showing the effect of ramiprilat (Rami; 10, 30, 100 nM) on PPARγ levels in nuclear extracts of human monocytes. Bar graph summarizes the results from three different experiments. **B,** Blot showing the increase in PPARγ levels by ramiprilat compared to rosiglitazone at 100 nM each. Blots were reprobed for equal loading on each lane for βactin. PPARγ levels were normalized to loading control βactin.

We next hypothesized that ramiprilat might increase the levels of 15-deoxy-D-12,14-prostaglandin J2 (15dPGJ2) which is a natural ligand for PPARγ via cyclooxygenase enzymes in monocytes and also it was previously demonstrated that binding of an ACE inhibitor leads to the activation of JNK/c-Jun pathway and increase in the expression of ACE and COX-2 (131). To determine whether COX-2 expression is increased in monocytes, human monocytes were cultured for four days with human serum and treated with ramiprilat for 24 and 48 h. Under basal conditions, COX-2 expression per se diminishes with time in culture, however ramiprilat prevented the diminution in the relative levels of COX-2 uptp 48 hours of treatment (Fig. 22). However we failed to observe any modulation in the levels of 15dPGJ2 as the 15dPGJ2 levels were too low to be detected by ELISA.

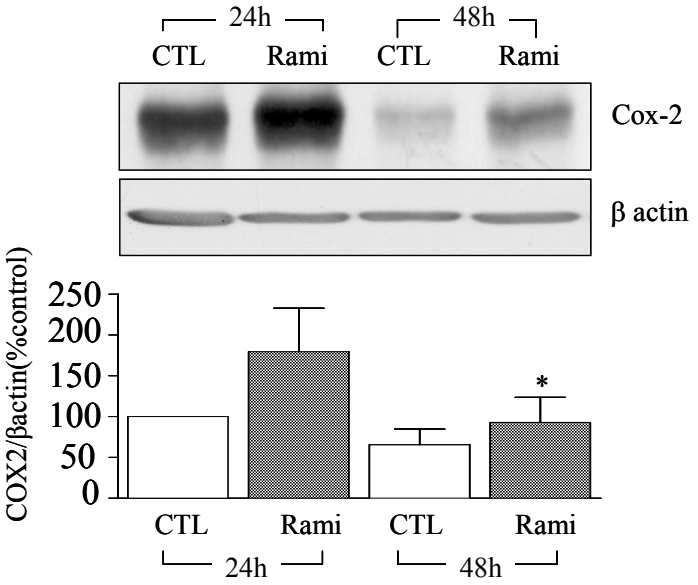

Fig. 22: Effect of ramiprilat on COX-2 expression in human monocytes. Human CD14+ monocytes were cultured in the presence of 5% human serum and treated with solvent (CTL) and ramiprilat (R; 100 nM; 24 and 48 h). Representative Western blots showing the expression of COX-2 under basal conditions and in the presence of ramiprilat. COX-2 levels were normalized to βactin. Bar graph summarizes the results obtained from five to seven different experiments.

3.12. Effect of ramiprilat on nuclear PPARγ levels in human endothelial cells

To explore the possibility of similar observation seen in human monocytes with regard to increase in nuclear PPARγ levels in endothelial cells as well, human umbilical vein endothelial cells overexpressing somatic ACE were treated with ramiprilat (100 nM) for 24 h. Ramiprilat increased the nuclear levels of PPARγ in endothelial cells as well. The effect of ramiprilat was more pronounced in increasing the PPARγ1 than γ2 levels (Fig. 23).

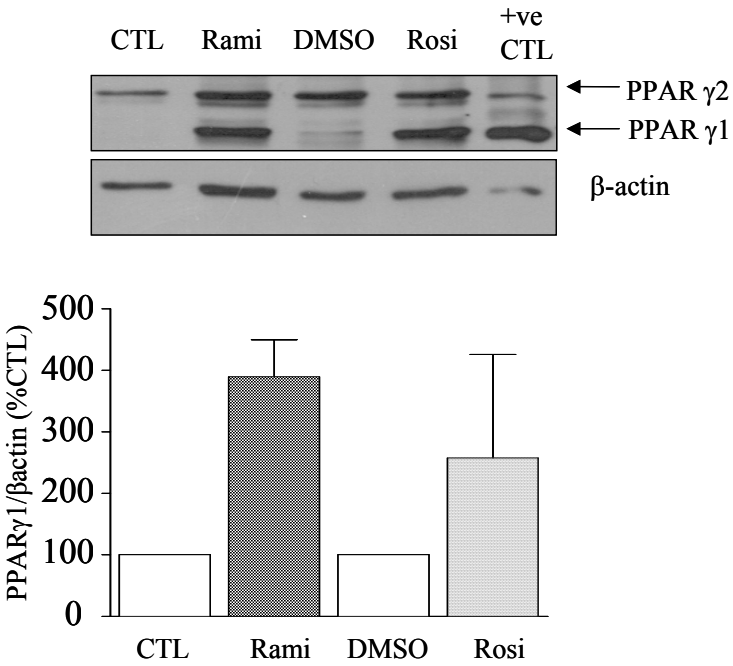

Fig. 23: Ramiprilat enhances PPARγ levels in human endothelial cells. Human umbilical vein endothelial cells overexpressing wild-type ACE were treated with ramiprilat (Rami) and rosiglitazone, (Rosi) each at 100 nM and treated for 24 h. Nuclear extracts of treated monocytes were analyzed for PPARγ. Human adipocyte whole cell lysate was used as a positive control. Blots were reprobed to check for equal loading for βactin. PPARγ1 levels were normalized to βactin. Bar graph represents data from 3 different experiments.

The substantial increase in nuclear PPARγ levels by ramiprilat as comparable to rosiglitazone was unfortunately not so reproducible owing to the very low levels of PPARγ in human endothelial cells and also due to high variability between each batch of cells from human umbilical cords.

To investigate the effects of ACE inhibitors on transcriptional activation of PPARγ, porcine aortic endothelial cells overexpressing ACE were transfected with a GAL4 reporter construct (i.e., 3X PPRE-TK-Luc). The transfected cells were treated with ramiprilat, rosiglitazone each at 100 nM and rosiglitazone (100 nM) in combination with PPARγ antagonist GW9662 (30μM) for 36 h. Compared with controls, rosiglitazone markedly activated the luciferase activity of the reporter gene, while combination treatment of PPARγ antagonist and rosiglitazone had a mild effect but ramiprilat did not have any effect on the promoter activity of PPAR response element (Fig. 24). These results suggest that the ramiprilat-induced activation of PPARγ levels could be through an independent mechanism and not by direct transcriptional activation of the promoter.

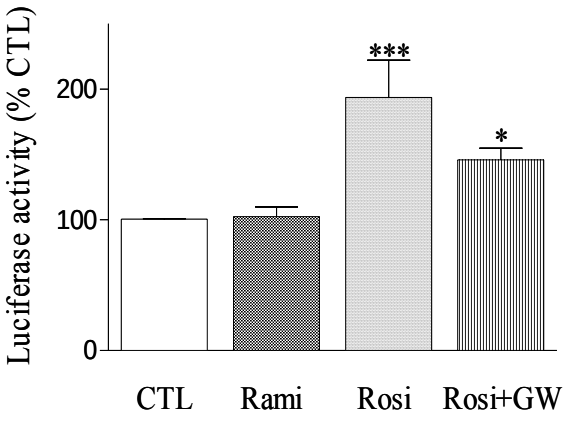

Fig. 24: No effect on PPRE activity by ramiprilat. Porcine aortic endothelial cells overexpressing ACE were transfected with PPRE X 3-TK-Luc were treated with ramiprilat (Rami; 100 nM), rosiglitazone (Rosi; 100 nM) and rosiglitazone (100 nM) in combination with PPARγ antagonist GW9662 (GW; 30μM) for 36 h. All cells were lysed for luciferase. The bar graph represents the relative luciferase activity, defined as the normalized luciferase activity in reference to that of vehicle- treated controls (CTL) and summarizes results from 4 different experiments: *, $p < 0.05$; ***, $p < 0.0001$.

