EXPRESSION AND FUNCTION OF ANGIOTENSINS IN THE REGULATION OF INTRAOCULAR PRESSURE - AN EXPERIMENTAL STUDY

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals, and reprinted with the permission of the copyright holders (II-IV), and on unpublished data (I):

I **Vaajanen A**, Lakkisto P, Virtanen I, Kankuri E, Oksala O, Vapaatalo H, Tikkanen I. Angiotensin receptors in the eyes of arterial hypertensive rats. Acta Ophthalmologica. Submitted.

II Luhtala S[*], **Vaajanen A**[*], Valjakka J, Oksala O, Vapaatalo H. Activities of angiotensin-converting enzymes 1 *(ACE1)* and 2 *(ACE2)* and inhibition by bioactive peptides in porcine ocular tissues. J Ocul Pharmacol. In press.
[*] equal contribution

III **Vaajanen A,** Vapaatalo H, Kautiainen H, Oksala O. Angiotensin (1-7) reduces intraocular pressure in the normotensive rabbit eye. Invest Ophthalmol Vis Sci 2008; 49: 2557-2562.

IV **Vaajanen A**, Mervaala E, Oksala O, Vapaatalo H. Is there a relationship between blood pressure and intraocular pressure? An experimental study in hypertensive rats. Curr Eye Res 2008; 33: 325-333.

MAIN ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme-related carboxypeptidase
AH	Aqueous humor
Ang I,II,III,IV	Angiotensin I,II,III,IV
Ang (1-10)	Angiotensin (1-10)= Ang I
Ang (1-8)	Angiotensin (1-8)= Ang II
Ang (2-8)	Angiotensin (2-8)= Ang III
Ang (3-8)	Angiotensin (3-8)= Ang IV
Ang (1-9)	Angiotensin (1-9)
Ang (1-7)	Angiotensin (1-7)
Ang (1-5)	Angiotensin (1-5)
Ang (3-7)	Angiotensin (3-7)
ARB	Angiotensin II receptor type 1 blocker
AT_1	Angiotensin II receptor type 1
AT_2	Angiotensin II receptor type 2
AT_4	Angiotensin II receptor type 4
BP	Blood pressure
dTGR	Double transgenic rat harboring human renin and human angiotensinogen genes
Ile-Pro-Pro (IPP)	Isoleucyl-prolyl-proline
IOP	Intraocular pressure
Leu-Pro-Pro (LPP)	Leucyl-prolyl-proline
Mas-receptor	Ang (1-7) receptor type
NPEC	Non-pigmented epithelial cells of ciliary body
OF	Outflow
RAS	Renin-angiotensin system
RT-PCR	Real-time reverse transcriptase polymerase chain reaction
SD	Sprague-Dawley rat
SHR	Spontaneously hypertensive rat
Val-Pro-Pro (VPP)	Valyl-prolyl-proline
WKY	Wistar Kyoto rat

ABSTRACT

An active intraocular renin-angiotensin system (RAS) has recently been shown to exist in the human eye and evidence is now accumulating that antihypertensive drugs acting on RAS can also lower intraocular pressure (IOP), though no agents are as yet in ophthalmological use. The aim of this experimental study was to elucidate the expression and function of RAS in the eye tissues and in the regulation of IOP.

The expression of ocular RAS was evaluated by RT-PCR, *in vitro* autoradiography and fluorometric assay. The functional RAS was investigated after administration of different RAS compounds by the two-level constant pressure method of Bárány and by IOP measurement using pneumatonometer or rebound tonometer. Experimental animals were ocular normotensive rabbits and rats. Enucleated fresh porcine eyes were used in enzyme activity determinations.The potential relationship between developing blood pressure and intraocular pressure as well as the effect of general anesthesia on IOP was evaluated using arterial hypertensive rat strains and their normotensive controls.

The main finding in this study was a heptapeptide angiotensin (1-7) (*Ang (1-7)),* which when administered intravitreally significantly reduced IOP in the normotensive rabbit eye. Its specific receptor, the Mas receptor, was for the first time found in the eye structures. A third finding in respect of intraocular RAS was the existence of *ACE2* in vitreous and ciliary bodies in addition to the earlier demonstration of its retinal activity.

The present findings suggest the potential as future antiglaucomatous agents of components which increase intraocular *ACE2* activity and the formation of *Ang (1-7),* or activate Mas receptors.

1 INTRODUCTION

The Finnish physiologist Tigerstedt and his coworker Bergman described for first time a pressor substance which they found in the rabbit kidney and named renin (Tigerstedt and Bergman 1898). In 1940 groups under Braun-Menéndez and Page reported that renin was the enzyme acting on a plasma protein substrate to catalyze the formation of the actual pressor peptide, first named hypertensin or angiotonin (Braun-Mendez et al. 1939; Page and Helmer 1940). Later the pressor substance was renamed angiotensin and the plasma substrate angiotensinogen (Jackson 2006).

Once angiotensin II (*Ang II)* was found, its important role in the regulation of blood pressure was soon recognized. In 1958 the German investigator Gross perceived a larger system when aldosterone synthesis and secretion were shown to be involved in the renin-angiotensin system (RAS) (Gross and Lichtlen 1958a,b; Gross and Schmidt 1958). In the 1970s the development of antihypertensive drugs commenced. First to be evolved were angiotensin-converting enzyme (ACE) inhibitors, which prevent the formation of *Ang II* (Ondetti et al. 1977), and in 1988 in the laboratory of DuPont Merck *Ang II* receptor type 1 blockers, which prevent the direct effects of *Ang II*. Both drugs acting on RAS are today the most widely used drugs in the treatment of hypertension (Hall 2003). In the future, renin blockers, agents inhibiting the whole renin-angiotensin system, may gain ground in the field of antihypertensive treatment (Triller et al. 2008).

Ang II is a potent vasoconstrictor and is traditionally regarded as the main effector peptide in the RAS. According to recent studies, however, the final effect of RAS activation is more complex, being based on the biological activity of *Ang II* and the activities of the other products of angiotensinogen metabolism, often exerting opposite effects on *Ang II* action (Kramkowski et al. 2006; Paul et al. 2006). Evidence is accummulating indicating the existence of local RAS systems which regulate long-term changes in a number of organs, e.g. the vasculature, adrenal gland, kidney, brain, testis and ovary via the activity of other angiotensins and their receptors (Deschepper et al. 1986; Derkx et al. 1987). An active intraocular RAS has also been described in the human eye (Sramek et al. 1992; Danser et al. 1994). Drugs acting on the RAS have been reported to be able to lower IOP (Costagliola et al. 1995; Costagliola et al. 2000; Shah et al. 2000; Inoue et al. 2001a; Wang et al. 2005a), but no RAS agents are as yet in ophthalmological use. These preliminary findings would suggest that the RAS not only regulates blood pressure but is also involved in the

regulation of IOP. However, the exact mechanism of this action is as yet not known.

The present study was sought to clarify in greater detail the expression and function of RAS in the eye tissues and in the regulation of IOP.

2 REVIEW OF THE LITERATURE

2.1 CIRCULATING RENIN-ANGIOTENSIN SYSTEM

The complexity of the present knowledge of RAS is depicted in *Figure 1*.

2.1.1 Angiotensins

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Angiotensinogen ***(NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Ser-R)***

The obligatory substrate for the whole RAS is angiotensinogen, an α-glycoprotein consisting of 255 amino acids, synthesized in and released from the liver and cleaved in the circulation by an enzyme called renin (Nasjletti and Masson 1971; Tewksbury et al. 1978). In addition to the main synthesis in the liver, angiotensinogen can also be synthesized at tissue level (Paul et al. 2006; Iusuf et al. 2008). Synthesis of angiotensinogen is stimulated, in addition to angiotensin II (*Ang II),* by for example inflammation, insulin, estrogens, glucocorticoids and thyroid hormone (Jackson 2006).

Renin

Renin is an aspartyl protease whose principal natural substrate is the circulating α-glycoprotein angiotensinogen. Renin is synthesized in the juxtaglomerular apparatus of the kidney as a preproenzyme of 406 amino acid residues that is attributed to prorenin, a mature but inactive form of the protein. The active form of renin consists of 340 amino acids, and is capable of cleaving the bond between residues 10 (=*Leu*) and 11 (=*Val*) at the amino terminus of angiotensinogen to generate the decapeptide angiotensin I *(Ang I)* (Morris 1986; Jackson 2006). Renin secretion is influenced by the pressure in the renal artery, by the activity of the sympathetic nervous system, and by the still hypothetical macula densa signal as well as by humoral factors. Renin-synthesizing cells are present not only in the kidney but also in a number of other organs, e.g. brain, pituitary and adrenal glands, heart, arterial smooth muscle, testis (Ganten et al. 1976; Hackenthal et al. 1990) and eye (Danser et al. 1989; Wagner et al. 1996). Renin is an important enzyme in the RAS for the cleavage of angiotensinogen to *Ang I* and further to more bioactive forms of

RAS (Satofuka et al. 2006; Iusuf et al. 2008). The inactive precursor of renin, prorenin, is released constitutively from the kidney. Its plasma levels are approximately 10-100-fold greater than those of renin and its action on RAS is probably marked not only via renin but also via renin receptors (Batenburg et al. 2007; Nguyen and Danser 2008). Prorenin can be activated in two ways: proteolytic or non-proteolytic, the first being irreversible and the latter reversible depending e.g. on temperature and pH (Nguyen and Danser 2008).

