Master's Thesis

ACTIVITIES OF ANGIOTENSIN-CONVERTING ENZYMES (ACE1, ACE2) AND INHIBITION BY BIOACTIVE LACTOTRIPEPTIDES (IPP, VPP, LPP) IN PORCINE OCULAR TISSUES

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PREFACE

This Master's Thesis was completed during 2007-2008 at the Research Center of Santen Oy in Tampere. Experimental procedures were partly performed at Institute of Biomedicine (Pharmacology), Biomedicum Helsinki at University of Helsinki.

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-Life is full of challenges that are created to be overcome-

In Tampere, June 7, 2009

Satu Luhtala

For the memory of my brother Marko

University of Jyväskylä
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Abstract of Master´s Thesis

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Abstract:

The renin-angiotensin system (RAS) is known to essentially contribute to the regulation of blood pressure. Angiotensin II (ANG II), a biologically active peptide product of this cascade, is considered as a remarkable hypertensive factor due to its vasoconstrictive effects. ANG II is formed by the action of angiotensin-converting enzyme 1 (ACE1) on the inactive peptideprecursor angiotensin I (ANG I). Angiotensin-converting enzyme 2 (ACE2) is responsible for the production of vasodilatory agent angiotensin (1-7) from ANG I and ANG II. ANG (1-7) has contrary effects to those of ANG II. Functional RAS has recently been identified also in the human eye. Evidence is accumulating of common mechanisms related to the regulation of blood pressure and intraocular pressure (IOP). Increased IOP is the major risk factor in the development of glaucoma, which is a sight-threatening eye disease. Antihypertensive drugs affecting RAS have been shown to reduce also IOP, although they are not yet in clinical use in the treatment of glaucoma. Certain milk β-casein –derived tripeptides, such as isoleucyl-prolyl-proline (IPP), valyl-prolyl-proline (VPP) and leucyl-prolyl-proline (LPP) have been shown to possess blood pressure lowering properties in previous preclinical and clinical studies. The efficacy of these substances is based mostly on their ACE1-inhibitory properties. In the present study, it was hypothesized that these lactotripeptides may affect RAS also in the eye. Firstly, the basal activities of ACE1 and ACE2 in certain porcine-derived ocular tissues (ciliary body, retina, vitreous body) were determined *in vitro* by fluorometric assay methods. Secondly, the inhibitory effects of synthetic IPP, VPP and LPP peptides on ACE1 and ACE2 were examined. ACE1 and ACE2 activities were detected in all tissues analysed. Basal ACE1 activity was much higher in the ciliary body (3.7 ± 0.7 mU/mg protein) compared to that in the retina (0.2 ± 0.02 mU/mg). The ACE2 activities were in the similar range in the retina (0.2 ± 0.01mU/mg) and the ciliary body (0.2 ± 0.02 mU/mg), but were lower than ACE1 activities measured in the corresponding tissue types. In the vitreous body the ACE1 activity (8.2 ± 0.31 nM/min/mL) was much higher compared to that of ACE2 (0.1 ± 0.02 nM/min/mL). Lactotripeptides IPP, VPP and LPP inhibited the ACE1 activity at one-thousandth (μM) of the concentration needed to inhibit the ACE2 activity (mM). All the peptides evinced equal ACE1- and ACE2-inhibitory potencies. Presence of catalytically active ACE2 in the ciliary and vitreous bodies was shown for the first time, in addition to retina wherein ACE2 has been localized also in previous studies. Concurrent expression of both ACE1 and ACE2 enzymes in the same ocular tissues suggests that both of them may participate with different functions in the physiology of the eye and also in various ophthalmic disorders, like glaucoma. According to the present findings, it can be suggested that intraocular RAS is involved in the regulation of ocular circulation and IOP. Tripeptides were shown to inhibit ACE1 activity at clinically relevant concentration *in vitro*, and can therefore be regarded as potential and attractive drug candidates in the treatment of glaucoma.

Keywords: ACE1, ACE2, renin-angiotensin system, porcine eye, bioactive peptide, lactotripeptide, Ile-Pro-Pro, Val-Pro-Pro, Leu-Pro-Pro, intraocular pressure, glaucoma

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Tiivistelmä:

Reniini-angiotensiini järjestelmän (RAS) tiedetään vaikuttavan oleellisesti verenpaineen säätelyyn. Angiotensiini II (ANG II), RAS-kaskadin tuottama bioaktiivinen peptidi, on merkittävä verenpainetta kohottava tekijä sen verisuonia supistavan vaikutuksen vuoksi. ANG II:ta muodostuu angiotensiiniä konvertoivan entsyymin 1 (ACE1) katalysoimana inaktiivisesta prekursoripeptidistä, angiotensiini I (ANG I) :stä. Angiotensiiniä konvertoiva entsyymi 2 (ACE2) puolestaan katalysoi verisuonia laajentavan angiotensiini (1-7):n tuotantoa ANG I:stä ja ANG II:sta. ANG II ja ANG (1-7) ovat toistensa vastavaikuttajia. Toiminnallinen RAS on hiljattain löydetty myös ihmisen silmästä. On osoitettu, että verenpaineen ja silmänpaineen säätelyyn liittyvät mekanismit ovat samankaltaisia. Kohonnut silmänpaine on merkittävä riskitekijä näkökykyä uhkaavan silmäsairauden, glaukooman synnylle. Verenpainetta alentavien, RAS:n toimintaan vaikuttavien lääkkeiden on todettu alentavan myös silmänpainetta, mutta näitä lääkkeitä ei vielä käytetä glaukooman hoidossa. Tiettyjen, maidon β-kaseiinista peräisin olevien tripeptidien, kuten isoleusyyli-prolyyli-proliinin (IPP), valyyli-prolyyli-proliinin (VPP) ja leusyyli-prolyyli-proliinin (LPP) verenpainetta alentavat ominaisuudet ovat osoitettu aiemmissa prekliinisissä ja kliinisissä tutkimuksissa. Näiden peptidien tehokkuus perustuu niiden ACE1 entsyymin aktiivisuutta estäviin ominaisuuksiin. Tämän tutkimuksen oletuksena oli, että nämä laktotripeptidit voisivat vaikuttaa RAS:n toimintaan myös silmässä. Tässä tutkimuksessa määritettiin ACE1 ja ACE2 entsyymien perusaktiivisuudet tietyissä sian silmäkudoksissa (sädekehä, verkkokalvo, lasiainen) *in vitro* fluorometrisellä menetelmällä. Lisäksi tutkittiin synteettisesti valmistettujen IPP, VPP ja LPP peptidien estovaikutusta näiden entsyymien aktiivisuuksiin. ACE1 ja ACE2 entsyymien todettiin olevan aktiivisia kaikissa määritettävänä olleissa kudoksissa. ACE1:n aktiivisuus oli merkittävästi korkeampi sädekehässä (3.7 ± 0.7 mU/mg proteiinia) kuin verkkokalvossa (0.2 ± 0.02 mU/mg). ACE2:n aktiivisuus oli samansuuruinen sekä verkkokalvossa (0.2 ± 0.01 mU/mg) että sädekehässä (0.2 ± 0.02 mU/mg), mutta se oli matalampi kuin ACE1:n aktiivisuus vastaavissa kudoksissa. Lasiaisesta mitattu ACE1 aktiivisuus (8.2 ± 0.31 nM/min/mL) oli moninkertainen verrattuna ACE2:n aktiivisuuteen (0.1 ± 0.02 nM/min/mL). Laktotripeptidit IPP, VPP ja LPP estivät ACE1:n aktiivisuutta tuhat kertaa pienemmällä pitoisuudella (μM), kuin mitä vaadittiin ACE2:n aktiivisuuden estymiseen (mM). Peptidien keskinäinen estovaikutus ACE1:n ja ACE2:n aktiivisuuksiin oli samankaltainen. Katalyyttisesti aktiivisen ACE2 entsyymin läsnäolo sädekehässä ja lasiaisessa osoitettiin ensimmäistä kertaa tässä tutkimuksessa. ACE2:ta on löydetty verkkokalvosta jo aiemmissakin tutkimuksissa. ACE1 ja ACE2 entsyymien samanaikainen ilmentyminen silmäkudoksissa ja niiden välittämät erilaiset vaikutukset viittaavat siihen, että molemmat osallistuvat silmän toimintaan ja vaikuttavat erilaisissa silmäsairauksissa, kuten glaukoomassa. Tämän tutkimuksen tulosten perusteella voidaan olettaa, että silmän paikallinen RAS osallistuu silmän verenkierron ja silmänpaineen säätelyyn. Tripeptidien todettiin estävän ACE1:n aktiivisuutta kliinisesti relevantilla pitoisuudella *in vitro*, ja näin ollen niitä voidaan pitää potentiaalisina lääkemolekyylikandidaatteina glaukooman hoidossa.

Avainsanat: ACE1, ACE2, reniini-angiotensiini järjestelmä, sian silmä, bioaktiivinen peptidi, laktotripeptidi, Ile-Pro-Pro, Val-Pro-Pro, Leu-Pro-Pro, silmänpaine, glaukooma

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ABBREVIATIONS

ACE	*Angiotensin-converting enzyme*
ACE1	*Angiotensin-converting enzyme 1*
ACE2	*Angiotensin-converting enzyme 2*
AH	*Aqueous humor*
ANG (1-7)	*Angiotensin (1-7)*
ANG (1-9)	*Angiotensin (1-9)*
ANG I	*Angiotensin I*
ANG II	*Angiotensin II*
ARB	*Angiotensin II receptor type 1 blocker (antagonist)*
AT_1	*Angiotensin II type 1 receptor*
AT_2	*Angiotensin II type 2 receptor*
cAMP	*Cyclic adenosine 3',5'-monophosphate*
cGMP	*Cyclic guanosine 3',5'-monophosphate*
DBP	*Diastolic blood pressure*
ET-1	*Endothelin-1*
HHL	*Hippuryl-L-histidyl-L-leucine (Hip-His-Leu)*
His-Leu	*L-histidyl-L-leucine*
IOP	*Intraocular pressure*
IPP	*Isoleucyl-prolyl-proline (Ile-Pro-Pro)*
KKS	*Kallikrein-kinin system*
LPP	*Leucyl-prolyl-proline (Leu-Pro-Pro)*
NO	*Nitric oxide*
NPE	*Non-pigmented ciliary epithelium*
POAG	*Primary open-angle glaucoma*
RAS	*Renin-angiotensin system*
RGC	*Retinal ganglion cell*
sACE	*Somatic cell –derived angiotensin-converting enzyme 1*
SBP	*Systolic blood pressure*
SHR	*Spontaneously hypertensive rat*
tACE	*Testicular angiotensin-converting enzyme 1*
VPP	*Valyl-prolyl-proline (Val-Pro-Pro)*

1 INTRODUCTION

The renin-angiotensin system (RAS) is known to substantially participate in the regulation of systemic blood pressure. Currently evidence is accumulating to confirm the existence of an active RAS cascade also in the eye. Pharmacotherapeutical inhibition of RAS function in the eye has been shown to reduce intraocular pressure (IOP) and appearance of disease-related cellular changes within the retina both in human and in animal models. Accordingly, intraocular RAS is suggested to be associated especially with the regulation of IOP and the pathogenesis of glaucoma. However, substances affecting RAS are not yet approved for therapeutic use in ophthalmology, but are still under research.

1.1 Overview of the renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is classically known as a circulating, coordinated hormonal cascade that participates in the regulation of blood pressure, electrolyte and fluid homeostasis along with neural and autoregulatory factors. RAS functions are principally mediated by its potent effects on vascular smooth muscle, renal reabsorption of electrolytes and water, and also via stimulation of aldosterone and vasopressin production. (For reviews see Lohmeier, 2001 and Hall, 2003.) This complex cascade consists of several components and enzymatic reactions resulting ultimately in the formation of bioactive angiotensin II and other angiotensins. The RAS cascade is depicted in Figure 1 (page 22) on ground of the present knowledge described in the literature. The balance between synthesis and degradation of bioactive actors, specifically angiotensins, is regulated by several enzymes and is prominent in determining RAS function (for review see Mitsui *et al*., 2004). Imbalance within the RAS is considered as a pathophysiological mechanism, and therefore enzymes acting within the RAS are substantial. Consequently, RAS is of clinical importance and is considered as an important actor especially in cardiovascular and renal (patho) physiology. (For review see Atlas, 2007.) In addition to the classical RAS, the presence of specific tissue-localized RAS has recently been described in many tissues, including eye. Depending on its residence within the body, physiological effects of local RAS are similar or differ from those of the circulating RAS. (For reviews see Kramkowski *et al*., 2006 and Paul *et al*., 2006.)

1.1.1 Angiotensinogen

(NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-Va1^{11}-Ile^{12}-His^{13}-Ser^{14}-R)

The primary substrate of the RAS cascade is angiotensinogen, an α-glycoprotein consisting of 255 amino acids. Most of the circulating angiotensinogen is synthesized in and released from liver to the circulation and is subsequently cleaved with high specificity by an enzyme called renin to form angiotensin I. Angiotensinogen is the only known inactive precursor protein for angiotensin peptides that have many physiological effects within the body. (For reviews see Griendling *et al*., 1993 and Carey & Siragy, 2003.)

1.1.2 Renin and its precursor prorenin

Renin is an aspartyl protease, which is synthesized mainly in the kidney and is secreted from the juxtaglomerular cells as an inactive preproenzyme. During transportation through the endoplasmic reticulum, a signal peptide is removed and the product is glycosylated resulting in formation of peptide consisting of 406 amino acid residues that is attributed to **prorenin**. Subsequently, prorenin is proteolytically converted into renin by several enzymes, such as trypsin, cathepsin G, tonin, tissue-type plasminogen activator (t-PA), kallikrein, and tonin. (For reviews see Griendling *et al*., 1993 and Belova, 2000.) Active renin consists of 340 amino acids and is capable of cleaving the Leu^{10}-Val^{11} –bond at the amino terminus within the angiotensinogen molecule to generate angiotensin I with high specificity (for review see Kobori *et al*., 2007). Renin secretion in the kidneys is principally regulated by renal baroreceptor mechanism, changes in delivery of NaCl, sympathetic nerve stimulation and through negative feedback by direct actions of ANG II (for review see Atlas, 2007). At the cellular level, renin secretion is controlled by classic signalling systems, including cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP) and Ca^{2+}/protein kinase C (PKC) pathways that inhibit renin secretion and their interaction with other contrary acting components, like protein kinase A (PKA) (for review see Kurtz & Wagner, 1999).

Prorenin is constitutively released from the kidney into the circulation and can be activated in two ways: proteolytic or non-proteolytic. In the proteolytic activation, a 43 amino acid propeptide is removed from the N-terminal of prorenin e.g. by kallikrein or serine protease.

The non-proteolytic activation is described as unfolding of the propeptide and can be induced by exposure to low pH or cold. (For review see Danser & Deinum, 2005.) Prorenin acts also by binding directly to the (pro)renin receptor. Receptor-bound prorenin displays angiotensin I-generating activity as a result of conformational change, induced by binding *per se* and thus is not dependent on cleavage of the propeptide. (Batenburg *et al*., 2007; for review see Nguyen & Danser, 2008.) Binding of the (pro)renin to its receptor triggers activation of the mitogen-activated protein (MAP) kinase-extracellular signal-regulated kinase (ERK) 1/2 signalling pathway (Nguyen & Danser, 2008).

1.1.3 Angiotensin I

(NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-COOH)

Angiotensin I (ANG I) is released from angiotensinogen by activation of renin. ANG I has no significant biological activity, except is a precursor for angiotensin II (ANG II). ANG I is cleaved to ANG II mainly by angiotensin-converting enzyme 1 (ACE1), which removes the carboxyterminal dipeptide His^9-Leu^{10} from ANG I. Additionally, other enzymes like chymase and cathepsin G are able to catalyze this cleavage. (For review see Belova, 2000.)

1.1.4 Angiotensin-converting enzyme 1 (ACE1)

Angiotensin-converting enzyme 1 (EC 3.4.15.1), abbreviated as ACE1, is a zinc metalloprotease (for review see Belova, 2000). *Ace1* gene is located on chromosome 17 (locus 17q23), similarly to other genes essential in the regulation of blood pressure (Mattei *et al*., 1989; for review see Knight *et al*., 2003). ACE1 has a significant role within the RAS as it acts as a dipeptidyl-carboxypeptidase by cleaving the C-terminal dipeptide His^9-Leu^{10} from ANG I to produce the potent vasoconstricting peptide ANG II. Furthermore, ACE1 inactivates other angiotensins to inactive form. (For review see Fleming, 2006.) ACE1 is also known as kininase II since it inactivates potent vasodilator bradykinin within the kallikrein-kinin cascade (see Figure 1, page 22) by degrading it into inactive peptides called bradykinin$_{(1\text{-}7)}$ and bradykinin$_{(1\text{-}5)}$ (for review see Schmaier, 2002). Bradykinin is formed from kininogen and is able to induce vasodilatation by stimulating the release of nitric oxide (NO) and prostacyclin from the vascular endothelium (for review see Su,

2006) or via direct effects through B_2-receptors (Berguer *et al*., 1993). Consequently, inhibition of the ACE1 results in an overall antihypertensive effect mediated by several mechanisms. In addition to cardiovasculature, ACE1 enzyme is present in nearly all human tissues and body fluids, e.g. in kidneys, gastrointestinal tract, liver, lung, brain, ovary, prostate and testis, wherein ACE1 is localized mainly in endothelial cells and epithelia (Lieberman & Sastre, 1983; Danilov *et al*., 1987; Tipnis *et al*., 2000).

Two distinct isoforms of ACE1 are expressed in mammalian tissues. The predominant isoform, somatic ACE (sACE), is composed of two homologous catalytic domains, called as N- and C-domains according to their positions in N-terminal and C-terminal, respectively (Soubrier *et al*. 1988; Wei *et al*., 1991). Both domains are catalytically active and function independently (Wei *et al*., 1991). Active domains are centered astride a zinc-binding site, wherein amino acid sequence homology between these domains is highest (Hubert *et al*., 1991). The other ACE isoform, testicular ACE (tACE), is exclusively expressed in male germ cells and differs structurally from sACE; it is composed of a single catalytic domain identical to the C-domain of sACE with the exception of a deviantly short N-terminal sequence (Ehlers *et al*., 1989; Lattion *et al*., 1989). Experimentally determined X-ray structures of human ACE1 enzyme are presented in Protein Data Bank (PDB IDs: 1o8a and 1uzf).

Natural ACE1 substrate ANG I can be cleaved by both domains (Wei *et al*., 1991; Michaud *et al*., 1997). According to Wei *et al*. (1991) and Georgiadis *et al*. (2003), ANG I binds preferentially to the C-domain *in vitro*. Fuchs *et al*. (2004 and 2008) have recently shown that the C-domain of sACE is the predominant site of ANG I cleavage also *in vivo* and that the presence of the C-domain is sufficient to maintain a functional RAS. The importance of two catalytic domains was investigated by mouse models, in which either the N- or C-terminal domain of ACE was specifically inactivated by introducing point mutations into the domain in order to eliminate its zinc-binding activity. Also van Esch *et al*. (2005) have confirmed the predominant role of the C-domain in the conversion of ANG I to ANG II *in vivo*. According to their study, selective inhibition of the C-domain within the ACE1 was sufficient to prevent ANG I -induced vasoconstriction in porcine femoral arteries.

Several significant biochemical differences between the two catalytic domains of ACE1 have been described *in vitro*. For instance, both domains are sensitive to chloride, but the C-domain requires much higher chloride concentration to achieve optimal activity than the N-domain (Wei *et al*., 1991; Deddish *et al*., 1996). Molecular mechanism of the chloride activation is not accurately known (Wei *et al*., 1991). Additionally, domains exhibit different substrate specificity (Jaspard *et al*., 1993) and inequal sensitivity to ACE inhibitors (Deddish *et al*., 1996; Michaud *et al*., 1997). Captopril, a well-known synthetic ACE inhibitor, binds preferentially to the N-domain, whereas lisinopril binds with higher affinity to the C-domain (Wei *et al*., 1992; Binevski *et al*., 2003). Ramipril and quinapril inhibit equally both domains (Deddish *et al*., 1996). The differences in the potencies of distinct inhibitors for the two domains depend both on the chloride concentration and on the structure of the inhibitory compound (Wei *et al*., 1992).

There is evidence that two active sites within the ACE1 derived from human (Skirgello *et al*., 2005), pig (Andújar-Sánchez *et al*., 2004) and bovine (Binevski *et al*., 2003) somatic cells exhibit negative cooperativity. This means that binding of a substrate or inhibitor at one active site makes the other site unavailable for either the same or different substrate or inhibitor (Skirgello *et al*., 2005). The exact mechanism of this interaction remains still unclear (Binevski *et al*., 2003).

1.1.5 Alternative pathways for ANG II production in tissues

In addition to classical ACE1, also other enzymes are able to cleave ANG I to ANG II, especially in tissues where ANG II is locally produced or alternatively derived from the circulation. Related to local production, many enzymes capable of producing ANG II from ANG I or directly from angiotensinogen have been identified, including chymase, chymostatin-sensitive ANG II generating enzyme (CAGE), cathepsin G, tonin, trypsin and chymotrypsin (described more closely below). These alternative routes are called as renin- or ACE –independent pathways for ANG II production and are evidenced as important in physiological and especially in pathophysiological conditions. (For reviews see Belova, 2000, Kramkowski *et al*., 2006 and Paul *et al*., 2006.) Furthermore, many differences between species and various organs are described concerning the appearance and function

of these alternative ANG II-forming pathways (Akasu *et al*., 1998; for review see Hollenberg, 2000).

Chymase is a chymotrypsin-like serine protease stored as an inactive enzyme within the secretory granules of mast cells, especially in heart, kidney and vascular smooth muscle. Chymase occurs as two distinct isoenzymes, α- and β-chymases, but humans have only a single α-chymase encoding gene. (Urata *et al*., 1990; for review see Bacani & Frishman, 2006.) Both α- and β –chymases are able to produce ANG II by hydrolyzing the Phe^{8}-His^{9} –bond in ANG I with high catalytic efficiency (Urata *et al*., 1990; Sanker *et al*., 1997). Additionally, β-chymase is able to degrade ANG II by hydrolyzing its Tyr^{4}-Ile^{5} –bond, albeit with very low catalytic efficiency (Sanker *et al*., 1997). Chymase-dependent ANG II production is presumed to have an important role in pathological cardiovascular conditions; it may be associated with vascular proliferative diseases (Nishimoto *et al*., 2001), atherosclerosis (Ihara *et al*., 1999), myocardial infarction (Jin *et al*., 2002) and diabetic nephropathy (Huang, X.R. *et al*., 2003). Additionally, chymase has been proposed to significantly contribute to the regulation of blood pressure (Li *et al*., 2004). Then, according to experiments of Kirimura *et al*. (2005) performed with spontaneously hypertensive rat strain (SHR), chymase has no particular significance in ANG II forming in the regulation of blood pressure.

Chymostatin-sensitive ANG II generating enzyme (CAGE) is a protease able to convert ANG I to ANG II. CAGE is found e.g. from human, monkey and dog aorta, and is predominantly located in the adventitia (Okunishi *et al*., 1987). The significance of CAGE in physiology is not yet known (for review see Kramkowski *et al*., 2006). *Cathepsin G*, a serine proteinase released by activated neutrophils, is able to produce ANG II by cleaving the Phe^{8}-His^{9} –bond both in ANG I and angiotensinogen (Klickstein *et al*., 1982; Owen & Campbell, 1998). Additionally, cathepsin G is able to degrade ANG II, which might be important in the regulation of ANG II activity within the tissue localized RAS, e.g. during inflammatory response (Owen & Campbell, 1998; Ramaha & Patston, 2002). Cathepsin G is also linked to vascular tissues, where it has been demonstrated to contribute to increased ANG II production at the site of atherosclerotic lesions in humans (Legedz *et al*., 2004) and in animal model (Daugherty *et al*., 2000). Another serine proteinase, *tonin*, is also able

to release ANG II directly from angiotensinogen (Grisé *et al*., 1981), likewise *tissue-type plasminogen activator (t-PA)*, *kallikrein* (for review see Belova, 2000), *chymotrypsin* (Schechter *et al*., 1983; Wintroub *et al*., 1986) and *trypsin* (Arakawa *et al*., 1976).

1.1.6 Angiotensin II

(NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-COOH)

Angiotensin II (ANG II) is an octapeptide that is regarded as the principal actor in the circulating RAS. ANG II has been demonstrated to possess a central position in the regulation of cardiovascular functions since it regulates blood volume and vascular resistance. ANG II acts as a potent vasoconstrictor and stimulates release of mineralocorticoid aldosterone from the adrenal cortex, which tends to elevate blood pressure by causing retention of extracellular sodium and water. (For review see Kramkowski *et al*., 2006.) In the kidneys ANG II constricts blood vessels and stimulates contraction of ambient mesangial cells, leading to reduction in renal blood flow and glomerular filtration rate. ANG II decreases blood flow also in the kidney medulla, thereby increasing passive sodium reabsorption in the loop of Henle. (For review see Carey & Siragy, 2003; Crowley *et al*., 2006.) Additionally, ANG II directly enhances activities of proximal tubule Na^+H^+-exchanger, Na^+/HCO_3^- -cotransporter and Na^+K^+ -ATPase (Garvin, 1991; Wang & Giebisch, 1996). All these vascular and tubular effects mediated by ANG II decrease sodium and water excretion, and thereby increase blood volume and blood pressure (Crowley *et al*., 2006; Sachetelli *et al*., 2006). ANG II regulates Na^+ absorption by cAMP-independent mechanism. PKC and intracellular calcium are crucial in modulating the effects of ANG II on sodium transport in the proximal tubule. (Du *et al*., 2003.)

In addition to circulation, ANG II is formed also locally in many tissues, such as kidney, heart, and vasculature. Consequently, it has been suggested that ANG II may also act in paracrine and autocrine way, and hence is able to induce cell growth and proliferation and control extracellular matrix formation. It has been shown that in addition to ANG II, also ANG II –derived metabolites, such as angiotensin III, angiotensin IV, and angiotensin (1-7) are biologically active. (For review see de Gasparo *et al*., 2000.) These other peptides

exert often opposite effects to those of ANG II, as discussed below (for reviews see Ardaillou & Chansel, 1998 and Atlas, 2007).

Vasoconstrictive effects of ANG II are closely related to its interaction with endothelin system. ANG II induces the synthesis and release of endothelin-1 (ET-1), a potent endogenous vasoconstrictor, present especially in vasculature. Accordingly, ET-1 also participates in the regulation of blood pressure when its synthesis is activated by ANG II. (Moreau *et al*., 1997; Hong *et al*., 2004; An *et al*., 2007.) The mechanisms behind the ANG II –induced ET-1 synthesis are complex and are not fully understood. For instance, reactive oxygen species (ROS), extracellular signal-regulated kinase (ERK) pathway, mitogen-activated protein kinase (MAPK) phosphorylation, and NADPH oxidase have been suggested to participate in this process (Cheng *et al*., 2003; Hong *et al*., 2004; An *et al*., 2007). ANG II stimulates also release of a vasoconstricting pituitary hormone vasopressin and reduces baroreceptor activity (Qadri *et al*., 1993; Yee & Struthers, 1998). Additionally, ANG II diminishes beneficial effects of vasorelaxing nitric oxide (NO) by inhibiting expression of nitric oxide synthase (NOS), an enzyme responsible for the production of NO (Nakayama *et al*., 1994; Ramseyer & Garvin, 2009). All these ANG II-induced incidences together contribute to the regulation of blood pressure and tend to increase the prevailing blood pressure level.

Effects of the RAS, primarily mediated by ANG II, are counterbalanced by interaction with the kallikrein-kinin system (KKS). Due to activation of this system, kallikrein enzyme produces a potent, naturally occurring vasodilator bradykinin by proteolytic cleavage of its kininogen precursor. (For review see Schmaier, 2002.) Binding of bradykinin to its B_2-receptor induces production and release of NO (Kuga *et al*., 1997; Danser *et al*., 1998), cGMP (Sheng *et al*., 1995), and also vasodilating prostanoids (Wotherspoon *et al*., 2005) and prostaglandin E_2 (Jose *et al*., 1981). Vasodilatation leads to improved blood flow (Su *et al*., 2000). KKS contributes also to activation of the RAS as kallikrein is able to convert prorenin to renin, which is important in catalyzing the production of angiotensins from angiotensinogen and by this way conducts the cascade. (For review see Schmaier, 2002.) Additionally, kallikrein is capable of converting angiotensinogen directly to ANG II and also ANG I to ANG II (for review see Belova, 2000).

1.1.7 Angiotensin II receptors

Angiotensin II receptors are divided into two subtypes: ANG II type 1 (AT_1) and type 2 (AT_2) receptors, both of which are also known as glycoproteins with seven transmembrane domains and are classified as G-protein coupled receptors (GPCR). Most known cardiovascular and other biological effects of ANG II are mediated through the **AT_1-receptors** and are reversed by specific antagonists acting on AT_1-receptor, widely used as antihypertensive drugs. (For review see de Gasparo *et al*., 2000.) Binding of ANG II to AT_1-receptor induces a conformational change in the receptor that promotes its interaction with G-protein(s), which in turn mediates signal transduction via several effector systems, such as phospholipases and ion channels. Cellular responses to AT_1-receptor signalling include vasoconstriction, aldosterone secretion, sodium and water reabsorption, release of vasopressin, cell growth and proliferation. In rodents, AT_1-receptors are divided into AT_{1A}- and AT_{1B} –receptor types, which have common properties both structurally and in ligand binding and action, but differ in their tissue distribution and transcriptional regulation. (Kakar *et al*., 1992; Kitami *et al*., 1992; for review see Hernández-Hernández *et al*., 2002.) AT_{1A}-receptors are predominantly expressed in most tissues (de Gasparo *et al*., 2000) and their expression has been suggested to be regulated by ANG II and cAMP and possibly by protein kinase C and tyrosine phosphorylation (for review see Griendling *et al*., 1993).