3.13. ACE inhibitor regulates adiponectin, a downstream target of PPARγ

Adiponectin is approximately 30 kDa plasma protein that is found in multimeric complexes in the circulation at relatively high levels in healthy human subjects. Adiponectin protein is secreted specifically from adipose tissue (149;150). Adipokine adiponectin is known to exert anti-inflammatory and insulin-sensitizing effects and is a downstream target of PPARγ. Since we failed to observe any activation of PPARγ responsive element we hypothesized that there might be an increased heterodimerization between PPARγ and RXRα probably via CRBP-1.

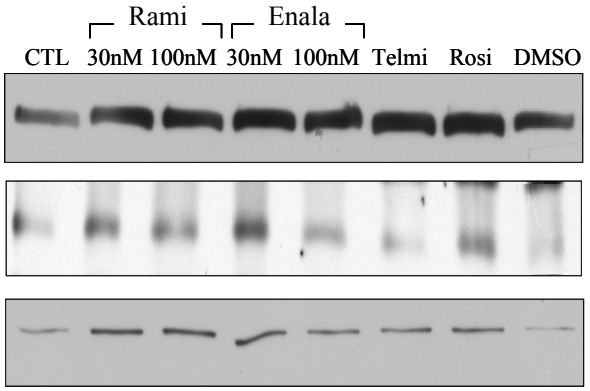

Fig. 25: Effect of ACE inhibitors on adiponectin in human adipocytes. Human adipocytes were treated with ACE inhibitors ramiprilat (Rami; 30 nM, 100 nM), enalaprilat (Enala; 30 nM, 100 nM), Angiotensin II receptor blocker-telmisartan (Telmi; 100 nM) and rosiglitazone (Rosi; 100 nM) for 24 hours respectively. The cell supernatent was harvested and analyzed for adiponectin by Western blotting.

Both the ACE inhibitors increased the relative amount of adiponectin secreted in the culture medium as analysed by Western blotting (Fig. 25). The supernatant harvested from the human adipocytes also had increased amount of soluble ACE compared to the ACE present in the whole cell lysate (Fig. 25).

We were able to translate the *in vitro* observation in *in vivo* experiments as well. Ramipril treatment also enhanced adiponectin levels in serum from C57 black mice treated for 5 days, as analyzed by ELISA (Fig. 26A). We also analyzed adiponectin levels in the serum samples from ob/ob mice treated with ramipril (Rami; 5mg/kg/day), rosiglitazone (Rosi; 3mg/kg/day) and in combination (Rami+Rosi). The adiponectin levels in serum of the combination treatment in ob/ob mice were significantly higher than rosiglitazone or ramiprilat alone suggesting a synergistic effect (Fig. 26B).

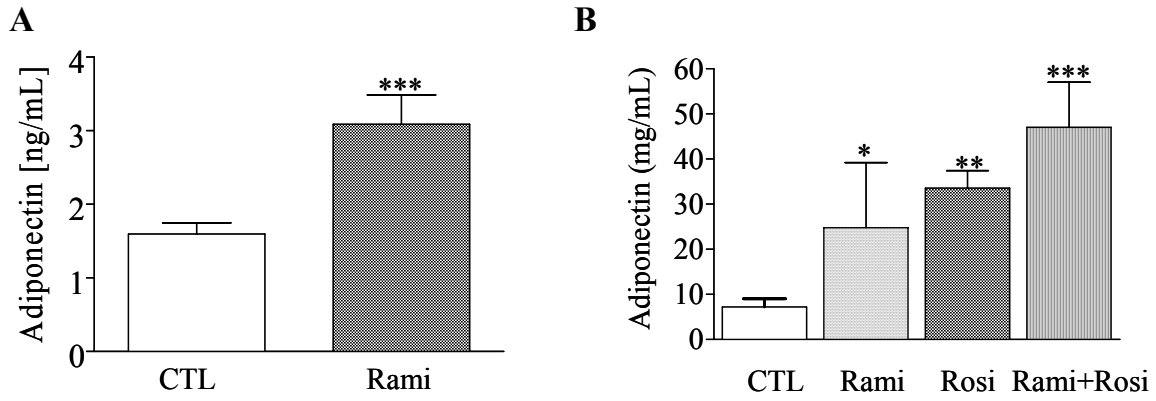

Fig. 26: Effect of ramipril treatment on plasma adiponectin levels. A, plasma from C57 black mice (which were treated for 5 days with ramipril, 5mg/kg/day), was analyzed by ELISA for adiponectin. **B,** plasma adiponectin levels in Ob/Ob mice, as analyzed by ELISA. Ob/Ob mice were treated for 10 weeks with ramipril (Rami; 5mg/kg/day), rosiglitazone (Rosi; 3mg/kg/day) and in combination by oral gavage. * $p< 0.05$ ** $p< 0.001$ *** $p< 0.0001$

Adiponectin levels have been reported to be lower in patients with coronary artery disease compared to healthy counterparts (151;152). We next determined whether ACE inhibitor has any effect on adiponectin levels in plasma samples from patients with coronary artery disease treated with or without ramipril (5mg/day) and compared to healthy subjects. Ramipril enhanced the relative amount of adiponectin in plasma from patients with coronary artery disease treated with ramipril by 3.5 fold compared to either untreated patients with coronary artery disease or by 7.5 fold when compared to plasma adiponectin levels in healthy subjects (Fig. 27).

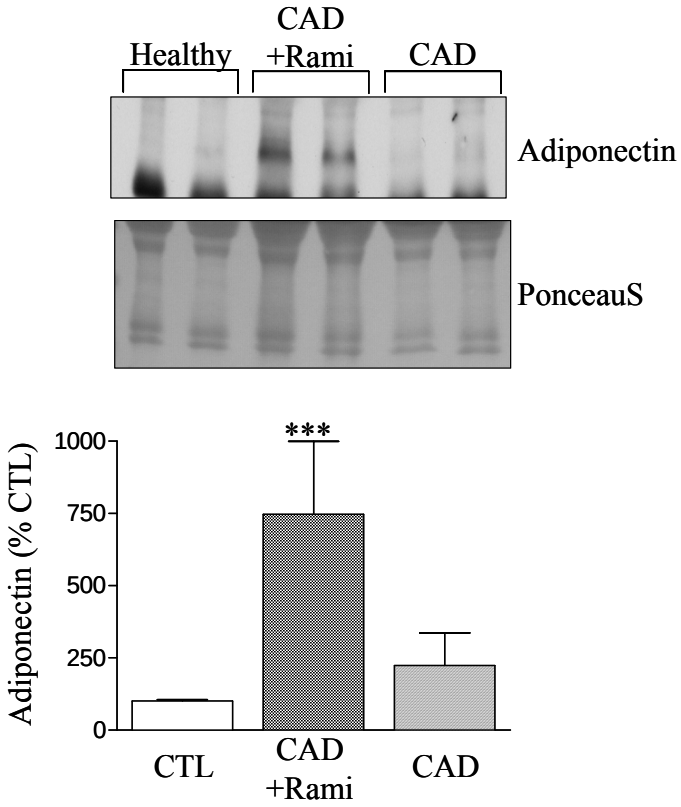

Fig. 27: Effect of ramipril treatment on adiponectin in plasma from patients with coronary artery disease. Plasma from healthy subjects, patients with coronary artery disease treated with or without ramipril was analyzed by Western blotting for adiponectin. Bar graph summarizes data obtained from five patients in each group. *** $p < 0.0001$.

3.14. NF- κB activation by ramiprilat in human monocytes

Nuclear factor-κb (NF-κB) is an inducible transcription factor that plays a role in the expression of spectrum of genes involved in immune and inflammatory responses and cell survival. It has been suggested that many anti-inflammatory effects of PPAR's might depend on the inhibition of the NF-κB signalling pathway (153). To elucidate the effect of ACE inhibitors on activation of NF-κB, electrophoretic mobility shift assay (EMSA) was performed. Nuclear extracts from human monocytes treated with ramiprilat (Rami; 10, 30, 100 nM; 24h) did not show any strong DNA binding activity for NF-κB (Fig. 28) as compared to monocytes treated with TNFα (10 ng/mL; 30 min). These results suggest that ramiprilat does have a slight inhibitory effect on activation of NF-κB.