Angiotensin I (Ang I, Ang 1-10) (*NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH)*

1 2 3 4 5 6 7 8 9 10

Ang I is a decapeptide formed from angiotensinogen by activation of renin. *Ang I* is a precursor for *Ang II* and a weak vasoconstrictor. It is further cleaved to the more potent octapeptide *Ang II* mainly by angiotensin-converting enzyme *(ACE)*, which removes the carboxyterminal dipeptide *His-Leu* of *Ang I* (Skeggs et al. 1956; Vickers et al. 2002). This cleavage can also be brought about by other enzymes such as CAGE, chymase and cathepsin G (*Figure 1)*. These alternative routes via other enzymes are called renin-independent or *ACE*-independent pathways for *Ang II* production (Kramkowski et al. 2006).

Angiotensin-converting enzyme (ACE, ACE1, kininase II, dipeptidyl carboxy-peptidase)

ACE, a membrane-bound proteinase containing 1277 amino acid residues, is predominantly expressed in high concentrations on the surface of endothelial cells in the pulmonary circulation and has a significant role in circulating RAS, forming *Ang II* from *Ang I,* and in degrading other angiotensins to inactive forms. Its important role is to catalyze the cleavage of the dipeptide *His-Leu* from the carboxyl terminus of *Ang I (*Skeggs et al. 1956; Ng and Vane 1967). Its main effect is strongly vasopressive (Sealey and Laragh 1990). *ACE* is also known as kininase II, as it also catalyzes the bradykinin cascade (Su 2006), having a degrading effect on the vasodilatory bradykinin (Jackson 2006; Kramkowski et al. 2006). Bradykinin is a nonapeptide formed from kininogens mainly produced by hepatocytes. It dilates blood vessels by stimulating the production e.g. of nitric oxide and prostacyclin in the vascular endothelium (Su 2006), or via direct effects through B_2 receptors (Berguer et al. 1993).

Angiotensin II (Ang II, Ang 1-8) (*NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH)*

Ang II (Braun-Mendez et al. 1939; Page and Helmer 1940) is a potent vasoconstrictor and is traditionally considered to be the main effector peptide in the circulating RAS. It is an octapeptide formed from *Ang I* mainly by *ACE* or other enzymes such as CAGE (chymostatin-sensitive *Ang II*-generating enzyme), chymase, cathepsin G or directly from the long polypeptide chain, angiotensinogen, by alternative pathways catalyzed by catepsin G, tonin, trypsin or chymotrypsin (Kramkowski et al. 2006; Paul et al. 2006). *Ang II* has three major physiological effects which are linked to blood pressure and electrolyte homeostasis: vasoconstriction, renal tubular sodium reabsorption and aldosterone biosynthesis.

Importantly *Ang II* has proinflammatory characteristics (Mervaala et al. 2000; Ruiz-Ortega et al. 2001). It stimulates free radical production, plasminogen activator inhibitor-1 release and tissue factor and adhesion molecule expression. It is considered to diminish the beneficial effects of nitric oxide by inhibiting nitric oxide synthase (eNOS). In blood vessels, it stimulates smooth muscle cell proliferation and leukocyte activation (Buczko 1999; Jackson 2006). These are essential factors in the pathogenesis of hypertension though the mechanism of RAS-induced hypertension has also been attributed to the direct effects of *Ang II* on angiotensin II type 1 (AT_1) receptors in vascular smooth muscle (Sealey and Laragh 1990; Paul et al. 2006) and stimulation of the release of aldosterone, a mineralocorticoid emanating from the adrenal cortex (Laragh et al. 1960; Sealey et al. 1978). Thus *Ang II* elevates blood pressure by releasing noradrenaline from adrenergic nerve endings, endothelin 1, a potent vasoconstrictor, from the endothelium (Sung et al. 1994) and vasopressin, a vasoconstricting pituitary hormone, as well as by reducing baroreceptor activity (Sealey and Laragh 1990; Ardaillou 1997). The half-life of *Ang II* is short, only a couple of seconds (Al-Merani 1978). Its vasopressive effects appear rapidly and are more long-lasting.

Angiotensin III (Ang 2-8, Ang III) (*NH_2-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH)*

Ang III is formed from *Ang II* or angiotensin (2-10) by aminopeptidase A and *ACE.* Similarly to *Ang II, Ang III* is also a vasoconstrictor, albeit less potent. *Ang III* is only 25 % as potent as *Ang II* in elevating blood pressure and 10 % in stimulating the adrenal medulla (Jackson 2006).

Angiotensin IV (Ang IV, Ang 3-8) (*NH_2-Val-Tyr-Ile-His-Pro-Phe-COOH)*

Ang IV is formed from *Ang III* or directly from *Ang II* by aminopeptidase activities. In contrast to *Ang II, Ang IV* is held to be a vasorelaxing agent. It also has cell-proliferative properties and may be involved in vascular inflammatory responses (Ruiz-Ortega et al. 2007). Its activation may also be involved in memory and neuronal development (Mustafa et al. 2001). The precise mechanism and function of *Ang IV* is not clear, but its vasodilatatory effect is explained by activation of endothelial nitric oxide synthase (Kramkowski et al. 2006).

Angiotensin-converting enzyme 2 (ACE2)

ACE2 is an important counter-regulatory factor in RAS especially at tissue level (Donoghue et al. 2000; Yagil and Yagil 2003). This is discussed in grater detail in section 2.2.2.

Angiotensin (1-9) (Ang (1-9)) (*NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-COOH)*

Ang (1-9) is formed from *Ang I* by activation of *ACE2,* which cleaves one amino acid (*Leu)* from the carboxyl terminus of *Ang I*. *Ang (1-9)* has recently been found and its function is not yet clear, but it is a strong inhibitor of *ACE* and serves as a substrate fot the formation of angiotensin (1-7) *(Ang (1-7))* (Mustafa et al. 2001; Iusuf et al. 2008). It activates bradykinin, increases nitric oxide formation and release of the eicosanoid precursor arachidonic acid, and is possibly involved in the inhibition of platelet function (Donoghue et al. 2000).

Angiotensin (1-7) (Ang (1-7)) (*NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH)*

Ang (1-7), formed from *Ang II* by *ACE*-independent enzymes (Welches et al. 1993; Santos et al. 2000), was first discovered more than 30 years ago (Semple et al. 1976a; Semple et al. 1976b; Ferrario et al. 1988) and is one of the products of RAS most extensively investigated in recent years (Ferrario and Chappell 2004). It can also be synthesized directly from *Ang I* or *Ang (1-9)*, bypassing the synthesis of *Ang II* (Kucharewicz et al. 2002), or from a prohormone angiotensin (1-12) *(Ang (1-12))*, which is proposed to serve as a precursor for *Ang (1-7)* (Nagata at al. 2006). The enzymes catalyzing the

degradation of *Ang I* and *II* to form *Ang (1-7)* are *ACE2* and endopeptidases such as neprilysin and prolylcarboxy-peptidase. These enzymes cleave *Phe-His-Leu* from *Ang I* and *Phe* from *Ang II*. *Ang (1-7)* is further metabolized to smaller peptides; to angiotensin (1-5) or to angiotensin (3-7) by *ACE* (Roks et al. 1999). *Ang (1-7)* is a biologically active heptapeptide with high selectivity. In most situations, *Ang (1-7)* and *Ang II* exert opposing actions, suggesting a primary role for *Ang (1-7)* as a counter-regulatory component for the vascular and proliferative actions of *Ang II* (Iwata et al. 2005; Kostenis et al. 2005). *Ang (1-7)* promotes release of prostanoids from endothelial and smooth muscle cells (Muthalif et al. 1998), release of nitric oxide (Seyedi et al. 1995), vasorelaxation and inhibition of vascular cell growth (Jaiswal et al.1992). *Ang (1-7)* also exhibits an important stimulatory interaction with the kallikrein-kinin system, and has thus a vasodilatory effect (Brosnihan et al. 1996). This mechanism is complex, involving bradykinin receptor activation and inhibition of *ACE*, and the release of nitric oxide and/or prostanoids. On the other hand, *Ang (1-7)* may be a component in the endogenous regulation of tissue growth (Santos et al. 2000).