AT_2-receptor, the other ANG II-binding receptor type, is clearly distinct from the AT_1-receptor in tissue-specific expression and signalling mechanisms. Biochemical and physiological function of AT_2-receptor is not yet well characterized, but with certainty AT_2-receptors are demonstrated to counteract the effects of ANG II mediated via AT_1-receptors and thus they contribute also to the regulation of blood pressure and renal function. (For review see de Gasparo *et al*., 2000.) By binding to AT_2-receptor, ANG II stimulates the release of NO that triggers cGMP production and induces vasodilatation, indirectly mediated by the modulation of bradykinin release. AT_2-receptor stimulation has also antiproliferative effects and it promotes apoptosis. (For review see Jöhren *et al*., 2004; Savoia *et al*., 2006.)

1.1.8 Angiotensin III

(NH_2-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-COOH)

Angiotensin III (ANG III) is formed from ANG II by aminopeptidase A or from angiotensin (2-10) by ACE1 (for review see Ardaillou & Chansel, 1998; Mitsui *et al*., 2004). Similarly to ANG II, ANG III is also a vasoconstrictor, albeit less potent, and may possess cell proliferating effects (for review see Kramkowski *et al*., 2006). ANG III exerts its effects via ANG II receptors AT_1 and AT_2 (Ardaillou & Chansel, 1998).

1.1.9 Angiotensin IV

(NH_2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-COOH)

Angiotensin IV (ANG IV) is formed from ANG II by aminopeptidase N or dipeptidylaminopeptidase III (DPA III) and also from ANG III by the hydrolytic action of aminopeptidases B and N (for reviews see Ardaillou & Chansel, 1998 and Mitsui *et al*., 2004). Effects of ANG IV are specifically mediated through **AT_4-receptors** that are found in many tissues from several mammalian species, including human and monkey (Harding *et al*., 1994; Bernier *et al*., 1998; Handa, 2001). AT_4-receptors are distinct from ANG II-binding AT_1- and AT_2-receptors. Existence of unique receptors implies to specific cellular and tissue responses of ANG IV mediated via AT_4-receptors that are affiliated with several signalling pathways. (Harding *et al*., 1994; Handa, 2001.) On the other hand, it has also been suggested that ANG IV may act as an allosteric activator of the AT_1-receptor in conjunction with ANG II *in vivo* (Lochard *et al*., 2004). Several physiological functions have been ascribed to ANG IV, although the precise function of ANG IV is still unknown. Vasodilatory effect of ANG IV is well known and is related to activation of endothelial nitric oxide synthase and stimulation of cGMP (Patel *et al*., 1998) and is dependent on intracellular calcium release (Chen *et al*., 2000). Similarly to ANG II, also ANG IV seems to have cell proliferative effects (Pawlikowski *et al*., 2001).

1.1.10 Angiotensin-converting enzyme 2 (ACE2)

ACE2 (originally termed ACEH) was idenfied in 2000 by two research groups Donoghue *et al*. (2000) and Tipnis *et al*. (2000) using distinct genomics-based methods in screening of human genome database. ACE2 is the only known enzymatically active ACE1 homologue (Donoghue *et al*., 2000; Vickers *et al*., 2002). The *Ace2* gene is located in X-chromosome (locus Xp22) and encodes ACE2 protein consisting of 805 amino acids with a single extracellular catalytic domain. Human ACE2 shares 42% sequence identity and 61% sequence similarity with the catalytic domain of its homologue ACE1. Comparison of genomic structures of ACE1 and ACE2 suggests that genes encoding these proteins arose by duplication of a common ancestor. (Donoghue *et al*., 2000; Tipnis *et al*., 2000.) ACE2 expression was initially found in the tubular epithelia of kidney, in the endothelial cells of renal blood vessels and heart, and in Leydig cells of testis (Donoghue *et al*., 2000; Tipnis *et al*., 2000). Later studies have shown widespread ACE2 distribution also e.g. in the vasculature, lung, liver, small intestine, brain and placenta (Hamming *et al*., 2004; Gembardt *et al*., 2005). Experimentally determined X-ray structure of human ACE2 is presented in Protein Data Bank (PDB ID: 1r42).

Like ACE1, ACE2 is a type 1 integral membrane glycoprotein (Tipnis *et al*., 2000). In spite of these similarities, ACE2 is distinct from ACE1. ACE2 acts as a carboxypeptidase that removes a single amino acid from the C-terminus of its substrate, whereas ACE1 acts predominantly as a peptidyl dipeptidase removing C-terminal dipeptides from its substrate. (Donoghue *et al*., 2000.) The catalytic mechanism of ACE2 closely resembles that of ACE1, but the active sites within these enzymes differ structurally, which explains the differences in substrate specificity between these enzymes (Guy *et al*., 2003; Towler *et al*., 2004). ACE2 is not inhibited by ACE1 inhibitors (Donoghue *et al*., 2000; Guy *et al*., 2003; Rice *et al*., 2004) because the S2' substrate-binding pocket within the active site of ACE2 differs from the corresponding inhibitor binding region in ACE1, noticed when the 3D-structure of ACE2 was recently solved with X-ray crystallography by Towler *et al*. (2004). Guy *et al*. (2005a) have demonstrated that ACE1 and ACE2 have differing sensitivity to chloride ions, which are known to be crucial in activation of these enzymes (Vickers *et al*., 2002; Huang *et al*., 2003), possibly because ACE2 has only one chloride-binding site in contrast to ACE1 which has two binding-sites for chloride (one in each subdomain).

ACE2 is considered as a balancing counter-regulator to the effects catalysed by its homologue ACE1 (for review see Guy *et al*., 2005b), and is commonly coexpressed with ACE1 (Rivière *et al*., 2005), although distribution of ACE2 is more restricted than that of ACE1 (Donoghue *et al*., 2000). ACE2 is able to cleave especially the bioactive ANG II to form ANG (1-7), and also ANG I to ANG (1-9), both of which have physiological effects opposite to those of ANG II. (Donoghue *et al*., 2000, Tipnis *et al*., 2000; Vickers *et al*., 2002.) It has been shown that ACE2 is highly efficient in the conversion of ANG II to ANG (1-7), over 400-fold greater than for the conversion of ANG I to ANG (1-9) (Vickers *et al*., 2002; Rice *et al*., 2004). As reflected by low catalytic efficiency, it is unlikely that ACE2 metabolizes ANG I *in vivo* (Guy *et al*., 2003). Unlike ACE1, ACE2 is not able to degrade bradykinin (Donoghue *et al*., 2000; Vickers *et al*., 2002), but has a role, albeit less significant than with ANG (1-7), also in the control of various vasoactive non-RAS peptides, including kinin metabolites (e.g. des-Arg bradykinin), neurotensin, apelins 13 and 36 and opioid peptide dynorphin A (Donoghue *et* al., 2000; Vickers *et al*., 2002).

In the absence of ACE2, the effects of ANG II predominate within the RAS cascade leading to vasoconstriction and eventually hypertension, as recently demonstrated e.g. by Gurley *et al*. (2006) with *Ace2*-deficient mice. Additionally, Diéz-Freire *et al*. (2006), Rentzsch *et al*. (2008), and Yamazato *et al*. (2007) have established that transgenic *Ace2* overexpression in blood vessels of hypertensive rat model named as spontaneously hypertensive rat (SHR), reduces blood pressure, attenuates hypertension-linked pathophysiological changes and consequently improves endothelial function. Furthermore, in hypertensive animal models expression of *Ace2* gene and ACE2 protein have been demonstrated as markedly reduced, and *Ace2*-knockout models have demonstrated higher circulating and tissue levels of ANG II in the absence of ACE2 (Crackower *et al*., 2002). On ground of these findings, it can be hypothesized that ACE2 is an important modulator of blood pressure (for review see Yagil & Yagil, 2003). Additionally, it has been evinced that ACE2 may contribute also to other cardiovascular diseases and to pathogenesis of various kidney diseases, as recently reviewed by Ferrario *et al*. (2005a), Danilczyk & Penninger (2006) and Wysocki *et al*. (2008), but is not further clarified in this context.

Currently, the exact mechanisms that regulate ACE2 in both physiological and pathological conditions are mostly unknown. ANG II has been demonstrated to decrease *Ace2* gene expression (down-regulation) through activation of AT_1-receptors. Conversely, the inhibition of ACE2 expression due to ANG II is associated with increased expression of *Ace1* gene (up-regulation) under hypertensive conditions both *in vivo* and *in vitro*. The mechanism by which ANG II affects expression of *Ace1* and *Ace2* genes is probably related to activation of extracellular signal-regulated kinase ERK1/ERK2 and p38 mitogen-activated protein (MAP) kinases. (Gallagher *et al*., 2008a; Koka *et al*., 2008.) Application of AT_1-receptor antagonists has been shown to increase expression of *Ace2* gene also in animal studies (Ferrario *et al*., 2005b; Igase *et al*., 2005 and 2008). ET-1, which synthesis is activated by ANG II, has also noticed to significantly reduce the amount of ACE2 mRNA in cardiac myocytes (Gallagher *et al*., 2008a).

1.1.11 Angiotensin (1-7)

(NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-COOH)

Angiotensin (1-7), abbreviated as ANG (1-7), is prominent component within the activated RAS along with previously described ANG II. ANG (1-7) is commonly produced from the cleavage of ANG II by ACE2. ANG (1-7) can be synthesized also directly from ANG I by neutral endopeptidase/neprilysin (NEP), prolyl oligopeptidase (POP) and thimet oligopeptidase (TOP). ANG (1-7) can be produced also from angiotensin (1-9) by NEP, prolyl-endopeptidase (PEP) and ACE1. (For reviews see Welches *et al*., 1993 and Trask & Ferrario, 2007; Rice *et al*., 2004.) ANG (1-7) can also be formed from recently found peptide, angiotensin (1-12) [*NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-Leu^{11}-Tyr^{12}-COOH*], which is is an intermediate precursor derived directly from angiotensinogen and is proposed to serve as a precursor for ANG (1-7) (Nagata *et al*., 2006; Trask *et al*., 2008). NEP is responsible for the cleavage of ANG (1-12) to form ANG (1-7) independently of renin (for review see Varagic *et al*., 2008).

Biological effects of ANG (1-7) are specifically mediated through the recently found G-protein –coupled **Mas receptor**, which is encoded by the *Mas* proto-oncogene (Santos *et al*., 2003). Mas receptor mediates the positive cardiovascular effects of ANG (1-7), such as vasodilation, antiproliferation and antifibrosis (for review see Trask & Ferrario, 2007).

Additionally, Mas receptor acts as a physiological antagonist of the AT_1-receptor and is also able to form hetero-oligomeric complex with AT_1-receptor, which hinders ANG II binding to its receptor and finally results in prevented actions of ANG II. The effects of ANG (1-7) mediated through Mas receptor are hypothesized as prominent in counteracting the effects of ANG II within the RAS, since mice lacking the *Mas* gene showed enhanced ANG II-mediated effects, especially vasoconstriction in mesentric microvessels. (Kostenis *et al*., 2005.) Additionally, binding of ANG (1-7) has not been observed in *Mas*-knockout mice (Santos *et al*., 2003). Effects of ANG (1-7) are defined in several studies by utilizing Mas receptor agonists, usually AVE 0991, and antagonists for Mas receptor, such as D-Ala^7-angiotensin (1-7), known also as A779, and Pro^7-angiotensin (1-7) (e.g. Lemos *et al*., 2005).

Many studies provide evidence that in most situations ANG (1-7) and ANG II have opposite actions within the RAS, suggesting a primary role for ANG (1-7) as a counter-regulatory component for the vascular and proliferative actions of ANG II (for review see Ferrario *et al*., 2005a). Consequently, imbalance between these two peptides is considered as a pathogenetic mechanism in certain cardiovascular diseases, especially in hypertension (for reviews see Trask & Ferrario, 2007 and Varagic *et al*., 2008). Antihypertensive effects of ANG (1-7) in SHR were demonstrated by Benter *et al*. (1995 and 2006). ANG (1-7) has also been indicated to influence blood flow distribution and systemic hemodynamics *in vivo* by increasing blood flow (Sasaki *et al*., 2001; Sampaio *et al*., 2003) and inducing vasodilatation (Gorelik *et al*., 1998; Sampaio *et al*., 2003) dose-dependently.

Mechanisms behind the vasodilatation associated with the active ANG (1-7) are complex. ANG (1-7) has been demonstrated to increase the release of bradykinin, which is specifically mediated by the endothelium-dependent release of NO both in human (Ueda *et al*., 2001) and in animals (Brosnihan *et al*., 1996; Castro *et al*., 2005). Thus, it can be presumed that KKS and RAS are linked via ANG (1-7) through the interaction of ANG (1-7) and bradykinin (Gorelik *et al*., 1998). ANG (1-7) contibutes also to the synthesis and release of vasodilating prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) via stimulation of arachidonic acid release (Jaiswal *et al*., 1992; Muthalif *et al*., 1998).

Additionally, ANG (1-7) has ability to inhibit ACE1 and by acting this way it prevents ANG II synthesis via ACE1-dependent pathway and hinders its vasoconstrictive effects (Li *et al*., 1997). The ACE1-inhibitory effect of ANG (1-7) is directed exclusively to the ACE1 C-domain (Tom *et al*., 2001). ANG (1-7) antagonizes also the vasoconstrictor effect of ANG II by acting as a competitive antagonist for AT_1-receptor (for review see Santos *et al*., 2001; Tom *et al*., 2001). In addition to Mas receptor, vascular effects of ANG (1-7) have been demonstrated mediated also via ANG II-binding AT_1- and AT_2-receptor types by mechanism involving activation of bradykinin-NO cascade. These findings refer to the possibility of functional interaction between ANG II and ANG (1-7) receptors. (Castro *et al*., 2005; Walters *et al*., 2005.) ANG II has been demonstrated to reduce *Ace2* gene expression (downregulation) and ACE2 activity, and these effects are prevented by ANG (1-7) (Zhu *et al*., 2002; Gallagher *et al*., 2006 and 2008a). It has been recently suggested that the MAP kinase-phosphatase pathway is the molecular mechanism behind the regulation of balance between ACE2, ANG (1-7) and ANG II (Gallagher *et al*., 2008b; Giani *et al*., 2008).

Once formed, ANG (1-7) is rapidly metabolised to smaller peptides; to angiotensin (1-5) by ACE1 (Chappell *et al*., 1998), to angiotensin (1-4) by NEP (for review see Varagic *et al*., 2008) and to angiotensin (3-7) by aminopeptidases. ANG (3-7) has been shown to bind to the AT_4-receptor described above (Handa, 2000).

1.1.12 Angiotensin (1-9)

(NH_2-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-COOH)

Angiotensin (1-9), abbreviated as ANG (1-9), is formed from ANG I by ACE2. Its function is not yet clear, but it has been demonstrated to be a strong inhibitor of ACE1 (Donoghue *et al*., 2000) and it serves as a substrate for the formation of ANG (1-7) by ACE1 and NEP (Rice *et al*., 2004). Furthermore, ANG (1-9) enhances activation of bradykinin and increases NO production (For review see Kramkowski *et al*., 2006).

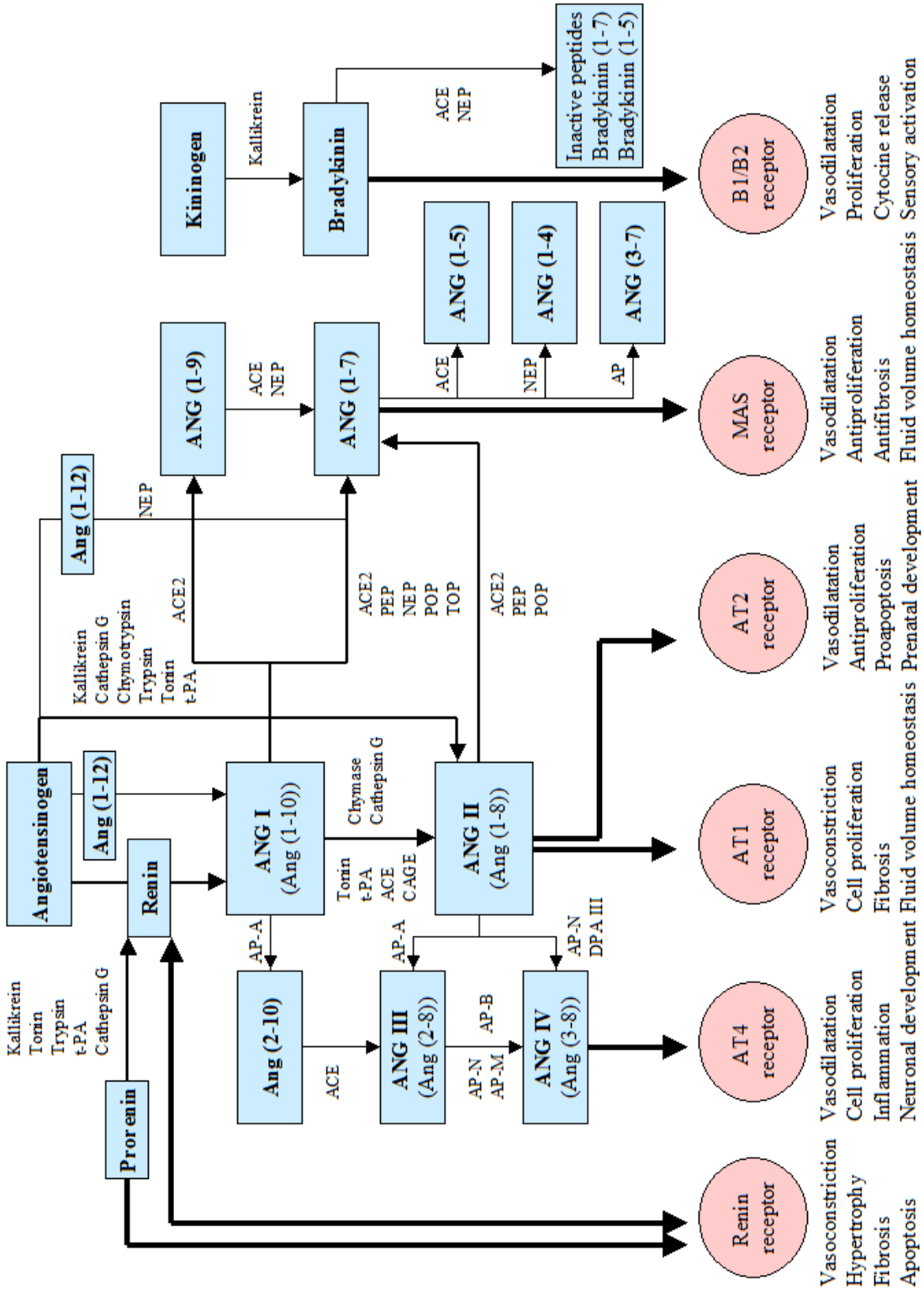

Figure 1. General view of the renin-angiotensin system (RAS). Abbreviations: ACE=angiotensin-converting enzyme 1, ACE2=angiotensin-converting enzyme 2, AT_1=angiotensin II type 1 receptor, AT_2=angiotensin type 2 receptor, AT_4=angiotensin type 4 receptor, AP=aminopeptidase (-A,-B,-M,-N), B1/B2=bradykinin receptor types 1 and 2, CAGE=chymostatin-sensitive ANG II-generating enzyme, DPA III=dipeptidylaminopeptidase III, Mas-receptor=ANG (1-7) –binding receptor, NEP=neprilysin, PEP=prolyl endopeptidase, POP=prolyl oligopeptidase, TOP=thimet oligopeptidase, tPA=tissue-type plasminogen activator. (Picture modified from review article by Vaajanen *et al*., 2008b.)

1.2 Inhibition of the RAS: pharmacotherapeutical approaches

ANG II is considered as prominent in the maintenance and regulation of blood pressure, aldosterone secretion and fluid homeostasis, and is substantial etiological factor in hypertension and other cardiovascular disorders. Consequently, prevention of ANG II formation or action is of major pharmacotherapeutical importance for combating cardiovascular diseases. Especially synthetic ACE inhibitors and AT_1-receptor blockers (ARBs) are widely used, and because of their antihypertensive efficacy are currently preferred as initial drugs for the treatment of hypertension. (For reviews see Moore, 2003 and Kobori *et al*, 2007.) It has been demonstrated that combined use of ACE inhibitors and ARBs is pharmacologically more efficient approach to inhibit RAS than monotherapy (Segura *et al*., 2003). Novel pharmacological alternatives accepted for the treatment of hypertension include renin inhibitors (for review see Pool, 2007). Recently found ACE2 has also been hypothesized as important contributor in the regulation of blood pressure and can therefore be considered as a novel therapeutic target for medication of hypertension (for review see Der Sarkissian *et al*., 2006). In this chapter, the focus is on RAS-inhibiting drugs that are used for antihypertensive purposes.

1.2.1 ACE inhibitors

ACE1 enzyme can be inactivated by blocking its active site with a selective inhibitor, which prevents binding of naturally occurring ACE substrate ANG I. As a result of inhibitor binding, synthesis and effects of vasoconstrictive ANG II are prevented. Consequently, synthetic ACE-inhibiting compounds, such as *captopril*, *enalapril* and *lisinopril* (see Figure 5 on page 59) are widely used as antihypertensive drugs. (For review see Turner & Hooper, 2002.)

It has been demonstrated that along with reduced ANG II formation, inhibition of kinin hydrolysis and consequent bradykinin accumulation may partly contribute to therapeutic effects accomplished by ACE inhibitors (Yasujima *et al*., 1984; Liu *et al*., 1997). However, opposite results are described aswell. In the studies by Waeber *et al*. (1989) and Campbell *et al*. (1999) ACE inhibitors were not demonstrated to modify bradykinin or other kinin

levels, probably because of the activity of carboxypeptidase N (kininase I), another pathway for bradykinin degradation, that remains constant regardless of ACE inhibition. ACE inhibitors do not inhibit the action of ACE2 due to structural differences in active sites of these two enzymes (Guy *et al*., 2003). ACE1 inhibition results in increased ANG I concentration, which subsequently leads to increased ANG (1-7) production. ACE1 is able to degrade vasodilating ANG (1-7), and therefore ACE1 inhibition can increase ANG (1-7) levels also by inhibiting its degradation. Accordingly, ANG (1-7) has been reported to contribute to the systemic antihypertensive effects of ACE inhibitors (Luque *et al*., 1996; Iyer *et al*., 1998; for review see Schindler *et al*., 2007a.) ACE inhibitors have been demonstrated to increase expression of *Ace2* gene and subsequently amount of ACE2 protein resulting also in concomitant increase of ANG (1-7) production from ANG II (Chappell *et al*., 2002; Jessup *et al*., 2006).

Because of alternative non-ACE enzymes that are capable of catalysing the formation of ANG II, ACE inhibitors do not completely prevent the formation of ANG II (for review see Nickenig *et al*., 2006). Medication with synthetic ACE inhibitors is generally well tolerated, but is nonetheless associated with some significant adverse effects, due to their high activity and specificity. Common adverse effects include hypotension, cough, renal impairment, hyperkalemia, and angioedema. (For reviews see Bicket, 2002 and Atlas, 2007.)

1.2.2 Angiotensin II AT_1-receptor antagonists

Angiotensin II AT_1-receptor antagonists, also called angiotensin receptor blockers (referred as ARBs) or sartans, are widely used antihypertensive drugs. ARBs are non-peptide compounds that are capable of binding to AT_1-receptors preventing subsequent binding action of ANG II to AT_1-receptor. By acting this way, ARBs block the effects of ANG II within the body, resulting eventually in reduction of blood pressure. Binding of ANG II antagonists to AT_1-receptor is independent of the pathway responsible for the synthesis of ANG II. Consequently, ARBs inhibit also effects of ANG II formed via alternative pathways, such as chymase. (For reviews see Israili, 2000 and Nickenig *et al*., 2006.) In addition to this advantage, ARBs possess good antihypertensive effects with low incidence

of adverse effects and are therefore well tolerated. For example, cough and angioedema are remarkably more common when ACE inhibitors are used. The most often reported adverse effects in hypertensive patients treated with ARBs are headache, dizziness and upper respiratory infection. (Hernández-Hernández *et al*., 2002; Matchar *et al*., 2008.)

Blockade of AT_1-receptors increases concentrations of renin, ANG I and ANG II that are supposed to activate AT_2-receptors that mediate effects opposite to those caused by ANG II binding to AT_1-receptor (for review see Unger, 2001). Stimulation of AT_2-receptors has therapeutic effect either directly or via enhanced release of bradykinin (Liu *et al*., 1997; Carey *et al*., 2001). It has also been shown that systemic administration of AT_1-receptor blockers induces *Ace2* gene expression and subsequent increase in amounts of ACE2 mRNA, active ACE2 protein and ANG (1-7) in the aorta of SHR (Igase *et al*., 2005) and in rat cardiac myocytes and fibroblasts (Gallagher *et al*., 2008a). Also Ferrario *et al*. (2005b) have demonstrated that blockade of ANG II binding to AT_1-receptor results in increased *Ace2* gene expression and ACE2 activity in rat cardiac tissues. Several studies (e.g. by Collister *et al*., 2003; Ishiyama *et al*., 2004; Schindler *et al*. 2007a) have recently demonstrated that use of AT_1-receptor antagonists increases the formation of ANG (1-7) from ANG II. Consequently, the antihypertensive action of ARBs may in part be due to increased metabolism of vasoconstrictive ANG II to ANG (1-7) by ACE2 (for review see Der Sarkissian *et al*., 2006; Schindler *et al*., 2007a).

1.2.3 Renin inhibitor

Renin is the main determinant of RAS activity, since it catalyses the first and rate limiting step in the RAS cascade, and is highly specific to its substrate angiotensinogen. Consequently, inhibition of renin offers pharmacological potential for blocking the RAS at its initial point of activation. Renin inhibition results in reduced concentrations of circulating ANG I and ANG II and other angiotensin peptides as their synthesis is prevented within the quiescent RAS. In contrast to ACE inhibitors, renin inhibitors have the potential to prevent ANG II production also via alternative non-ACE pathways, and are therefore considered far more effective than ACE inhibitors. *Aliskiren* is the first nonpeptide renin inhibitor with proved antihypertensive efficacy and is recently approved

for the treatment of hypertension by US Food and Drug Administration (FDA) and European Medicines Agency (EMEA). (For reviews see Fisher & Hollenberg, 2005 and Pool, 2007.) For instance, Gradman *et al*. (2005) have demonstrated that Aliskiren is as efficient as AT_1-receptor antagonist irbesartan in reducing blood pressure dose-dependently in patients with mild to moderate hypertension. In addition to its great antihypertensive efficiency, Aliskiren displays also good tolerability. Alongside with Aliskiren, several other renin inhibitors are currently under research. (For review see Pool, 2007.)

1.2.4 Future views of RAS-inhibiting pharmacological treatment options

ACE2 activators and ACE2 gene therapy

Recent studies e.g. by Diéz-Freire *et al*. (2006), Gurley *et al*. (2006), and Rentzsch *et al*. (2008) have provided evidence that (over)expression of *Ace2* gene may be beneficial due to its blood pressure lowering effects. Additionally, it has been shown that expression of *Ace2* gene is significantly reduced in hypertensive animal models (Crackower *et al*., 2002) and corresponding studies performed on *Ace2*-knockout mice have demonstrated that in the absence of ACE2, the effects of ANG II predominates leading to deleterious effects (Gurley *et al*., 2006). Treatment with ACE inhibitors and ARBs is noticed to increase *Ace2* expression (for review see Ferreira & Raizada, 2008). Consequently, *Ace2* gene transfer may be therapeutically potential strategy for long-term control of hypertension. For the specificity based on the particular genetic target, gene therapy would also minimize adverse effects related to RAS-inhibiting drugs. In addition to ACE2, overexpression of ANG (1-7)-coding gene or its receptor would be potential targets for gene therapy. (For reviews see Katovich *et al*., 2005 and Der Sarkissian *et al*., 2006.)

Unsoeld *et al*. (2008) have very recently shown that exogenously applied recombinant human ACE2 (rhACE2) attenuates the negative hemodynamic effects induced by ANG II in mice. Thus specific activators of ACE2 may also have potential therapeutic utility. Hernández Prada *et al*. (2008) have just reported that they have found two molecules: *xanthenone* (*XNT*) and *resorcinolnaphthalein* that are able to activate ACE2 in a dose-

dependent manner *in vitro* using a novel conformation-based rational drug discovery strategy. They have demonstrated also that these novel molecules, especially XNT, are able to considerably reduce blood pressure *in vivo* in SHRs and Wistar-Kyoto rats. They have suggested that these effects may be, at least partially, mediated by an increased ANG (1-7) production. Finally, as Ferreira and Raizada (2008) have recently concluded in their review article, ACE2 is strongly proposed as the target for the next generation of antihypertensives.

ANG (1-7) agonists

AVE 0991 (*AVE*), the first nonpeptide analog of ANG (1-7) available, has recently been demonstrated to mimic the effects of ANG (1-7) by binding to Mas receptor. Similarly to ANG (1-7), AVE enhances synthesis and release of bradykinin and subsequently production of NO by activating NO synthase, but does not alter the effects of ANG I and ANG II *in vitro* (Wiemer *et al*., 2002) and *in vivo* (Carvalho *et al*., 2007). Consequently, stimulation of ANG (1-7) receptor has been shown to improve endothelial function in normotensive rats, although concurrent antihypertensive effect was not observed (Faria-Silva *et al*., 2005). These effects are demonstrated in bovine aortic endothelium *in vitro* (Wiemer *et al*., 2002), and also in rat vasculature (Faria-Silva *et al*., 2005), kidneys (Pinheiro *et al*., 2004) and heart (Benter *et al*., 2006) *in vivo*. In addition to the Mas receptor, also AT_1- and AT_2 –receptors involve in mediating the effects of AVE by oligomerization or crosstalk (Pinheiro *et al*., 2004). Accordingly, AVE is suggested as potential drug candidate for the treatment of disorders related to increased ANG II production.

Prorenin inhibition

Inhibition of prorenin by PRAM-1 has recently been demonstrated to prevent cardiovascular and renal damages associated with hypertension in SHRs, although arterial pressure remained unaffected. On ground of these findings, it has been suggested that activation of prorenin may be a major mechanism that mediates cardiovascular and renal damages in hypertension. (Susic *et al*., 2008.) Currently, prorenin inhibitors are not

approved for medicinal use, but may offer new pharmacological approaches to RAS inhibition in the future.