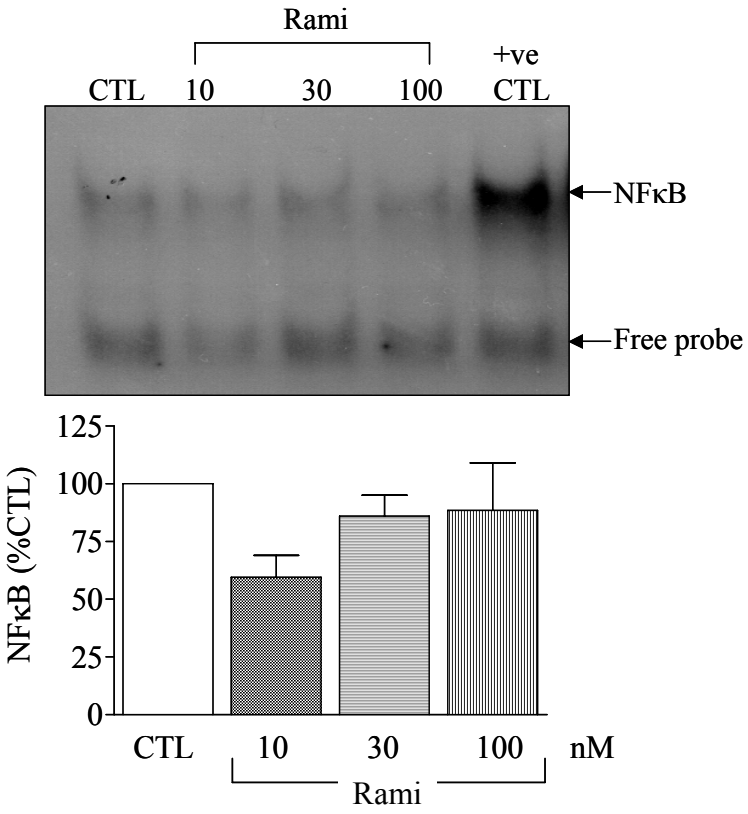

Fig. 28: Effect of ramiprilat on NF-κB activation: Human primary monocytes were cultured for 4 days in the presence of human serum and exposed to with or without ramiprilat (Rami; 10, 30, 100 nM; 24h). Cells were harvested, nuclear extracts prepared, and EMSA performed using a radiolabelled oligonucleotide containing the consensus site for NF-κB. Representative autoradiograph demonstrating that ramiprilat, has no significant effect on NF-κB activation compared to positive control. Nuclear extracts from monocytes treated with TNFα for 30 min was used as a positive control. Specific NF-κB band is indicated by arrow. Bar graph is representative of two to three separate experiments.

4. Discussion

4.1. ACE dimerization initiates ACE signalling in endothelial cells

The findings of this study demonstrate that ACE occurs as a homodimer as well as a monomer in endothelial cells. Binding of an ACE inhibitor to the enzyme significantly enhances ACE dimer formation. The integrity of C-terminal active site of ACE seems to be the determining factor for ACE dimerization as the ACE-inhibitor induced dimerization as well as the ACE inhibitor-induced phosphorylation of ACE on Ser^{1270} and the subsequent activation of JNK pathway was abrogated by the mutation of two critical histidyl residues that bind zinc ions. Given that dimer formation correlated with the time course of the inhibitor-induced ACE phosphorylation on Ser^{1270}, the dimerization of ACE thus may be the initial step in the ACE signalling pathway recently identified in endothelial cells.

Protein-protein interactions resulting in dimerization and heterodimerization are of central importance in the control of gene expression and cell function. Analysis of the quaternary structure of several membrane-bound and secretory proteins has demonstrated that dimer or oligomer formation is an important event that is required for efficient intracellular transport of these proteins, enhances their efficacy and also might be important for signal transduction (154-156). Other type I membrane glycoproteins, such as the β-secretase (β-site amyloid precursor protein cleaving enzyme) are also able to dimerize and thus regulate their intracellular and/or extracellular functions (157). Similar to ACE, β-site amyloid precursor protein cleaving enzyme can be phosphorylated on a serine residue near its C-terminus and can also be cleaved from the cell surface (158).

High molecular mass aggregates of ACE that might represent homodimers have been found during the purification of the enzyme from human lung (159) and also in *in vitro* experiments by using reverse micelles and ACE-expressing COS cells (143;160). Mature somatic ACE has a calculated molecular mass of 149.7 kDa, due to the glycosylation of the enzyme, the molecular mass of the enzyme is between 170 and 180 kDa revealed by denaturing electrophoresis (161;162). Other forms of human somatic ACE exists as an immature form in intracellular compartments (~170 kDa) and a soluble form (~ 175 kDa) which is cleaved from the plasma membrane (163). ACE is synthesized as a single polypeptide precursor that matures in the Golgi apparatus by acquisition of complex-type oligosaccharides and is then inserted into the membrane. ACE has been shown to exist in dimeric form *in vitro* (64;160)

Native gel electrophoresis is an excellent tool to detect oligomers and aggregates. By means of native gel electrophoresis, wherein the electrophoretic mobility of proteins depends primarily on both the protein's charge and its hydrodynamic size, we found that ACE migrates not only as monomers but also as dimers. Results obtained from the split-ubiquitin assay revealed that ACE dimerization is more pronounced in living cells (yeast) than in *in vitro* assay system confirming that ACE forms functional dimers. Cross-linking of ACE on the surface of the endothelial cells indicated that ACE forms dimers on the cell-surface, albeit that the population of ACE dimers is relatively small as we could observe relatively low level of dimers formed and could be attributed to methodological problems related to the detection of ACE dimers by the antibodies used in this study. Dimerization of proteins usually involves covalent and non-covalent interactions. The mechanism of ACE dimerization seems to be similar to the noncovalent interactions involved in dimerization of membrane proteins such as P-selectin (164) and ICAM-1 (165) but differs from dimerization of other membrane proteins, where disulfide links are involved, for example, platelet derived growth factor (PDGF) (166).

Given that ACE forms dimers in endothelial cells, we next determined whether the binding of an ACE inhibitor to ACE has any effect on dimer/monomer population in the endothelial cells. Our results demonstrated that the treatment of endothelial cells with ramiprilat, increased the dimer/monomer ratio. The formation of ACE dimers also correlated well with the ACE inhibitor induced phosphorylation of the enzyme on Ser^{1270}. The ACE inhibitor-induced phosphorylation of ACE is biphasic, that is the phosphorylation increased from 2 to 7 minutes and elevation is maintained again after 6 to 48 hours (130). ACE-induced dimerization thus preceded in the phosphorylation of the Ser^{1270} in the cytoplasmic domain as previously demonstrated (130) and activation of the signalling cascade culminating in changes in the endothelial gene expression (131). The formation of ACE dimers was also observed when cells were stimulated with enalaprilat, quinaprilat and captopril indicating that this is a specific effect by this class of compounds. ACE dimer formation was unequivocally observed in an endothelial cell line overexpressing human somatic ACE as well as in primary cultures of human umbilical vein endothelial cells that endogenously express ACE, indicating that the effects observed cannot be attributed to an artifact associated with the overexpression system.

Somatic ACE is principally N-glycosylated with a mixture of both N-acetyl-lactosaminyl (complex) sugars and oligomannosidyl or hybrid sugars (65;84;167). Somatic ACE harbours 17 potential N-linked glycosylation sites. Ten such sites occur in the N domain, for which

carbohydrates constitute 37% of its molecular mass, while the C-domain has seven such sites (66;142;167;168). It has been previously suggested that ACE dimerization has been attributed to interactions between its carbohydrate side chains and a carbohydrate recognition domain at its N-domain that are sensitive to treatment with galactose (160), we therefore investigated the effects of galactose, glucose or mannitol on basal or the ramiprilat-induced dimerization of ACE. However, we were unable to find any significant interference of these monosaccharides with ACE dimerization. It has also been suggested that the 9B9 monoclonal antibody directed against the putative carbohydrate recognition domain region can interfere with ACE self-association (143). Therefore we tested various antibodies directed to the epitopes of N-domain of ACE on dimer formation. The monoclonal antibodies had no effect on the basal or ramiprilat-induced dimerization of ACE but displayed differential effects on shedding of the enzyme. It has been reported previously that the monoclonal antibodies directed to different epitopes of human ACE, induced shedding of the enzyme and also has been linked to inhibition of dimerization (143). In our study, we observed that the shedding of the enzyme was apparent with monoclonal antibodies 9B9 and 3A5 as reported previously (143) but we failed to see any link between shedding and dimerization. The differences in the results observed, related to the effects on dimerization, by the authors (143) and our results is possibly due to the experimental model used to study dimerization as we used endothelial cells in culture and not reverse micelles (64) which might be a pseudo-physiological system to study dimerization.