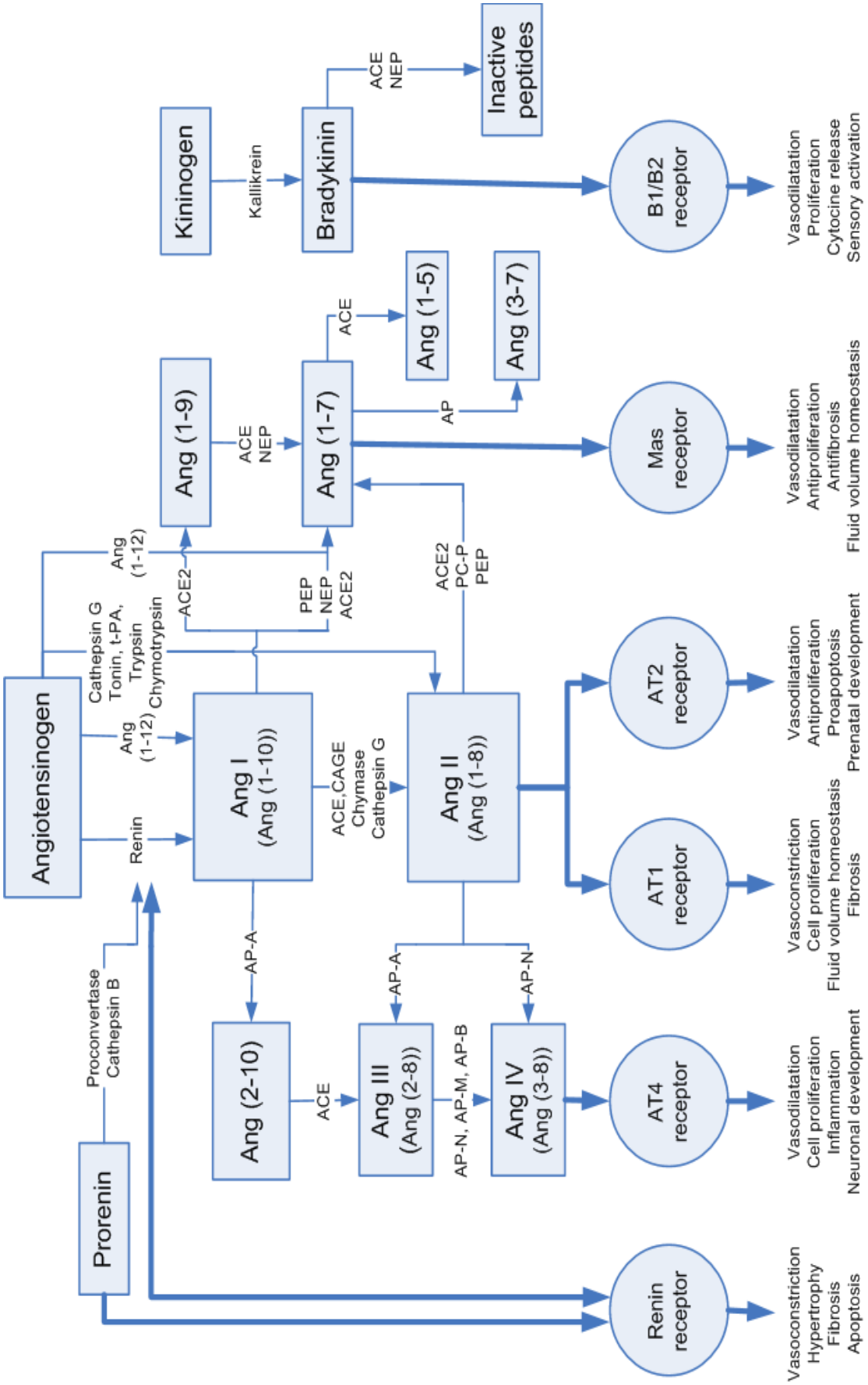

Kininogen
Kallikrein
Bradykinin
ACE
NEP
Inactive peptides
B1/B2 receptor
Vasodilatation
Proliferation
Cytocine release
Sensory activation
Ang (1-9)
ACE
NEP
Ang (1-7)
ACE
Ang (1-5)
AP
Ang (3-7)
Mas receptor
Vasodilatation
Antiproliferation
Antifibrosis
Fluid volume homeostasis
Ang (1-12)
ACE2
PEP
NEP
ACE2
ACE2
PC-P
PEP
Angiotensinogen
Cathepsin G
Tonin, t-PA,
Trypsin
Chymotrypsin
Ang (1-12)
Renin
Ang I
(Ang (1-10))
ACE,CAGE
Chymase
Cathepsin G
Ang II
(Ang (1-8))
AT2 receptor
Vasodilatation
Antiproliferation
Proapoptosis
Prenatal development
AT1 receptor
Vasoconstriction
Cell proliferation
Fluid volume homeostasis
Fibrosis
Proconvertase
Cathepsin B
AP-A
AP-A
AP-N
Ang (2-10)
ACE
Ang III
(Ang (2-8))
AP-N, AP-M, AP-B
Ang IV
(Ang (3-8))
AT4 receptor
Vasodilatation
Cell proliferation
Inflammation
Neuronal development
Prorenin
Renin receptor
Vasoconstriction
Hypertrophy
Fibrosis
Apoptosis

Figure 1.

Figure 1. The renin-angiotensin system. ACE = angiotensin-converting enzyme, ACE2 = angiotensin-converting enzyme-related carboxypeptidase, Ang I,II,III,IV= angiotensin I,II,III,IV, Ang (1-10) = angiotensin (1-10), Ang (1-8) = angiotensin (1-8), Ang (2-8) = angiotensin (2-8), Ang (3-8) = angiotensin (3-8), Ang (1-9) = angiotensin (1-9), Ang (1-7) = angiotensin (1-7), Ang (1-5) = angiotensin (1-5), Ang (3-7) = angiotensin (3-7), AT1 = angiotensin II type 1 receptor, AT2 = angiotensin II type 2 receptor, AT4 = angiotensin II type 4 receptor, AP =aminopeptidase (-A,-N,-M,-B), B1/B2 = bradykinin receptors, CAGE = chymostatin-sensitive Ang-II generating enzyme, Mas-receptor = Ang (1-7) receptor type, Nep = neprilysin, PEP = prolyl endopeptidase, PCP = prolylcarboxy-peptidase, tPA = tissue-type plasminogen activator (Vaajanen et al. 2008, modified version).

2.1.2 Angiotensin receptors

The effects of angiotensins are exerted through specific heptahelical G-protein-coupled receptors which contain seven transmembrane regions (de Gasparo et al. 2000; Burnier 2001). *Ang II* receptors in the cardiovascular system are classically divided into two main subtypes: *Ang II* type 1 (AT_1) and 2 (AT_2) receptors, but evidence is accumulating to indicate the importance of other receptor types such as Mas- and AT_4 receptors (Santos et al. 2003, Ruiz-Ortega et al. 2007). Generally adult tissues contain primarily AT_1 receptors, AT_2 receptors being represented especially in developing fetal tissues, and their number decreases rapidly in the postnatal period (Timmermans et al. 1993a). The AT_1 receptor is 359 amino acids long, and has only about 30 % sequence homology to the AT_2 receptor type (Burnier 2001; Jackson 2006).

Ang II receptor type 1 (AT_1 receptor)

Most of the known biological effects of *Ang II* are mediated by the AT_1 receptors in cardiovascular, renal, neuronal, endocrine, hepatic and other target cells, which are specifically blocked by AT_1 receptor antagonists, widely used as antihypertensive drugs, “sartans” (de Gasparo et al. 2000; Burnier 2001). The first angiotensin receptors were cloned in 1991 (Murphy et al. 1991). *Ang II* binding to the AT_1 receptor induces a conformational change in the receptor molecule which promotes its interaction with the G-protein(s), which in turn mediate signal transduction via several plasma membrane effector systems (de Gasparo et al. 2000). In rodents, the AT_1-receptors are further divided into AT_{1a} and AT_{1b} receptors (Kakar et al. 1992), which are 95% identical in amino acid sequence (de Gasparo et al. 2000). These two subtypes have been reported to have similarities in their ligand binding and activation properties but to differ in their tissue distribution. The AT_1 receptor contains a polymorphism reportedly

associated with hypertension (Timmermans et al.1993b; Kainulainen et al. 1999).