ACE-inhibitory peptides

In addition to synthetic ACE inhibitors described above, certain bioactive peptides with suitable structures are also able to inhibit the activity of ACE1 enzyme by binding to its active site. Many ACE1-inhibitory peptides have recently been discovered from enzymatic hydrolysates of different food proteins (for review see Möller *et al*., 2008), such as milk casein (FitzGerald *et al*., 2004). These casein-derived peptides have been demonstrated to possess ACE1-inhibitory properties and exhibit antihypertensive effects in spontaneously hypertensive rats (SHR) and hypertensive humans (more details in the chapter 1.4 on page 58). Additionally, no harmful adverse effects reported for synthetic ACE inhibitors, such as dry cough and angioedema, have been detectable during ingestion of these peptides, probably due to lower ACE-inhibitory activity (for review see Erdmann *et al*., 2008). These so called 'biologically active peptides' may provide alternative approach for the classical ACE1-inhibitory drugs because of their scientifically proven beneficial effects and safety, especially in humans suffering from mild hypertension and also in prevention of hypertension (for reviews see Erdmann *et al*., 2008; Hong *et al*., 2008).

1.3 RAS in the eye

Presence of a functional RAS has recently been found in several tissues, including eye, and is demonstrated to act independently of circulatory RAS. An essential requirement for a local tissue RAS is that all of the components necessary for the biosynthesis of both ANG II and ANG (1-7) reside in the tissue. (For reviews see Carey & Siragy, 2003 and Varagic *et al*., 2008.) In detail, for existence of local RAS, the elements described in Table I (below) should all exist in the certain tissue.

Table I. Requirements for the presence of a local RAS within the tissue according to Carey & Siragy, 2003.

- Presence of mRNAs for all the RAS substances necessary for synthesis of bioactive products
- Biologically active components are synthesized within the tissue
- Presence of receptors for binding of the active products formed within the cascade
- The biosynthesis of active products is regulated locally, independently of the systemic circulation
- Reduction or elimination of action evoked by the cascade products induce a physiological response

1.3.1 Localization of RAS components in ocular tissues

Existence of a local RAS has been demonstrated by biochemical, immunohistochemical and molecular biological techniques in ocular tissues of several species. Table II (pages 34-35) summarizes the accumulated data on the evidence for the existence of intraocular RAS. All the main components of the RAS are expressed in mammalian ocular tissues enabling local production of ANG II within the eye. Furthermore, other requirements for a local RAS (see Table I) are demonstrated being fulfilled, as clarified in this chapter. In this context, the focus is on the presence of RAS molecules especially in ciliary body, vitreous body and retina (see Figure 2 on page 30).

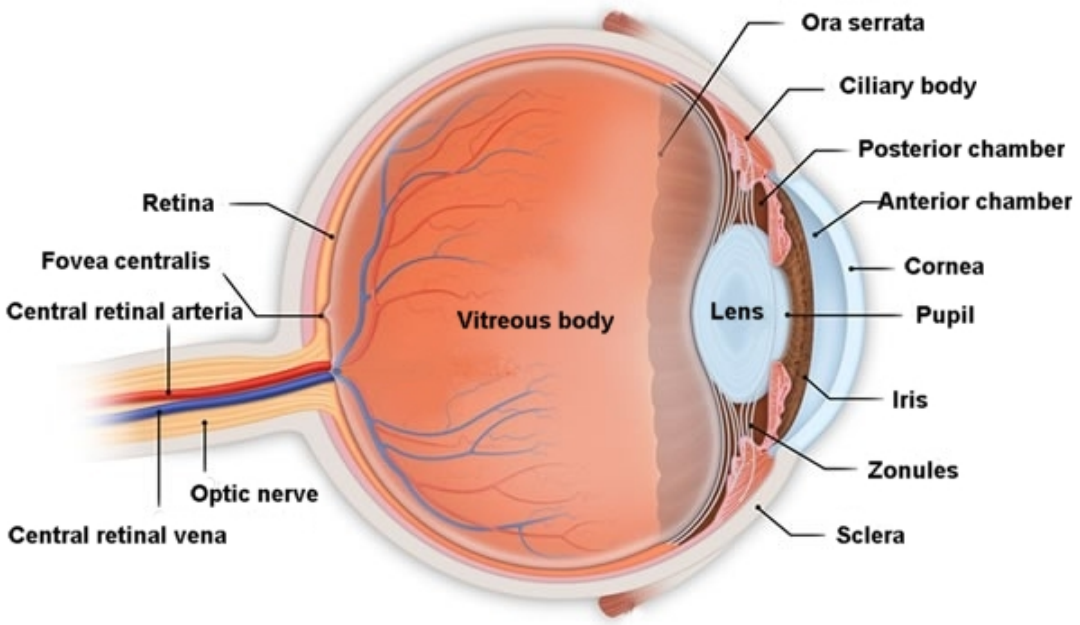

Figure 2. Anatomical structure of the human eye.
(Picture modified from: http://sheinman.com/images/eye-anatomy.jpg)

Ciliary body comprises of ciliary muscle and ciliary epithelium. Ciliary epithelium is bilayer and consists of non-pigmented epithelial (NPE) cell layer that borders the posterior chamber of the eye and pigmented epithelial (PE) cell layer that faces the stroma (see Figure 3, page 38). Sramek *et al*. (1988) and later Wallow *et al*. (1993) have provided immunohistochemical evidence for the presence of prorenin in the NPE. Angiotensinogen has also been localized in the NPE layer (Sramek *et al*. 1992; Cullinane *et al*., 2002). Brandt *et al*. (1994) and Berka *et al*. (1995) have demonstrated the presence of renin mRNA in the same cell layer. Laliberte *et al*. (1988) have used immunohistochemical techniques to indicate the presence of ACE1 in the ciliary body, trabecular meshwork, and Schlemm's canal, eye structures that are involved in the aqueous humor regulation. According to Strittmatter *et al*. (1989) and Savaskan *et al*. (2004), also ACE1 is primarily localized in the NPE layer. ACE1 activity within this eye structure has also been detected in several studies (e.g. Ramirez *et al*., 1996; Geng *et al*., 2003).

Danser *et al*. (1994) have demonstrated that ANG I and ANG II are formed in the anterior uveal tract. Afterwards, Savaskan *et al*. (2004) have defined localization of ANG II in the

NPE. Lin *et al*. (1990) demonstrated the presence of specific ANG II-binding sites in human NPE cells using autoradiographic binding techniques. Later, Wheeler-Schilling *et al*. (1999) and Ramirez *et al*. (1996) have confirmed the presence of AT_1- and AT_2-receptor types within the ciliary body. Active chymase has been detected in the uveal tract comprising of ciliary body, iris and choroid in monkey and dog eye by Shiota *et al*. (1997), indicating the existence of alternative ANG II –generating pathways in the eye. Chymase is produced by mast cells, present also in the ciliary body and other eye parts comprising the uvea (May, 1999).

Vitreous body (also called as vitreous humor or fluid) is a highly hydrated, transparent, viscous extracellular matrix that is bound to the inner limiting lamina (ILL) layer of the retina and the posterior capsule of the lens. Vitreous body is mainly acellular, apart from hyalocytes, and comprises of hyaluronan and many proteins, like collagen, opticin, vitrin, fibulin-1 and nidogen-1. Vitreous proteins are mainly produced in and actively secreted from non-pigmented ciliary epithelium to the vitreous body. (For review see Bishop *et al*., 2002.) Prorenin (Danser *et al*., 1989; Wallow *et al*., 1993), renin (Danser *et al*., 1989; Ramirez *et al*., 1996) and angiotensinogen (Ramirez *et al*., 1996) have been detected in the vitreous body. Additionally, low amounts of ANG I and ANG II have been measured by Danser *et al*. (1994). Later the presence of ANG II has also been confirmed e.g. by Funatsu *et al*. (2002) and Senanayake *et al*. (2007). ACE1 activity has been detected in vitreous body in several studies (Shiota *et al*., 1997; Maruichi *et al*., 2004). In addition to ACE1, chymase, an alternative ANG II –generating enzyme has been detected in the human vitreous body (Maruichi *et al*., 2004), but has not been detected in vitreous bodies of monkey and dog eye (Shiota *et al*., 1997).

Retina is a tissue lining the inner surface of the eye and consists structurally of ten distinct cell layers in which different neuronal cell types interconnected by synapses are localized. Retinal pigment epithelium (RPE) forms the outermost cell layer towards the choroid. The innermost layer from the interior eye part is composed of glial cells, primarily Müller cells. RAS components are primarily found in neurons and glia cells in the inner retina and in blood vessels. Prorenin, renin and angiotensinogen are specifically localized to macroglial Müller cells using immunohistochemistry (Berka *et al*., 1995) and real-time polymerase

chain reaction (RT-PCR) technique (Kida *et al*., 2003). Sramek *et al*. (1992) indicated the presence of angiotensinogen also in retinal blood vessels. Wagner *et al*. (1996) have also confirmed the existence of retinal RAS by utilizing RT-PCR in demonstrating renin and angiotensinogen mRNAs in RPE and neural retina.

By using immunocytochemical staining and autoradiography, the presence of ACE1 has been demonstrated to confine primarily in neural retina (Ferrari-Dileo *et al*., 1988), including photoreceptors, bipolar cells, amacrine cells, ganglion cells and Müller glial cells and also in retinal blood vessels (Wheeler-Schilling *et al*., 2001; Savaskan *et al*., 2004). Wagner *et al*. (1996) have also confirmed the expression of *Ace1* gene within the retina by measuring ACE mRNA. Presence of active ACE1 protein in the retina has been demonstrated in several studies (e.g. Shiota *et al*., 1997; Geng *et al*., 2003). Chymase has not been detectable within the monkey and canine retina in a study by Shiota *et al*. (1997).

In the retina, ANG II is localized in Müller cells (Senanayake *et al*., 2007), ganglion cells, photoreceptors and retinal vessels (Kohler *et al*., 1997; Savaskan *et al*., 2004). Also ANG I has been localized in neural retina (Danser *et al*., 1994). Autoradiographic binding techniques have demonstrated the presence of specific ANG II-binding receptors in the retinal vasculature and optic nerve head (Ferrari-Dileo *et al*., 1987 and 1991; Sato *et al*., 1993). More recently, those receptors were recognized as AT_1- and AT_2-receptors and both are found mainly in neurons in the ganglion cell layer and also in the inner nuclear layer (amacrine cells) and endothelial cells of retinal vasculature (Wheeler-Schilling *et al*., 1999; Senanayake *et al*., 2007). Kida *et al*. (2003) and Senanayake *et al*. (2007) have demonstrated the presence of mRNAs for AT_1- and AT_2-receptor types in cultured human Müller cells, and perceived also that AT_1-receptors are more abundant than AT_2-receptors in the retina. Striker *et al*. (2008) have confirmed the presence of AT_1- and AT_2-receptor types also in human retinal pigment epithelium (RPE). These specific receptors enable local actions of ANG II within the retina (Wheeler-Schilling *et al*., 1999; Senanayake *et al*., 2007).

The presence of ACE2 mRNA and active ACE2 protein have recently been confirmed and localized in rodent (Tikellis *et al*., 2004) and human (Senanayake *et al*., 2007) retina by *in*

situ hybridisation, Western blot analysis and immunohistochemistry. According to these studies, ACE2 is predominantly present in inner nuclear layer consisting of Müller cells but also in photoreceptors. Until now, retina is the singular eye part in which the presence of ACE2 has been confirmed. Additionally, Senanayake *et al*. (2007) have recently found ANG (1-7), formed from ANG II by ACE2, in Müller cells in the human retina. Vaajanen *et al*. (2009) have established the expression of specific ANG (1-7) –binding Mas-receptor in the rat retina.

There has been debate, whether the RAS components found in the eye, originate from local ophthalmic production or are they derived from the circulation. It has been demonstrated that neither ANG I, ANG II nor angiotensinogen are able to pass the blood-brain barrier (BBB) (Schelling *et al*., 1980; for review see Meisenberg & Simmons, 1983), which is comparable to the barriers, as blood-retina barrier (BRB), present in the eye (Steuer *et al*., 2005). In porcine ocular tissues levels of ANG I and ANG II have proved to be 5 to 100 – fold higher than could be accounted for admixture with blood or diffusion from blood (Danser *et al*., 1994). The ACE1 activity has also been determined to be much higher in ocular tissues than in plasma (Ramirez *et al*., 1996; Geng *et al*., 2003). Additionally, inter-tissue differences in the levels of various RAS components suggest high degree of compartmentalization between distinct ocular structures possibly related to minor diffusion between these tissues. As a conclusion, it is evident that the levels of angiotensins and other RAS molecules within the eye are too high to be originated from the blood circulation, and are therefore locally produced by intraocular RAS.

The function and physiological significance of the intraocular RAS is not yet entirely understood, but may have special clinical importance since it has been shown to have a regulatory role in the intraocular pressure and thus may contribute to development of glaucoma (for reviews see Kramkowski *et al*., 2006 and Paul *et al*., 2006).

Table II. Detailed overview of the localization of identified RAS components in the eye.

RAS component	*Part of the eye*	*Species*	*References*
Prorenin	Ciliary body[1,4] Retina[3,5] Subretinal fluid[2] Uveal tract[3] Vitreous humor[2-4]	Human[1,2,4,5] Bovine[3]	[1] Sramek *et al*., 1988 [2] Danser *et al*., 1989 [3] Deinum *et al*., 1989 [4] Wallow *et al*., 1993 [5] Berka *et al*., 1995
Renin	Aqueous humor[5*] Bulbar conjunctiva[5] Choroid[5] Ciliary body[3,4+,5] Iris[3,5] Optic nerve[5] Retina[2,4-7] Retina-choroid complex[6] Subretinal fluid[1] Uveal tract[2] Vitreous humor[1,5]	Human[1,4,6,7] Bovine[2] Rabbit[5] Rat[3,4+]	[1] Danser *et al*., 1989 [2] Deinum *et al*., 1989 [3] Brandt *et al*., 1994 [4] Berka *et al*., 1995 [5] Ramirez *et al*., 1996 [6] Wagner *et al*., 1996 [7] Kida *et al*., 2003
Angiotensinogen	Aqueous humor[2] Bulbar conjuctiva[2] Choroid[1,2] Ciliary body[1,2] Iris[2] Optic nerve[2] Retina[2-4] Retina-choroid complex[3] Sclera[3] Uvea[1] Vitreous humor[1,2]	Human[1,3,4] Rabbit[2]	[1] Sramek *et al*., 1992 [2] Ramirez *et al*., 1996 [3] Wagner *et al*., 1996 [4] Kida *et al*., 2003
ACE1	Anterior uveal tract[10] Aqueous humor[1,2,7,10] Bulbar conjunctiva[7] Choroid[4+,6,7,9-11,15] Ciliary body[4+,5-7,11,15] Cornea[4+,15] Iris[4+,7,11] Lens[4+] Optic disc[4+] Optic nerve[4+,7] Retina[4,4+,7-12,14,15] Retina-choroid complex[8] Schlemm's canal[5] Sclera[4+,6,8,10] Tear fluid[1,3] Trabeculum[5] Uvea[4+] Vitreous humor[7,10,13]	Human[1-4,8,12,13,15] Monkey[10] Porcine[11] Bovine[4] Canine[10] Feline[4+] Rabbit[1,7,9] Rat[5,6,14]	[1] Vita *et al*., 1981 [2] Weinreb *et al*., 1985 [3] Immonen *et al*., 1987 [4] Ferrari-Dileo *et al*., 1988 [5] Laliberte *et al*., 1988 [6] Strittmatter *et al*., 1989 [7] Ramirez *et al*., 1996 [8] Wagner *et al*., 1996 [9] Kohler *et al*., 1997 [10] Shiota *et al*., 1997 [11] Geng *et al*., 2003 [12] Kida *et al*., 2003 [13] Maruichi *et al*., 2004 [14] Tikellis *et al*., 2004 [15] Savaskan *et al*., 2004

(Table continues on next page)

RAS component	*Part of the eye*	*Species*	*References*
ACE2	Retina[1,2]	Human[2] Rat[1]	[1] Tikellis *et al*., 2004 [2] Senanayake *et al*., 2007
Chymase	Anterior uveal tract[1] Choroid[1] Sclera[1] Vitreous humor[2]	Human[2] Monkey[1] Canine[1]	[1] Shiota *et al*., 1997 [2] Maruichi *et al*., 2004
ANG I	Aqueous humor[1+] Retina[1] Subretinal fluid[1+] Uveal tract (anterior)[1] Vitreous humor[1,1+]	Human[1+] Porcine[1]	[1] Danser *et al*., 1994
ANG II	Aqueous humor[1+,2] Choroid[4,6] Ciliary body[6] Cornea[6] Retina[1,4,6,7] Subretinal fluid[1] Uveal tract (anterior)[1] Vitreous humor[1,1+,3-5,7]	Human[1+,2,5-7] Porcine[1] Rabbit[3,4]	[1] Danser *et al*., 1994 [2] Osusky *et al*., 1994 [3] Ramirez *et al*., 1996 [4] Kohler *et al*., 1997 [5] Funatsu *et al*., 2002 [6] Savaskan *et al*., 2004 [7] Senanayake *et al*., 2007
ANG II receptor type 1 (AT_1)	Bulbar conjunctiva[1] Choroid[1,3] Ciliary body[1,3,8] Iris[1,3] Ciliary body-iris complex[6] Optic nerve[1] Retina[1,2,3***,4-7,8***] Retina-choroid complex[6]	Human[4-7] Rabbit[1] Rat[2,3,8]	[1] Ramirez *et al*., 1996 [2] Murata *et al*., 1997 [3] Wheeler-Schilling *et al*., 1999 [4] Kida *et al*., 2003 [5] Savaskan *et al*., 2004 [6] Senanayake *et al*., 2007 [7] Striker *et al*., 2008 [8] Vaajanen *et al*., 2009b
ANG II receptor type 2 (AT_2)	Bulbar conjunctiva[1*] Choroid[1*,2] Ciliary body[1**,2,5] Iris[1,2] Optic nerve[1] Retina[1**2-5] Retina-choroid complex[3]	Human[3,4] Rabbit[1] Rat[2,5]	[1] Ramirez *et al*., 1996 [2] Wheeler-Schilling *et al*., 1999 [3] Senanayake *et al*., 2007 [4] Striker *et al*., 2008 [5] Vaajanen *et al*., 2009b
ANG 1-7	Retina	Human	Senanayake *et al*., 2007
Mas receptor	Ciliary body Retina	Rat	Vaajanen *et al*., 2009b

[*] Found only in the eye of Normal *Fauve de Bourgogne* pigmented rabbit.
[**] Found only in the eye of Normal *New Zealand White* albino rabbit.
[***] Only AT_{1A} receptor type localized (not AT_{1B}).
[+] The reference data includes characterization of tissue localization for several animal species. Superscript is used to particularize results separately for each species.

1.3.2 Significance of the intraocular RAS: (patho)physiological and pharmacological aspects for glaucoma

The presence of a functional RAS has recently been demonstrated in the eye of several species, including human. In many studies RAS is associated with pathophysiology of certain ocular diseases, especially glaucoma. Circulatory RAS is known as an essential actor in the regulation of blood pressure by controlling sodium balance, vascular tone and blood volume (for review see Hall, 2003). Mechanisms related to regulation of intraocular pressure are demonstrated to be similar to those of systemic blood pressure, and are therefore substantially associated with the RAS (Langman *et al*., 2005). Consequently, inhibition of the RAS in both of these conditions is evidenced as beneficial and is therefore of high clinical significance. Until now, no drugs that affect the RAS are in clinical use in ophthalmic diseases, but they may emerge as potential pharmaceuticals in the future once their efficiency is adequately shown. In this chapter, the evidence for RAS participation in the regulation of intraocular pressure is provided.

1.3.2.1 Regulation of intraocular pressure and glaucoma

Aqueous humor dynamics: formation and outflow

Intraocular pressure (IOP) is determined as the balance between aqueous humor production (inflow) and secretion (outflow), the process collectively called as aqueous humor (AH) dynamics. AH is a transparent fluid containing various electrolytes, organic solutes, growth factors and many proteins. AH is produced and secreted by bilayer epithelium within ciliary body (ciliary process) in consequence of several cellular functions. The exact mechanisms of AH dynamics are not fully understood. (For reviews see Fautsch *et al*., 2006 and Do *et al*., 2008.) Active ion transfer, particularly of chloride ions, through the bilayered ciliary epithelium is demonstrably crucial in AH formation and secretion (Kong *et al*., 2006; Law *et al*., 2009).

The first phase in the AH formation is uptake of NaCl from the stroma by pigmented ciliary epithelial cells. Ion transport is principally mediated by either paired Na^+/H^+- and Cl^-/HCO_3^--antiporters (Avila *et al*., 2002) or by $Na^+/K^+/2Cl^-$-cotransporter (Kong *et al*.,

2006). Ion transfer mechanisms may vary species-dependently. Subsequently, ions pass through gap junctions (Avila *et al*., 2002; Kong *et al*., 2006) and possibly also via aquaporins 1 and 4 (Patil *et al*., 2001; Zhang *et al*., 2002) from pigmented epithelium to the nonpigmented ciliary epithelial (NPE) cell layer. Na^+/K^+-ATPases are approved as important mediators in ion transport. Na^+/K^+-ATPases act as primary active transport system that generates sodium gradient enabling the deployment of other secondary transport systems. (Shahidullah *et al*., 2003.) Expression of three various Na^+/K^+-ATPase isoforms (α1, α2, α3) in the ciliary body has been confirmed e.g. by Coca-Prados & López-Briones (1987), Ghosh *et al*. (1990) and Shahidullah *et al*. (2007). Transepithelial transport of chloride ions within the ciliary epithelium generates an osmotic gradient, which drives water movement by osmosis (Shahidullah *et al*., 2003). Transepithelial fluid and ion transport is modulated by locally produced cyclic adenosine 3´,5´-monophosphate (cAMP) that activates chloride ion transfer, and is therefore considered as a prominent regulator of AH production (Bausher & Horio, 1995; Ni *et al*., 2006).

NPE cells within the ciliary body secrete Na^+ and Cl^- ions actively into AH in the posterior chamber through activated Na^+K^+-ATPases and Cl^--channels (for review see Civan & Macknight 2004). Then AH flows around the lens and through the pupil into the anterior chamber, wherefrom it leaves the eye through two main pathways at the angle formed by the junction of iris and cornea. AH flows principally through the trabecular meshwork into Schlemm's canal, collector channels and finally reaches aqueous veins (see Figure 4, page 38). This pathway is called as *trabecular or conventional outflow*. (For review see Fautsch *et al*., 2006.) Recently it has been demonstrated that extracellular matrix and cell functions within the trabecular meshwork (TM) contribute also actively to outflow through the TM. Contractility properties of TM and ciliary muscle are shown to be opposite to each other: contraction of ciliary muscle leads to distension of TM with subsequent reduction in outflow, whereas contraction of TM leads to opposite effect. Dysregulation of subcellular structures or trabecular cell loss itself is expected to have adverse effects on AH dynamics and IOP. (For reviews see Wiederholt *et al*., 2000 and Tan *et al*., 2006).

A smaller portion of AH passes through the ciliary muscle and leaves the eye through the sclera (see Figure 3 on next page) or through vessels of ciliary muscle draining into vortex

veins, or into choroidal vessels. This alternative pathway is called as *uveoscleral or unconventional outflow* and is less well understood than the firstly mentioned pathway. The proportion of total AH outflow attributed to the uveoscleral pathway is normally substantially smaller than that of the conventional pathway. (For review see Fautsch *et al*., 2006.) However, as reviewed by Alm & Nilsson (2009) the relative importance of the unconventional pathway varies significantly with age and from species to species. In addition to regulated active ion transport, also ultrafiltration and passive diffusion has been shown to contribute to AH formation, albeit with significantly lower input (Shahidullah *et al*., 2003). It has been demonstrated very recently that bestrophin-2 (Best2), a putative Ca^{2+} chloride channel, is expressed in nonpigmented ciliary epithelium and appears to prevent both the formation and outflow of AH despite the actual IOP level, and is thefore a critical modulator of AH dynamics (Bakall *et al*., 2008; Zhang *et al*., 2009).

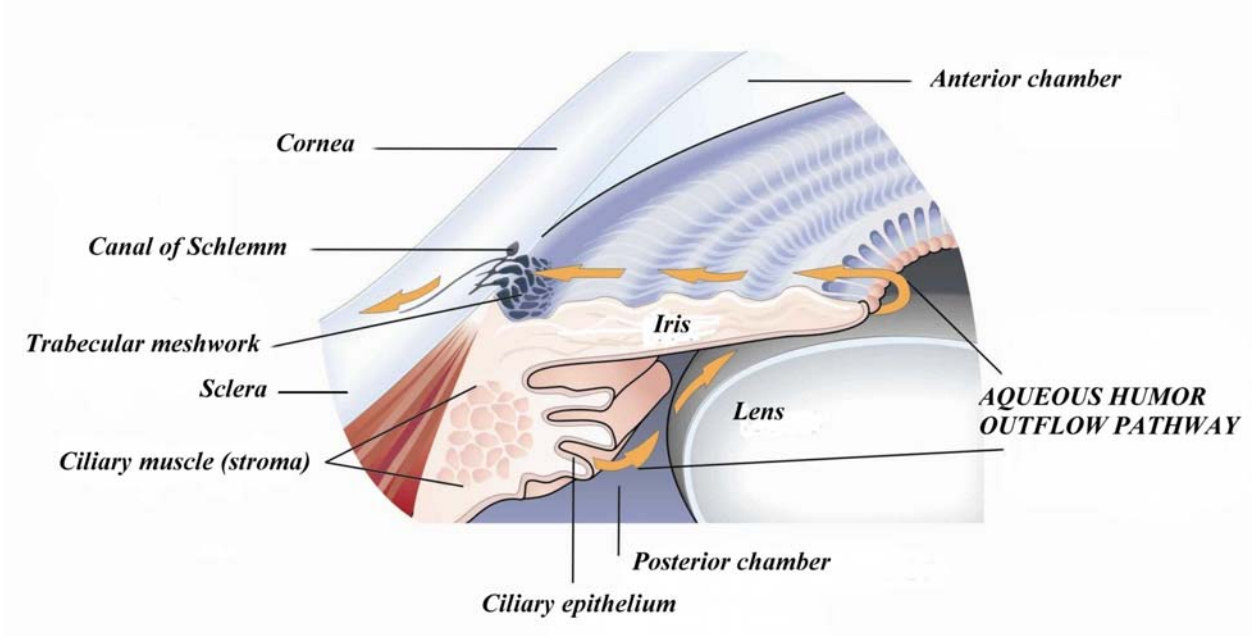

Figure 3. Aqueous humor dynamics. Processes within the ciliary epithelium are responsible for the production of aqueous humor. Outflow pathway is depicted with arrows. (Compare to the anatomical eye structure presented in Figure 2 on page 30.) Picture modified from Vaajanen, 2009a.

Normally, IOP in man is approximately 15 mmHg and is maintained by the balanced aqueous humor dynamics (for review see Do *et al*., 2008). This pressure level is necessary for the maintenance of structural integrity and optical functions of the eye. The AH flow is needed to supply nutrients and remove metabolic wastes from the avascular structures of the anterior eye part and in cellular and humoral immune responses under inflammation. (For review see To *et al*., 2002.) Increase in the IOP is caused by altered AH dynamics,

principally by inhibited or obstructed outflow of AH, although excessive production of AH promotes also increase in the IOP level (Kong *et al*., 2006; Do *et al*., 2008).

Glaucoma: definition, pathophysiology, incidence and current pharmacotherapy

Glaucoma is a common progressive ocular neuropathy associated with deterioration of the visual field, and often elevated IOP caused by impaired outflow of aqueous humor. Glaucoma is classically divided into primary and secondary open-angle glaucoma, angle-closure glaucoma and congenital glaucoma, according to its etiology and pathophysiological mechanisms. The primary open-angle glaucoma (POAG) is the most common type of glaucoma. Secondary form of open-angle glaucoma is associated with concomitant incidence of other ocular or systemic disease, e.g. high blood pressure, which is the actual cause of this disorder. In open-angle glaucoma (OAG), elevation in IOP level is caused by impaired AH outflow resulting from dysfunctions in the outflow pathway. As for angle-closure glaucoma (ACG), increased IOP is caused by physically obstructed anterior chamber angle, which impaires access of AH to the outflow pathway.

In normal-tension glaucoma (NTG), the IOP is consistently normal despite of the presence of otherwise characteristic glaucomatous nerve damages. Contrary, high IOP does not necessarily lead to glaucoma. OAG is often symptomless and appears as an incidental finding during eye evaluation performed for other indications. (For reviews see Distelhorst & Hughes, 2003 and Fraser & Manvikar, 2005.) Primary congenital glaucoma (PCG) is the most common type of glaucoma in small children though its incidence is still relatively rare. PCG is caused by developmental defects of trabecular meshwork and anterior chamber angle that prevents adequate AH outflow and subsequently the amount of AH increases inside the eye and raises the IOP level. (For review see Ho & Walton, 2004.) PCG is an autosomal recessive disorder caused predominantly by mutations in the cytochrome P4501B1 gene (*CYP1B1*) (Chavarria-Soley *et al*., 2008). The precise pathophysiological mechanisms of glaucoma are unknown. (For reviews see Distelhorst & Hughes, 2003 and Fraser & Manvikar, 2005.)

Structural abnormalities in optic nerve and optic disc (optic nerve head) characteristic for glaucoma leads to visual field abnormalities and finally even vision loss. Blindness is

associated with damage of retinal ganglion cell axons in the optic nerve that transmits signals from the eye to the brain. Cell damages lead to ganglion cell death with resultant optic nerve atrophy and finally irreversible vision loss. Elevated IOP causes axonal damage either by direct nerve compression or by diminishing blood flow to the optic nerve head. (For reviews see Distelhorst & Hughes, 2003 and Fraser & Manvikar, 2005.)