Dimerization plays an important role in the functioning of many membrane proteins (169;170). To determine whether dimerization of ACE is linked to the recently described ACE signalling cascade, which involves the ACE inhibitor-induced phosphorylation of the enzyme on Ser^{1270} and subsequent activation of JNK pathway (129;130), we compared the effects of ramiprilat on the dimerization of the wild-type ACE as well as of the S1270A ACE mutant. Ramiprilat elicited dimerization of both the wild-type and the S1270A ACE, indicating that the phosphorylation of the cytoplasmic tail of the enzyme does not influence dimer formation but requires intact extracellular domains to form dimers.

Although the N- and C-domains of ACE resulted as a consequence of gene duplication (73), there are marked functional differences between these two domains of ACE, despite the high degree of sequence homology. The rate of hydrolysis of various synthetic and natural substrates by the two domains differs, with the C-domain of human ACE having higher catalytic constants for Hip-His-Leu, angiotensin I, bradykinin, and substance P, whereas the N-domain hydrolyzes LH-RH and the negative hematopoietic regulator N-Ac-Ser-Asp-Lys-Pro at much higher rates (55;57;171). The C-terminal domain is more sensitive to chloride

activation (57;172). Most of the competitive ACE inhibitors bind tightly with the C-domain active center (173). The two domains of ACE differ also in conformation (174;175). The striking functional difference between the N- and the C-domains of ACE is found to be the conversion of angiotensin I which takes place preferentially within the C-domain and selective C-domain inhibition is adequate to prevent angiotensin I-induced vasoconstriction (176). Also evidence points out that the N-domain of ACE may be functionally less significant, as the RXP407 peptide which specifically inhibits the N-domain of ACE has no effect on blood pressure (177).

We therefore sought to determine whether interfering with the dimerization of ACE affected the ACE inhibitor-induced phosphorylation of the enzyme and/or subsequent activation of JNK by using different C-or N-domain inactive ACE mutants. Different C- or N-domain inactive ACE mutants overexpressed in CHO cells revealed that the C-domain is more important for dimerization rather than the N-domain. Analysis of the inactivation of the C-domain by mutation of the two essential Zn^{2+} complexing histidyl residues of the HEMGH consensus sequence completely abrogated the enzyme's ability to dimerize either under basal conditions or in response to ramiprilat. Mutation within the C-domain also abolished the inhibitor-induced signalling, in as much as we failed to observe ramiprilat-induced increase in Ser1270 phosphorylation and/or subsequent activation of JNK pathway. However, mutation of the N-domain did not affect ACE-inhibitor induced dimerization and subsequent ACE signalling. Given that N-domain bears a carbohydrate recognizing domain and has more putative glycosylation sites than C-domain (59;69;142), in this case N-domain was not found to be essential for dimerization. Although we do not know how ACE inhibitors can effect ACE dimerization via the C-domain, it is possible that their binding induces conformational changes that eventually result in the exposure of a dimerization domain, as has been reported for the ligand-induced dimerization of the epidermal growth factor receptor (178).

Results of the present study suggests that the ACE inhibitor-induced dimerization of ACE, via the C-domain of the enzyme commences the ACE signalling pathway that involves subsequent activation of the JNK/c-Jun pathway and leads to changes in endothelial cell gene expression illustrated in Fig. 26.

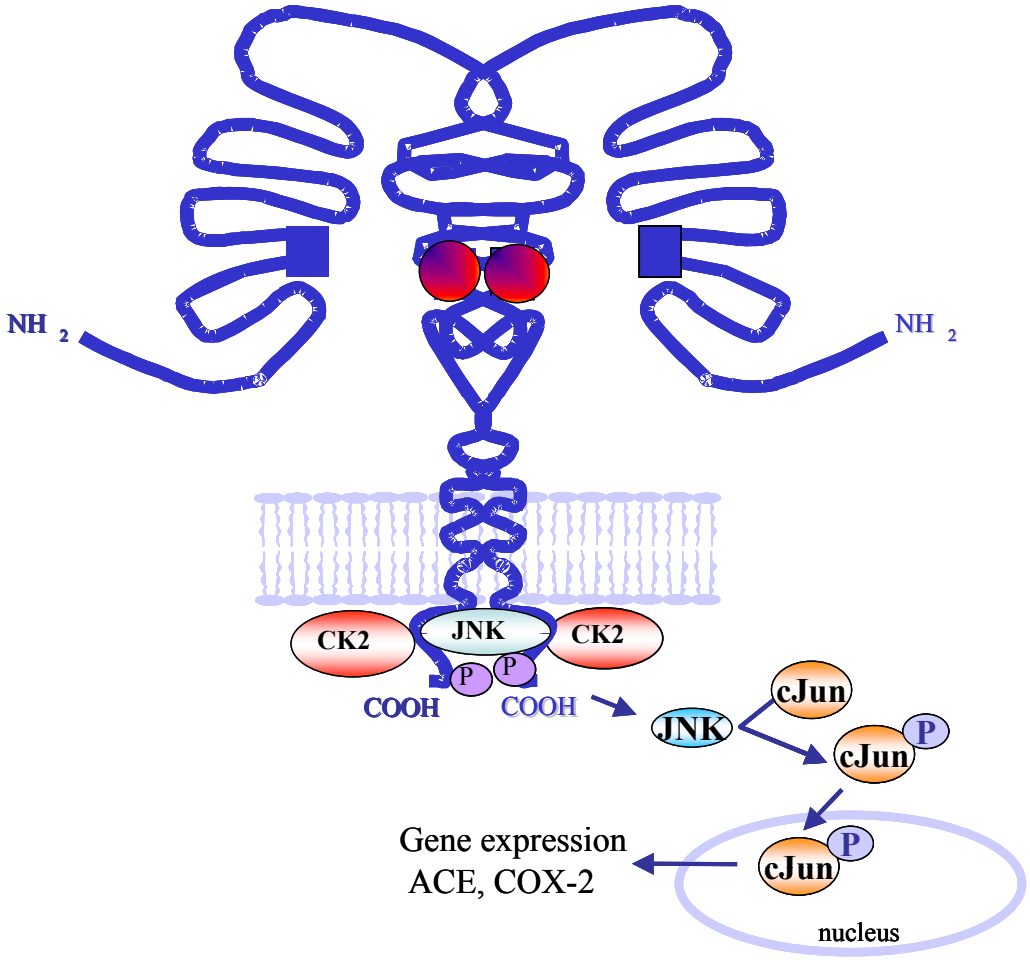

Fig. 26: Scheme summarizing the ACE inhibitor-induced dimerization of ACE, via the C-domain of the enzyme, representing the initial step in the ACE signalling pathway that involves the activation of the JNK/c-Jun pathway and leads to changes in endothelial cell gene expression.

4.2. Downstream effectors of ACE signalling pathway in endothelial cells

ACE signalling results in the translocation of phosphorylated c-Jun to the nucleus and enhanced binding of the activator protein (AP)-1 transcription factor to the DNA, followed by the increased expression of ACE (130) and COX-2 (131). Increased ACE and COX-2 levels could be associated with improvement in endothelial cell function and has physiological relevance as increased ACE levels have been demonstrated in lung tissue and plasma from rats treated with ACE inhibitor (179) and in the serum from patients who distinctly benefit from ACE inhibitor therapy (180). Prostacyclin production is significantly increased in

patients treated with ACE inhibitors and selective COX-2 inhibition attenuates the positive effects of ACE inhibitors on blood pressure (181).