Ang II receptor type 2 (AT_2 receptor)

AT_2 receptors are less well characterized than AT_1 receptors, but are considered to be cardiovascular protective receptors which antagonize the effects of *Ang II* mediated via AT_1 receptors. They were first found and cloned in the 1990s (Kambayashi et al. 1993; Nakajima et al. 1993). The AT_2 receptors are clearly distinct from the AT_1 receptors in tissue-specific expression and in signalling mechanism, but like other angiotensin receptors they belong to the superfamily of G-protein-coupled receptors (de Gasparo et al. 2000). AT_2 receptors may exert the antiproliferative, proapoptotic, vasodilatory and antihypertensive effects of angiotensins, and they evidently have an important role in prenatal development (de Gasparo 2000; Jackson 2006). These receptors seem to be re-expressed and up-regulated in some pathological conditions in adults, for example cardiac hypertrophy, myocardial infarction and wound healing (Mizoue et al. 2006; Oishi et al. 2006). PD123,319 is a selective AT_2 receptor antagonist (Ford et al. 1996) and CGP 42112A is a selective agonist for this receptor type (Ewert et al. 2003).

Ang II receptor type 3 (AT_3 receptor)

The role and function of AT_3 receptors is not known, but they are reported to be present in neuroblastoma cells in amphibians (Burnier 2001).

Ang II receptor type 4 (AT_4 receptor)

An AT_4 receptor type is known to be involved in cardiovascular pathology. It is considered to be a target receptor especially for *Ang IV*, which can be generated by degradation of *Ang II*, by aminopeptidases or by other proteases, which in turn could be activated during tissue damage, suggesting that elevated *Ang IV* levels will be found in pathologic conditions. (Mustafa et al. 2001; Ruiz-Ortega et al. 2007). On the other hand, AT_4 is also a target receptor for *Ang (3-7)*, which is a break-down product of vasorelaxing *Ang (1-7)* (Handa 2000).

Mas receptor

The Mas-receptor was first found in the mouse kidney and subsequently in other organs, e.g. heart, brain and vasculature (Santos et al. 2003; Iwata et al. 2005). *Ang (1-7)* is held to be an endogenous ligand for this receptor type (Santos et al. 2003), which is distinct from the AT_1 and AT_2 receptors. It is a G protein-coupled receptor encoded by the Mas protooncogene. It mediates a number of the positive cardiovascular effects of *Ang (1-7),* namely vasodilatation, antiproliferation and antifibrosis, and it has a role in fluid volume homeostasis. *In vivo* the Mas receptor acts antagonistically to the AT_1 receptor, and in addition can hetero-oligomerize with the AT_1 receptor and thereby inhibit the actions of *Ang II* (Kostenis et al. 2005). AVE 0991 is another known agonist for the Mas receptor and it can mimic some effects of *Ang (1-7)* (Pinheiro et al. 2004; Lemos et al. 2005). At least two known antagonists for the Mas receptor have been identified as D-Ala^7-angiotensin (1-7) (A779) and Pro^7-angiotensin-(1-7) (Silva et al. 2007).

2.1.3 Blood pressure

The circulating RAS has an essential role in the regulation of blood pressure and body fluid balance. RAS acts as a feedback system, in which *Ang II* is traditionally considered the main regulatory peptide and *ACE* the main regulatory enzyme and AT_1 the main regulatory receptor. In respect of regulation of blood pressure the most important actions of *Ang II* are vasoconstriction, sympathetic nervous stimulation, increased aldosterone biosynthesis and renal actions (Fyhrquist et al. 1995; Luft 2001). These *Ang II* effects elicit tissue responses mainly via AT_1 receptors (Hirsch et al. 1990; Crowley et al. 2007). In fact, according to recent evidence the circulating RAS is not held to be directly responsible for the rise in blood pressure especially in essential hypertension in elderly people, there being more important non-circulating “local” renin-angiotensin systems, which have important roles in regulating blood pressure and regional blood flow (Beevers et al. 2001). Local tissue RAS is discussed in section 2.2. In other words, there are angiotensins and enzymes other than *Ang II* and *ACE* at tissue level which are involved in the regulation of blood pressure as counter-regulatory factors to *Ang II*, e.g. *ACE2* and *Ang (1-7) (*Grobe et al. 2007; Ferreira and Raizada 2008).

Hypertension

Arterial hypertension is a major modifiable risk factor for cardiovascular, cerebrovascular and renal disease and mortality. The worldwide prevalence of hypertension in the adult population was about 26% in 2000, and is increasing in economically developed countries concomitantly with increasing age, obesity and less physical activity (Kearney et al. 2005). About 95 % of hypertensive subjects suffer from an essential, idiopathic hypertension whose etiology remains unknown. The remaining 5 % are secondary to a specific reason for high pressure. The most important etiological factors are renal and renovascular reasons. There is no specific level of blood pressure where end organ complications set in, but a pressure level over 140/90 mmHg in repeated measurements is regarded as a risk level for most individuals (Carretero and Oparil 2000a; Hemmelgarn et al. 2006). On the other hand, genetic factors have been estimated to account for about 30 % of variation in blood pressure (Beevers et al. 2001). According to the World Health Organization and the International Sociey of Hypertension (WHO-ISH 2003) the limits of normal hypertension are more strict the upper limits being 130/85 mmHg (Whitworth 2003).

As the name of the RAS (renin-angiotensin system) would indicate, renin plays an important role in the regulation of blood pressure via control of systemic *Ang II* levels. Measured renin levels correlate with the circadian rhythm of normal blood pressure, which is at a lower level during the night (Stern et al. 1986; Hamada et al. 2008). Observations to the contrary have been reported: especially in elderly hypertensive people RAS and renin activity seem to be at lower levels (Beevers et al. 2001). Although the precise etiology of hypertension is not known, drugs acting on RAS, e.g. ACE-inhibitors and AT_1 receptor blockers, are among the most potential, clinically used antihypertensive agents.

2.1.4 Angiotensin-converting enzyme (*ACE*)- inhibitors

The development of *ACE* inhibitors began over 40 years ago when *teprotide* was first discovered in the poison of *Bothrops jararaca* snake in Brazil (Ferreira 1965). *Teprotide* was perceived to inhibit *kininase II,* but only when administered intravenously. About ten years later the first oral *ACE* inhibitor, captopril, was developed (Ondetti et al. 1977). Nowadays *ACE* inhibitors like captopril are widely used in the treatment of hypertension as well as of heart insufficiency. Their positive cardiovascular effects are especially advantageous in hypertensive patients with diabetes mellitus type 2 and nephropathy, in that

they reduce proteinuria and delay the development of renal diseases (Schmieder et al. 2007). The mechanism of action of *ACE* inhibitors is targeted to inhibit the function of angiotensin-converting enzyme, formation of *Ang II* being inhibited (Ruskoaho 1984), this also, however, leading to increased plasma bradykinin levels. *ACE* inhibitors do not inhibit the action of *ACE2*, and thus the vasorelaxing effects of *Ang (1-7)* and bradykinin cascade remain intact (Carretero and Oparil, 2000b; Burnier 2001). On the other hand, bradykinin can be involved in adverse effects of *ACE* inhibitors, for example cough and angioedema (Nussberger et al. 1998).

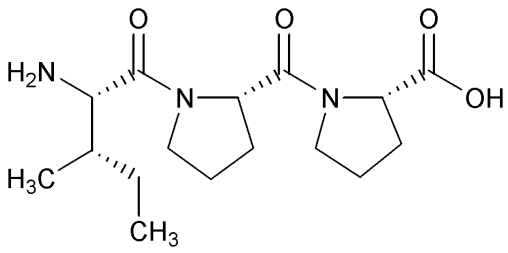
2.1.5 *Ang II* receptor type 1 blockers (ARB)

The very first AT_1 receptor blocker was *saralasin*, a non-selective peptidic antagonist of *Ang II* which when administered intravenously made it possible to investigate angiotensin receptors even at the beginning of the 1970s. The first oral AT_1 receptor antagonist, losartan, was developed by DuPont Merck Laboratories in 1988 after the finding of *ACE* inhibitors. In recent years, numerous orally active AT_1 receptor antagonists have been synthesized. These antagonists, also called *Ang II* receptor type 1 blockers (ARB), are used especially in the treatment of hypertension, heart failure and renal disease, and they have overall a high affinity to the AT_1 receptors when the function of these receptors is inhibited and *Ang II* action is diminished, leading e.g. to vasorelaxation (Kööbi et al. 2003). On the other hand, they have no affinity to AT_2 receptors, but they have reported to exhibit high protein binding rates in plasma (Carretero and Oparil, 2000b; Burnier 2001). The advantage of ARBs is their good antihypertensive effect with minor adverse effects.