Nowadays glaucoma is the second leading cause of blindness worldwide. As the global population continues to become older, it is estimated that in 2010 there will be 60.5 million people with glaucoma, increasing even to 79.6 million by 2020, and of these 74% will have primary open-angle glaucoma. (Quigley & Broman, 2006.) A major risk factor for glaucoma is high IOP, and the other factors include e.g. age, black race, male sex, high blood pressure, positive family history of glaucoma, diabetes, severe myopia, migraine and vasospasms (Tielsch *et al*., 1995; Bonomi *et al*., 2000; for review see Distelhorst & Hughes, 2003; Deva *et al*., 2008).

Treatment strategy for glaucoma is based on delaying the progression of the disease. Accordingly, pharmaceuticals that are used for glaucoma aim primarily at lowering the IOP in order to minimize cell death and thus to delay appearance of vision impairments. Lowering the IOP can be achieved either by reducing the formation of AH or by increasing its outflow. (For review see Saxena *et al*., 2002; Kass *et al*., 2002.) Current pharmacotherapy for glaucoma comprises conventionally of β-adrenergic receptor antagonists and prostaglandin analogues and also, albeit less frequently, of α-adrenergic receptor agonists, carbonic anhydrase inhibitors, cholinergic agonists and combinations of these compounds (for reviews see Distelhorst & Hughes, 2003 and McKinnon *et al*., 2008). These drugs are administered mainly topically and their IOP-lowering effects are accomplished either by reducing the formation of AH in the ciliary body (e.g. β-adrenergic antagonists) or by increasing AH outflow through the uveoscleral pathway (e.g. prostaglandin analogues) (for reviews see Distelhorst & Hughes, 2003 and Alm & Nilsson, 2009). According to a meta-analysis carried out by van der Valk *et al*. (2005), prostaglandin analogues and β-adrenergic antagonists are the most effective IOP-reducing agents in POAG and ocular hypertension. Surgical operations are performed only for patients who do not respond to antiglaucoma medications (for review see Distelhorst &

Hughes, 2003). Although drugs that are used in the treatment of glaucoma are generally effective, many adverse effects are revealed, and therefore new potent pharmacological agents are certainly needed (for review see Saxena *et al*., 2002).

1.3.2.2 Evidence for the involvement of RAS in the pathogenesis of glaucoma

The precise pathophysiological mechanisms behind the glaucoma are unknown. Many implications presented in the literature strongly suggest that the intraocular RAS participates in the regulation of IOP and cellular damage associated with glaucoma (for reviews see Coca-Prados *et al*., 1999 and Paul *et al*., 2006). Expression of RAS components in the eye structures involved in the AH dynamics, essentially in the ciliary body, has been demonstrated (see Table II, pages 34-35). Additionally, topical application of RAS-inhibiting drugs, like ACE inhibitors has been shown to lower IOP in many animal and human studies, as will be introduced in this chapter. Furthermore, ANG II –induced increase in vascular tone and subsequent reduction of ocular blood flow are suggested as pathogenetic mechanisms in glaucomatous cellular damages in the optic nerve (for review see Buckley *et al*., 1997).

RAS in the regulation of aqueous humor dynamics and IOP

Several signalling mechanisms and molecules are described as regulators of AH dynamics and IOP (for review see Coca-Prados & Escribano, 2007). Intraocular RAS has been suggested to significantly contribute to this process by its direct effects and by closely mediating other mechanisms via several other signalling pathways related e.g. to NO, KKS and endothelin system. Evidence from comprehensive research that corroborates that novel concept will be presented in this chapter. RAS-related regulatory mechanisms of blood pressure and IOP seem to be similar, and are principally related to sodium transport in the tubular and ciliary epithelium, respectively (Langman *et al*., 2005). Accordingly, kidneys contribute significantly to blood pressure via regulation of extracellular volume, and a complete intrarenal RAS is described in the glomerulus and the proximal tubule with potential for local production of bioactive angiotensins, ANG II and ANG (1-7) (for reviews see Caruso-Neves *et al*., 2001; Kobori *et al*., 2007). For this reason, it is of

importance to compare mechanisms associated with regulation of IOP and blood pressure by RAS cascade.

In a recent study by Cullinane *et al*. (2002), it was demonstrated that ANG II induces calcium signalling system in human ciliary non-pigmented epithelium (NPE) *in vitro*, which subsequently increases potassium channel activity in the plasma membrane resulting in potassium efflux via BK-channels. These effects were noticed to accompany simultaneously by cell volume reduction. ANG II effects were shown to mediated via AT_1-receptors since losartan, an AT_1-receptor antagonist, significantly blocked ANG II induced signal transduction pathways and ion secretion mechanisms. (Cullinane *et al*., 2002.) Hou and Delamere (2002) have also demonstrated that ANG II increases the concentration of cytoplasmic sodium, an intracellular mediator of secretion, dose-dependently in cultured rabbit NPE cells by mechanism related to activation of Na^+/H^+-exchange. By activating ion secretion mechanisms in the NPE cells within the ciliary body, ANG II may subsequently influence the IOP level.

Administration of aldosterone in the eye has been shown to increase the IOP level (for review see Mirshahi & Agarwal, 2003). In the eye, aldosterone induces sodium retention resulting in increased amount of AH, and simultaneously increases IOP level by decreasing the facility of AH outflow. Consequently, the concentrations of aldosterone and sodium ions in AH are ascended in ocular hypertension. (Stokes *et al*., 2000; for review see Mirshahi & Agarwal, 2003.) Expression of aldosterone-binding mineralocorticoid receptor (MR) and pre-receptor metabolizing enzymes 11β-hydroxysteroid dehydrogenases (11 β-HSD) have been detected in human and rat nonpigmented ciliary epithelium and trabecular meshwork (Stokes *et al*., 2000). Rauz *et al*. (2003) have demonstrated the expression of epithelial sodium channels (EnaCs) and serum- and glucocorticoid-regulated kinase isoform-1 (SGK1) in ciliary epithelium by aldosterone-induction and have suggested that these are important in sodium transport signalling cascade. Accordingly, via RAS activation aldosterone may have significance in the regulation of AH dynamics (for review see Mirshahi & Agarwall, 2003) and is very recently suggested as an important mediator also in retinal vascular pathology (Wilkinson-Berka *et al*., 2009).

In the kidneys ANG II regulates extracellular volume either directly by modulating renal sodium and water reabsorption by activation of Na^+/H^+-antiporter, Na^+/HCO_3^--cotransporter and Na^+K^+-ATPase or indirectly by stimulating the production and release of aldosterone from adrenal gland (for review see Caruso-Neves *et al*., 2001; Crowley *et al*., 2005). ANG II activates cytosolic Ca^{2+} production in adrenal glomerulosa cells increasing simultaneously potassium ion channel activity and aldosterone production (Capponi *et al*., 1984; Kramer, 1988; Foster & Rojas, 1999). Aldosterone enhances reabsorption of sodium and water in the distal tubules and collecting ducts and thereby promotes potassium excretion, increases intracellular Na^+ concentration and consequently leads to antinatriuresis and systemic hypertension (Olsen *et al*., 1985; for review see Atlas, 2007). Blockade of ANG II formation has been demonstrated to increase the capacity of renal Na^+ excretion and thus urine formation (Johns, 1987; Burnier *et al*., 1996). Intracellular sodium ion concentration ($[Na^+]_i$) modulates ANG II effects in renal epithelium by regulating the availability of AT_1-receptors at the plasma membrane. Increases in ($[Na^+]_i$) lead to reduced abundance of AT_1-receptors and induce recruitment of receptors for dopamine (D1 receptor) that mediate antagonistic effects on renal sodium and water excretion in contrast to ANG II. (Efendiev *et al*., 2003.) In conclusion, mechanisms related to ion exchange by angiotensin peptides in ciliary and renal tubular epithelia are comparable.

Effect of ACE inhibitors on IOP in animals

ACE inhibitors have been demonstrated to possess oculohypotensive effects. Topical application of ACE inhibitors has been reported to reduce IOP in several animal studies. Watkins *et al*. (1987) and Abrams *et al*. (1991) have proved the IOP reduction by spiraprilat (SCH 33861) in the ocular hypertensive rabbit and canine eye, respectively. According to Abrams *et al*. (1991), spriraprilat decreased IOP in amounts similar to β-blocker timolol, which is clinically used in the pharmacotherapy of glaucoma. Experiments performed with conscious rabbits (Watkins *et al*., 1988) have shown that topically administered spiraprilat does not produce systemic blood pressure lowering effects when used at concentrations (0.01% and 0.1%) which are 10 to 100-fold greater than needed to lower IOP twice a day during five days period. Lotti and Pawlowski (1990) established that administration of enalaprilat produces significant decrease in IOP level in monkeys.

Shah *et al*. (2000) demonstrated IOP reduction in rabbits by topical administration of various ACE inhibitors (ramipril, enalapril, fosinopril) in acute and chronic models of ocular hypertension. They ensured also that all these three drugs inhibited ACE1 activity in AH *in vitro*. Hashimoto *et al*. (2002) have confirmed IOP reduction by captopril in anesthetized dogs, when captopril was administered intravenously as a dosage of 1.5mg/kg. However, captopril caused marked reduction also in the blood pressure level, which may be undesirable during ophthalmic medication (Hashimoto *et al*., 2002).

In a study by Sharma *et al*. (2006), IOP-reducing effects of benazepril (0.1%) were shown in ocular normotension and in experimentally induced acute and chronic glaucoma in rabbit. Significant IOP reduction was achieved during the first hour postdose and the effect lasted for more than four hours in glaucomatous animals and only one and half hour in ocular normotensive animals. The magnitude of IOP reduction was also higher in animals with experimentally induced glaucoma. During the experiment, aqueous humor samples were also withdrawn from the anterior chamber one hour after instillation of the drug for determination of ACE1 activity. ACE1 activity in AH was reported significantly reduced due to topically admistered benazepril. Sharma *et al*. (2006) investigated also transcorneal permeation of benazepril from 0.1% solution in goat cornea *in vitro*, and found that permeation is in maximum 15 minutes after topical instillation, after which the permeability rate decreases steadily. This finding suggests that benazepril has good lipophilic properties and can cross the cornea in substantial amounts following topical administration.

Effect of ACE inhibitors on IOP in humans

ACE inhibitors have been demonstrated to lower IOP also in ocular normotensive humans and in patients with glaucoma or ocular hypertension. Constad *et al*. (1988) have studied the effects of topically instilled ACE inhibitor spiraprilat on IOP in ocular hypertension and POAG. They elicited that spiraprilat was well tolerated and effective, albeit less potent than timolol (0.5%), in lowering the IOP in continuous use. IOP-reducing effect of captopril in man is proved in many studies. According to study by Costagliola *et al*. (1995), oral dose of 25 mg captopril lowered IOP significantly both in ocular normotensive

and hypertensive humans (IOP was recorded up to three hours after dosing). In contrast to these findings, Al-Sereiti and Turner (1989) demonstrated that a single oral dose (50mg) of captopril had no significant effect on IOP over a four-hour period. In a study performed by Chiseliță *et al*. (1996), captopril was indicated to act as ocular hypotensive agent when captopril (2.5%) solution was topically administered four times a day. IOP-lowering effect of captopril lasted for 6-8 hours and the average relief in IOP was 6.5 mmHg in ocular normotensive and 8.9 mmHg in glaucomatous patients. Captopril was well tolerated and it had no adverse influences on ocular circulation and systemic arterial pressure. In a study by Friström *et al*. (1997), ACE1-inhibiting cilazapril was shown to have significant IOP-lowering effect (13%) two to four hours postdose when administered *per os*.

Effect of ANG II AT_1-receptor antagonists on IOP in animals

Oculohypotensive effects have also been accomplished by administration of ANG II AT_1-receptor antagonists. Inoue *et al*. (2001a) indicated that topically applied olmesartan (CS-088) reduced IOP in rabbits with experimentally induced glaucoma with a maximum IOP reduction of 10.1 mmHg (by 2% CS-088), which is comparable to the effect accomplished by timolol. Later, Wang *et al*. (2005) have also demonstrated dose-dependent ocular hypotensive effect of CS-088 following multiple-dose topical applications in glaucomatous monkey eyes. In that study, the maximum reduction in IOP was slightly lower than in the study of Inoue *et al* (2001a), but the magnitude and duration of the ocular hypotensive effect were increased with repeated dosing. Shah *et al*. (2000) have established the ocular hypotensive effects of losartan in the rabbit eye.

Animals that have been used in studies elucidating the effects of ACE inhibitors and AT_1-receptor antagonists on IOP were congenitally glaucomatous (e.g. Inoue *et al*., 2001a) or more commonly those with artificially high IOP induced e.g. by chymotrypsin-injection (Inoue *et al*., 2001a; Sharma *et al*., 2006), glucose infusion (Shah *et al*., 2000; Sharma *et al*., 2006) or episcleral vein cauterization (Ruiz-Ederra *et al*., 2005). Generally the other (untreated) eye of the same animal has been used as a control for different experimentations. Especially animals that are induced glaucomatous by episcleral vein cauterization are considered as suitable for glaucoma-related studies since retinal changes,

such as retinal ganglion cell (RGC) loss, seem consistent with findings in human glaucoma (Mittag *et al*., 2000; Naskar *et al*., 2002; Ruiz-Ederra *et al*., 2005).

Effect of ANG II AT_1 –receptor antagonists on IOP in humans

In a randomised double-blinded human study (Costagliola *et al*., 2000), oral administration of losartan (50mg) reduced IOP in ocular normotensive humans and patients with POAG. In ocular normotensive subjects the magnitude of the IOP reduction (about 15-18%) was less extensive when compared to values recorded in glaucomatous patients (about 20%). Three hours postdose also systolic (SBP) and diastolic (DBP) blood pressure values decreased significantly in hypertensive patients, but losartan was not noticed to appreciately affect SBP and DBP in arterial normotensive humans. Similarly, Hashizume *et al*. (2005) demonstrated the IOP reduction by topical application of another AT_1-receptor antagonist, candesartan, in humans. However, there are also contrary results of IOP reduction by AT_1 -receptor antagonists. Kaiser *et al*. (1997) have reported that no significant effects on IOP were noticed after topical application of valsartan (CGP 48933) in the human eye. Altogether, there are still many studies providing strong evidence that AT_1-receptor antagonists and ACE inhibitors could represent new group of antiglaucomatous compounds. Furthermore, specific renin inhibitor enalkiren has been shown to accomplish reduction in intraocular pressure (Giardina *et al*., 1990), and may therefore also provide potential alternative to glaucoma treatment.

Mechanisms related to IOP reduction accomplished by RAS inhibition

The mechanisms behind the IOP reduction by ACE inhibitors and AT_1-receptor antagonists are noticed to be associated principally with increased uveoscleral outflow (Costagliola *et al*., 1995; Inoue *et al*. 2001a, 2001b; Wang *et al*., 2005), although the exact mechanism is not fully clarified (see Figure 4 on page 53). As for ANG II, it has been demonstrated to affect AH dynamics by reducing outflow dose-dependently in rabbits with normal IOP. These effects of ANG II are shown to appear via AT_1-receptors (Inoue *et al*., 2001b), and are reversed by ARBs with subsequent reduction in IOP (Costagliola *et al*., 2000). By preventing bradykinin breakdown, ACE inhibitors activate the NO pathway via

bradykinin (for review see Buckley *et al*., 1997) or activate directly bradykinin B1-receptors (for review see Ignjatovic *et al*., 2002). NO is the primary mediator of bradykin-induced vasodilatory response in opthalmic circulation (Meyer *et al*., 1993) and is demonstrated to substantially contribute to the AH dynamics principally by increasing AH outflow (Kotikoski *et al*., 2003; Schneemann *et al*., 2003). It has been accentuated that the NO signalling cascade may regulate the ion transport across the ciliary epithelium, and is therefore able to contribute to the rate of AH formation, although the exact mechanism is still unclear (Shahidullah *et al*., 2005; for review see Do *et al*., 2006&2007).

Other mechanisms proposed to be associated to NO- and bradykinin-induced reduction of IOP are modulation of ocular blood flow (for review see Schmetterer & Polak, 2001) and relaxation of trabecular meshwork and ciliary muscle (Wiederholt *et al*., 1994; Meyer *et al*. 1995a). Physiologically, increase in the IOP enhances NO production by nitric oxide synthases (NOS), expressed predominantly in the nonpigmented ciliary epithelium and trabecular meshwork (Meyer et al., 1999; Schneemann *et al*., 2003; Shahidullah et al., 2007). NO levels in the AH of glaucomatous dog eye are shown significantly increased in contrast to normal dog eye (Källberg *et al*., 2007). Galassi *et al*. (2004) perceived that levels of NO_2^-, endogenous nitric oxide metabolite, were reduced in plasma and AH obtained from POAG patients. According to study by Tsai *et al*. (2002), NO levels in plasma and AH may vary depending on the type of glaucoma, possibly reflecting their differences in the pathogenesis. *In vivo*-studies, e.g. by Deussen *et al*. (1993), Jacot *et al*. (1998) and Kiel *et al*. (2001) have shown that administration of NOS inhibitors results in decreased blood flow in the ciliary body. Prevented NO production results in ciliary vasoconstriction, which is accompanied with decreased AH production and consequent reduction in the IOP level (Kiel *et al*., 2001). Schneemann *et al*. (2002) and Shahidullah *et al*. (2005) have suggested that NO-induced effects on AH dynamics are mediated via cGMP, the levels of which are reduced in plasma and AH of POAG patients (Galassi *et al*., 2004).

According to Toris *et al*. (2002), increased IOP in ocular hypertensive patients is caused by reduction in trabecular outflow facility and uveoscleral outflow, while AH production remains normal. ACE inhibitors significantly reduce ANG II synthesis, and they have been

shown to lower ANG II levels also in the AH (Osusky *et al*., 1994; Sharma *et al*., 2006), which refers to reduced ANG II formation in the ciliary body and that will subsequently lead to normalized AH outflow. According to the literature, drugs that antagonize the RAS seem to be more efficient in glaucomatous than in normotensive eyes, suggesting that intraocular RAS is probably more activated in pathological conditions (Costagliola *et al*., 2000; Sharma *et al*., 2006). For example, topical application of captopril (Chiseliţâ *et al*., 1996) and olmesartan (Inoue *et al*., 2001a, 2001b; Wang *et al*., 2005) has been reported to lower IOP, the ocular hypotonic effect being more prominent in ocular-hypertensive subjects (Inoue *et al*., 2001a, 2001b; Wang *et al*., 2005).

In addition, ACE inhibitors promote synthesis of prostaglandins by preventing the breakdown of bradykinin. Prostaglandins are known to decrease effectiveness of ANG II, as demonstrated by Michibayashi (1983), and are shown to increase glomerular filtration in the kidneys (Milot *et al*., 1996). Accordingly, prostaglandins are known to lower IOP by increasing the uveoscleral outflow in monkey eye (Nilsson *et al*., 1989; Lotti & Pawlowski, 1990), although the precise mechanism is still unknown (for review see Weinreb *et al*., 2002). Synthetic prostaglandin analogs, like latanoprost ($PGF_{2\alpha}$ analog) are important in pharmacotherapy of glaucoma (for review see Saxena *et al*., 2002), and their IOP-reducing effects are mediated via FP-receptors (Sharif *et al*., 2003). In studies by Lotti and Pawlowski (1990) and Shah *et al*. (2000), enalaprilat was shown to reduce IOP in the eye, and the effect was reversed by indomethacin, which inhibits prostaglandin synthesis and activity (Nakata *et al*., 1981), indicating that prostaglandins may contribute also to ocular hypotensive effect of ACE inhibitors.

Increased uveoscleral outflow induced by topical prostaglandin treatment is associated with increased expression of certain matrix metalloproteinases (MMP-1, MMP-2, and MMP-3) in the human and monkey eye, especially within the regions of uveoscleral outflow pathway. Matrix metalloproteinases are capable of degrading extracellular matrix proteins, and process many bioactive molecules. (Gaton *et al*., 1999 and 2001.) Other proposed mechanisms that may contribute to the increased uveoscleral outflow are prostaglandin-induced relaxation of the ciliary muscle and subsequent reduction of extracellular matrix components, especially collagens, within the ciliary body (Nilsson *et*

al., 1989; Lindsey *et al*., 1996; Sagara *et al*., 1999). Krauss *et al*. (1997) have confirmed that prostaglandins influence also contractility of the trabecular meshwork (TM) by binding to distinct receptor types resulting in opposite effects; relaxation is mediated by EP_2-receptor and contraction by TP-receptors. Additionally, Shen *et al*. (2001) have reported that ANG II is able to induce cell proliferation of bovine TM cells and increase synthesis of collagen *in vitro* in these cells. Liu *et al*. (2008) have recently confirmed these observations in their experiments with rat hepatic stellate cells.

It has also been proposed that beneficial effects of ACE inhibitors on IOP may be due to reduced AH production caused by reduced blood flow in the ciliary body (Reitsamer & Kiel, 2003). In the kidneys, exogenous administration of ANG II elicits decrease in renal blood flow and glomerular filtration rate (Yamamoto *et al*., 2001). Nonetheless, most of the *in vitro* studies imply that the RAS has only minor effects on ocular blood flow. ANG II has been shown to induce weak contraction in bovine (Nyborg *et al*., 1990), porcine (Meyer *et al*., 1995a), and human (Nyborg & Nielsen, 1990) ciliary arteries, and the response decreases rapidly (tachyphylaxis). Similarly, Reitsamer and Kiel (2003) have noticed in their experiments performed with rabbits that AH production is dependent on blood flow in the cliary body only when the blood flow is declined below 74 % of the baseline.

Endothelin-1 (ET-1), a potent vasoconstrictive peptide induced by ANG II (Hong *et al*., 2004; An *et al*., 2007) has also been proposed to participate in the local regulation of AH outflow and IOP (for reviews see Buckley *et al*., 1997 and Haefliger *et al*., 1999). Expression of ET-1 and its receptors ET_A and ET_B in NPE cells, ciliary muscle, trabecular meshwork and Schlemm's canal have been confirmed by Prasanna *et al*. (1998), Tao *et al*. (1998) and Fernández-Durango *et al*. (2003). Endothelin-converting enzyme 1 (ECE-1) is capable of catalyzing the formation of ET-1 from its precursor Big ET-1, and has also been localized in human NPE cells in the ciliary body (Prasanna *et al*., 1999). Afterwards, Choritz *et al*. (2005) have demonstrated that ET-1 –induced contraction of ciliary muscle and trabecular meshwork is predominantly mediated by ET_A –receptors. Emre *et al*. (2005) have detected increased plasma concentrations of ET-1 in humans during glaucoma,

likewise Källberg *et al*. (2002) and Prasanna *et al*. (2005) who demonstrated increased ET-1 levels in the AH of dogs and rats with experimentally induced glaucoma.

Taniguchi *et al*. (1994) have studied the effects of ET-1 on AH dynamics and IOP in the rabbit eye. They indicated that intravitreal injection of ET-1 significantly reduced IOP by mechanisms related to reduction in the AH formation and simultaneous increase in the AH outflow. Results from the studies by MacCumber *et al*. (1991) and Sugiyama *et al*. (1995) are comparable. ET-1 has been suggested to inhibit activity of the Na^+/K^+-ATPase, a key enzyme responsible for the AH formation, within the nonpigmented ciliary epithelium, leading to reduced AH formation and subsequent reduction in IOP (Prasanna *et al*., 2001; Krishnamoorthy *et al*., 2003). Bausher (1995) has demonstrated that endothelins inhibit production of cAMP, an important regulator of AH formation, and are therefore able to lower IOP by interfering signal transduction pathway. Wu *et al*. (2003) have recently shown that ET-1 inhibited metabolism of vasorelaxing NO in a concentration-dependent manner, which was observed by measuring nitrite (a stable metabolite of NO) levels in isolated porcine ciliary processes.

ET-1 has been shown to elicit contraction in porcine ciliary arteries (Haefliger *et al*., 1993; Meyer *et al*., 1995b), which may provide explanatory to reduced AH production due to diminished blood flow in the ciliary body. ET-1 –induced contraction of ciliary arteries has been shown to be selectively reversed by calcium channel blockers, suggesting that effects of ET-1 are, at least partly, mediated by Ca^{2+} ion transport (Meyer *et al*., 1995b; Strenn *et al*., 1998). On the other hand, ET-1 induces contractility of human trabecular meshwork (TM) cells *in vitro*, which interferes with the AH outflow through TM and may therefore increase the IOP level (Cellini *et al*., 2006). All these observations suggest that ET-1, which is endogenously present in the eye (Taniguchi *et al*., 1994), may play a role in the regulation of IOP possibly via ANG II-induction. Accordingly, ACE inhibitors reduce the formation of vasoconstrictor peptide ET-1, at least partly, by preventing ANG II synthesis (for review see Buckley *et al*., 1997).

Chymase, an alternative ANG II –generating enzyme, is considered as prominent actor in the RAS cascade, especially in tissues in pathological conditions. The presence of active

chymase has been confirmed also in the ocular tissues (Shiota *et al*., 1997), and may thefore influence regulation of the IOP via ANG II production. In a recent study by Konno *et al*. (2005), intraocular injection of chymase resulted in increased IOP in rabbits, and the effect was attenuated by a specific chymase inhibitor Suc-Val-Pro-Phe^{p} $(OPh)_2$. They demonstrated also that ET-1 increased IOP, and the effect was reversed by a selective endothelin ET_A-receptor antagonist (BQ-123). Chymase is able to modify endothelins into ET-1, and therefore increased chymase activity may result in enhanced production of ET-1, which may be involved in the development of ocular hypertension as a pathogenetic mechanism. Accordingly, chymase and ET-1 inhibitors were not shown to affect IOP in ocular normotensive rabbits, indicating that under normal physiologic conditions endogenous chymase and ET-1 are not involved in the maintenance of IOP. (Konno *et al*., 2005.)

Especially at tissue level, recently found ACE2 and ANG (1-7), a vasorelaxing breakdown product of ANG II, are considered as prominent regulators of blood pressure by balancing the effects of ANG II (for review see Ferreira & Raizada, 2008). In the kidneys ACE2 is predominantly expressed in the proximal tubule, where it is co-localized with ACE1 and ANG (1-7), evidencing active ACE2-dependent processing of ANG II. Proximal tubule is crucial in conversion of ANG I to ANG II. (Shaltout *et al*., 2007.) According to Tikellis *et al*. (2006), expression of *Ace2* gene is reduced in the kidney of hypertensive rat strain (SHR) when compared with normotensive Wistar Kyoto (WKY) rat. Furthermore, genetic studies on *Ace2*-knockout animal model have shown that in the absence of ACE2 protein, the effects of ANG II predominate, and may result in hypertension (Crackower *et al*, 2002; Gurley *et al*., 2006). Moreover, Benter *et al*. (1995, 2006) have demonstrated antihypertensive actions of ACE2 degradation product ANG (1-7) *in vivo* in SHRs. In conclusion, it can be assumed that ACE2 contributes to the regulation of blood pressure in the kidneys, especially in pathophysiology possibly by counteracting the effects of ACE1 (for review see Wysocki *et al*., 2008).

Systemic effects of ACE inhibitors have also been suggested to be related to increased concentration of vasodilatory ANG (1-7) (Schindler *et al*., 2007b). Vaajanen *et al*. (2008a) have recently indicated that ANG (1-7) reduces IOP in rabbits, when injected directly into

the vitreous fluid. Additionally, it was shown that only a specific Mas receptor antagonist (A-779) was able to revoke the IOP-lowering effect induced by ANG (1-7). This indicates that ANG (1-7) acts probably via its own receptor type (Mas) in the eye, as reported also for other tissues, like heart (Castro *et al*., 2005). Vaajanen *et al*. (2008a) have hypothesized that ANG (1-7) lowers IOP probably by reducing AH formation, since ANG (1-7) had no effect on outflow of AH in that study. As for ANG II, it has been demonstrated to affect AH dynamics by reducing outflow dose-dependently in rabbits with normal IOP. These effects of ANG II are appeared via AT_1-receptors. (Inoue *et* al., 2001b; Vaajanen *et al*., 2008a.) Despite of the reduction in AH outflow, ANG II had no direct influence on IOP in the study of Vaajanen *et al*. (2008a). However, referring to earlier studies, it may be possible that ANG (1-7) could also increase AH outflow in the eye, since it has been demonstrated to increase glomerular filtration rate in the kidneys (DelliPizzi *et al*., 1994; Heller *et al*., 2000) possibly by inhibiting Na^+K^+-ATPase activity (Handa *et al*., 1996; Lara *et al*., 2002) and via stimulation of prostaglandins, especially PGI_2, known to inhibit sodium and water reabsorption in the kidneys (Hilchey & Bell-Quilley, 1995).

As a conclusion, findings presented above substantiate the presumption that RAS is involved in the regulation of IOP, providing at least a partial explanation for the regulatory mechanisms behind the IOP. The exact mechanism of this action remains still unknown. It is clearly shown that the mechanisms related to regulation of IOP and blood pressure in the ciliary and renal tubular epithelia are common. Additionally, pathogenetic mechanisms in glaucoma and hypertension appear to be comparable, which may explain demonstrated coincidence of these diseases in humans and animals. (Mitchell *et al*., 2004; Langman *et al*., 2005; Vaajanen *et al*., 2008c.) With regard to the studies referred herein, it can be strongly suggested that compounds inhibiting RAS may also be potential in ophthalmic use, especially in the treatment of glaucoma.