To identify the downstream effectors of the many beneficial effects of ACE inhibitors which cannot be attributed only to inhibition of angiotensin II generation (as angiotensin II levels are not decreased during long term ACE inhibitor therapy) and bradykinin degradation, we used DNA microarray technology. We identified 21 genes (in addition to ACE and COX-2) that are differentially regulated (7 upregulated and 14 downregulated) (Table. 1) by ramiprilat in endothelial cells expressing wild-type ACE but not in cells expressing a non-phosphorylatable ACE mutant (wherein Ser^{1270} is replaced with alanine) which was the criteria for ACE signalling. In contrast to the gene array results, we failed to detect any difference between the solvent control and ramiprilat treated endothelial cells in the regulation of modulated genes. We failed to see any modulation of the expression levels of the selected genes by ramiprilat even after prolonged exposure to ramiprilat at the protein level, although the expression levels of the selected genes were substantial enough to be detected by Western blot considering that the protein expression levels take much longer time than the expression of genes.

Cellular retinol binding protein 1 (CRBP-1) was upregulated by ramiprilat in endothelial cells overexpressing wild-type ACE by four fold in the microarray. However there was no difference in CRBP-1 expression per se between endothelial cells expressing wild-type ACE or the non-phosphorylatable ACE. Moreover we failed to see any modulation by ramiprilat on CRBP-1 levels in endothelial cells expressing either the wild-type ACE or the S1270A mutant ACE.

Local functional RAS has been described in other cells and tissues so we screened for the plasma samples from healthy volunteers as well from patients with coronary artery disease for the genes identified in the microarray. We observed that CRBP-1 was detectable at low levels in the plasma samples and also ramipril markedly increased the levels of CRBP-1. CRBP-1 belongs to the fatty-acid binding protein (FABP) family. In humans, CRBP-1 is highly expressed in the ovaries, pituitary gland, testes, adrenal glands, and liver where it is much more abundant in hepatic stellate cells than in hepatocytes. Although the physiologic importance of CRBP-1 in the disease processes is largely unknown, it has been proven that CRBP-1 levels is decreased in various cancers (182;183). Therefore considering the molecular function and post-translation profiles of the regulated genes, we found Cellular retinal binding protein-1 (CRBP1) to be regulated distinctly by ramiprilat in human plasma from patients manifest with coronary artery disease, treated with ramipril. CRBP-1 levels were also diminished in plasma from diabetic patients as compared to the healthy subjects.

However we do not know yet as to whether diabetic patients treated with ramipril also have enhanced levels of plasma CRBP-1 similar to coronary artery patients treated with ramipril. Although CRBP-1 levels were not regulated by ramiprilat in cultured endothelial cells, it is tempting to speculate that the plasma CRBP-1 might be contributed by other tissues such as adipose tissue, wherein a functional local RAS is present. It needs to be investigated how ramiprilat regulates plasma CRBP-1 levels in human patients or whether ramiprilat acts on other tissues where there might be an active ACE signalling pathway.

Retinoid signalling requires a prenuclear phase, which is regulated by CRBP's. CRBP-1 is considered essential for vitamin A homeostasis. The most important physiologic functions of CRBP-1 is to bind to retinol from blood, esterification and intercellular transfer of retinol between cells, more so in between liver cells (184). CRBP-1 facilitates the retinoid transport from the cytoplasm into the nucleus and influences the ligand binding of the nuclear receptors (185;186). Therefore we assessed whether overexpressing CRBP-1 would have any effect on the activity of PPRE and RXRE. We found that in endothelial cells overexpressing CRBP-1, 9-cis-retinoic acid increased the RXR activity and rosiglitazone increased the activity of PPRE suggesting that overexpression of CRBP-1 favours the conversion of retinol into retinoic acid which is the ligand for PPRE/RXR/RAR and promotes gene expression. However, the regulation of CRBP-1 by ramiprilat still needs further investigation.

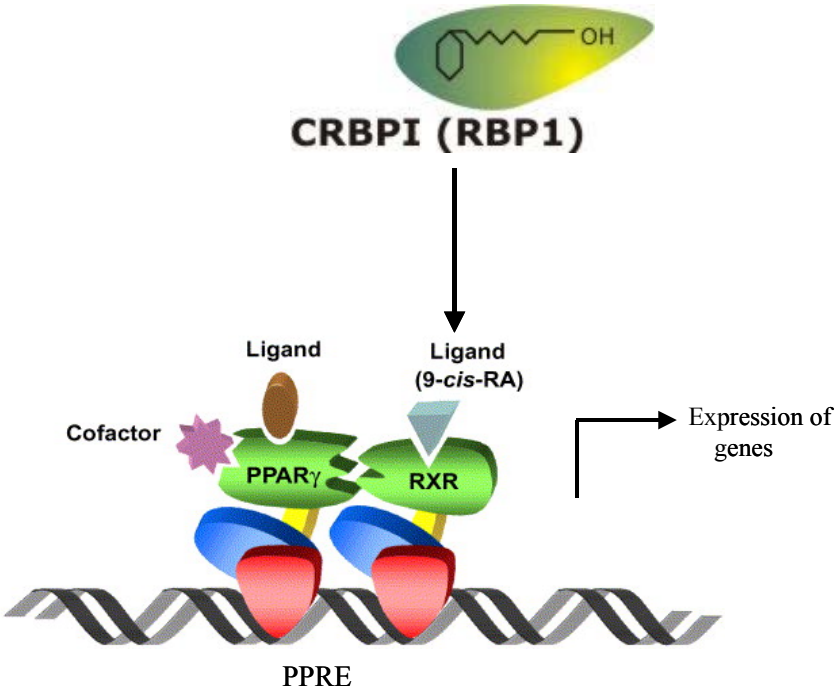

Fig. 27. Scheme illustrating the reliance of PPAR signalling on RXR/RAR. Retinol is transported bound to CRBP-1 and is converted to retinoic acid (RA), Retinoic acid binds to RA receptors (RARs) and the retinoid X receptors (RXRs). RXRs function as obligate heterodimer partners for PPARs. Upon binding to ligands, PPAR heterodimerizes with RXR and regulates the expression of genes.

4.3. ACE signalling in monocytes

Atherosclerosis is initiated by dysfunction of endothelial cells at lesion-prone sites in the walls of arteries, promoting a proinflammatory vasculature and results in monocyte infiltration into the arterial intima (187). These monocytes then differentiate into macrophages, which then internalise large amounts of low-density lipoprotein forming cholesterol-laden macrophages called foam cells (188). Given the clinical significance of activated monocytes, and that angiotensin II activates monocytes and stimulates the expression of tissue factor as well as the release of pro-inflammatory cytokines (189), it is most likely that ACE in monocytes would aggravate inflammatory responses. To determine if the ACE signalling pathway exists in monocytes, we first examined ACE expression in monocytes derived from human blood. ACE expression significantly increased in monocytes cultured for 4 days with human serum and on exposure to ramiprilat we observed JNK activation. Taken together, these results indicate that there is ACE signalling in monocytes. On binding of ACE inhibitor, ACE functions as a signal transduction molecule in human monocytes.

One of the most relevant findings from clinical trials related to either ACE inhibitors (190) or AT_1 receptor antagonists (191;192)is their ability to delay the onset of type 2 diabetes. The mechanisms underlying this effect are unclear but may be related to the activation of PPARγ, a nuclear receptor that influences the expression of multiple genes involved in carbohydrate and lipid metabolism (40). Enalapril was shown to activate PPARγ in aortae from apolipoprotein E knockout mice (146) and also AT_1 receptor blocker telmisartan acts as a selective PPARγ modulator (147;148).

Human monocyte derived macrophages have been found to express PPARγ (193);198). PPARγ activation in monocytes and macrophages is associated with anti-inflammatory actions. PPARγ agonists were shown to inhibit monocyte cytokine production and macrophage activation (194;195). It has been reported that PPARγ activation leads to attenuation of early and advanced atherosclerosis (196).