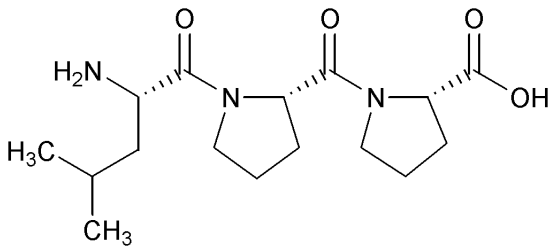
2.1.6 Bioactive tripeptides

In addition to the *ACE* inhibitors and *ARBs*, accumulating evidence would indicate that small bioactive peptides, e.g. casein-derived peptides have positive cardiovascular effects even when added to food (Jauhiainen and Korpela 2007; Möller et al. 2008; Erdmann et al. 2008). According to animal (Jauhiainen et al. 2005a) and human (Jauhiainen et al. 2005b) studies long-term oral treatment with milk products containing small tripeptides has lowered blood pressure and reduced arterial stiffness in hypertensive patients. Investigations have focused especially on tripeptides containing amino acids *Ile-Pro-Pro* (IPP), *Val-Pro-Pro* (VPP) and *Leu-Pro-Pro* (LPP); for molecule

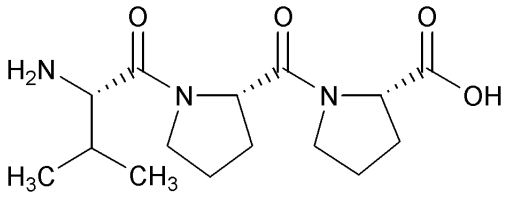
structures, see *Figure 2*. IPP has been shown to have most powerful effects on blood pressure. The antihypertensive mechanism of bioactive peptides is not exactly known but it has been surmised to be related to inhibition of *ACE,* but also to calcium, potassium and magnesium metabolism (Hong et al. 2008).



IPP



LPP



VPP

Figure 2. Molecule structures of bioactive tripeptides (Bachem Distribution Services GmbH).

2.2 TISSUE RENIN-ANGIOTENSIN SYSTEM

In addition to the circulatory RAS there is a tissue-localized system which has been known for some time and which is seen to regulate long-term changes in a variety of organs (Metsärinne et al. 1996; Bader et al. 2001). In the other words, RAS is not only endocrine but also complicated autocrine system. In tissues *Ang II* is derived either from the circulation, or from its local production. Local *Ang II* formation can also be catalyzed by enzymes other than the classical *ACE,* actions termed renin-independent or *ACE*-independent pathways for *Ang II* production (Kramkowski et al. 2006). By blocking the activity of these enzymes *Ang* II production can be reduced. In addition to *ACE*- independent enzymes, there is an important recently discovered RAS component: angiotensin-converting enzyme 2 (*ACE2)*. *ACE2* can degrade *Ang I* to *Ang (1-9)* and *Ang II* to form the biologically active *Ang (1-7)*, which in turn acts in many respects opposite to *Ang II.* According to the literature these alternative pathways for *Ang II* production and for degradation of *Ang II* are important in both physiological and pathophysiological conditions (Urata et al.1990; Bacani and Frishman 2006).

2.2.1 Alternative pathways for *Ang II* production

Chymostatin-sensitive Ang II generating enzyme (CAGE)-dependent pathway of Ang II production

CAGE is a protease able to convert *Ang I* to *Ang II*. It is found e.g. in the human, monkey and dog aorta, distributed predominantly in the adventitia, while *ACE* is found localized mainly in the endothelium. Such a contrasting distribution may indicate the distinct functional role of these two enzymes. The exact role of *CAGE* in physiology is yet unknown (Okunishi et al. 1987; Kramkowski et al. 2006).

Chymase-dependent pathway of Ang II production

Chymases (α- and β-chymase) are chymotrypsin-like serine proteases found in the heart, kidney, vascular smooth muscle and secretory granules of mast cells. They are able to cleave *Ang I* to produce *Ang II*, but not to form *Ang II* direct from angiotensinogen (Urata et al. 1990; Miyazaki and Takai 2006). Chymase-mediated *Ang II* production may have an important role especially in pathological conditions (Bacani and Frishman 2006). Chymase may be

associated with the development of diabetic and hypertensive nephropathy (Huang et al. 2003), vascular proliferative diseases (Nishimoto et al. 2001) and myocardial infarction (Jin et al. 2002).

Cathepsin G-dependent pathway of Ang II production

Membrane-bound cathepsin G expressed on neutrophils is a serine protease able to convert *Ang I* to *Ang II*, but also to produce *Ang II* direct from angiotensinogen (Klickstein et al. 1982; Belova 2000). Cathepsin G may evince potent local vasoactive and chemoattractant properties in inflammation (Owen and Campbell 1998). An other serine protease, called tonin (Grisé et al.1981), as well as the tissue-type plasminogen activator, trypsin and chymotrypsin, are also able to release *Ang II* directly from angiotensinogen (Kokkonen at al. 1998).

2.2.2 ACE 2 dependent pathway of *Ang II* metabolism

Angiotensin-converting enzyme 2 (ACE2)

The human angiotensin-converting enzyme-related carboxypeptidase (*ACE2*) is a structurally related homolog of *ACE* with 42% protein sequence identity (Donoghue et al. 2000; Vickers et al. 2002), but it acts contrary to the carboxypeptidases, and increases *Ang (1-9)* and *Ang (1-7)* formation. Unlike *ACE, ACE2* is not able to degrade bradykinin. *ACE2* is mainly expressed in cardiac blood vessels, kidneys and testis (Tipnis et al. 2000). It is considered to be a balancing counter-regulator in the RAS, as it is able to convert especially the bioactive *Ang II* to form vasorelaxing *Ang (1-7)* with high affinity, and *Ang I* to form *Ang (1-9)*, which in turn serves as a substrate for the generation of *Ang (1-7)* (Donoghue et al. 2000; Mustafa et al. 2001). It is of importance in that both *Ang (1-7)* and *Ang (1-9)* have physiological effects opposite to those of *Ang II*. In the absence of *ACE2*, the predominant effects of *Ang II* lead to vasoconstriction and hypertension. In the light of such findings, *ACE2* can be regarded as an important modulator of blood pressure (Yagil and Yagil 2003).

2.2.3 Ocular RAS expression

RAS in ocular tissues has also been under investigation during recent years. Most of the recognized RAS components have already been detected in the human eye (Danser at al. 1994; Wagner et al. 1996), except for the recently described Mas receptor for *Ang (1-7)* and novel peptidases degrading angiotensins. Prorenin, the precursor of renin, has been identified in the human ciliary body responsible for aqueous humor formation (Sramek et al. 1988). Renin mRNA has been detected in the retinal pigment epithelium and choroid (Wagner et al. 1996). Angiotensinogen has also been found in the non-pigmented epithelial cells (NPEC) of ciliary body (Sramek et al. 1992), and its gene expression has been demonstrated in the retina, choroid and sclera (Wagner et al. 1996). *Ang I* has been found in aqueous humor (Danser et al. 1994) and *Ang II* in many human ocular tissues: in the NPEC, in cells of the cornea, in epithelial cells of the conjunctiva, in trabecular meshwork (TM) cells as well as in ganglion cells, and photoreceptor cells of the retina, in addition to endothelial cells in retinal and choroid vessels (Savaskan et al. 2004). *ACE* has been identified in the human NPEC but also in the retina and choroid (Savaskan et al. 2004). ACE has also been found in the human tear fluid (Immonen et al. 1987). *ACE2* has been localized in Műller cells and photoreceptors in the retina (Tikellis et al. 2004) and *Ang (1-7)* has very recently been found in the human retina (Senanayake et al. 2007). *Ang II* receptors (predominantly type 1) are present in the retina, e.g. in Műller cells and blood vessels (Senanayake et al. 2007) and in ganglion cells as well as in the cornea (Savaskan et al. 2004). AT_2 receptors are also localized in Műller cells, in ganglion cells and in the inner nuclear layer of the retina (Senanayake et al. 2007). For details, see *Table 1.*

Expression of ocular RAS has also been investigated in several animal studies. For details, see *Table 1.*

Table 1. Localization of RAS components in ocular tissues of different species (Vaajanen et al. 2008).