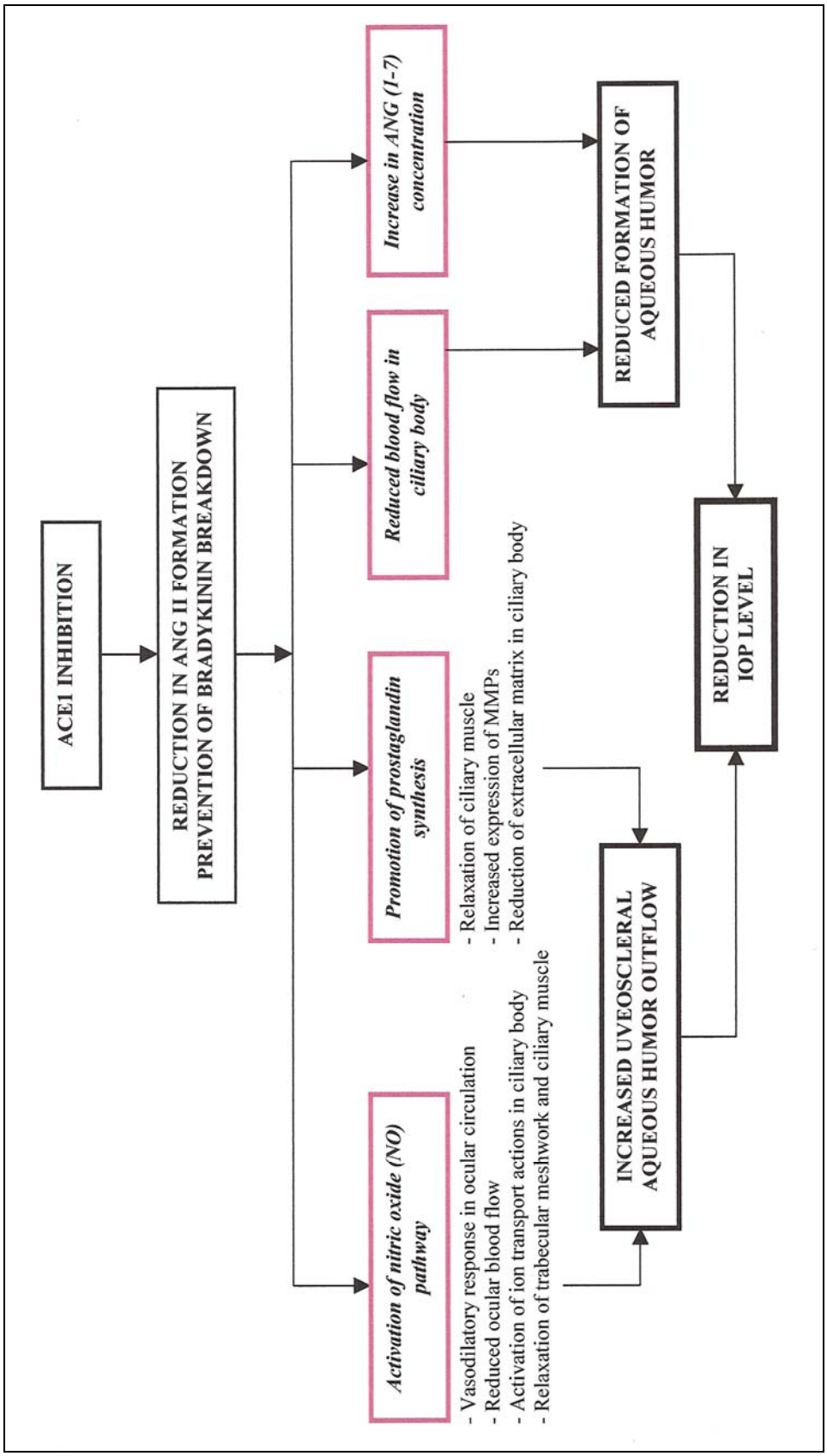

Figure 4. Mechanisms related to the IOP reduction by ACE inhibitors.

RAS and cellular changes characteristic of glaucoma

RAS may play important role in the pathogenesis of optic nerve damage characteristic of glaucoma. Chronic and progressive optic nerve neuropathy involving the death of retinal ganglion cells (RGCs) is essentially affiliated to increased IOP related to glaucoma both in animal models (Ruiz-Ederra *et al*., 2005; Urcola *et al*., 2006) and in human (Harwerth & Quigley, 2006). ANG II is able to enhance apoptosis by binding to AT_2-receptors (Yamada *et al*., 1996), and may therofore contribute to glaucomatous optic nerve neuropathy and RCG death in which apoptosis plays an important role (Guo *et al*., 2005). As a response to glaucoma-related cellular damages, glial cells, especially astrocytes and Müller cells, are activated within the retina (Wang *et al*., 2002) possibly via MAPK/ERK-signalling pathway (Tezel *et al*., 2003). Glial cells play a central role in the regulation of retinal blood flow, and thefore overactivation of these cells results in many detrimental vascular changes mediated by complex mechanisms (For reviews see Flammer & Mozaffarieh, 2007 and Fletcher *et al*., 2008).

Systemic microcirculation and ocular blood flow in retina, optic nerve head and choroid are demonstrated reduced in glaucoma (for review see Flammer *et al*., 2002; Fuchsjäger-Mayrl *et al*., 2004). Many factors are described to influence retinal blood flow. The role of endothelial-derived vasoactive agents, like angiotensins, NO and endothelins, produced at least partly by the activated RAS, have recently been highlighted (for review see Hayreh, 2001). It has been proposed that ANG II-induced vasoconstriction may regulate blood flow via AT_1-receptors in the retinal vasculature. Vasoconstrictive effects have been shown prevented by AT_1-receptor antagonists. Accordingly, vasoconstriction of ocular blood veins has been considered as a pathogenic mechanism in reduced retinal blood flow. (Rockwood *et al*., 1987; Maruichi *et al*., 2004.) Also the IOP level affects the blood flow (for review see Hayreh, 2001), and topical ocular hypotensive medication in general has been demonstrated effective in reducing the incidence of glaucomatous visual field abnormalities and optic disc deterioration (Kass *et al*., 2002).

Based on these observations, it is reasonable to assume that inhibition of RAS function may have neuroprotective effects and it possibly prevents the progression of visual field

defects (Hirooka *et al*., 2005) by influencing directly blood flow and by reducing the IOP level. According to the preliminary study by Inoue *et al*. (2003), RAS inhibition with AT_1-receptor antagonist might be beneficial in reconstitution of the blood flow in the optic nerve nead. Kulkarni *et al*. (1999) have demonstrated that the retinal vascular endothelium releases vasodilating NO and prostaglandin I_2 as a response to ANG II-induced vasoconstriction. Vasoconstrictive effects due to ANG II were most prominent in the smallest vessels (Kulkarni *et al*., 1999).

By preventing ANG II formation and bradykinin breakdown, ACE inhibitors activate NO production and simultaneously reduce formation of ET-1 (Yao *et al*., 1991; Meyer *et al*., 1993). NO is characterized as endothelium-derived relaxing factor (EDRF), and is formed from L-arginine by nitric oxide synthases (NOS). Vasodilating NO regulates retinal vascular tone and therefore significantly contributes to the retinal blood flow by mechanism related to production of cyclic GMP. (For review see Toda & Nakanishi-Toda, 2007.) Accordingly, inhibition of NOS by L-NAME and L-NMMA has been shown to reduce retinal circulation *in vitro* (Haefliger *et al*., 1992; Meyer *et al*., 1993) and *in vivo* in animals (Jacot *et al*., 1998) and in humans (Dorner *et al*., 2003). It appears that also NO regulates blood flow in the optic nerve head (Sugiyama *et al*., 2000).

In the human and porcine retina, ET-1 has been localized mainly in the retinal pigment epithelium, astrocytes in the optic nerve, photoreceptors and retinal vasculature (Ripodas *et al*., 2001; Narayan *et al*., 2004). Enzymes that catalyze the formation of ET-1 from the precursor Big ET-1 are called endothelin-converting enzymes (subtypes: ECE-1 and ECE-2) and have also been found in the bovine retina and optic nerve (Dibas *et al*., 2005). ET-1 is suggested to regulate retinal and choroidal blood flow by activating its receptors located in the retinal and choroid blood vessels (Narayan *et al*., 2004). In contrast to NO, ET-1 has been shown to elicit vasoconstriction in porcine ophthalmic and ciliary arteries (Yao *et al*., 1991; Meyer *et al*., 1993) and in the human ophthalmic artery (Haefliger *et al*., 1992). Consequently, endothelium-derived NO and ET-1 affect profoundly vascular tone of the ophthalmic veins and are involved in the local regulation of ophthalmic microcirculation via RAS activation (Haefliger *et al*., 1992; Meyer *et al*., 1993). Administration of exogenous ET-1 has been shown to elicit significantly decreased retinal (Polak *et al*.,

2003) and choroidal blood flow (Polska *et al*., 2002) in humans. ET-1 induced reduction in blood flow in retina and optic nerve head has also been noticed in several animal studies (Chauhan *et al*., 2004; Sasaoka *et al*., 2006). Furthermore, endothelins are suggested to have wider actions besides regulation of blood flow relating to extracellular matrix changes in the optic nerve head and impacts on neuronal signalling that contributes to retinal ganglion cell (RGC) loss (for review see Chauhan, 2008). Lau *et al*. (2006) have demonstrated RGC loss in the rat eye as a consequence of acute application of ET-1 via intravitreal injection. These findings provide evidence that in excess ET-1 can be considered as a potential contributor to ocular neuropathy in glaucoma (for review see Prasanna *et al*., 2003) and is associated with dysfunction of the ET-1 regulation, possibly due to overactivity within the RAS.

Retinal glial cells, principally astrocytes and Müller cells, significantly contribute to regulation of retinal blood flow in response to neural function, and ensure adequate blood flow in retina (for review see Fletcher *et al*., 2008). Activation of retinal glial cells in glaucoma may also be regulated by RAS, since the localization of RAS molecules within the retina is confirmed predominantly into glial cells, like Müller cells (Berka *et al*., 1995). Furthermore, Prasanna *et al*. (2002) have demostrated that ET-1, activated at least partly by ANG II, induces proliferation of cultured astrocytes derived from human optic nerve head through ET_A/ET_B receptor activation. These findings suggest that ET-1, the levels of which in plasma (Emre *et al*., 2005) and AH (Källberg *et al*., 2002; Prasanna *et al*., 2005) are elevated in glaucoma, may accomplish activation of glial cells within the retina in optic nerve damage during progressive glaucoma, as recently reviewed by Chauhan (2008). Liu and Neufeld (2000) have noticed that astrocytes are capable of inducing the expression of NOS type 2 (NOS2) and contribute to RGC death by producing excessive NO in glaucomatous optic neuropathy.

In the medication of glaucoma, it is essential for its pathogenetic mechanisms that both reduction of IOP and improvement of ocular blood flow, and subsequently prevention of optic neuropathy are considered (for reviews see Grieshaber & Flammer, 2005 and Mozaffarieh & Flammer, 2007). Despite of many animal models of retinal injury, there are only few studies (e.g. Kass *et al*., 2002) evidencing that drugs used currently for the

treatment of glaucoma are able to protect retinal ganglion cells in humans (for review see Woodward & Gil, 2004). According to all the studies descrided above, compounds inhibiting RAS seem to have beneficial effects both on IOP and ocular blood flow, and presumably to vitality of retinal ganglion cells and may therefore be particularly potential drug alternatives for glaucoma.

1.3.2.3 Challenges in the pharmacotherapy of ophthalmic diseases

Medical treatment of ocular diseases is challenging since the eye protecting ocular barriers hinder efficient absorption of drugs, particularly to the posterior eye part. The blood-retina barrier (BRB) is comprised of retinal pigment epithelium and tight junctions that are formed between retinal capillary endothelial cells to prevent free diffusion of substances between the circulating blood and the neural retina. Ophthalmic drugs are primarily administered topically, but this delivery route is inefficient, specifically when the purpose is to reach retinal cells. Currently, posterior eye part diseases are treated by high doses of drug administered intravenously or by injecting drug directly to the vitreous fluid. However, these methods are not optimal due to increased risk of treatment-related adverse reactions. Drug delivery to the anterior eye part, e.g. to the ciliary body, is also challenging. (For reviews see Urtti, 2006 and Gunda *et al*., 2008.) In order to reach therapeutic concentrations, the drug molecule has to pass through the anterior segment barriers, such as corneal and conjunctival epithelia (Gunda *et al*., 2008) and the blood-aqueous barrier (BAB) comprised of nonpigmented epithelium layer of the ciliary body, posterior iridial epithelium and endothelium of the iridial vessels (Cunha-Vaz, 1979).

When pathophysiological glaucoma-related mechanisms are taken into account, optimal drug molecule is necessitated to reach both the anterior (ciliary body) and posterior (retina) eye part at concentrations needed to interfere with AH dynamics in order to reduce the IOP level and to accomplish neuroprotection of RGCs (for review see Woodward & Gil, 2004). Accordingly, new efficient drug-delivery systems for targeted pharmacotherapy exploited e.g. by RAS-inhibiting substances should be developed in order to apply these novel promising therapeutic possibilities. (For review see Urtti, 2006; Gunda *et al*., 2008.)

1.4 Characterization of bioactive ACE-inhibitory lactotripeptides

In addition to previously described ACE inhibitors and ARBs, accumulating evidence indicates that certain small peptides have also beneficial cardiovascular, especially antihypertensive effects (for review see Erdmann *et al*., 2008). Isoleucyl-prolyl-proline (Ile-Pro-Pro, IPP), valyl-prolyl-proline (Val-Pro-Pro, VPP) and leucyl-prolyl-proline (Leu-Pro-Pro, LPP) are proline-rich tripeptides derived from milk protein casein, and are therefore called casokinins and lactotripeptides (for reviews see FitzGerald *et al*., 2004 and Korhonen, 2009). Amino acid sequences corresponding of these peptides are found in the primary structure of the bovine β-casein as follows: IPP f(74-76), VPP f(84-86) and LPP f(151-153) (UniProtKB/Swiss-Prot database: P02666). IPP and VPP have been demonstrated to possess blood pressure-lowering properties associated with inhibition of ACE1 activity, which may provide pharmacological applications for these nutraceuticals (Seppo *et al*., 2002 and 2003; Jauhiainen *et al*., 2005b; for review see Hong *et al*., 2008).

Inhibitory activity of molecules possessing ACE1-inhibitory properties is mainly dependent on a specific chemical structure (for review see Hong *et al*., 2008). Lactotripeptides IPP, VPP and LPP have common structural features that substantially resemble also chemical structures of general ACE inhibitors, like captopril, enalapril and lisinopril (see Figure 5 on page 59). Generally ACE1-inhibitory peptides are characterized as short-chain peptides with hydrophobic amino acid residues at the C-terminus that determine binding properties to ACE1 and thus their ACE1-inhibitory potency (for review see Hong *et al*., 2008). All the amino acids present in the structures of IPP, VPP and LPP are characterized as hydrophobic. C-terminal amino acid residues of ACE1 inhibitors have been assumed to specifically interact with the subsites S1, S1’ and S2’ at active site of ACE1 (for review see Cushman & Ondetti, 1999). The central structural requirements for ACE1 inhibition (pharmacophore) include: a) a terminal carboxyl group for ionic interactions with a positively charged residue assumed in the active site of ACE1 b) an amidic carbonyl group for assumed hydrogen bonding with the ACE1 active site and c) a functional group, such as carboxylate, ketone or thiol, which is supposed to interact with zinc-ion in the ACE1 active site (Kuster & Marshall, 2005; Tzakos & Gerothanassis, 2005). No relationship between the N-terminal structure and the ACE1-inhibitory activity

has been found (for review see López-Fandiño *et al*., 2006). According to distinct pharmacophore points present in the chemical structures of IPP, VPP and LPP peptides, they could be pharmacologically potential as ACE1-inhibitory molecule. The relationship between structural properties and functional activities of ACE1-inhibitory lactotripeptides has not yet been completely elucidated (for review see Hong *et al*., 2008).

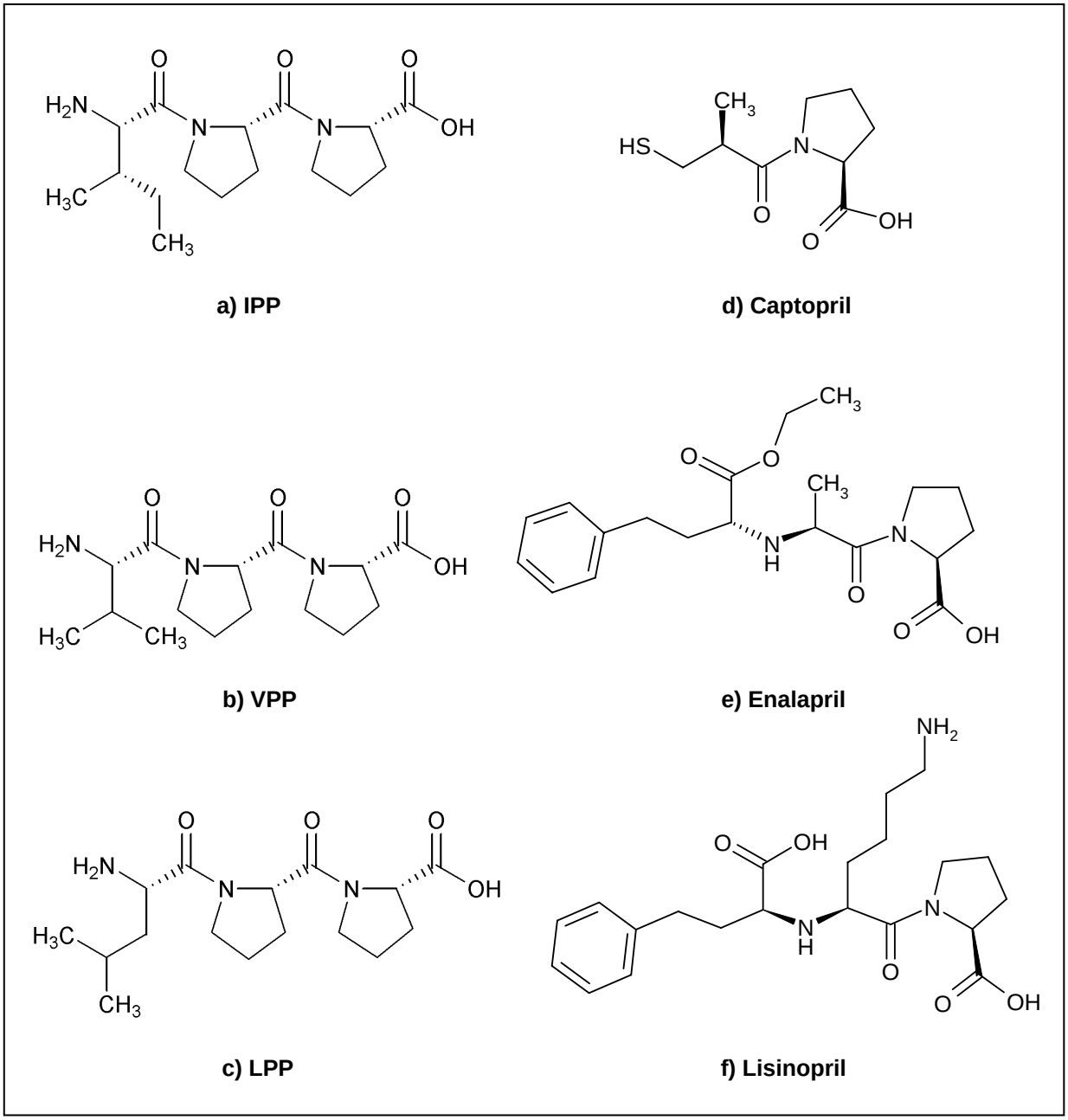

Figure 5. Chemical structures of IPP (a), VPP (b), LPP (c) and general ACE inhibitors captopril (d), enalapril (e) and lisinopril (f). Structures of IPP, VPP and LPP are reproduced with the permission of Bachem Distribution Services GmbH, Germany. Models for the structures of ACE inhibitors were adapted from the DrugBank database: captopril (DB01197), enalapril (DB00584) and lisinopril (DB00722) and were redrawn with MDL ISIS™/Draw Version 2.5.

Bioactive tripeptides IPP, VPP and LPP are inactive within the sequence of the precursor protein, milk casein, but can be released in the gastrointestinal tract by digestive enzymes or by fermentation of milk with proteolytic starter culture or in proteolysis by enzymes derived from certain microorganisms. (For reviews see FitzGerald *et al*., 2004 and López-Fandiño *et al*., 2006). Lactic acid bacteria and their proteolytic activity are well characterised and are consequently widely used as starter strains for applications in production of various fermented dairy products. Certain *Lactobacillus* strains, especially *Lactobacillus helveticus* bacterium and *Saccharomyces cerevisiae* yeast have been demonstrated to produce bioactive peptides from milk protein during fermentation processes on account of their proteolytic systems and are therefore of industrial relevance. (For reviews see FitzGerald *et al*., 2004 and Savijoki *et al*., 2006.) Evolus® (Valio, Finland) and Calpis™ (Calpis Food Industry, Japan) are commercially available functional dairy products supplemented with bioactive peptides IPP and VPP. These peptides are formed industrially from milk β- and κ-caseins during microbial fermentation by combination of *Lb. helveticus* CP790 strain and *S. cerevisiae* (Calpis™) or exclusively by *Lb. helveticus* LBK-16H strain (Evolus®). (For review see López-Fandiño *et al*., 2006; Korhonen, 2009.)

In order to function physiologically *in vivo*, orally administered ACE1-inhibitory peptides should resist digestive proteases and should be absorbed in an active form from the intestine into the circulation to achieve the target organ (for review see Vermeirssen *et al*., 2004). Short peptides containing a C-terminal proline-proline bond are usually resistant to human proteolytic gastrointestinal enzymes, and therefore also IPP and VPP peptides can be assumed being absorbed intact in the intestine (for review see Vermeirssen *et al*., 2004; Ohsawa *et al*., 2008). IPP and VPP peptides have been demonstrated to transport across the human intestinal Caco-2 cell monolayer as intact, mainly via paracellular diffusion (Satake *et al*., 2002; Foltz *et al*., 2007 and 2008). Also Jauhiainen *et al*. (2007a) have provided evidence that IPP absorbs through the intestinal epithelium and reaches the circulation at least partially undegraded without being markedly bound to plasma proteins. Masuda *et al*. (1996) demonstrated that IPP and VPP were detectable in the abdominal aorta of SHR following oral administration of fermented milk, substantiating that these peptides absorb undegraded and have good bioavailability. In addition, short peptides consisting of two or

three amino acids, such as lactotripeptides, are absorbed more rapidly from the small intestine than free amino acids (for review see Webb, 1990). Pijl *et al*. (2008) have recently characterized the pharmacokinetic behaviour of synthetic IPP, VPP and LPP peptides *in vivo* in porcine, and confirmed that these tripeptides are absorbed intact into the blood circulation. They noticed also that the half-life of elimination was maximally 15 minutes, suggesting that bioactive effects related to these peptides are rather acute.

1.4.1 Evidence for antihypertensive effects of IPP and VPP *in vivo*

Efficacy and safety of antihypertensive milk casein-derived tripeptides IPP and VPP have been extensively studied both in hypertensive animal models and in humans in long-term clinical intervention trials (for reviews see López-Fandiño *et al*., 2006 and Korhonen, 2009). Results and details of these studies are summarised in Tables III and IV (pages 66-67).

1.4.1.1 Animal experiments

Several animal experiments have shown that IPP and VPP peptides possess both acute and long-term antihypertensive effects when ingested orally as pure peptides or as ingredients in fermented sour milk products. Yamamoto *et al*. (1994a) have found that ingestion of α- and β-casein hydrolysate of proteinase derived from *Lactobacillus helveticus* CP790 has antihypertensive effects in SHR by oral administration with simultaneous inhibition of ACE1 activity. In another study, Yamamoto *et al*. (1994b) perceived that most fermented milk prepared by strains of *Lactobacillus helveticus* showed significant antihypertensive effects in SHR by oral administration, but the effect was not demonstrable after consumption of milk fermented with other species of lactic acid bacteria. Nakamura *et al*. (1995b) have shown that single oral administration of sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Calpis™) significantly lowers the blood pressure in SHR from 6 to 8 hours after administration. Nakamura *et al*. (1995a) demonstrated also that antihypertensive ACE1-inhibitory effects of fermented sour milk (Calpis™) are mainly attributed to IPP and VPP peptides. Additionally, Masuda *et al*. (1996) noticed that short-term oral administration of that product accomplished significant

decrease in ACE1 activity in the abdominal aorta of SHR that was related to local presence of ACE1-inhibitory tripeptides IPP and VPP. These differences were not seen in the aorta extracts of normotensive Wistar Kyoto control rats. Antihypertensive effects have not been shown in animals with normal blood pressure level, but are restricted to hypertensive animal models (Nakamura *et al*., 1995b; for review see FitzGerald *et al*., 2004).

In experiments by Sipola *et al*. (2001 and 2002), IPP and VPP have been shown to attenuate the development of hypertension in SHR also after long-term (12- and 14-week) oral administration of *Lb. helveticus* LBK-16H fermented milk rich in IPP and VPP peptides. Results from the recent experiments by Jauhiainen *et al*. (2005a) are in accordance with these previous findings.

1.4.1.2 Clinical studies

Antihypertensive effects of IPP and VPP in humans have been confirmed in several randomised, placebo-controlled studies and were also substantiated in two recent meta-analyses performed by Pripp *et al*. (2008) and Jauhiainen *et al*. (2009). Ingestion of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* fermented sour milk containing IPP and VPP has been shown to lower both systolic and diastolic blood pressure levels in hypertensive subjects during eight-week (Hata *et al*., 1996; Kajimoto *et al*., 2001b) and four-week (Mizushima *et al*., 2004) intervention periods. Similarly, continuous consumption of sour milk fermented with *Lactobacillus helveticus* strain LBK-16H has been demonstrated to accomplish reduction of blood pressure during eight-week (Seppo *et al*., 2002), ten-week (Jauhiainen *et al*., 2005b) and 21-week (Seppo *et al*., 2003) consumption periods. Antihypertensive effects have also been shown when peptides were administered orally in tablet form in clinical studies by Kajimoto *et al*. (2001a), Aihara *et al*. (2005) and Mizuno *et al*. (2005).

In human studies, reduced blood pressure levels were noticed only in subjects with mild to moderate hypertension. No marked changes have been observed in healthy subjects with normal blood pressure, when exposed even to high doses of IPP and VPP (e.g. Itakura *et al*., 2001; Yasuda *et al*., 2001). Additionally, no significant adverse effects related to

ingestion of lactotripeptide-enriched sour milk products (e.g. Hata *et al*., 1996; Jauhiainen *et al*., 2005b) or tablets containing pure IPP and VPP (e.g. Kajimoto *et al*., 2001a) have been detected in these studies. Similarly, toxicological studies have not revealed any deleterious effects associated with application of ACE1-inhibitory lactotripeptides IPP and VPP, even at very high doses (4000 mg/kg/day), and can therefore be consireded safe (Kurosaki *et al*., 2005; Nakamura *et al*., 2005; Dent *et al*., 2007). Despite of referred clinical studies that provide evidence for antihypertensive effects of lactotripeptides IPP and VPP, there are also some very recently published studies (Engberink *et al*., 2008; van der Zander *et al*., 2008) claiming that these peptides do not significantly change the blood pressure level.

1.4.2 Mechanisms related to antihypertensive effects of IPP, VPP and LPP

Inhibition of angiotensin-converting enzyme 1 (ACE1)

Mechanisms behind the antihypertensive effects of IPP, VPP and LPP are not exactly known. ACE1 inhibition is admittedly one and the most studied of those mechanisms, and these peptides are regarded as competitive substrates for ACE1 (for reviews see Erdmann *et al*., 2008 and Hong *et al*., 2008). The ACE1-inhibitory effects of these peptides have been shown *in vitro*. IC_{50}-values (the concentration at which ACE1 activity is inhibited by 50% *in vitro*) determined for milk casein –derived IPP and VPP peptides are 5 μM and 9 μM, respectively (Nakamura *et al*., 1995a; Pan *et al*., 2005). Antihypertensive ACE1-inhibitory effect of γ-zein –derived LPP peptide (IC_{50} 9.6 μM) was reported by Maruyama *et al*. (1989). Lehtinen *et al*. (2008) have confirmed that inhibitory potencies of synthetic IPP, VPP and LPP peptides on activity of pure ACE1 enzyme are equal. Also *in vivo*-experiments provide evidence for ACE1 inhibition by IPP and VPP. ACE1 activities in tissues of SHR have been shown reduced after a single and long-term oral administration of *Lb. helveticus* and *S. cerevisiae* fermented milk rich in IPP and VPP peptides (Masuda *et al*., 1996; Nakamura *et al*., 1996). However, when compared to captopril, a widely used ACE1 inhibitor, lactotripeptides have lower ACE1-inhibitory potency *in vitro* (for review see López-Fandiño *et al*., 2006). Captopril is even a thousand times more efficient in ACE1 inhibition than synthetic IPP, VPP and LPP peptides (Lehtinen *et al*., 2008). Foltz *et al*. (2007) have reported that during daily consumption of lactotripeptide-enriched milk

containing high doses of IPP (20.4 mg), VPP (20.0 mg) and LPP (16.5 mg) peptides, the maximum plasma concentration of e.g. IPP was only about 1 nM, which is far below its IC_{50}-value determined *in vitro*. In a study by Pijl *et al*. (2008), maximum plasma concentrations of IPP, VPP and LPP were shown to be about 10 nM for all peptides following daily administration of synthetic peptides as a mixture containing 4.0 mg IPP, 4.0 mg VPP and 4.0 mg LPP per kilogram of body weight. However, *in vivo* comparative studies with captopril have also shown that antihypertensive ACE1-inhibitory peptides exhibit higher *in vivo* inhibitory potency than could be expected from their *in vitro* activity (for review see Erdmann *et al*., 2008). However, it is possible that lactotripeptides lower blood pressure also via other mechanisms than ACE1 inhibition (Foltz *et al*., 2007).

Other possible mechanisms

Additional mechanisms related to antihypertensive effects of lactotripeptides include e.g. opiod-like activity, mineral-binding and antithrombotic properties (for reviews see López-Fandiño *et al*., 2006 and Jauhiainen & Korpela, 2007). Release of vasodilatory substances, like NO, could also contribute to blood pressure-lowering effects of IPP and VPP, as recently demonstrated by Kim *et al*. (2008). When administered in milk products, milk minerals like calcium (Griffith *et al*., 1999) and magnesium (Kawano *et al*., 1998; Jee *et al*., 2002) may also contribute to blood pressure level along with bioactive lactotripeptides, as shown by Jauhiainen *et al*. (2005a). According to Foltz *et al*. (2007), plasma concentrations of IPP and LPP increased significantly when ingested in the form of lactotripeptide-enriched dairy products. In addition to ACE1, also other ANG II-forming enzymes, like chymase may contribute to blood pressure lowering effects associated with bioactive lactotripeptides (Yamamoto *et al*., 1999).