We next investigated the ability of ramiprilat to activate PPARγ in monocytes. Ramiprilat increased the PPARγ levels in the nuclear extracts of human monocytes but not in whole cell lysates. The increase in nuclear PPARγ levels by ramiprilat was similar to that of rosiglitazone which is a known PPARγ agonist and used to treat Type II diabetes. The

possible mechanism of the result observed, could be that ramiprilat might increase the PPARγ levels in the nucleus where it binds to its heterodimeric partner RXR and increases the gene expression. We next investigated whether this phenomenon could be reproduced in human endothelial cells, wherein ACE signalling pathway was demonstrated. We found that ramiprilat increased PPARγ levels in the nuclear extracts of human endothelial cells as well. This observation seems to be complex to understand as human umbilical vein endothelial cells generally have very low levels of PPARγ and probably depends on the genetic make-up of the donor which may or may not be sensitive to treatment with ramiprilat and therefore the results observed were not so reproducible. We next sought to determine if ramiprilat has any effect on the promoter activity of PPAR's as reported for telmisartan, an angiotensin II receptor blocker which increased the activity of PPRE. Endothelial cells expressing ACE and transfected with 3 copies of PPRE did not show any increase in activity on treatment with ramiprilat while rosiglitazone increased the PPRE activity and also PPARγ antagonist GW9662 along with rosiglitazone had a marginal effect. These results indicated the increase in PPARγ by ramiprilat is not through transcriptional activation but by indirect means which still needs further investigation.

As we failed to see any promoter activation of PPRE by ramiprilat we next hypothesized that ramiprilat might increase the levels of 15-deoxy-D-12,14 prostaglandin J2 (15dPGJ2) which is a natural ligand for PPARγ by means of cyclooxygenase (COX) enzymes. Therefore we next determined COX-2 expression in monocytes treated with ramiprilat and we observed that ramiprilat was able to decrease the diminution of COX-2 levels upto 48 hours but the levels of 15dPGJ2 were too low to be detected by ELISA. Taken together these results suggest that ACE inhibitors initiate ACE signalling and leads to enhanced expression of ACE, COX-2, CRBP-1 which in turn favours the heterodimerization of PPARγ with RXR.

Angiotensin II has been shown to activate NF-κB mediated genes and thereby leading to the downregulation of PPAR's (197). It has also been suggested that many of the anti-inflammatory effects of PPAR's may be due to the inhibition of the NF-κB signalling pathway (153). We therefore assessed whether ramiprilat downregulates NF-κB in human monocytes expressing ACE. Ramiprilat did not enhance DNA binding to NF-κB in human monocytes compared to TNFα. Further investigation is needed to address this issue as to how ramiprilat interferes with NF-κB pathway.

4.4 ACE inhibitor regulates adiponectin, downstream target of PPARγ

Since ramipril enhanced the plasma levels of CRBP1 in patients with type 2 diabetes or coronary artery disease and CRBP1 potentially influences the nuclear receptor proteins (RAR/RXR), which are abundant in adipose tissue and regulates adipocyte gene expression together with PPAR, we therefore analysed the effect of ACE inhibitor on adipokine expression as adipose tissue does have components of RAS. ACE inhibitors increased the adiponectin levels in the culture supernatant of adipocytes isolated from visceral fat. Plasma samples from mice (C57 black mice; ob/ob mice) treated with ramipril also demonstrated enhanced adiponectin levels compared to untreated control mice.

Adiponectin levels are decreased in patients with obesity, diabetes and coronary artery disease. Treatment with ACE inhibitor restored the plasma adiponectin levels in patients with coronary artery disease. The mechanisms by which ramiprilat enhances adiponectin levels however is yet to be investigated. Taken together these data suggest that ACE inhibitor affects adipocyte homeostasis, probably through activation of RAR/RXR-PPARγ signalling. Enhanced plasma levels of anti-inflammatory and anti-atherogenic adipokine adiponectin may be the one of the ways by which ACE inhibitor exerts positive effects in patients with type 2 diabetes and thereby preventing the onset of the disease.

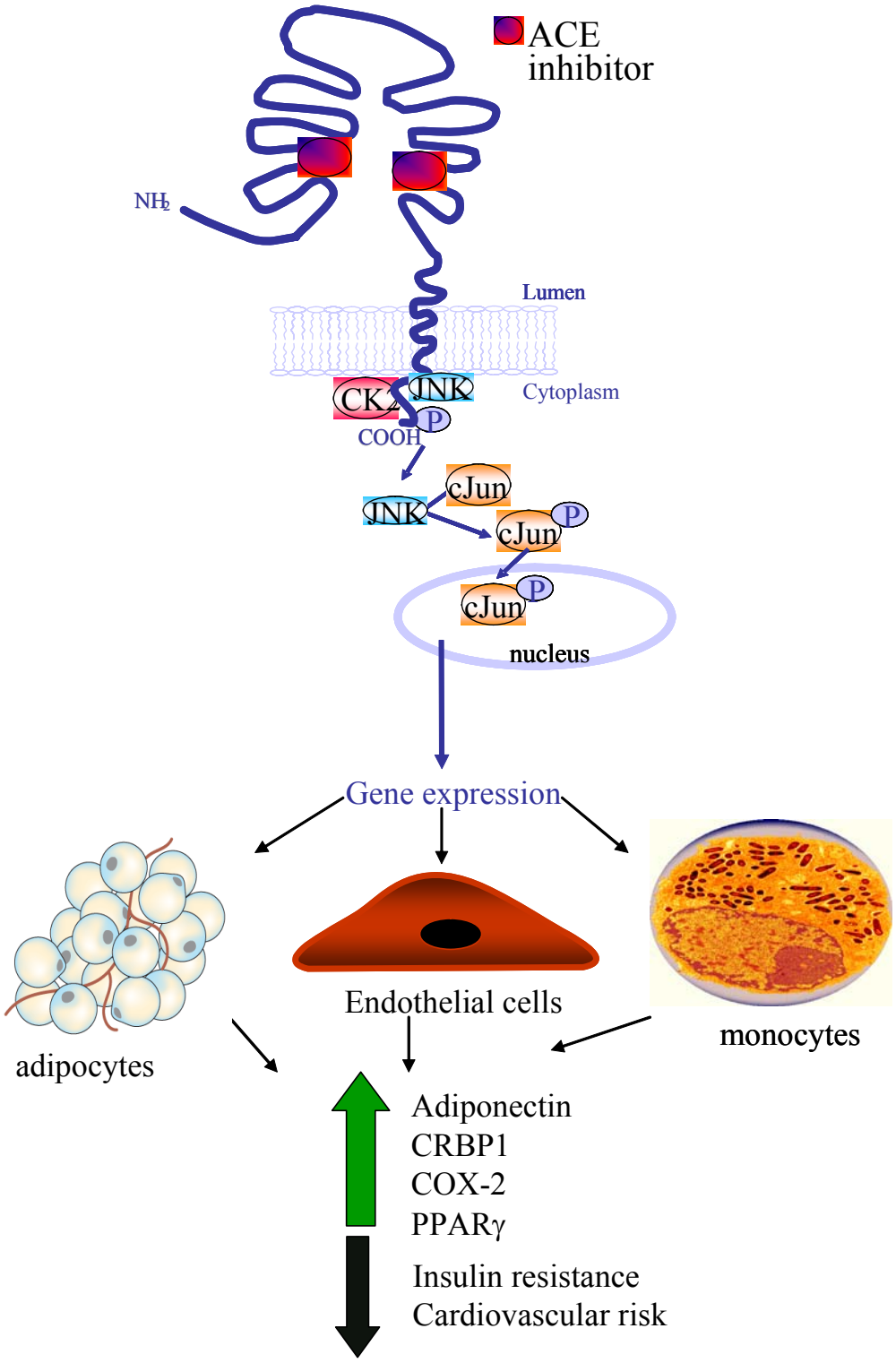

Fig. 27: Scheme summarizing the effects of ACE inhibitor through ACE signalling.