RAS molecule	*Eye part*	*Species*	*References*
Prorenin	Retina	Human	Sramek et al., 1988
	Ciliary body	Human	Danser et al., 1989
	Vitreous body	Human	
Renin	Retina	Human, rabbit	Danser et al., 1989
	Ciliary body	Rabbit	Wagner et al., 1996
	Choroid	Human, rabbit	Ramirez et al., 1996
	Iris	Rabbit	
	Vitreous body	Human, rabbit	
	Aqueous humor	Rabbit	
Angiotensinogen	Retina	Human, rabbit	Sramek et al., 1992
	Ciliary body	Human, rabbit	Ramirez et al., 1996
	Choroid	Human, rabbit	Wagner et al., 1996
	Iris	Human, rabbit	
	Vitreous body	Human, rabbit	
	Aqueous humor	Rabbit	
ACE1	Retina	Dog, monkey, human, rabbit, porcine	Vita et al., 1981 Weinreb et al., 1985
	Ciliary body	Human, rabbit, porcine	Immonen et al., 1987
	Choroid	Dog, monkey, human, rabbit, porcine	Ramirez et al., 1996 Wagner et al., 1996
	Sclera	Dog, monkey	Shiota et al., 1997
	Iris	Rabbit, porcine	Geng et al., 2003
	Cornea	Human	Savaskan et al., 2004
	Vitreous body	Dog, monkey, rabbit	
	Aqueous humor	Human, dog, monkey, rabbit	
	Tear fluid	Human, rabbit	
ACE2	Retina	Rodent	Tikellis et al., 2004
		Human	Senanayake et al., 2007
Chymase	Choroid	Dog	Shiota et al., 1997
	Sclera	Dog	Maruichi et al., 2004
	Vitreous body	Human	
Ang II receptor type 1	Retina	Human	Savaskan et al., 2004
	Cornea	Human	Senanayake et al., 2007
Ang II receptor type 2	Retina	Human	Senanayake et al., 2007
Ang I	Retina	Porcine	Danser et al., 1994
	Choroid	Porcine	
	Vitreous body	Porcine, human	
	Aqueous humor	Human	
Ang II	Retina	Human, porcine, rabbit	Danser et al., 1994
	Ciliary body	Human, rabbit	Ramirez et al., 1996
	Choroid	Porcine, human, rabbit	Savaskan et al., 2004
	Iris	Rabbit	Senanayake et al., 2007
	Cornea	Human	
	Vitreous body	Porcine, human, rabbit	
	Aqueous humor	Human, rabbit	
Ang 1-7	Retina	Human	Senanayake et al., 2007

2.3 INTRAOCULAR PRESSURE

The average volume of the adult human eye globe is about 6.5 cm^3 and the average globe dimensions are 24 mm (anterior-posterior), 23 mm (vertical) and 23.5 mm (horizontal). The vitreous body comprises about 80 % and aqueous humor (AH) 20 % of the globe volume (Sherman et al. 2006). In the healthy human eye, the flow of AH against resistance generates an IOP of about 15 mmHg, which is necessary for the proper shape. The circulating AH nourishes unvascularized eye structures such as the cornea and lens and it has an important role in the optical system (Brubaker 1982; Millar et al. 2006). IOP is maintained by a homeostatic balance between formation and outflow of AH. For anatomy and AH pathway, see *Figure 3.*

2.3.1 Aqueous humor formation

AH is secreted by the ciliary epithelium lining the ciliary processes mainly by active ionic transport across the epithelium against a concentration gradient. (Hoy and Delamere 2002; Millar et al. 2006). The anatomy of the ciliary process is depicted in *Figure 4.* Active secretion requires energy, normally provided by the hydrolysis of adenosine triphosphate by Na^+/K^+ ATPase (Caprioli 1992). Energy-dependent active transport of sodium into the posterior chamber by the non-pigmented ciliary epithelial cells (NPEC) results in water movement from the stromal pool into the posterior chamber. Active transport of Cl^- and HCO^-_3 (formed in the reaction sequence catalyzed by carbonic anhydrase) occurs to a lesser extent (Caprioli 1992). In addition to active secretion there are two essential physiological processes in the formation of AH: diffusion from the blood compartment and ultrafiltration. These two processes are passive and require no active cellular participation. Diffusion of solutes across cell membranes occurs down a concentration gradient, and substances with high lipid solubility coefficients which can easily penetrate biological membranes move readily in this way. Ultrafiltration is the term used to describe the bulk flow of blood plasma across the fenestrated ciliary capillary endothelia into the ciliary stroma; it can be increased by augmentation of the hydrostatic driving force (Millar et al. 2006). Recent findings such as the discovery of anti-angiogenic factors in the human ciliary body may open up new prospects for an understanding of AH secretion, IOP and the progression of glaucoma. The ciliary body should be regarded as a multifunctional and interactive tissue (Coca-Prados and Escribano 2007).

The AH formation rate in the healthy human eye is 2.5-2.8 µl/min and the entire volume is replaced every 100 min. It is known to reduce in certain circumstances: during sleep, with ageing and in some systemic diseases such as diabetes (Brubaker 1991). There is a circadian rhythm of flow, with the highest rate during morning hours and the lowest during night hours especially in a sleep. The nighttime reduction of AH flow has been reported to be even 45% (Reiss et al. 1984), but the suppression of flow is greater than the change of intraocular pressure (Ericson 1958). On the other hand, IOP depends on the body position: it is higher in head - down vs. head - up position while aqueous flow is same in both body positions (Carlson et al. 1987). AH formation is almost stable up to the age of 60, but thereafter it decreases with advancing age (Becker 1958). A slight decline of flow rate occurs after even age 10, about 3 % per decade (Brubaker 1981). In addition, there has been reported to be a tendency toward less AH formation eg. in the advanced stages of diabetic retinopathy (Larsson et al. 1995).

Under normal conditions active secretion accounts for 80% to 90% of total AH formation (Weitzman and Caprioli 2006). Active secretion is essentially pressure-insensitive at near-physiological IOP. However, the ultrafiltration component in AH formation is sensitive to changes in IOP, decreasing as this increases. This phenomenon is quantifiable and is termed pseudofacility, because a pressure-induced decrease in inflow appears as an increase in outflow when techniques such as constant-pressure perfusion are used to measure outflow facility (Bàrany 1963; Beneyto et al. 1995). From the posterior chamber AH flows around the lens and through the pupil into the anterior chamber, from which it leaves the eye through two main pathways at the anterior chamber angle.

2.3.2 Aqueous humor drainage

AH exits the eye principally through the trabecular meshwork in the chamber angle and Schlemm's canal into the aqueous veins. This is called trabecular or conventional outflow (Lütjen-Drecoll et al. 2001). The state of the actin cytoskeleton and adhesions of trabecular meshwork cells are important determinants of fluid outflow through the trabecular meshwork (Tan et al. 2006). On the other hand, fluid flow through the inner wall endothelium of Schlemm's canal is controlled by the location of the giant vacuoles and pores present in cells of the endothelium, but the flow resistance itself is more likely to be generated either in the extracellular matrix of the juxtacanalicular connective

tissue or the basement membrane (Johnson 2006). A smaller proportion of AH makes its way directly into the ciliary body and is drained by way of the ciliary muscle, the suprachoroidal space, and the sclera, a process termed uveoscleral or unconventional outflow (Lütjen-Drecoll et al. 2001). In addition there is an uveo-vortex route for AH drainage ie. a route via the iris blood vessels and the vessels of ciliary muscle draining to the vortex veins. AH can also move by bulk flow to the suprachoroidal space from which it is picked up by the choroidal blood supply concerned with drainage of the anterior uvea and reaches the vortex veins (Green et al. 1977). The main route (90%) of drainage in the normal eye is that through the trabecular meshwork. This outflow channel is pressure-dependent (Millar et al. 2006). Uveoscleral outflow constitutes approximately 10% of total outflow, and it is virtually independent of IOP levels greater than 7 to 10 mmHg. The other alternative, albeit minor, pathways of outflow are those through iris vessels, corneal endothelium or anterior vitreous body (Weinreb 2000).