It is known that ACE1-inhibitory drugs, like ramipril reduce arterial stiffness by functional mechanisms including mean pressure reduction, alterations in composition of extracellular matrix and also by reducing production of vasoconstrictive ANG II and subsequently aldosterone production (Ahimastos *et al*., 2005). Aldosterone has been demonstrated to have deleterious effects on vasculature by increasing collagen content and fibrosis (for review see Mahmud & Feely, 2004). Lactotripeptides IPP and VPP have also been shown

to have beneficial effects on vascular endothelium and arterial tone in *in vitro* experiments (Jäkälä *et al*., 2008). Jauhiainen *et al*. (2007b) have demonstrated that consumption of lactotripeptide (IPP and VPP) –enriched *Lb*. *helveticus* LBK-16H fermented milk reduces arterial stiffness in hypertensive subjects. Continuous intake of casein hydrolysate containing abundantly antihypertensive peptides IPP and VPP has recently been shown to improve vascular endothelial function independent of blood pressure-lowering effects in a clinical study accomplished by Hirota *et al*. (2007). Accordingly, by improving vascular function, lactotripeptides might also have beneficial effects on blood pressure. In conclusion, IPP and VPP provide alternative to pharmacotherapeutical treatment of hypertension and could be applied as initial treatment in mildly hypertensive individuals or as supplemental treatment (for review see Erdmann *et al*., 2008).

Table III. Animal studies on the antihypertensive effects of lactotripeptides IPP and VPP in SHR.

Product(s)	*Duration (h/wk)*	*Peptide dose (mg/kg of body weight/day)*	*(Maximal) reduction in SBP (mmHg)*	*References*
Casein hydrolysate of purified proteinase from *Lb. helveticus* CP790	10 h	15 and 75	Dose of 15 mg/kg/day 21.6 ± 4.9 after 6h Dose of 75 mg/kg/day 29.8 ± 8.9 after 4h	Yamamoto *et al.*, 1994a
I Fermented milk containing IPP and VPP (*Lb. helveticus*, *S. cerevisiae*) II Pure peptides IPP and VPP	24 h	IPP 0.3 and VPP 0.6	I Fermented milk: 21.8 ± 4.2 after 6h II Pure peptides: VPP: 32.1 ± 5.3 after 4h IPP: 28.3 ± 4.8 after 8h	Nakamura *et al.*, 1995b
I Fermented milk containing IPP and VPP (*Lb. helveticus* LBK-16H) II Pure peptides IPP and VPP	12 wk	IPP and VPP 2.5-3.5	I Fermented milk: 17 *vs control* II Pure peptides: 12 *vs control*	Sipola *et al.*, 2001
Milk fermented by *Lb. helveticus* strains (I) CHCC641 and (II) CHCC637, contains various ACE inhibitory peptides	8 h	Not reported	Mean arterial pressure: 11 (I) and 12 (II) *vs control*	Fuglsang *et al.*, 2002
Fermented milk containing IPP and VPP (A *Lb. helveticus* LBK-16H) (B *Lb. helveticus*, *S. cerevisiae*)	14 wk	IPP 0.4 (A) and 0.2 (B) mg/day VPP 0.6 (A) and 0.3 (B) mg/day	21 *vs control* (A) 10 in group A *vs* group B	Sipola *et al.*, 2002
I Fermented milk containing IPP and VPP (*Lb. Helveticus* LBK-16H) II Pure peptides IPP and VPP III Peptides IPP and VPP + minerals (potassium, sodium, calcium, magnesium) IV Minerals (potassium, sodium, calcium, magnesium)	9 wk	I Fermented milk: IPP and VPP 1.5 ± 0.1 mg/day II Pure peptides: IPP and VPP 2 ± 0.2 mg/day III Peptides + minerals: IPP and VPP 1.7 ± 0.3 mg/day IV Minerals: No IPP and VPP	I Fermented milk: 16.9 *vs control* II Pure peptides: 8.3 *vs control* III Peptides + minerals: 12.7 *vs control* IV Minerals: 13.5 *vs control*	Jauhiainen *et al.*, 2005a

SBP = systolic blood pressure

Table IV. Clinical studies on the antihypertensive effects of lactotripeptides IPP and VPP.

Product(s)	*Peptide dose (mg/day)*		*Duration (wk)*	*Subjects (n)*	*(Maximal) decrease in SBP/DBP (mmHg)*		*References*
	IPP	*VPP*			*SBP*	*DBP*	
Fermented milk containing IPP and VPP (*Lb. helveticus* CM4, *S. cerevisiae*)	1.1	1.5	8	30	14.1 ± 3.1	6.6 ± 2.5	Hata *et al.*, 1996
Fermented milk containing IPP and VPP (*Lb. helveticus, S. cerevisiae*)	1.1	1.5	8	18+26[H]	12.1 ± 4.7 7.6 *vs control*	Not reported	Itakura *et al.*, 2001
Tablets containing IPP and VPP (*Lb. helveticus* CM4)	1.64	2.52	8	81	12.4 ± 10.9	8.1 ± 12.7	Kajimoto *et al.*, 2001a
Fermented milk containing IPP and VPP (*Lb. helveticus* CM4, *S. cerevisiae*)	1.52	2.53	8	30	13.7 ± 4.3 *vs control*	7.4 ± 1.1 *vs control*	Kajimoto *et al.*, 2001b
Tablets containing IPP and VPP (*Lb. helveticus* CM4)	11.5	17.7	1	20[H]	2.7 ± 0.7	1.2 ± 0.8	Yasuda *et al.*, 2001
Fermented milk containing IPP and VPP (*Lb. helveticus* LBK-16H)	2.25	3-3.75	8	17	7.3% *vs control*	7.3% *vs control*	Seppo *et al.*, 2002
Fermented milk containing IPP and VPP (*Lb. helveticus* LBK-16H)	2.25	3-3.75	21	39	6.7 ± 3.0 *vs control*	3.6 ± 1.9 *vs control*	Seppo *et al.*, 2003
Fermented milk containing IPP and VPP (*Lb. helveticus, S. cerevisiae*)	1.15	1.98	4	46	5.2 3.7 *vs control*	2 1.7 *vs control*	Mizushima *et al.*, 2004
Fermented milk containing IPP and VPP (*Lb. helveticus* LBK-16H)	2.4-2.7	2.4-2.7	8-10 (P1) 5-7 (P2)	59 (P1) 39 (P2)	6.4 *vs control*	1.5 *vs control*	Tuomilehto *et al.*, 2004
Fermented milk containing IPP and VPP (*Lb. helveticus* LBK-16H)	22.5	30	10	94	4.1 ± 0.9 *vs control*	1.8 ± 0.7 *vs control*	Jauhiainen *et al.*, 2005b
Tablets containing powdered fermented milk rich in IPP and VPP (*Lb. helveticus* CM4)	4.7	8.3	4	40+40[x]	*High-normal blood pressure* **SBP**: 3.2 mmHg, **DBP**: 5 mmHg *vs control* *Mild hypertension* **SBP**: 11.2 mmHg, **DBP**: 6.5 mmHg *vs control*		Aihara *et al.*, 2005
Tablets containing IPP and VPP (*Aspergillus oryzae*)	1.8-3.6	1.8-3.6	6	131	*High-normal blood pressure* **SBP**: 2.8 mmHg **DBP**: 1.4 mmHg *Mild hypertension* **SBP**: 13 mmHg **DBP**: 4.2 mmHg (at highest doses of peptides)		Mizuno *et al.*, 2005

[H] Healthy subjects with normal blood pressure level (mild to moderate hypertension if not marked)
[x] 40 subjects with high-normal blood pressure and 40 with mild hypertension
SBP = systolic blood pressure, DBP = diastolic blood pressure

2 AIMS OF THE STUDY

An active local renin-angiotensin system (RAS) has recently been found in the human eye. Increasing evidence appeals to its significance in the regulation of ocular functions, especially intraocular pressure and blood flow. Consequently, RAS is suggested to be associated with pathophysiology of glaucoma.

Aims of the present study were as follows:

1. To determine activities of angiotensin-converting enzymes 1 (ACE1) and 2 (ACE2) *in vitro* in certain tissues (retina, ciliary body, vitreous body) of porcine eye, morphologically and physiologically close to the human eye.

2. To test inhibitory effects of three bioactive peptides IPP, VPP and LPP on activities of ACE1 and ACE2 in porcine-derived ocular tissues.

3. To provide an overview of the current knowledge related to presence of intraocular RAS and its implications on ocular pathophysiology, especially in glaucoma.

3 MATERIALS AND METHODS

3.1 Sample preparation

Fresh porcine eyes obtained from a local abattoir (Paimion teurastamo Oy, Paimio) were used in this study. The experimental procedures for animals were in accordance with the statement given by the ARVO (Association for Research in Vision and Ophthalmology) for the use of animals in ophthalmic research. Eyeballs were carefully enucleated from domestic pigs at an age of approximately 5-6 months (weight about 80 kg), and put on ice within ten minutes after CO_2 narcosis and sacrifice. Enucleated eyes were handled in pairs and placed in Minigrip® plastic bags, which were sealed tightly and kept on ice in a closed styrox box during transportation (duration two hours) to the laboratory (Santen Oy, Research Center, Tampere).

Eyes were carefully prepared under a stereoscopic dissecting microscope within five hours post mortem. Firstly, excess tissues, muscles and conjunctiva were removed from the sclera with scissors and tweezers. Vitreous body samples were aspirated with a needle (Microlance™ 3, 18G × 1 ½"; Beckton Dickinson, NJ, USA) attached to a disposable 1ml plastic syringe. Approximately 250 µl of vitreous fluid per eye was collected. After that the eye was hemisectioned at the ora serrata into anterior and posterior segments with scalpel and scissors. Remaining vitreous mass was carefully removed from the posterior segment. Then incisions were performed to the posterior eyecup and the retina was gently scraped off from its bed and removed by cutting out from papilla. After that the lens, capsule and excess vitreous mass were isolated from the anterior bisection of the eye, and the iris was removed by cutting along the line in its root. The ciliary body was pulled loose from the sclera with a pair of forceps.

Preparation of the eyes was performed on a plastic platform placed in a glass Petri dish, which was kept on ice. In order to prevent drying of ocular tissues during the preparation, they were moisturised with sterilized, physiological (0.9 % [w/v]) NaCl solution (Fresenius Kabi Ab, Helsinki, Finland), if necessary. Preparation was performed by an ophthalmologist (Anu Vaajanen, M.D., Ph.D.). The respective tissue types from both eyes

of the same animal were pooled by transferring them into the same preweighed and chilled Eppendorf tube. Tubes were weighed with an analytical balance (GR-202; A&D Company, Limited, Tokyo, Japan) to get wet weights of tissues. Pooled samples were used in order to ensure detectable levels of ACE enzymes. Tissue pieces were crudely chopped with a pair of scissors and after that were homogenized in ice-cold 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3) with a homogenizer (Silent Crusher S; Heidolph Instruments GmbH & Co.KG., Schwabach, Germany) for 20-30 seconds. Samples for measurement of ACE1 activity were diluted in 0.6 ml of aforementioned buffer, whereas samples for measurement of ACE2 activity were diluted in 1.5 ml of that buffer. The purpose of the homogenization was to enhance shattering of tissues and thus release of RAS enzymes. Exceptionally, vitreous body samples were not homogenized, but used as such in enzyme activity measurements because of its liquid appearance. All samples were kept on ice during the homogenization procedure. Samples were mixed thoroughly with a vortex mixer (REAX 1R 54113; Heidolph Instruments GmbH & Co.KG., Schwabach, Germany) and divided into aliquots to three Eppendorf tubes, which were immediately transferred to a deep-freezer (-80ºC) for storage until measurement of enzyme activities and protein concentration.

3.2 ACE1 activity measurement

The ACE1 activity was determined from the amount of the dipeptide L-histidyl-L-leucine (abbreviated as His-Leu) formed by enzymatic breakdown of a synthetic substrate hippuryl-L-histidyl-L-leucine (abbreviated as Hip-His-Leu or HHL), according to the applied method described by Friedland and Silverstein (1976). Hip-His-Leu corresponds the terminal sequence of ANG I, a natural substrate for ACE1 enzyme. Substrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). ACE1 catalyses the degradation of the substrate and the extent of His-Leu release from HHL is directly related to the activity of ACE1 enzyme within the sample.

Principle of the assay:

$$\text{Hip-His-Leu} + H_2O \xrightarrow{\text{ACE1}} \text{Hippuric acid} + \text{His-Leu}$$

Modifications and enhancements to the method first described by Friedland and Silverstein (1976) were done by taking into account methodological aspects noticed in studies of Santos *et al*. (1985) and Oliveira *et al*. (2000) published afterwards. In accordance with these observations, optimal reaction conditions for measurement of ACE1 activity in porcine ocular tissues were determined and used in this study (see Table V on page 74). ACE1 activity measurements were performed in Santen Oy Research Laboratory.

For measurement of ACE1 activity, tissue homogenates (retina, ciliary body) were thawed and centrifuged at 2000 × *g* for 20 minutes in a cooled (+4ºC) centrifuge (Minifuge T; Heraeus-Christ GmbH, Osterode am Harz, Germany). Supernatants were separated and kept on ice before the assay. For the assay, supernatants from the ciliary body homogenates (*n=6*) were diluted in proportion of 1:2 in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3). Vitreous body samples (*n=6*) were solely thawed and immediately used as such in reactions. Supernatants from the retina homogenates (*n=6*) were also analysed as undiluted. Reactions were performed in brown, light-impervious glass tubes enabling the use of light sensitive fluorescent detection reagent. During the reaction procedure tubes were tightly covered with caps in order to prevent evaporation of reaction mixtures. After each reagent addition, reaction mixtures were shaken with vortex mixer or by pipeting back and forth. All reagents used were of analytical grade.

In the present study, 10 µl of the sample was incubated with 240 µl of assay buffer containing 5 mM HHL (in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3) in a water bath (TurboVap® LV Evaporator; Zymark Ltd., Runcorn, Cheshire, UK) at +37ºC for 60 minutes. Before initiating the reaction by addition of freshly prepared substrate solution, test tubes containing sample and buffer were pre-incubated three minutes in the water bath. After the incubation with the substrate the reaction was stopped by addition of 1.5 ml of 0.3 M NaOH. For fluorometric quantitation of His-Leu, 100 µl of freshly prepared 2% ([w/v], in methanol) *ortho*-phthaldialdehyde OPA (Sigma-Aldrich,

Vienna, Austria) was added to the reaction mixture, and tubes were allowed to stand for ten minutes at room temperature. Primary amines (herein His-Leu) form fluorescent adducts when reacted with OPA in alkaline conditions. After the incubation, the reaction mixture was acidified by addition of 200 µl of 3 M HCl to halt the reaction and to stabilize the fluorescence. Finally, the reaction mixtures were centrifuged (Rotixa P; Hettich Instruments, Lp., Beverly, MA, USA) at 3000 rpm for ten minutes at room temperature to pellet precipitated proteins.

The reaction product His-Leu was measured fluorometrically with LS55 Luminescence spectrometer in a cuvette (Luminescence Spectroscopy Cells, Part No. B0631104), both purchased from Perkin Elmer Life and Analytical Sciences Inc., Boston, MA, USA. Excitation and emission wavelengths used were 360 nm and 500 nm respectively and the instrument was set to zero against above mentioned assay buffer. Each sample was determined in duplicate within 20-second intervals and the mean was used in calculations. To correct for the intrinsic fluorescence derived from the each tissue, tissue blanks were included and were performed in a similar manner as described above but the sample was not incubated with the substrate, but was added after NaOH treatment. Fluorescence of tissue blank was subtracted from the fluorescence of the sample. Additionally, reaction blank (buffer added instead of a sample) were analysed as a control for the determination of background fluorescence caused solely by reagent ingredients. Reaction blank fluorescence was subtracted from standards.

The specificity of the assay was determined by including captopril (Sigma-Aldrich, Schnelldorf, Germany), a commonly used ACE1 inhibitor, as a control at a final concentration of 1 µM for each sample. Inhibitor was added to the reaction by replacing 25 µl of buffer with it (see Table V on page 74). Captopril-inhibited samples were analysed as duplicate. Pure ACE1 enzyme extracted from rabbit lung (Sigma-Aldrich, St. Louis, MO, USA) was also analysed as a positive control in the assay. ACE1 enzyme was added to the control instead of unknown sample by the same volume. A known quantity of His-Leu (Sigma-Aldrich, Buchs, Switzerland) diluted in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3), was used for establishing the standard curve from which the quantity of His-Leu released in the assay can be converted by comparing fluorescence

of standard with known His-Leu concentration to fluorescence of the samples. Different dilutions of His-Leu standard (a range from 0.08 to 100 µM as final concentrations in the reaction) were prepared and those were treated in the same way as samples and were determined in duplicate. Basal enzyme activity was characterized as micromoles of liberated dipeptide His-Leu per one minute under the assay conditions. Calculation of results is described accurately in the chapter 3.5 on page 80.

Measurement of ACE1 inhibition

The synthetic peptides (IPP, VPP, LPP) were assayed *in vitro* for their capacity to inhibit the ACE1 activity within ocular tissues according to the method described above. Inhibitory effects of tripeptides were tested at final concentrations from 1 to 330 µM and captopril, a widely used synthetic ACE inhibitor, at concentrations from 1 to 100 nM. These concentrations were designated on ground of the results of preliminary experiments with pure ACE1 enzyme performed by Lehtinen *et al*. (2008). Inhibitors were diluted to appropriate concentrations in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3). Inhibitors were added to the reaction by replacing 25 µl of the assay buffer solution (see Table V, page 74) by certain inhibitor. Inhibitor was pre-incubated with sample for 5 minutes at room temperature before the addition of substrate. Tripeptides H-Ile-Pro-Pro-OH (IPP), H-Val-Pro-Pro-OH (VPP) and H-Leu-Pro-Pro-OH (LPP) were purchased from Bachem AG (Bubendorf, Switzerland) and captopril from Sigma-Aldrich (Schnelldorf, Germany). Samples used in the assay were pooled from several supernatants of certain tissue homogenate after being centrifuged at 2000 × *g* for 20 minutes (+4ºC) and each pooled tissue sample was determined duplicate (*n=2*) for basal ACE1 activity and quadruble (*n=4*) for inhibited ACE1 activity by each devised concentration of the inhibitor. In the inhibitory assay ciliary body sample was used as a dilution of 1:2 (in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3), whereas retina and vitreous body samples were analyzed as undiluted. Pooled samples were used in order to ensure detectable levels of basal and inhibited ACE1 activity. As ACE1 catalyses the degradation of the substrate, the inhibitory effect could be derived directly from the decrease in fluorescence during the reaction. Calculation of the results is described in the chapter 3.5 on page 80.

Table V. Procedure for the assay of basal and inhibited ACE1 activity in porcine ocular tissues.

	S (sample)	**I** (inhibited sample)	**TB** (tissue blank)	**RB** (reaction blank)	**St** (standard)
Sample (µl)	10	10	-	-	-
His-Leu Standard (µl)	-	-	-	-	10
Inhibitor (µl)	-	25	-	-	-
Assay buffer (µl)	115	90	115	125	115
Incubated at +37ºC for 3 min					
10 mM HHL substrate solution (µl)	125	125	125	125	125
Incubated at +37ºC for 60 min					
Sample (µl)	-	-	10	-	-
0.3 M NaOH (ml)	1.5	1.5	1.5	1.5	1.5
2 % [w/v] OPA (µl)	100	100	100	100	100
Incubated at room temperature for 10 min					
3 M HCl (µl)	200	200	200	200	200
Centrifuged at 3000 rpm for 10 min					
Supernatant separated for analysis and then measured fluorometrically (λ_{ex} =360 nm and λ_{em} = 500 nm)					

(-) Not added

3.3 ACE2 activity measurement

ACE2 activity in certain porcine ocular tissues was determined by using commercial assay kit: SensoLyte™ 390 ACE2 Activity Assay Kit (AnaSpec, Inc., San Jose, CA, USA), which is based on the fluorescence resonance energy transfer (FRET). Synthetic peptide Mc-Ala/Dnp is used as a substrate, which is cleaved into two separate fragments Mc-Ala and Dnp by ACE2 enzyme. Upon cleavage, Dnp quenches the fluorescence of Mc-Ala resulting in increased fluorescence, which can be monitored fluorometrically at wavelengths of 330 nm (excitation) and 390 nm (emission). The intensity of the fluorescence is related to the activity of the ACE2 enzyme in the sample.

Principle of the assay:

$$\text{Mc-Ala/Dnp} \xrightarrow{\text{ACE2}} \text{Mc-Ala + Dnp}$$

ACE2 activity measurements were performed at Institute of Biomedicine (Pharmacology), Biomedicum Helsinki, University of Helsinki. Samples were taken out from a deep-freezer (-80ºC) and were kept frozen during transportation (Tampere-Helsinki, duration 2.5 hours) by transferring samples immediately to a styrox box containing half of its volume of carbon dioxide snow (Oy AGA Ab, Finland). After the transportation homogenates were thawed and centrifuged at maximum speed of 20 000 × *g* for 10 minutes in a cooled (+4ºC) Eppendorf-centrifuge, model 5417R (Eppendorf AG, Hamburg, Germany). Supernatants were separated, diluted 1:30 (retina, *n=5*) and 1:5 (ciliary body *n=6*) in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3). Vitreous body samples (*n=5*) were analysed undiluted. Samples were kept on ice until used in enzyme activity measurement. All reagents used were of analytical grade.

Determination of ACE2 activity was performed according to instructions given by the manufacturer of the assay kit. Exceptionally, samples used in the assay were homogenized and diluted in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3) instead of the assay buffer included in the kit. Inhibitors, controls and standards were also diluted in the aforementioned buffer for the standardization of the assay and thus enabling the

comparison of the fluorescence values from unknown samples, standards and controls. Effect of buffer used and assay buffer recommended for the use in the kit instructions on ACE2 activity was tested by homogenizing the same tissue sample to both of these buffers. No significant differences were observed between these buffers and therefore 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl (pH 8.3) was accepted for the use since the amount of the assay buffer included in the kit was insufficient to be used both for sample preparation and performing the assay as instructed.

Assays were performed in black 96-well microplates (OptiPlate-96 F from Perkin Elmer, Inc., Boston, MA, USA) at room temperature. In basal ACE2 activity measurements, 50 µl of each sample was added to a bottom of a well with 50 µl of Mc-Ala/Dnp substrate diluted 1:100 in the kit assay buffer (at the final concentration of 25 µM) as instructed. Immediately after that the plate was gently shaken for 10 seconds. The fluorescence of Mc-Ala was determined with fluorescence plate reader (Wallac Victor^2 1420 Multilabel Counter; Perkin Elmer, Inc., Boston, MA, USA) by measuring continuously and recording at five-minute intervals the increase in fluorescence upon substrate hydrolysis during the 40 minutes time course (measurement time 1.0 second/well). Deviated from the kit instructions, used wavelengths were: excitation 340 nm and emission 405 nm instead of 330 nm/390 nm, due to filters that were available for the instrument used in measurements.

Samples were measured both in the absence and presence (at a final concentration of 100 nM) of a highly specific ACE2 inhibitor DX600 to confirm the specificity of used technique for measuring ACE2 activity in tissues. Each sample was analyzed in duplicate and the mean was used in calculations. Intrinsic fluorescence derived from each tissue type (substrate replaced by the assay buffer) was also measured and proved to be non-significant. Thus, only fluorescence of reaction blank control (sample replaced by the same volume of buffer used in sample dilutions) was subtracted from the fluorescence values of samples. Pure ACE2 enzyme obtained from Calbiochem (San Diego, CA, USA) was also used as a positive control in the assay (sample volume replaced by ACE2).

Mc-Ala fluorescence reference standard (included in the kit) was used for establishing the standard curve from which the amount of Mc-Ala released in the assay can be quantified

by comparing the blank control-corrected fluorescence of standard dilutions (relative fluorescence units RFU) with known Mc-Ala concentration to those of samples. Standard solutions containing a range of 0.075 to 5 µM of Mc-Ala at final concentrations were prepared according to kit instructions. Enzymatic activity was characterized as micromoles of formed reaction product Mc-Ala per one minute under the assay conditions. Calculation of results is described accurately in the chapter 3.5 on page 80.

Measurement of ACE2 inhibition

ACE2-inhibitory activity of three synthetic tripeptides (IPP, VPP and LPP) was assayed *in vitro* by the method described above. Inhibitory effects of tripeptides were tested at concentrations from 1 to 10 mM. These concentrations were designated on ground of the results obtained in preliminary experiments with pure ACE2 enzyme performed by Lehtinen *et al*. (2008). Specific ACE2 inhibitor DX600 (included in the assay kit) was tested at a concentration of 100 nM. pH values from each tripeptide dilution used in the inhibitory assay was checked with BlueLine pH 23 Electrode (Schott Instruments GmbH, Mainz, Germany). Even at highest tripeptide concentrations, they were not shown to alter pH in the dilution buffer. Samples used in assay were pooled from several supernatants of certain tissue homogenates after being centrifuged at 20 000 × *g* for 10 minutes (+4ºC). Pooled samples representing each tissue were diluted 1:30 (retina), 1:5 (ciliary body) and 1:2 (vitreous body). Samples were determined sixfold (*n=6*) for basal ACE2 activity and also for inhibited ACE2 activity by tripeptides IPP, VPP and LPP and DX600 (*n=6*) and in duplicate (*n=2*) for inhibited activity by captopril at each devised concentration and in each tissue. Pooled samples were used in order to ensure detectable levels of basal and inhibited ACE2 activity.

In the measurement of basal ACE2 activity in the pooled tissue sample, 40 µl of each tissue sample and 10 µl of buffer (0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3) were added to bottom of a well in 96-well plate. Then 50 µl of substrate dissolved 1:100 in assay buffer (at final concentration of 25 µM) was added to each well. Inhibited ACE2 activity was determined by adding certain inhibitor to the reaction by

replacing 10 µl of the buffer solution with the inhibitor by the same volume. Inhibitor was pre-incubated with sample for 5 minutes at room temperature before addition of substrate.

3.4 Measurement of protein concentration

The protein concentration of samples were determined by the widely used method described by Lowry *et al*. (1951). This method is based on two different reactions. In the first reaction (called Biuret reaction), divalent copper ions react with the nitrogens in the peptide bonds of proteins forming complexes and reduced copper (Cu^{+}) under alkaline conditions. This reaction is stabilized by the addition of tartrate. In the second reaction (called Folin-Ciocalteau reaction) protein-copper complexes react with the Folin-phenol reagent, which is a mixture of phosphotungstic acid and phosphomolybdic acid in phenol. In this reaction the reagent becomes reduced to molybdenum/tungsten blue by the copper-catalyzed oxidation. The reaction product can be detected colorimetrically with a spectrophotometer at wavelength of 750 nm. The intensity of the blue colour is proportional to the sample protein concentration, albeit it depends partly upon the presence of tyrosine and tryptophan residues in the sample proteins.

Protein concentrations were used for the calculation of the ratio of enzyme activity to the sample protein level (units [U]/grams [g] of protein). Only retina and ciliary body samples were analysed because the volumes of vitreous body samples were inadequate to this analysis. Accordingly, ACE1 and ACE2 activities in the vitreous body were calculated as the amount of formed reaction product in one minute per unit of volume (mol/min/ml). Protein concentration measurements were performed at Institute of Biomedicine (Pharmacology), Biomedicum Helsinki, University of Helsinki. All reagents used were of analytical grade. The aliquots of samples (supernatants from tissue homogenates) representing those used earlier in basal ACE1 and ACE2 activity measurements were taken out from a deep-freezer (-80ºC) and thawed. For the protein quantitation retina samples were diluted 1:50 and ciliary body samples 1:25 in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3.

At the beginning, 100 µl of each sample dilution and 1000 µl of the Reagent 1 were added into test tubes, mixed thoroughly with VF2 –vortex (Janke & Kunkel, IKA Labortechnik, Stauffen, Germany) and incubated for ten minutes at room temperature. Reagent 1 was prepared by mixing the following solutions in the proportion of 100:1:1 (by volume); 2% [w/v] Na_2CO_3 in 0.1 M NaOH, 1% [w/v] $CuSO_4 \cdot 5\ H_2O$ in distilled water and 2% [w/v] NaK-tartrate in distilled water, respectively. After that, 100 µl of freshly prepared dilution of Reagent 2 was added to the reaction mixture, mixed immediately and incubated for 30 minutes at room temperature. Reagent 2 denotes 2 N Folin-Ciocalteu's phenol reagent (Merck AG, Darmstadt, Germany) diluted 1:2 in distilled water. Finally, ten minutes before the ending of incubation phase, 200 µl of the reaction mixture from each sample was transferred replicate into the wells of F96-MicroWell™ plate (Thermo Fisher Scientific Inc., Roskilde, Denmark). After 30 minutes incubation period, absorbance values were measured with microplate reader Labsystems Multiskan RC (Thermo Electron Corporation, Brussels, Belgium) at 750 nm. All measurements were performed in duplicate. Additionally, blank was included in the assay. Blank was prepared by replacing a sample with the same volume of buffer (0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3). The blank absorbance representing background absorbance caused by reagent ingredients was subtracted from the sample absorbance values.

To enable the protein quantitation of samples, bovine serum albumin BSA (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. Standard solutions containing a range of 50-800 µg protein/ml were prepared by diluting from the BSA-stock solution (1 mg/ml) in 0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3. Standards were treated similarly as other samples and were used to create a standard curve from which the protein quantity of unknown samples can be determined. In practice, a standard curve was obtained by plotting the blank-corrected absorbance at 750 nm (x-axis) *vs* BSA concentration (y-axis). Protein concentrations of the samples were estimated by comparing absorbance values to the regression equation for the standard curve.

3.5 Statistics and calculation of results

Basal enzyme activities in the retina and ciliary body are characterized as units (U)/mg protein and in the vitreous body as units (U)/milliliter of sample. One unit of enzyme activity is defined as the amount of enzyme required to release 1µmol of His-Leu in one minute. The results describing basal ACE1 and ACE2 activities in the porcine ocular tissues are expressed as mean ± SEM (standard error of the mean).