Summary

The angiotensin converting enzyme (ACE) is an important component of the renin-angiotensin system (RAS) and is crucially involved in the homeostasis of fluid and electrolyte balance and thus in the regulation of blood pressure. The zinc metallopeptidase is involved in the generation of angiotensin II, a potent vasoconstrictor and in the degradation of bradykinin, a potent vasodilator. It is worth noting that ACE more readily hydrolyzes bradykinin than it does angiotensin I thus culminating in the net physiological effect of the production of a vasoconstrictor and the decrease in the availability of a vasodilator. ACE inhibitors have become one of the most successful therapeutic approaches as a first line of therapy in hypertension, and are also widely used in treating heart failure, myocardial infarction, stroke, coronary artery disease and impaired left ventricular function. However, one unexpected clinically relevant finding related to ACE inhibitors is their ability to delay the onset of type II diabetes that was revealed by various large clinical trials. However, the mechanisms underlying these beneficial effects of ACE inhibitor therapy are currently unclear and cannot be explained by the prevention of angiotensin II formation or the attenuated degradation of bradykinin. Thus the potential beneficial effects attributed to ACE inhibitors may occur independent of reductions in blood pressure paving way for new and/or unknown mechanism.

Our group has recently redefined ACE as a signal transduction molecule which upon binding to ACE inhibitor turns on a signalling cascade leading to phosphorylation of Ser^{1270} by CK2, activation of JNK and changes in gene expression in endothelial cells. However the mechanism by which ACE inhibitor initiates the signalling cascade was not clear. It was hypothesized that ACE, which is anchored to the membrane with a single transmembrane domain should dimerize prior to initiating further downstream signalling events in endothelial cells. Therefore, we sought to explore whether or not ACE forms dimers in endothelial cells and whether ACE dimerization is essential for the initiation of ACE signalling in endothelial cells. Using native gel electrophoresis, we found that ACE forms dimers in endothelial cells and that there is an increase in the dimer formation upon treatment of endothelial cells with ACE inhibitors. ACE homodimerization was also demonstrated using the split-ubiquitin system and chemical cross-linking experiments. ACE dimers are also formed in endothelial cells overexpressing the non-phosphorylatable ACE, wherein ACE signalling was abolished indicating that dimerization process is not influenced by the phosphorylation of the serine residue residing in the cytoplasmic tail. Monosaccharides like glucose, galactose and mannitol

did not have any influence on ACE-inhibitor induced dimerization. Making use of different monoclonal antibodies directed to the epitopes of N-domain which harbours carbohydrate recognizing domain, also did not affect dimerization. However, inactivation of the C-domain active site by introducing mutation of the key histidine residues in HEMGH consensus sequences, which complexes the zinc ions, abolished enzyme dimerization both in the basal state and in response to ramiprilat. Mutation of the C-domain also resulted in the loss of ACE inhibitor-induced ACE signalling, that is we failed to observe ramiprilat-induced increase in the phosphorylation of the Ser^{1270} and the subsequent JNK activation. ACE-inhibitor induced dimerization precedes the phosphorylation of Ser^{1270} and activation of JNK. Thus the ACE-inhibitor induced dimerization via the C-domain of ACE represents the initial step in the ACE signalling pathway which involves the activation of JNK/c-Jun pathway and leading to the changes in the gene expression in endothelial cells.

Our group previously identified ACE itself as well as cyclooxygenase-2 (COX-2) as two "ACE signalling-regulated" genes. To screen for additional genes regulated in a similar manner we used DNA microarray technology, to assess ramiprilat-induced changes in the endothelial cell gene expression. 21 genes were identified to be differentially regulated of which, 7 were upregulated and 14 were downregulated by ramiprilat. However, when screened at the protein level, we found no significant differences between the untreated control cells and those treated with ramiprilat. As several other cells and tissues possess a fully functional RAS we screened plasma samples from healthy volunteers as well as from patients with coronary artery disease for the proteins identified in the microarray. We observed that the cellular retinal binding protein-1 (CRBP-1) was detectable at low levels in plasma from patients and that ramipril markedly increased serum levels of this protein. Endothelial cells overexpressing CRBP-1 demonstrated increased RXRE and PPRE activity when stimulated with 9-cis retinoic acid and rosiglitazone respectively suggesting that CRBP-1 might affect gene expression via heterodimerization of PPAR elements with RXR elements by virtue of its function as a transport protein of retinoic acid. Studies aimed at determining the consequences of elevated CRBP-1 expression on endothelial cell homeostasis are ongoing.

Although the RAS has been described in many other tissues apart from endothelial cells, ACE signalling has not yet been addressed in tissues such as monocytes/macrophages, which have an increased ACE expression in an atherosclerotic setting. We observed that upon stimulation of cultured ACE expressing monocytes with ramiprilat, JNK is activated suggesting the

occurrence of ACE signalling in human monocytes. It is worth noting that ACE inhibitors delay the onset of type II diabetes in spite of moderate decrease in blood pressure. To further elucidate the mechanism underlying this effect, we found that ACE inhibitors increase the PPARγ levels in the nuclear extracts of ACE expressing monocytes which were also reproduced in human endothelial cells overexpressing human somatic ACE. However, ramiprilat did not have any direct effect on the activity of a luciferase-coupled promoter containing several copies of the PPRE in human endothelial cells. These results contrasted with the actions of the PPARγ agonist suggesting that ramiprilat enhances PPARγ levels through an indirect mechanism. We next hypothesized that ramiprilat might increase the levels of 15-deoxy-D12,14-prostaglandin J2 (15dPGJ2) which is a natural ligand for PPARγ via COX enzymes in monocytes. We observed that ramiprilat was able to decrease the diminution of COX-2 levels upto 48 hours of treatment but the levels of 15dPGJ2 were too low to be detected by ELISA. However ramiprilat enhanced the plasma levels of adiponectin, a downstream target of PPARγ, which is a anti-atherogenic and anti-inflammatory adipokine, in patients with coronary artery disease. Though adiponectin is a PPARγ-regulated gene, the observed increase in adiponectin might be attributed to the increase in RXR rather than via PPARγ.

Taken together, the results of this investigation have revealed that ACE inhibitors initiate ACE signalling by eliciting the dimerization of the enzyme, more specifically via its C-domain active centers. The ACE signalling cascade when activated leads to the enhanced expression of ACE, COX-2 and CRBP-1 which in turn favours the heterodimerization of PPARγ with RXR and thus results in the increased expression of “PPARγ regulated” genes such as adiponectin. The latter results provide a molecular basis for the observation that ACE inhibitors can delay the onset of type 2 diabetes in as much as it was possible to link ramipril with CRBP-1, RXR activity and the expression of adiponectin, an adipokine associated with improved insulin sensitivity. Further work is however required to elucidate the consequences of ACE inhibitors in monocytes and adipocytes as well as in intact animals.

6. Zusammenfassung

Das Angiotensin-konvertierende Enzym (ACE) ist eine zentrale Komponente des Renin-Angiotensin-Systems (RAS) und ist insbesondere für die Regulation des Wasser-Elektrolyt-Haushaltes sowie für die Kontrolle des Blutdrucks von großer Bedeutung. Das Enzym wandelt zum einen Angiotensin I in das stark vasokonstrikitorisch wirkende Hormon Angiotensin II um und hydrolysiert zum anderen das vasodilatorisch wirkende Hormon Bradykinin in inaktive Fragmente. Die Hemmung des RAS durch ACE-Inhibitoren ist zu einer wichtigen, erfolgreichen therapeutischen Maßnahme geworden, nicht nur zur Behandlung des Bluthochdrucks, sondern auch bei Myokardinfarkten, Diabetes mellitus Typ II, Schlaganfällen, koronarer Herzkrankheit (KHK) sowie bei Links-Herz-Insuffizienz. Interessanterweise lässt sich eine Reihe der positiven Effekte der ACE-Inhibitoren nicht allein durch Senkung des Angiotensin II-Spiegels bzw. Anstieg der Bradykinin-Konzentration erklären. Vielmehr scheint es noch andere Wege zu geben, mittels derer ACE-Inhibitoren ihre positiven Wirkungen entfalten.