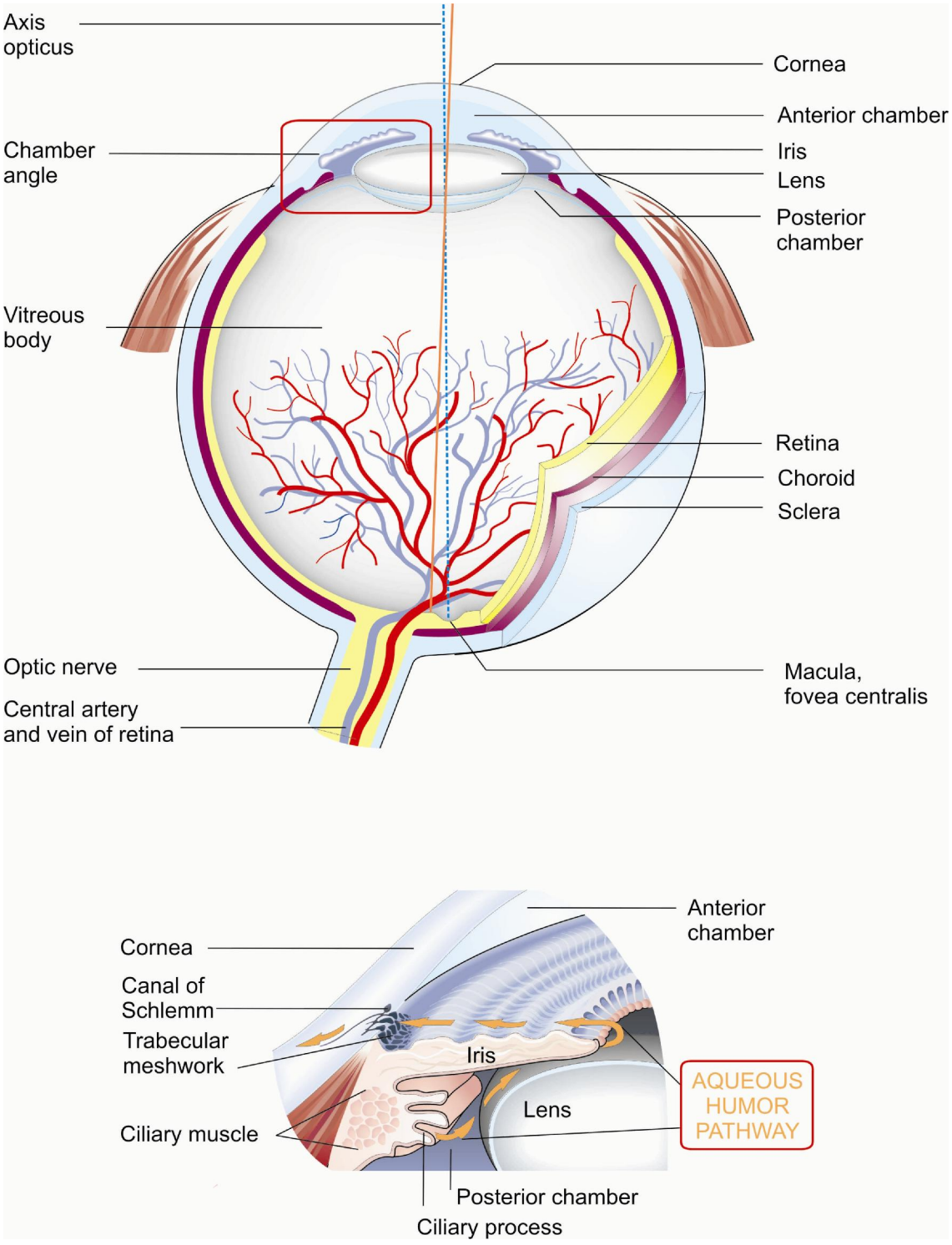


Figure 3. Anatomy of the human eye and aqueous humor pathway.

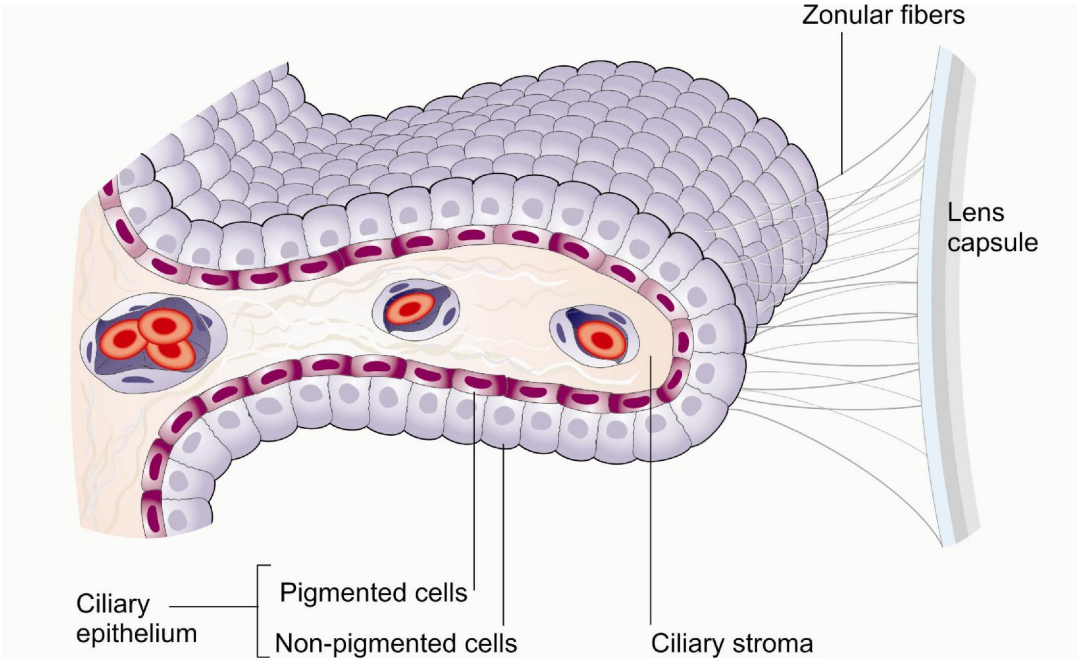


Figure 4. Anatomy of ciliary process.

2.3.3 Goldmann`s equation

As noted above, IOP is maintained by a homeostatic balance between formation and outflow of AH. The tissues of the anterior chamber angle offer a resistance to fluid outflow. IOP builds up, in response to the inflow of AH, to a level sufficient to drive fluid across that resistance at the same rate as it is produced by the ciliary body. This is the steady-state IOP. In the glaucomatous eye this resistance is unusually high, causing elevated IOP (Millar et al. 2006). Goldmann`s equation has served for over 50 years as an adequate description of aqueous humor dynamics (Goldmann 1951; Brubaker 2004).

$$F=(P_i - P_e) \times C$$

F= the rate of aqueous humor formation
P_i= intraocular pressure
P_e= episcleral venous pressure
C= tonographic facility of outflow

2.3.4 Regulation of intraocular pressure

The precise mechanisms in the regulation of IOP as well as underlying reasons for glaucomatous optic nerve damage are not known. The autonomic nervous system may have a major role in the regulation of IOP by reason of the existence and function of its receptors for the relevant structures involved in AH formation (Ruskell 1982) and drainage (Millar et al. 2006). In addition to IOP, ocular perfusion instability and vascular dysregulation are both contributed to glaucomatous optic neuropathy. The main cause of the perfusion instability is a disturbed autoregulation in the context of a general vascular dysregulation which can be caused by dysfunction of autonomic nervous sytem and vascular endothelial cells (Gherghel et al. 2004; Grieshaber and Flammer 2005). Circulation and blood pressure are partly regulated by the autonomic nervous system but also by RAS, which acts via vasoconstriction but also via body sodium and fluid balance mechanisms (Jackson 2006). Thus local RAS may be the other major player in the regulation of IOP, the mechanism of action being involved more probably in the formation of aqueous humor, but also having a role in its drainage.

Autonomic nervous system

In general, parasympathomimetics (cholinergic drugs) acting via muscarine receptors cause vasodilation in the anterior segment, resulting in increased blood flow to the choroid, iris, ciliary processes and ciliary muscle (Sato and Sato 1995; Barbelivien 1995). Opinions vary as to the direct influence of cholinergics on AH formation but their IOP-lowering effects are assumed to be mediated by a decrease in the resistance in aqueous outflow. The action is mediated entirely by ciliary muscle contraction and alteration in the trabecular meshwork configuration, leading to reduced resistance to AH outflow with no direct pharmacological effect on the trabeculat meshwork itself (Kaufman and Bárány 1976). Parasympathomimetics are also reported to diminish drainage through the uveoscleral route (Weitzman and Caprioli 2006).

Sympathetic (adrenergic) drugs act via α_1, α_2, β_1 or β_2 receptors, which have opposite actions. *Activation* of α receptors by sympathomimetics improves AH outflow and probably also its formation, while *inhibition* of β receptors by sympathetic receptor blockers reduces AH formation, both actions leading to reduced IOP. Timolol, one of the most effective antiglaucomatous agents, acts via non-selective β-receptor blocking (Zimmerman et al. 1977; Yablonski et al. 1978) Sympathominetics affect smooth-muscle tone in the iris and ciliary body

and their receptor stimulation may alter intraocular, intrascleral and extrascleral vascular tone, while also having possible direct effects on the endothelium lining the outflow pathways, all of which may alter total outflow facility (Townsed and Brubaker 1980; Millar et al. 2006).

Other mechanisms

Several other mechanisms may be involved in the regulation of IOP: serotonergic (Krootila et al. 1987), dopaminergic (Siegel et al. 1987), adenosinergic (Crosson 1995), and prostaglandinergic (Camras et al. 1996) as well as corticosteroid- and glycosaminoglycans-mediated mechanisms (Millar et al. 2006, Coca-Prados and Escribano 2007).

The prostaglandin mechanism may be one of most important ones in that exogenous prostaglandin analogues are among the most potent antiglaucomatous drugs. They enhance uveoscleral outflow (Weitzman and Caprioli 2006). Endogenous prostaglandins may be involved in low IOP in eye inflammation processes (Goldstein and Tessle 2006). The corticosteroid mechanism is also clinically important, since topical or systemic glucocorticoids may induce elevation of IOP in susceptible individuals (Yamamoto et al. 2008).