The extent of inhibition on ACE1 and ACE2 enzyme activity (%) for each inhibitor was calculated as follows:

$$\frac{S-I}{S} \times 100\ \%$$, where S = fluorescence intensity of non-inhibited sample
I = fluorescence intensity of the sample in the presence of inhibitor

Inhibition was expressed also as the inhibitor concentration at which the enzyme activity is inhibited by 50% (IC_{50}) under assay conditions, assuming that the basal (non-inhibited) activity is 100%. IC_{50}-values were determined by regression analysis of enzyme inhibition (%) *vs* log inhibitor concentration. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test were used for statistical comparisons of basal activity *vs* inhibited activity. SPSS® software Version 16.0 was used for these statistical analyses. Comparison between inhibition of enzyme activity in various tissues (retina, ciliary body, vitreous body) was performed by unpaired t-test (Sigma Plot Version 10.0) for all the studied inhibitors and used concentrations. *p*-values smaller than 0.05 were considered statistically significant and those differences were denoted by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4 RESULTS

4.1 Basal activities of ACE1 and ACE2

Activities of both ACE1 and ACE2 enzymes were detected in all porcine ocular tissues analysed *in vitro* (see Table VI below). ACE1 activity was significantly higher, almost twentyfold, in the ciliary body when compared to the retina. The activities of ACE1 and ACE2 were at the same level in the retina, whereas in the ciliary body the ACE2 activity was considerably lower than ACE1 activity. The activity of ACE1 was indicated to be much higher than the ACE2 activity also in the vitreous body.

Table VI. Activities of ACE1 and ACE2 in the porcine ocular tissues. Enzyme activities in retina and ciliary body are expressed as mean ± SEM mU/mg protein, whereas activities in vitreous body are given as mean ± SEM nmol/min/ml of sample.

Enzyme	*Retina*	*Ciliary body*	*Vitreous body*
ACE1	0.2 ± 0.02 (*n=6*)	3.7 ± 0.70 (*n=6*)	8.2 ± 0.31 (*n=6*)
ACE2	0.2 ± 0.01 (*n=5*)	0.2 ± 0.02 (*n=6*)	0.1 ± 0.02 (*n=5*)

4.2 Inhibition of ACE1 activity

All the tripeptides (IPP, VPP, LPP) analysed inhibited the ACE1 activity dose-dependently, reaching considerable inhibitory effect when used at micromolar concentrations in retina, ciliary and vitreous bodies (IC_{50} 9-25 μM). All the peptides were evinced to have about equal inhibitory potential. Captopril was indicated to be more potent ACE1 inhibitor in ocular tissues compared to IPP, VPP and LPP. Determined IC_{50}-value for captopril was 5-10 nM depending on the tissue type, implying that inhibitory efficiency of captopril is thousandfold compared to that of the aforementioned peptides. For the inhibition of the ACE1 activity in the ciliary body, higher peptide concentrations were needed than in the two other tissues, which was indicated to be consistent also with the inhibitory effect of captopril in tissues being analysed. See Figures 6-9 (pages from 82 to 83) and Table VII (page 84).

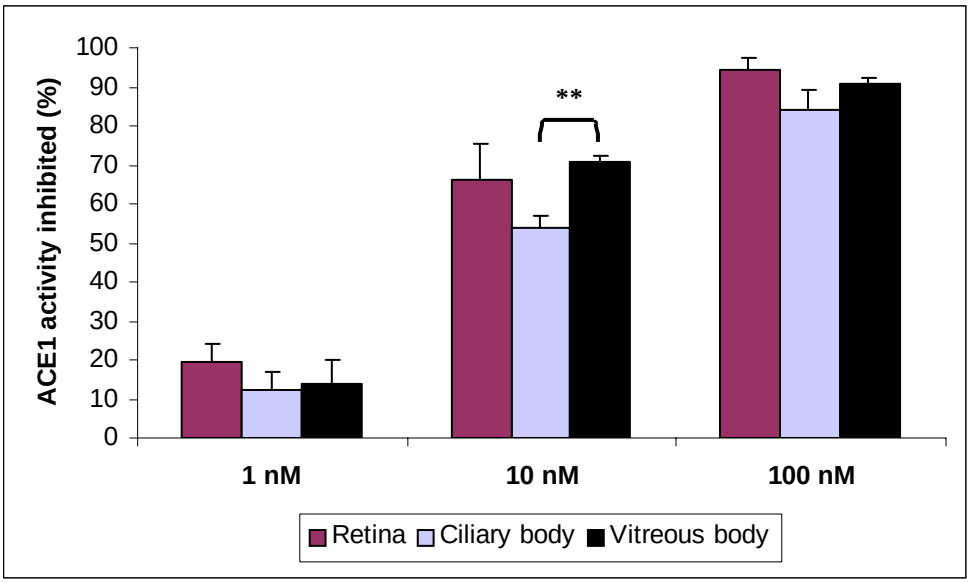

Figure 6. Inhibition of ACE1 activity by **captopril** in porcine ocular tissues (mean ± SEM, n=4). Significant inhibition ($p<0.05$-0.001) *vs* basal activity at 1 nM and higher concentrations in vitreous body and at 10 and 100 nM in retina and ciliary body. Significance of inter-tissue differences is marked as ** ($p<0.01$).

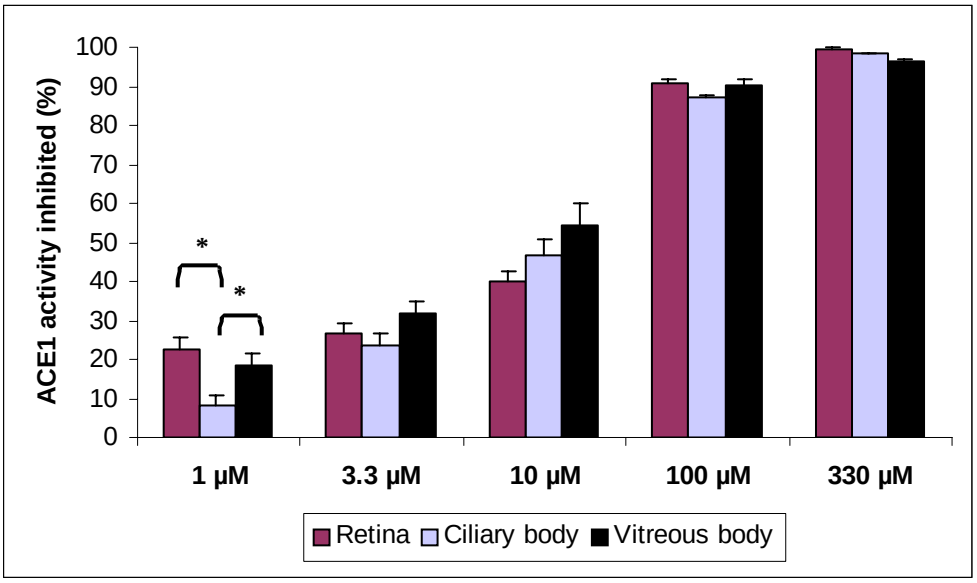

Figure 7. Inhibition of ACE1 activity by **IPP** in porcine ocular tissues (mean ± SEM, n=4). Significant inhibition ($p<0.01$-0.001) *vs* basal activity at 1 µM and higher concentrations in retina and vitreous body and at concentrations ≥ 3.3 µM in ciliary body. Significance of inter-tissue differences is marked as * ($p<0.05$).

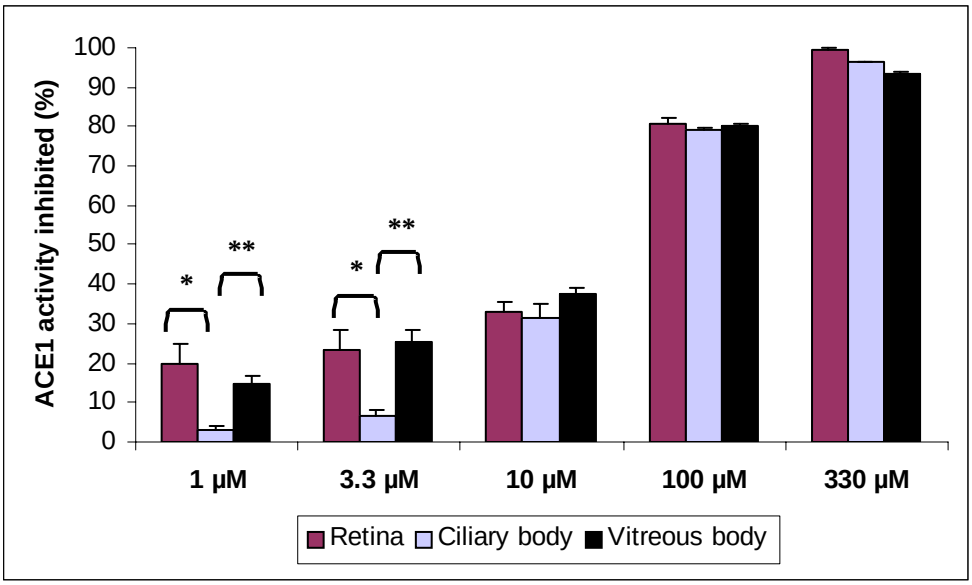

Figure 8. Inhibition of ACE1 activity by **VPP** in porcine ocular tissues (mean ± SEM, n=4). Significant inhibition ($p<0.05$-0.001) *vs* basal activity at 1 µM and higher concentrations in retina and vitreous body and at concentrations ≥10 µM in ciliary body. Significance of inter-tissue differences is marked as * ($p<0.05$) and ** ($p<0.01$).

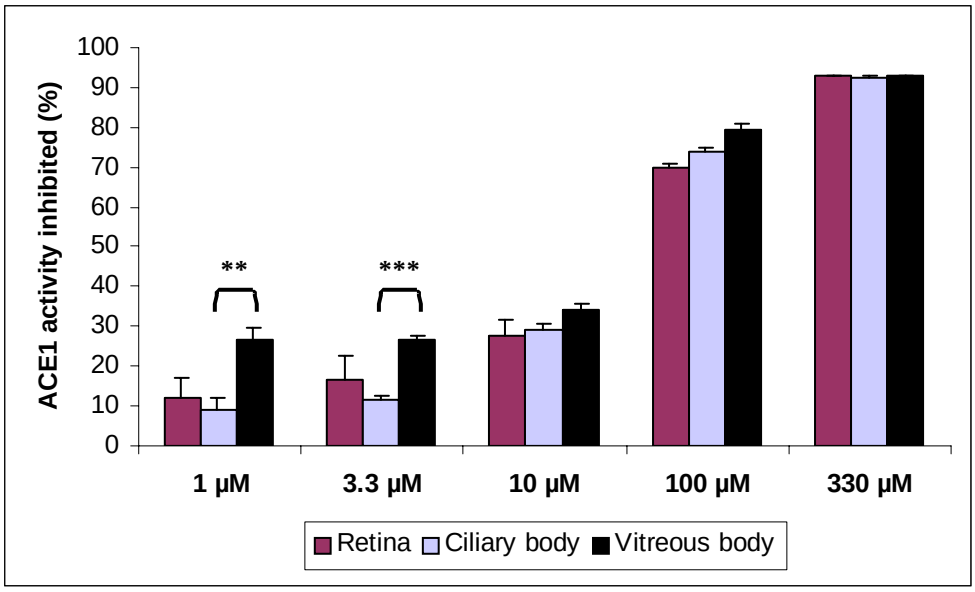

Figure 9. Inhibition of ACE1 activity by **LPP** in porcine ocular tissues (mean ± SEM, n=4). Significant inhibition ($p<0.05$-0.001) *vs* basal activity at 1 µM and higher concentrations in ciliary and vitreous bodies and at concentrations ≥10 µM in retina. Significance of inter-tissue differences is marked as ** ($p<0.01$) and *** ($p<0.001$).

Table VII. IC_{50}-values for captopril, IPP, VPP and LPP describing their ACE1-inhibitory potency in distinct porcine ocular tissues.

Inhibitor		*Retina*	*Ciliary body*	*Vitreous body*
Captopril	(nM)	5.4	9.9	6.0
IPP	(μM)	10.8	13.6	9.1
VPP		14.7	23.5	15.8
LPP		24.5	24.8	13.8

4.3 Inhibition of ACE2 activity

Inhibition of ACE2 activity by IPP, VPP and LPP was obtained dose-dependently at millimolar concentrations (IC_{50} 4-22 mM) in retina, ciliary and vitreous bodies. All the peptides were evinced to have about equal inhibitory potential on the ACE2 activity. Occurrence of inter-tissue variability between retina, ciliary and vitreous bodies in inhibitory potencies of tripeptides were distinctly perceived. ACE2 activity was inhibited most efficiently in the ciliary body. Results related to inhibitory effect of DX600, a specific ACE2 inhibitor, in ocular tissues were uncertain and refers to poor repeatability. The ACE2 activity was not reduced by captopril up to concentration of 3.3 mM. See Figures 10-13 (pages from 84 to 86) and Table VIII (page 86).

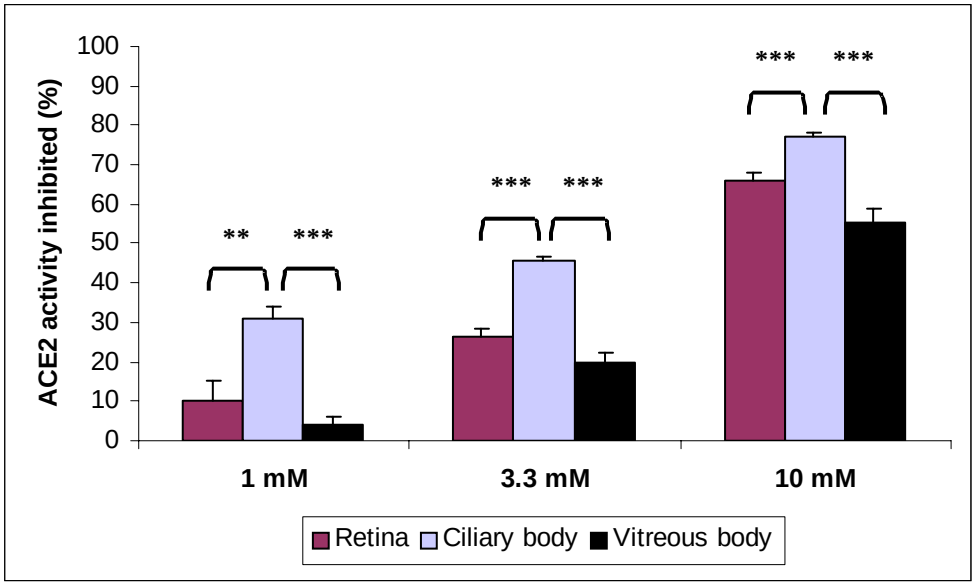

Figure 10. Inhibition of ACE2 activity by **IPP** in porcine ocular tissues (mean ± SEM, n=6). Significant inhibition ($p<0.01$-0.001) *vs* basal activity at 1 mM and higher concentrations in ciliary body and at concentrations ≥3.3 mM in retina and vitreous body. Significance of inter-tissue differences is marked as ** ($p<0.01$) and *** ($p<0.001$).

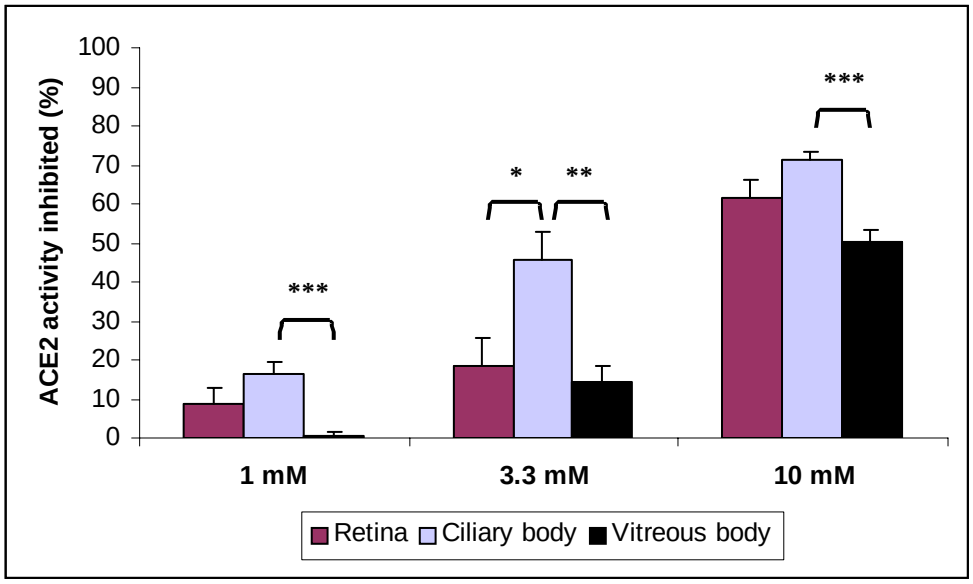

Figure 11. Inhibition of ACE2 activity by **VPP** in porcine ocular tissues (mean ± SEM, n=6). Significant inhibition ($p<0.05$-0.001) *vs* basal activity at 1 mM and higher concentrations in ciliary body, at 10 mM concentration in retina and at concentrations ≥3.3 mM in vitreous body. Significance of inter-tissue differences is marked as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

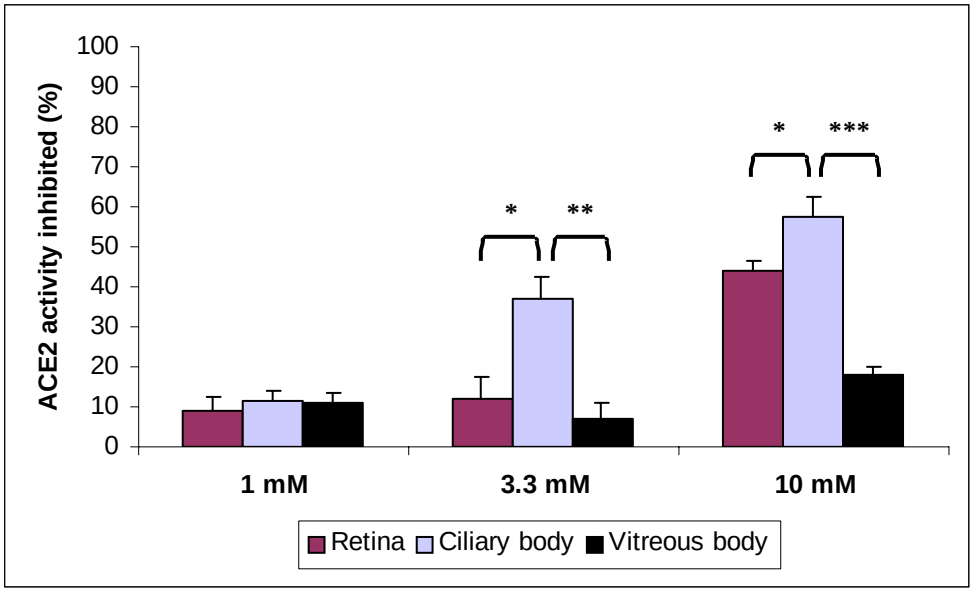

Figure 12. Inhibition of ACE2 activity by **LPP** in porcine ocular tissues (mean ± SEM, n=6). Significant inhibition ($p<0.01$-0.001) *vs* basal activity at 3.3 mM and higher concentrations in ciliary body and at 10 mM concentration in retina and vitreous body. Significance of inter-tissue differences is marked as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

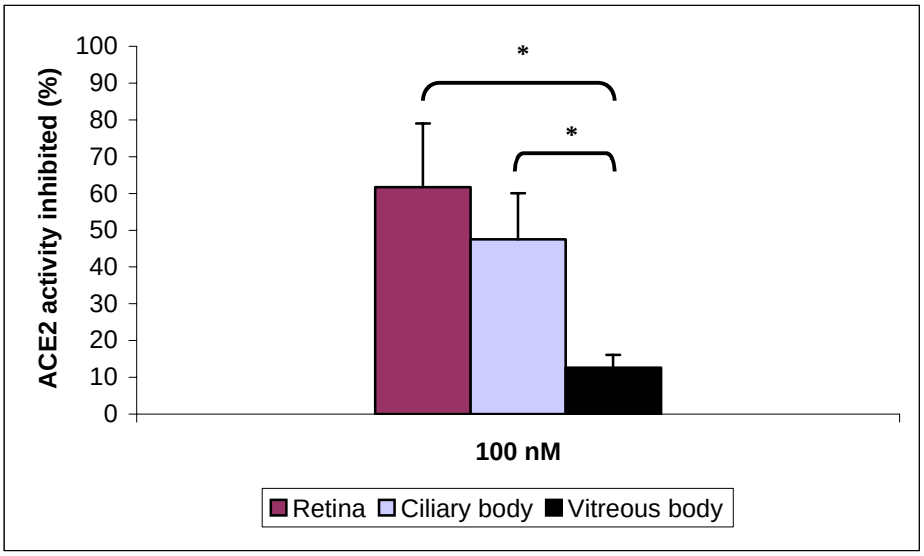

Figure 13. Inhibition of ACE2 activity by **DX600** in porcine ocular tissues (mean ± SEM, n=6). Significance of inter-tissue differences is marked as * ($p<0.05$).

Table VIII. IC_{50}-values for IPP, VPP, LPP and captopril describing their ACE2-inhibitory potency in distinct porcine ocular tissues.

Inhibitor		*Retina*	*Ciliary body*	*Vitreous body*
IPP	(mM)	7.3	4.6	9.0
VPP		8.2	4.0	9.9
LPP		11.7	6.7	22.3
Captopril		-	-	-
DX600		*	*	*

(-) ACE2 inhibition by captopril was not detected
(*) IC_{50} for DX600 was not determined because its ACE2-inhibitory effect was analysed solely at concentration of 100 nM

5 DISCUSSION

Affiliation of the intraocular RAS to the eye function and pathophysiology of certain ophthalmic diseases, such as glaucoma, has recently been the intriguing object of research. Additionally, new RAS-blockade molecules that could be applied as pharmaco-therapeutical agents are under extensive research. Consequently, the aim of the present study was to measure the activities of central RAS enzymes, ACE1 and recently described ACE2, in certain porcine ocular tissues (retina, ciliary body and vitreous body) *in vitro*. In addition, the inhibitory potential of bioactive lactotripeptides IPP, VPP and LPP shown to reduce blood pressure mainly by ACE1 inhibition, was analysed within these tissues. There is accumulating evidence that supports the concept that RAS substantially participates in the regulation of both blood pressure and intraocular pressure. Accordingly, ACE inhibitors have been demonstrated to have oculohypotensive effects, which gave basis to the presumption that these ACE-inhibitory peptides could have beneficial effects also in the ocular tissues and could possibly be used e.g. in the treatment of glaucoma.

5.1 Methodological aspects

The source of ocular tissues and sample preparation

Fresh porcine eyes were used in this study because the anatomy and physiological features of the pig eye have a close resemblance to the human eye (McMenamin & Steptoe, 1991; Chandler *et al*., 1999). Previous studies (e.g. by Wagner *et al*., 2004; Ruiz-Ederra *et al*., 2005; Bachmann *et al*., 2006) have substantiated that porcine eyes are suitable as human models also in functional studies that clarifies ocular physiology and pathological mechanisms related to ophthalmic disorders and dysfunction due to the distinctive similarities to the human eye. Porcine eyes are also affordable and easily available from an abattoir compared to postmortem human eyes, which are difficult to obtain for research use, although they are of first priority in experiments aiming at human applications. Effects of tripeptides IPP, VPP and LPP on ACE1 activity in ocular tissues were determined for the first time in the present study. Analyses were performed *in vitro* in order to guide prospective *in vivo*-studies in experimental animals and in humans. Specific tissue types

from both eyes of the same animal were pooled to enable detectable levels of ACE1 and ACE2 enzymes and consequently performance of inhibition assays by certain inhibitors. Pooled samples have been used also in earlier experiments in which the presence of active RAS components in ocular tissues of porcine (Geng *et al*., 2003) and rabbit (Ramirez *et al*., 1996) were determined. Vita *et al*. (1981) have noticed that the level of ACE1 activity in ocular fluids varied when eye colour was considered. Therefore, all the studied porcine eyes were of same colour (blue).

Samples were stored in a deep-freezer until measured for basal and inhibited ACE1 and ACE2 activities and for protein concentration. Due to practical reasons, it was not possible to perform all these three determinations during the same day and therefore samples were kept frozen until assayed. Before transferred to the freezer, samples were readily divided into aliquots for each protocol and in this way freeze-thaw cycles were minimized, which is important for ensuring sample quality. It has been shown that there is no remarkable change in the ACE1 activity before and after freezing of samples (Vita *et al*., 1981). Additionally, Cushman and Cheung (1971) have demonstrated that ACE1 activity in crude tissue extracts is stable for at least six months when stored at temperature of 5ºC at pH between 5 and 9. However, basal activities were measured in every determination batch and these results were used in defining the proportions of basal and inhibited enzyme activities in order to minimize errors in assessment of inhibitory potencies for various inhibitors.

ACE1 activity measurement

In the present study, ACE1 activity in ocular tissues was measured by the widely used fluorometric method described first by Friedland and Silverstein (1976). This method has also been utilised in the measurement of ACE1 activities in distinct ocular tissues (e.g. Shiota *et al*., 1997 and Tikellis *et al*., 2004) and in ocular fluids (e.g. Vita *et al*., 1981 and Weinreb *et al*., 1985). In addition to this method, several other methods for measurement of ACE1 activity have been introduced in the literature, including spectrophotometric (e.g. Cushman & Cheung, 1971) and radiochemical assays (e.g. Ramirez *et al*., 1996), high-performance liquid chromatography HPLC (e.g. Meng *et al*., 1995) and mass spectrometry

(e.g. Elased *et al*., 2005). Fluorometric method based on the measurement of HHL-derived His-Leu was utilised in the present study because it proved to be sufficiently sensitive, is easy to perform and because all applicable equipment was readily available. Spectrophotometric assay described by Cushman and Cheung (1971) was also tested for ACE1 activity measurement. This method is based on the measurement of hippuric acid derived from the hydrolysis of HHL. By exploiting this method, ACE1 activities were measured in rabbit tissues (kidney, heart, lung) shown to have high activity of that enzyme, and subsequently also in ocular tissues. Nevertheless, this method proved to be overly insensitive for detection of presumable low amounts of active enzyme in ocular tissues, and was excluded.

In addition to ACE1, the presence of other ANG II-forming enzymes, such as chymase, has been established in ocular tissues (Shiota *et al*., 1997). Similarly to ACE1, also chymase cleaves the Phe^{8}-His^{9} –bond of ANG I to produce ANG II (Sanker *et al*., 1997). Apparently, chymase is not able to cleave the Gly-His –bond within the synthetic substrate HHL used in this study, and thus is not able to produce dipeptide His-Leu, being subsequently quantified by fluorometric assay method. In contrast, ACE1 attacks also peptides having Gly-His –bond in their sequence (Hooper, 1990; Laurent & Salzet 1996). Due to the structural differences in the active sites of ACE1 and ACE2, the binding of HHL to ACE2 is prevented and therefore ACE2 is not able to degrade it (Rice *et al*., 2004). Consequently, exclusively ACE1 can release the C-terminal dipeptide His-Leu from the substrate and hence this method can be considered as specific for the activity measurement of that enzyme. Additionally, the sensitivity of ACE1 activity to captopril, a specific ACE1 inhibitor, indicates also that the detected activity is due to ACE1 present in the sample, as chymase activity is not inhibited by captopril (Urata *et al*., 1990).

Optimal assay conditions were defined to validate the assay protocol suitable for ACE1 activity measurement in tissue homogenates of porcine retina and ciliary body and untreated vitreous fluid samples. The activity of ACE1 is shown to be highly dependent on reaction conditions regarding especially chloride ion concentration (Cheung *et al*., 1980) and pH (Rohrbach *et al*., 1981). Cushman and Cheung (1971) stated that an optimal ACE1 activity of the rabbit lung acetone extract is achieved at pH 8.1-8.3 and in the presence of

300 mM NaCl. The same chloride concentration is shown to be optimal for hydrolysis of HHL by ACE1 also in other studies (e.g. Michaud *et al*., 1997). Cushman and Cheung (1971) used 0.1 M phosphate buffer containing 0.3 M NaCl (pH 8.3) in their activity assay and under these conditions HHL had a relatively low affinity for ACE1. Vita *et al*. (1981) used also that buffer in the measurement of ACE1 activity in ocular fluids. According to Ryan *et al*. (1980), phosphate decreases the affinity of HHL to ACE1 and may also affect enzyme activity and therefore other buffer constituents are preferred when measuring the ACE1 activity. Oliveira *et al*. (2000) have compared ACE1 activities in phosphate and borate buffers and the activity was shown to be maximal when the sample is diluted in borate buffer. Optimal reaction conditions regarding the pH may depend on the origin of the enzyme being measured, as noticed by Rohrbach *et al*. (1981); for the hydrolysis of HHL by bovine ACE1, the maximum pH (8.75) was higher compared to that (8.3) of rabbit ACE1. In experiments of the present study, the pH of reaction buffer was 8.3 according to recommendation of Santos *et al*. (1985), albeit optimal pH for porcine ACE1 may deviate from that (was not determined).

Captopril, IPP, VPP and LPP were tested for their efficiency to inhibit ACE1 activity in ocular tissues. Inhibitors were not shown to change the buffer pH, even at the highest concentration of stock solution. In fluorometric methods, in which HHL is used as a substrate, the final substrate concentration in the reaction is mostly 5 mM (e.g. Vita *et al*., 1981). This substrate concentration was used also in the present study based on the experimentations performed at different HHL concentrations. It has been shown by Cushman and Cheung (1971) and Rohrbach *et al*. (1981) that ACE1 is subject to substrate inhibition when the substrate concentration is three to four times greater than the K_M (a constant representing the substrate concentration at which the velocity of an enzyme-catalyzed reaction is half maximal). In the present study, the formation of His-Leu was linear at substrate concentration of 5 mM. However, for better accuracy of results describing inhibitory potential of tripeptides and captopril on ACE1 activity, the kinetic parameters (k_{cat}, K_M, and V_{max}) should have been determined.