Im Hinblick darauf ist von besonderem Interesse, dass das Enzym ACE erst kürzlich als Signaltransduktionsmolekül identifiziert werden konnte. Die Bindung von ACE-Inhibitoren an ACE löst in Endothelzellen eine Signaltransduktionskaskade aus, bei der zunächst die ubiquitäte Proteinkinase CK2 ACE an einem intrazellulären Serin-Rest (Ser^{1270}) phosphoryliert. Infolge dieser Phosphorylierung wird der c-Jun/JNK-Signalweg und der Transkriptionsfaktor AP-1 aktiviert, was zur Steigerung der Expression von ACE und Cyclooxygenase-2 (COX-2) führt. In Endothelzellen, die eine nicht-phosphorylierbare Mutante von ACE (S1270A) überexprimieren, findet das ACE-Signalling nicht statt. Da mittels der Technik inverser Micellen gezeigt werden konnte, dass ACE als Dimer vorliegen kann, sollte die physiologische Relevanz dieser Dimerisierung und deren Einfluß auf das ACE-Signalling in Endothelzellen untersucht werden. Mit Hilfe nativer Gelelektrophorese, sowie Experimenten mit dem Split-ubiquitin System oder chemischem „Cross-linking“ konnte im Rahmen dieser Doktorarbeit gezeigt werden, dass ACE in Endothelzellen als Dimer vorliegt und die Dimerisierung durch Gabe von ACE-Inhibitoren gesteigert wird. Da die nicht-phosphorylierbare, inaktive ACE-Mutante auch dimerisieren kann, konnte ein Einfluss der ACE-Phosphorylierung auf die Dimerisierung ausgeschlossen werden. Obwohl eine Dimerisierung des Enzyms über eine N-terminale Zucker-Seitenkette postuliert worden war, konnte im Rahmen dieser Arbeit die ACE-Inhibitor-induzierte Dimerisierung weder durch die Gabe von Monosacchariden wie Glukose, Galactose und Mannitol unterdrückt

werden, noch durch die Inkubation der Endothelzellen mit verschiedenen N-terminalen, monoklonalen ACE-Antikörpern. Die basale und ACE-Inhibitor-induzierte Dimerisierung von ACE konnte jedoch durch die Inaktivierung des C-terminalen, aktiven Zentrums von ACE durch Mutation der Histidinreste in der HEMGH-Konsensussequenz verhindert werden. Dies führte zur völligen Aufhebung des ACE-Signaltransduktion weges, d.h. weder die Ramiprilat-vermittelte Phosphorylierung an Ser^{1270} noch die JNK-Aktivierung erfolgte. Somit konnte die durch ACE-Inhibitoren induzierte Dimerisierung über die carboxyterminale Domäne des Enzyms als Initiationspunkt der ACE-Signaltransduktion bestätigt werden.

Mittels DNA-Microarray Technologie wurde ferner der Einfluss der ACE-Dimerisierung und der dadurch initiierten Signaltransduktion auf die Expression weiterer Gene untersucht, wobei 21 durch Ramiprilat regulierte Gene identifiziert wurden. Sieben Gene wurden in ihrer Expression gesteigert, 14 abgeschwächt. Zahlreiche weitere Experimente zeigten jedoch, dass Ramiprilat keinen Einfluss auf die Menge der durch diese Gene kodierten Proteine in Endothelzellen hatte. Da eine Reihe anderer Zellen und Gewebe ein funktionelles RAS exprimieren, untersuchten wir auch das Blutplasma von gesunden Probanden und Patienten mit KHK. Interessanterweise fand sich im Plasma von Patienten unter Ramipril-Therapie eine erhöhte Konzentration des „Cellular retinol binding protein-1“ (CRBP-1), welches auch mittels Microarray als durch Ramiprilat-hochreguliertes Gen ermittelt worden war. In Endothelzellen führte eine Überexpression von CRBP-1 zu einem Anstieg der Promotoraktivität von RXRE und PPRE nach Stimulation durch 9-cis Retinolsäure und Rosiglitazon, was möglicherweise auf das erhöhte, zelluläre Angebot von Retinol-Produkten und einer daraus resultierenden vermehrten Dimerisierung von PPAR und RXR zurückzuführen ist.

Obwohl das RAS nicht nur in Endothelzellen, sondern auch in anderen Geweben eine Rolle spielt, konnte das ACE-Signalling bislang noch nicht in anderen Geweben nachgewiesen werden. Insbesondere die Untersuchung des ACE-Signallings in Monozyten bzw. Makrophagen könnte von besonderem medizinischem Interesse sein, da in diesen Zelltypen die ACE-Expression im Rahmen der Entwicklung einer Atherosklerose deutlich ansteigt. Es scheint jedoch ein ACE-Signalling in Monozyten zu existieren, da Ramiprilat in diesen Zellen eine Aktivierung der JNK sowie eine Erhöhung der COX-2 Expression auslöst.

Eine weiterer positiver Aspekt einer ACE-Inhibitor-Therapie ist, dass diese Medikamente die Ausbildung eines Diabetes mellitus Typ II verzögern. Experimente zur Ermittlung des zugrundeliegenden Mechanismus zeigten, dass die Behandlung von ACE-exprimierenden Monozyten oder Endothelzellen mit ACE-Inhibitoren zu einem erhöhten PPARγ-Level in den

Kernextrakten dieser Zellen führte. Allerdings scheint Ramiprilat die PPARγ-Expression nur indirekt zu beeinflussen, denn die PPRE-Promotoraktivität wurde durch die Behandlung von Endothelzellen mit Ramiprilat, im Gegensatz zu der Inkubation mit dem PPARγ-Agonisten Rosiglitazon, nicht beeinflusst. Eine Wechselwirkung zwischen ACE und PPARγ scheint in der Tat naheliegend, da Patienten mit KHK infolge einer Ramipril-Behandlung eine erhöhte Plasmakonzentration von Adiponectin aufweisen, das in seiner Expression durch PPARγ gesteigert wird und als Adipokin anti-inflammatorische und anti-atherogene Eigenschaften aufweist.

Zusammenfassend lässt sich sagen, dass das ACE-Inhibitor-vermittelte ACE-Signalling durch eine Dimerisierung des ACE initiiert wird und zum Anstieg der Expression von ACE, COX-2 und CRBP-1 führt. Die gesteigerte CRBP-1-Bildung erhöht vermutlich die Bildung von PPAR/RXR-Heterodimeren, was in der vermehrten Expression von PPAR-regulierten Genen (z.B. Adiponectin) resultiert.

7. List of Abbreviations

ACE	Angiotensin I converting enzyme
ACE2	Angiotensin converting enzyme-2
ATP	Adenosine tri-phosphate
BSA	Bovine serum albumin
CK2	Casein kinase 2
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	Human umbilical vein endothelial cells
JNK	c-Jun amino terminal kinase
NMMHC	Non muscle myosin heavy chain
PAEC	Porcine aortic endothelial cells
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNFα	Tumor necrosis factor-α

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Posterpräsentationen:

- First meeting of European Vascular Genomics Network of Excellence, Summer School als Doktorandin der vaskulären Biologie, Maastricht, Netherlands, Juli 3-7, 2005.

- 62nd ADA Scientific sessions. Juni 2002. Biochemical effects of the dual PPAR α and γ agonist ragaglitazar on glucose and lipid metabolism. Reeba Vikramadithyan, Jagadeeshan Hiriayan, Cynthia Gershome, Ramanujam Rajagopalan, Ranjan Chakrabarti.

- Vorsitz und Koordination eines Symposiums zum Thema : Role of Nitric oxide in Health and Disease, Society of Biological chemists, Pondicherry chapter. Januar 2000.

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Chairman's Excellenz Preis –Innovationspreis für die Entdeckung von **DRF-10945** (Wirkstoff zur Behandlung metabolischer Störungen, derzeit in Testphase II einer klinischen Studie).

Schriftliche Erklärung

Ich erkläre, daß ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel,

"Molecular mechanism of intracellular signal transduction by the angiotensin-converting enzyme."

in der **KARDIOVASKULÄREN PHYSIOLOGIE** unter Betreuung und Anleitung von **PROF. DR. INGRID FLEMING** mit Unterstützung durch **PROF. DR. RUDI BUSSE** ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.
Ich habe bisher an keiner in-oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.
Teile der vorliegenden Arbeit werden in folgendem Publikationsorgan veröffentlicht:

Molecular Pharmacology

Kohlstedt K, Gershome C, Friedrich M, Muller-Esterl W, Alhenc-Gelas F, Busse R, Fleming I. Angiotensin-converting enzyme (ACE) dimerization is the initial stepp in the ACE inhibitor-induced ACE signalling cascade in endothelial cells. Mol. Pharmacol. 2006 May; 69(5): 1725-32.

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