In the present study samples were incubated for 60 minutes with substrate solution. It was shown that distinct tissue types demanded different periods of time for revealing the

optimal activity. With 60 minutes incubation time fluorescence values for all the tissues being analyzed were in reliable range. In contrary, Vita *et al*. (1981) incubated ocular fluid samples and substrate only for 15 minutes in their protocol. As a conclusion, modified method of Friedland and Silverstein (1976) utilised in this study proved to be suitable for ACE1 activity measurement with high sensitivity and good reproducibility both in the determination of basal and inhibited activities.

ACE2 activity measurement

ACE2 activity was analyzed using commercially available kit intended for that purpose. According to the kit manufacturer AnaSpec Inc., the kit can be used for ACE2 activity measurement in various biological samples, including tissue homogenates. This assay is specific to ACE2 activity because the fluorogenic substrate Mca-APK(Dnp) included in the kit is not suggested to be cleaved by other enzymes than ACE2. Differing from that substrate, another substrate Mca-YVADAPK(Dnp) utilized also in many ACE2 activity assays is substrate also for caspase-1/interleukin-converting enzyme (ICE) and is therefore unspecific when used in the measurement of ACE2 activity (Enari *et al*., 1996; Vickers *et al*., 2002).

The amount of the unspecified assay buffer included in the kit pack proved to be insufficient to be used for reactions and sample homogenization. Constitution and chemical properties of that buffer solution have not been reported by its manufacturer and it was not possible to buy any extra buffer. Consequently, another buffer (0.1 M disodiumtetraborate buffer containing 0.3 M NaCl, pH 8.3) was used in the assay. Applicability of this buffer was tested to ensure that constituents and properties of this buffer do not interfere with the assay. Rice *et al*. (2004) and Wysocki *et al*. (2006) used 50 mM Hepes/NaOH buffer containing 150 mM NaCl (pH 7.4) with Mca-APK (Dnp) substrate in their experiments to measure ACE2 activity. As with ACE1, chloride ions affect the ACE2 activity in a substrate- and pH-dependent manner (Cheung *et al*. 1980; Guy *et al*. 2003). Therefore Rice *et al*. (2004) performed ACE2 activity assays in reaction conditions that closely resemble the salt concentration and pH of the extracellular environment. According to studies performed by Vickers *et al*. (2002), proteolytic activity of ACE2 is in optimum at pH 6.5,

but ACE2 maintains substantial catalytic activity at pH range from 7 to 9. Furthermore, ACE2 activity was noticed significantly enhanced by high concentrations of chloride and the catalytic activity was demonstrated to be optimal in the presence of 1M NaCl. Chloride concentration and pH of the buffer used in the present study differ from above mentioned (herein 300 mM NaCl, pH 8.3). Consequently, present results describing ACE2 activity should be considered rather directional than commensurate to *in vivo* –conditions.

DX600, a specific ACE2 inhibitor, included in the assay kit was determined as a positive control in each ACE2 activity assay to ensure that the detected enzyme activity is due to presence of ACE2 within the sample. DX600 does not inhibit ACE1 activity (Huang, L *et al*. 2003). For some reason, DX600 inhibited ocular tissue-derived ACE2 by varying efficiency when used at final concentration of 100 nM in accordance with the kit instructions, although DX600 has been indicated as very potent ACE2 inhibitor (Huang, L *et al*., 2003). This discrepancy may result from the differences in reaction conditions, such as the buffer pH or chloride ion concentration, both known to affect ACE2 activity (Guy *et al*., 2003). Another explanation may be the presence of inhibitory substances in the tissue homogenates that may cause dissociation of the enzyme-inhibitor complex leading to overestimation of ACE2 activity. Tissue samples may also contain constituents which prevent binding of DX600 to ACE2 or that degrade inhibitor rendering it defunctioning. However, DX600 is established as a stable peptide, which is not hydrolyzed by ACE2 (Huang, L *et al*., 2003). This interference effect would possibly been abolished by raising the sample dilution level, but then the activity values would have descended out of the detection limit. On the other hand, dilution increases the possibility of dissociation of inhibitor from the enzyme (Gorski & Campbell, 1991). In addition, DX600 proved to be viscous even when diluted appropriately, that possibly have resulted in insufficient amount of inhibitor in the reaction as inhibitor solution may stick into the pipette tip.

Protein quantitation

A widely used method described by Lowry *et al*. (1951) was utilised for the determination of protein content in the ocular tissue samples in order to calculate the ratio of enzyme activity to total protein concentration. This method is considered sensitive, inexpensive and

simple to perform. Reactions in Lowry's procedure result in strong blue color, which depends partly on the sample's tyrosine and tryptophan content. Thus, the amount of color formed is not strictly proportional to the total protein concentration, unless is dependent on the amino acid composition of the proteins present in the sample. (Waterborg, 2002) Presence of tyrosine and/or trypthopan –rich proteins in distinct ocular tissues is not known. It is also very likely that there are inter-tissue differences in protein composition. However, Lowry's method has been used also in other studies (e.g. Weinreb *et al*. 1985) directed to eye-derived tissues.

In the present study, bovine serum albumin (BSA) was used to construct the standard curve. BSA is a commonly used standard material in determination of protein concentration. However, in order to achieve absolute values for protein concentration, it is recommendable to use the same protein being analyzed (here ACE1 and ACE2) also as a standard material (Waterborg, 2002). Partly for economic reasons, BSA was used to achieve approximate measure of the sample protein concentration. This was reasonable because the objective in this study was to compare relative activities of ACE1 and ACE2 in distinct ocular tissue types, rather than determine absolute activity values. Constituents of the buffer used for sample homogenization are not recognized as interfering substances in this method (not reported in the literature). Consequently, Lowry's method can be considered suitable for protein quantitation in retina, ciliary and vitreous bodies.

5.2 Basal and inhibited activities of ACE1 and ACE2

Basal activities of ACE1 and ACE2 in the porcine eye

Results of this study clearly show the presence of catalytically active ACE1 and ACE2 in all tissues analysed and represent an important contribution to the present conception of local RAS in the eye. Activity of ACE2 in the ciliary and vitreous bodies is demonstrated for the first time in any species in the present study. ACE2 has previously been detected exclusively in the human (Senanayake *et al*., 2007) and rodent (Tikellis *et al*., 2004) retina. No significant deviations were found in the basal enzyme activities individually. However, regarding the physiological aspects, the enzyme activities could have been varied

individually according to animal's IOP, existence of certain ophthalmic diseases (e.g. retinopathy) or other disease state (e.g. high blood pressure).

Activities of both ACE1 and ACE2 were demonstrated to vary tissue-specifically in ocular tissues being analysed. The ACE1 activity was markedly higher in the ciliary body than in the retina, whereas ACE2 activities were at the same level in both of these tissues. Accordingly, the ACE1/ACE2-proportion was greater in the ciliary body compared to the retina, in which the activities of these two enzymes were at the same level. ACE1 and ACE2 activities were detected also in the vitreous body. However, these activities cannot be compared to the values obtained in retina and ciliary body by reason of the difference in calculation of enzyme activity (tissue: units/mg of protein *vs* liquid: units/ml of sample). Similar to ciliary body, the ACE1 activity in the vitreous body was much higher compared to the ACE2 activity in that tissue type.

Regardless of the inter-tissue differences, both ACE1 and ACE2 were shown to be functional in the same tissues, which supports for its part the concept that these enzymes may have counterbalancing functions in the RAS cascade also in the eye. Furthermore, Vaajanen *et al*. (2009) have very recently established the presence of novel Mas receptor in the eye. Effects of ANG (1-7), a breakdown product of ANG II formed by ACE2 catalysis, are specifically mediated through this receptor type (Santos *et al*., 2003). This finding together with the results of the present study suggest that the existence of ACE2 in the eye is physiologically significant, and confirms for its part that ANG II and ANG (1-7) are the principal bioactive mediators also in the intraocular RAS.

Comparison of ACE1 and ACE2 activities among distinct tissues and in different species is not reasonable for methodological aspects (e.g. varying reaction conditions, sensitivity, sample dilution, dipeptidase activity) and for inter-species differences in the structure and function of RAS cascade. For instance, Hara *et al*. (1982) noticed in their study that human ACE1 has chemical properties similar to mouse ACE1, but the affinity of human ACE1 for synthetic substrate HHL and naturally occurring substrate ANG I was two times higher than that of mouse ACE1.

Geng *et al*. (2003) have also studied ACE1 activities in distinct porcine ocular tissues. In addition, ACE1 activies have been determined in ocular tissues of several other species, including human, bovine, feline (by Ferrari-Dileo *et al*., 1988), rabbit (by Ramirez *et al*., 1996), monkey and dog (by Shiota *et al*., 1997). Comparison of the results obtained in the present study to those accomplished earlier is not realistic for prominent methodological differences and for other reasons clarified above. Nonetheless, relative ACE1 activities in distinct tissues can be compared. Geng *et al*. (2003) have demonstrated that the ACE1 activity in the ciliary body was approximately threefold to that detected in the retina. Relative ACE1 activities are in accordance with the present study, albeit herein the ACE1 activity of the ciliary body was shown nearly twentyfold compared to that of retina. Relative ACE1 activities in the ciliary body and retina of cat and rabbit are also comparable (Ferrari-Dileo et *al*., 1988; Ramirez *et al*., 1996). Retinal ACE1 activity was still lower in the present study than previously measured in the monkey and dog eye (Shiota *et al*., 1997), although inter-species differences were notable also in the previous study. Taken together, ACE1 activities in the porcine ciliary body and retina are in agreement with previous results described in the literature. ACE1 activities in the vitreous body have been determined also in several species, like human (Maruichi *et al*., 2004), monkey and dog (Shiota *et al*. 1997). ACE1 activities detected in these studies are expressed as mU/mg protein. In the present study protein content were not quantified in the vitreous body samples, and therefore results are not comparable. ACE2 activities in ocular tissues have not been reported earlier in the literature, and therefore comparison to previous results could not be performed.

In ACE1 activity assay based on the measurement of fluorescence emitting dipeptide His-Leu, the extent of His-Leu release from HHL substrate is considered directly related to the ACE1 activity (Friedland & Silverstein, 1976). Removal of His-Leu from the C-terminus of HHL is analogous to the action of ACE1 on its physiological substrate ANG I (Hooper, 1990). However, ACE1 activities determined with synthetic substrate have been shown to be threefold higher than those determined with naturally occurring substrate ANG I (Shiota *et al*., 1997). In the experiment of Ryan *et al*. (1980), it was shown that reaction velocity, indicated as V_{max} (μM of formed reaction product/min/mg of enzyme protein), increases when lower C-terminal homologues of ANG I are used as a substrate for ACE1 instead of

intact ANG I. HHL is a synthetic substrate mimicking the C-terminus of ANG I. Therefore, results obtained by means of *in vitro* –experiments can certainly not be directly applied to *in vivo* –conditions, but can be considered rather directional.

When assessing ACE1 activity in tissues by using the method based on the measurement of dipeptide His-Leu, it must be emphasized that samples may contain dipeptidases, which are able to degrade His-Leu produced by ACE1 through hydrolysis of HHL (Santos *et al*., 1985; Hooper, 1990). Consequently, dipeptidase activity present in the sample leads to underestimation of ACE1 activity because single amino acids His and Leu do not significantly react with OPA. Vita *et al*. (1981) have tested human and rabbit tear and aqueous humor samples for the presence of peptidase activity. They perceived that peptidase activity in human ocular fluid was very low or absent, in contrast to rabbit ocular fluids, wherein high peptidase activities were detected. Accordingly, ACE1 activity in rabbit ocular fluids is underestimated by nearly 50% when assessed from the breakdown of HHL to His-Leu. According to Santos *et al*. (1985), dipeptidase activity depends on the reaction buffer and is minor when sodium borate buffer is used.

Koiter *et al*. (1998) have noticed interdependence between the ACE1 activity and sample dilution level, and the dilution effect was observed especially in tissue homogenates. Dipeptidase activity may explain the dilution effect, since degradation of the compoud that emits the fluorescence (His-Leu) is more rapid in concentrated samples resulting in underestimation of ACE1 activity. On account of these observations, ACE1 activities determined in the present study may be underestimated. Spectrophotometric assay based on the measurement of amount of hippuric acid formed in the breakdown of HHL is not affected by dipeptidase activity –related errors. Consequently, this method is recommended for the ACE1 activity measurement in tissues, but was not applicable in the present study because of its insensitiveness to detect low amounts of active ACE1 in ocular tissues.

Results obtained in this study cannot be directly applied to RAS action in the human eye because of described species differences. However, the porcine eye is morphologically and physiologically closest to the human eye after the monkey eye and is therefore widely utilised in ophthalmic research (McMenamin & Steptoe, 1991). Additionally, existence of

a functional intraocular RAS in the porcine eye has been demonstrated also in earlier studies (e.g. Danser *et al*., 1994; Meyer *et al*., 1995; Geng *et al*., 2003).

It was not possible to estimate, whether ACE1 and ACE2 enzymes are originally localized and produced in ocular tissues or are they released from circulation and consequently sequestered into ocular tissues to generate e.g. ANG II. As well, enzymes may be produced in certain tissues and are subsequently released to other eye parts. For instance, Bishop *et al*. (2002) have accentuated the role of posterior ciliary body in the biosynthesis of various proteins eventually released to the vitreous humour. On the other hand, amounts of RAS components, especially angiotensins and ACE1 enzyme in ocular tissues have proved to be too high to be originated from circulation when tissue and plasma concentrations were compared (Danser *et al*., 1994; Geng *et al*., 2003). Consequently, intraocular RAS can be considered independent of the circulating RAS. However, disruption of the blood-retinal barrier, e.g. during enucleation procedure could allow nonspecific leakage of protein – including ACE1 and ACE2– from circulation. Contamination by other tissue type, e.g. by vascularized choroid tissue, wherein high ACE1 activity has been determined (Geng *et al*., 2003), during sample preparation could distort results. For these reasons, it is recommendable e.g. to establish cell cultures representing only certain cell types for the further examination of RAS expression in distinct ocular cells.

Inhibitory potential of IPP, VPP and LPP on ACE1 and ACE2 activities

It has been demonstrated in many clinical studies that long-term administration of dairy products containing ACE1-inhibitory tripeptides IPP and VPP lowers blood pressure both in animal models and in man (for review see López-Fandiño *et al*., 2006). Additionally, there is strong evidence that regulatory mechanisms behind the blood pressure and IOP have common features (Langman *et al*., 2005). Inhibition of the RAS cascade by ACE inhibitors and ANG II AT_1-receptor antagonists has been shown to reduce both blood pressure and IOP levels. Based on these observations, it was justifiable to test the effects of tripeptides IPP, VPP and LPP on activities of ACE1 and ACE2 in the ocular tissues *in vitro*.

As it was hypothesized, tripeptides were demonstrated to reduce the activities of both ACE1 and ACE2 dose-dependently in tissue homogenates of retina, ciliary and vitreous bodies, but only ACE1 inhibition was achieved at clinically relevant concentrations. IPP, VPP and LPP evinced approximately equal inhibitory potency. Inhibition of the ACE1 and ACE2 activities was established to be dependent on the tissue type. For the inhibition of the ACE1 activity in the ciliary body, higher peptide concentrations were needed than in the retina and vitreous body, whereas ACE2 was inhibited most efficiently in the ciliary body. Captopril was also established as more potent ACE1 inhibitor in the retina and vitreous body than in the ciliary body. In contrary to this finding, Geng *et al*. (2003) have demonstrated that captopril was more potent inhibitor in the porcine ciliary body than in the retina. However, basal ACE1 activity in the retina was very low, which could have made it difficult to detect inhibitory effect by the method that was utilised in the study of Geng *et al*. (2003). Noticed inter-tissue differences in ACE1 inhibition can be factual or may be related to presence of endogenous ACE inhibitors, like ANG (1-7) that may have disturbed the assay. According to findings of Udupa and Rao (1997), ACE inhibitors may block ACE1 function to varying degrees in distinct tissues, depending on tissue characteristics, that should also be taken into account.

Prominent inhibition of ACE2 activity by IPP, VPP and LPP was achieved at very high concentration (mM) when compared to that needed to significantly inhibit ACE1 activity (μM) in all the tissues being analysed. Accordingly, at clinically relevant concentrations IPP, VPP and LPP were shown to have ACE1-inhibitory effect without concurrent inhibition of the ACE2 activity. In the clinical trial (by Jauhiainen *et al*., 2005b) in which the blood pressure lowering effect of IPP and VPP were shown, calculated theoretical concentrations of these peptides in the circulation were maximally at micromolar level. In pharmacokinetic studies by Foltz *et al*. (2007) and Pijl *et al*. (2008), it was shown that maximum plasma concentrations of IPP, VPP and LPP were at nanomolar level in human and in pig. Consequently, inhibition of the ACE2 activity is suggested not to occur *in vivo* in humans. Concurrent inhibition of both ACE1 and ACE2 is not even desirable because they play opposite roles in the RAS cascade; inhibition of ACE1 is clearly beneficial, whereas ACE2 should be activated rather than inactivated.

Results of the current study can be considered consistent with earlier results (Lehtinen *et al*., 2008) obtained by using purified commercial ACE1 and ACE2 enzymes in the determination of inhibitory effects of IPP, VPP and LPP on these enzymes. IC_{50}-values for inhibition of ocular tissue-derived ACE1s by IPP, VPP and LPP were approximately two to fourfold higher than those determined with pure ACE1 enzyme. In the study of Lehtinen *et al*. (2008), IC_{50}-values were determined by spectrophotometry using HHL as a substrate at final concentrations of 1, 3.3 and 10 mM. Therefore, IC_{50}-values determined at the same substrate concentration as in the present study (5 mM) are not available. To compare results of the present and previous study (Lehtinen *et al*., 2008), IC_{50} at 5 mM substrate concentration was roughly estimated by regression analysis of IC_{50} *vs* substrate concentration for IPP, VPP and LPP.

Ocular tissue samples may contain proteins that interfere with the assay e.g. by inhibiting ACE1 itself, degrading inhibitors or substrate or by altering reaction conditions resulting in weaker ACE1 inhibition. Differences may also be derived from methodological divergence. For instance, Gorski and Campbell (1991) have perceived that fluorometric assay with HHL as a substrate grossly underestimated ACE1 inhibition when compared to spectrophotometric assay with 3-(2-furylacryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) as a substrate. Additionally, it is approved that FAPGG is more active substrate than HHL in terms of kinetic constants K_m and k_{cat} (Buenning *et al*., 1983), and is therefore recommended for assessment of ACE1-inhibitory effects of various substances (Vermeirssen *et al*., 2002). Previous studies that directly compare accuracy of ACE1 activity determinations by fluorometric (His-Leu measurement) and spectrophotometric (hippuric acid measurement) methods were not found. Furthermore, Michaud *et al*. (1997) and Lehtinen *et al*. (2007, 2008) have shown that the efficacy of ACE inhibitor is clearly dependent on substrate concentration.

ACE inhibitors may act differently against ACE1s obtained from different sources, which has to be considered, especially when comparing results obtained with purified enzymes and tissue-derived enzymes. IC_{50}-values for milk casein-derived IPP and VPP peptides are 5 μM and 9 μM, respectively (Nakamura *et al*., 1995a) and 9.6 μM for LPP (Maruyama *et al*., 1989). In the present study, ACE1-inhibitory effects were determined by synthetic

tripeptides, and their IC_{50}-values were shown to be only slightly higher than reported earlier for casein-derived tripeptides. This indicates that the reaction conditions used in the present study were applicable to measurement of ACE1 inhibition by these peptides. Furthermore, results suggest that both casein-derived and synthetic tripeptides act in a corresponding manner independently of ACE1 source (purified enzyme, ocular tissue).

In contrast to ACE1, IPP, VPP and LPP were denoted to inhibit ACE2 activity more efficiently in ocular tissues than in previous assays performed with purified enzyme. When tripeptides were used at a final concentration of 10 mM, inhibition of pure ACE2 was only 10 to 20 % (Lehtinen *et al*. 2008). In ocular tissues 10 mM tripeptide concentration caused much higher (44 to 77 %) mean inhibition of ACE2 activity depending on the tripeptide and tissue type (exception: mean inhibition of ACE2 in vitreous body by LPP was only 18%). However, due to very high tripeptide concentration that is required for ACE2 inhibition *in vitro*, tripeptides are not suggested to have any significance as ACE2 inhibitors *in vivo*.

Captopril, a widely used antihypertensive drug, inhibited ACE1 activity efficiently in all ocular tissues and the inhibitory potency determined in the present study corresponds well with earlier results obtained by using ocular tissues (Ferrari-Dileo *et al*., 1988). Captopril was demonstrated to be much more potent ACE1-inhibitor (IC_{50} at nanomolar level) than tripeptides. In ocular tissues ACE1 inhibition by captopril was two to fourfold better than inhibition of purified ACE1 enzyme (Lehtinen *et al*. 2008). In comparison with results from the study by Geng *et al*. (2003), the ACE1-inhibitory potential of captopril was considerably better in the present study. In experiments of Geng *et al*. (2003), 50 % reduction in ACE1 activity in porcine ocular tissues was achieved when captopril was used at a final concentration of 2 μM in both ciliary body and retina. In the present study the IC_{50}-value for ACE1 inhibition by captopril was at nanomolar level. Differences in these results can be explained, at least partly, by varied test conditions, e.g. substrate, assay method and its sensitivity, and the way of result calculation. In contrast to ACE1, the activity of ACE2 was not reduced by captopril up to concentration of 3.3 mM. Either in previously reported studies e.g. by Tipnis *et al*. (2000), Huang *et al*. (2003), Rice *et al*. (2004) and Wysocki *et al*. (2006), several ACE inhibitors were not noticed to inhibit ACE2

activity. These observations are also consistent with the recently described model of the active site of ACE2 in which the S2' pocket differs from the corresponding pocket in ACE1, that prevents ACE inhibitors from binding to ACE2 (Towler *et al*., 2004).

When interpreting results describing inhibitory effects of certain substances on enzyme activity, one has to consider that kinetics of enzyme inhibition *in vitro* may not necessarily reflect the action of inhibitor *in vivo*. Most ACE inhibitors are demonstrated to inhibit ACE1 activity in a competitive way (Catravas *et al*., 1990; Weisser & Schloos, 1991). Georgiadis *et al*. (2003) noticed that specific inhibition of one active domain within the ACE1 does not prevent the hydrolysis of ANG I by the other active site, which supports the concept of a mixed-type inhibition, at least in *in vitro* conditions. Mixed-type inhibition is demonstrated also in several other studies for captopril (Okuda & Arakawa, 1985; Baudin & Bénéteau-Burnat, 1999), lisinopril (Uçar & Özer, 1992), enalapril and ramipril (Baudin & Bénéteau-Burnat, 1999) on activity of ACE1 derived from tissues of several mammalian species. Moalli *et al*. (1985) found that captopril and enalapril behaved as noncompetitive ACE inhibitors *in vivo*, unless are generally considered as competitive ACE inhibitors.

According to Wei *et al*. (1991) and Skirgello *et al*. (2005), synthetic substrate HHL binds specifically to the C-domain of the human sACE, unless both domains are able to hydrolyse HHL. Contrary, Binevski *et al*. (2003) demonstrated that HHL was hydrolyzed by both domains present in the bovine sACE with equal affinity. Consequently, despite of homology between ACEs derived from distinct species, they may exhibit unlike substrate specificity. Captopril is shown to preferentially bind to the N-domain of the ACE1 (Michaud *et al*., 1997; Binevski *et al*., 2003). However, binding *in vitro* is affected by reaction conditions, such as chloride concentration. For example, Wei *et al*. (1992) have noticed that captopril inhibits the N-domain only slightly better than the C-domain with HHL as a substrate at 300 mM NaCl. As a comparison, at low chloride concentration captopril prefers substantially the N-domain. The activity of C-domain increases even up to concentration of 800 mM of NaCl (Wei *et al*., 1991). According to Bevilacqua *et al*. (1996), binding characteristics of ACE inhibitors to N- and C-domains of human ACE1 are

different in various tissues. ACE1 is glycosylated enzyme and it is therefore assumed that binding pattern may vary due to organ-specific glycosylation (Bevilacqua *et al*., 1996).

Binding of the inhibitor depends also on whether the ACE1 is in soluble form (as in plasma), or membrane-bound (as in tissue). A recent study by Georgiadis *et al*. (2003) demonstrated that total inhibition of ANG I cleavage demands blockade of both active domain when soluble ACE is used, while only the C-domain contributes to conversion of ANG I to ANG II by membrane-bound ACE. Results of Van Esch *et al*. (2005) are in accordance with these observations. Also these findings substantiate the concept that ACE1 may interact differently in *in vivo* and *in vitro* conditions.

Regarding all these aspects presented herein, inhibition of ACE1 activity by captopril *in vitro* is not merely competitive because the substrate and inhibitor are not necessarily competing for binding to the same active site within the enzyme. Additionally, it has been indicated that two active sites within the ACE1 exhibit negative cooperativity (Binevski *et al*., 2003; Andújar-Sánchez *et al*., 2004; Skirgello *et al*., 2005). Binding of the naturally occurring substrate ANG I to the C-domain within the ACE1 is considered most prominent in *in vivo* –related conditions (van Esch *et al*., 2005; Fuchs *et al*., 2004 and 2008), unless ANG I is able to bind to both domains of ACE1 (Michaud *et al*. 1997). Presence of the C-domain is evidenced to be sufficient to maintain active RAS (Fuchs *et al*. 2004 and 2008). Currently, there is no evident conception of the binding characteristics of ACE inhibitory tripeptides IPP, VPP and LPP to N- and C-domains of the ACE1. Presumably, binding of these bioactive peptides may also be dependent on various circumstances, as clarified above.

5.3 Conclusions and future prospects

The presence of active ACE1 enzyme has previously been confirmed in several ocular tissues of various species. In accordance with these earlier findings, ACE1 activity was currently detected in porcine retina, ciliary and vitreous bodies. The presence of active ACE2 enzyme in the porcine vitreous and ciliary bodies is shown for the first time in any species in the present study. ACE2 was found also from the porcine retina, consistently

with the previous findings in the human and rodent retina (Senanayake *et al*., 2007; Tikellis *et al*., 2004). Within the eye, ANG II is involved in the regulation of retinal circulation and may also be related to the maintenance of IOP since application of ACE inhibitors and ANG II AT_1-receptor antagonists is demonstrated to lower the prevailing IOP level. ACE2 with its balancing effects in the RAS may also has a significant role in ocular physiology and in ophthalmic disorders, such as glaucoma by being involved in the regulation and maintenance of IOP and retinal microcirculation.

Results of this study provide additional evidence for the existence of intraocular RAS, but simultaneously many other research objects concerning the function of complex and fascinating RAS come into sight. Commonly, ACE1 and ACE2 colocalize in the same tissue (Rivière *et al*., 2005), as demonstrated also in the present study. Increasing evidence substantiates that these enzyme homologues may have counterbalancing interaction in physiological regulation of IOP and ocular circulation. Consequently, it has been hypothesized that imbalance between the regulation of ACE1/ACE2 –ratio may lead to ophthalmic disorders, like glaucoma. Therefore, it is of particular importance to assess activities of these enzymes concurrently. Huang *et al*. (2003) have recently indicated that e.g. Mca-YVADAPK (Dnp) is a substrate that can be cleaved by both ACE1 and ACE2 and is suitable for this purpose, as has already been applied by Wysocki *et al*. (2006).

To extend the present study, concurrent activity measurement of ACE1 and ACE2 could be performed e.g. by glaucoma models of experimental animals. Concurrently, it would be intriguing to assess, how e.g. ACE2 activators alter the balance between these enzymes. In addition, it is of significance to conduct enzyme activity measurements in corresponding human ocular tissues or in ocular cell cultures. In prospective experiments other methodological approaches, such as immunohistochemistry and *in situ* hybridization are recommended to define more accurate cellular localization of ACE2 and to assess *Ace2* gene expression (mRNA) in ocular tissues. Measurement of both ACE2 mRNA and ACE2 activity is essential since pathophysiological changes may occur only at posttranscriptional level, perceived as altered enzyme activity, but the gene expression remains unchanged, as noticed by Wysocki *et al*. (2006) in their experiments related to expression of *Ace2* gene and ACE2 activity in the kidney of diabetic mice.

Bioactive tripeptides IPP, VPP and LPP, which are capable of lowering human blood pressure, were shown to exert ACE1-inhibitory effects *in vitro* in ocular tissues at clinically relevant concentration (μM) without coincidental inhibition of ACE2 activity. Prominent ACE2 inhibition by these peptides required up to thousandfold consentration (mM) and is therefore not suggested to have any significance in *in vivo* –conditions. Consequently, applied at low concentrations these tripeptides may have ACE1-inhibitory effects also *in vivo* and may therefore have potential as pharmacotherapeutic agents e.g. in the treatment of raised IOP in glaucoma. It is also worth of noticing that structurally these peptides consist of only three amino acids and are generally considered resistant to degradation by digestive enzymes (for review see Vermeirssen *et al*., 2004). This may offer benefit in penetration through cornea into the inner eye parts providing good bioavailability in ophthalmic use (topical administration route) of these substances. Accordingly, these results are encouraging and provide theoretical rationale for further research, such as *in vivo* –studies. Still, there are many pharmacokinetic challenges associated to development of this kind of RAS-inhibiting ophthalmic drugs that should be encountered. E.g. physical properties of drug molecule, delivery system that enables penetration through cornea and blood-retina barrier, sheltering from degrading peptidases, administration route and bioavailability are things that remain to be investigated in the future.

Results of the experimental part of this study are presented in an article "Activities of angiotensin-converting enzymes, ACE1 and ACE2, and inhibition by bioactive peptides in porcine ocular tissues", published recently in *Journal of Ocular Pharmacology and Therapeutics* 2009, Vol. 25 (1), pp. 23-28 by Luhtala *et al*. (see the References, page 121). A review of literature concerning the presence of intraocular RAS and its role in the regulation of IOP is published by Vaajanen, Luhtala *et al*. in *Annals of Medicine* 2008, Vol. 40 (6), pp. 418-427 (see the References, page 131).

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