2.3.5 Glaucoma

Definition

Glaucoma is a multifactorial long-term ocular neuropathy which is associated with a progressive loss of the visual field, retinal nerve fiber structural abnormalities and optic disc changes (Bathija et al. 1998; McKinnon et al. 2008). Normal (mean ± SD) IOP is 15.5 (±2.57) mmHg, but due to a gaussian distribution in which two standard deviations include the values of about 95% of the population, an IOP over 20.5 (±2) mmHg could be considered as upper limit for normal IOP. Before settling on a glaucoma diagnosis in patients with elevated IOP, it is essential that characteristic optic nerve head cupping or visual field abnormalities have appeared, otherwise high IOP is to be regarded as ocular hypertension (Kwon and Caprioli 2006). Optic nerve cupping (=excavation) means that the nerve head cup:disc ratio is 0.5 or greater. Also a difference in cup: disc ratio of 0.2 or more between the right and left eye is a pathognomic disturbance caused by glaucoma (Dielemans et al. 1994). Other signs attributable to glaucoma are increased pallor of the nerve head, changes

in vessels, splinter hemorrhage, peripapillary atrophy and retinal nerve fiber layer defects (Infeld and O`Shea 1998). On the other hand, a glaucoma diagnosis can be reached even in ocularly normotensive patients if optic nerve cupping or typical visual field defects are manifested. This situation is seen in low-tension ie. normotensive glaucoma eyes (Grosskreutz and Netland 1994). Primary open-angle glaucoma is usually a symptomless and progressive illness which if left untreated leads to visual disability and eventual blindness (Weinreb and Khaw 2004).

Epidemiology and risk factors

Worldwide glaucoma is the second leading cause of blindness after cataract (Weinreb and Khaw 2004). Incidence data on true glaucoma are limited; according to the population-based Barbados Incidence Study of Eye Diseases (1992-1997, n=3427), the observed four-year incidence of open-angle glaucoma was 1.2 % (95% CI: 0.6, 2.1%), being highest in elderly persons (70 or more years) 4.2% (95% CI: 2.6, 6.3%) (Wu et al 2001). There will be 60.5 million people with glaucoma in 2010, increasing to 79.6 million by 2020, and of these, 74% will have OAG. Asians will represent 47% of those with a glaucoma diagnosis, and with angle-closure glaucoma even up to 87%. Bilateral blindness will be present in 4.5 million people with OAG in 2010, rising to 5.9 million people in 2020 (Quigley and Broman 2006). A major modifiable risk factor for glaucoma is (elevated) IOP, others including increasing age, black race, male sex, positive family history (Sommer 1996; Deva et al. 2008) and in addition lean body mass and a cataract history (Leske et al. 1995). Factors considered as minor, are myopia, diabetes mellitus, systemic hypertension (Bonomi et al. 2000), migraine / vasospasms and vascular dysfunction (Tielsch et al. 1995; Grieshaber and Flammer 2005).

Pathogenesis

There are several theories with respect to the pathogenesis of glaucoma diseases, but the precise mechanism of POAG is unknown. The mechanical theory envisages direct pressure-induced damage to the retinal ganglion cell axons at the level of the lamina cribrosa. The vascular theory proposes microvascular changes and resultant ischemia in the optic nerve head. Cellular and molecular events conceivably leading to glaucomatous retinal ganglion cell death have also been proposed in the pathogenesis of glaucoma (Kwon and Caprioli 2006). It may be concluded that although elevated IOP is the major known risk factor for glaucoma, the condition is linked at least to altered ocular

Table 2. Classification of glaucoma subtypes according to Duane`s Ophthalmology (2006).

A	**Developmental glaucoma** 1. Primary congenital glaucoma 2. Glaucoma associated with congenital anomalies 3. Secondary glaucoma in infants
B	**Primary open-angle glaucoma** 1.Primary open-angle glaucoma 2.Ocular hypertension 3.Normotensive glaucoma
C	**Primary angle-closure glaucoma** 1.Pupillary block glaucoma 2.Plateau iris 3.Ciliary block glaucoma (malignant glaucoma)
D	**Lens-related glaucoma**
E	**Exfoliative glaucoma**
F	**Pigmentary glaucoma**
G	**Glaucoma following trauma**
H	**Uveitic glaucoma**
I	**Corticosteroid-induced glaucoma**
J	**Glaucoma associated with retinal disorders**
K	**Glaucoma associated with corneal disorders**
L	**Glaucoma in aphakia and pseudophakia**

blood flow; fluctuations in blood flow are more harmful in glaucomatous optic neuropathy than a steady reduction in ocular blood flow (Tielsch et al. 1995; Grieshaber and Flammer 2005). In addition, e.g. fluctuations in systemic blood pressure (episodic nocturnal hypotension) can increase the susceptibility of the optic nerve head to damage (Mitchell et al. 2004).

Glaucoma subtypes

The glaucoma diagnosis comprises heterogeneous groups of diseases causing elevated IOP or typical ocular damage, and it can be divided into subtypes according to its etiology, pathophysiological mechanisms or anatomical properties. One mode of classification is shown in *Table 2*. Primary open-angle glaucoma is the most common form (McKinnon et al. 2008). On the other hand, up to 50% of POAG patients have normal IOP and thus so-called normotensive glaucoma (Tielsch et al. 1991; Grosskreutz and Netland 1994).

Current pharmacotherapy

All therapies currently used for the treatment of glaucoma are aimed at lowering IOP or preventing a rise in IOP in order to minimize cell death. Therapeutic agents under wide investigation are neuroprotectants, which target the disease process manifested in the death of retinal ganglion cells, axonal loss and irreversible loss of vision (Khaw et al. 2004; McKinnon et al. 2008). A reduction in IOP by 30% reduces disease progression from about 10% to 35%, even in normotensive glaucoma patients (Tielsch et al. 1995; Bonomi et al. 2000). The target IOP level in the treatment of glaucomatous eyes is about 25% to 30% lower than the baseline pressure before treatment, or even greater if there is substantial damage in the visual field (Jampel 1997). Current pharmacotherapy comprises drugs acting on adrenergic α - and β- receptors or on cholinergic muscarine receptors, prostaglandin analogues and carbonic anhydrase inhibitors and combinations of these compounds (Vapaatalo 1995; McGinnon et al. 2008). They are administered mainly topically and targeted either to reduce the formation of aqueous humor in the ciliary body or to increase outflow through uveoscleral pathways (*Table 3).*

Table 3. Effects of ocular hypotensive agents on intraocular pressure and aqueous humor dynamics (Weitzman and Caprioli 2006).

Compound	*IOP*	*Aqueous production*	*Conventional outflow facility*	*Uveoscleral outflow facility*
Non-selective β-blocker	↓ 20%-30%	↓ 35%		
β1 selective β-blocker	↓ 15%-25%	↓ 25%		
Direct miotic	↓ 15%-25%		↑ 25%	↓
Non-selective adrenergic agonist	↓ 15%-25%		↑	↑
α2-agonist	↓ 20%-30%	↓ 35%		?
Carbonic anhydrase inhibitor	↓ 20%-35%	↓ 35%		
Prostaglandin analogue	↓ 25%-35%			↑ 100%

Blood-ocular barriers

Blood-ocular barriers are important in protecting the eyes as is the blood-brain barrier in protecting the brain i.e. compartments in the systemic circulation have to penetrate blood-ocular barriers in order to penetrate the eye, which can occur at least if the barriers are broken. This is also important in respect of systemic RAS and drug penetration from the circulation into the eye structures. Two blood-ocular barriers are clinically significant: the blood-retina barrier (BRB) and the blood-aqueous barrier (BAB). The BRB may be seen to comprise two major components: the endothelium of retinal blood vessels (inner barrier) and the retinal pigment epithelium (outer barrier) (Cunha-Vaz 2004). The BAB is formed by an epithelial barrier located in the non-pigmented layer of the ciliary epithelium and in the posterior iridial epithelium, and by the endothelium of the iridial vessels (Cunha-Vaz 1979).

2.3.6 Relationship between BP and IOP

A number of human studies have been carried out on the relationship between ocular hypertension or glaucoma damage and systemic hypertension, but no clear consensus prevails as to whether IOP is related to the level of BP. There seems to be no relation between systemic and ocular hypertension (Tarkkanen et al. 2008). On the other hand, in some studies BP has been described as having a modest positive association with POAG or IOP (Tielsch et al. 1995; Bonomi et al. 2000). Particularly, poorly controlled hypertension seems to be related to a modestly increased risk of OAG, but independently of the effect of BP on IOP and other glaucoma risk factors (Mitchel et al. 2004). Low systemic BP has been found to be associated with reduced IOP (Klein et al. 2005), and arterial hypertension has been associated with increased IOP and high tension glaucoma (Dielemans et al. 1994). On the other hand, observations to the contrary have also been reported (Leske et al. 1995; Sommer et al. 1996).

2.3.7 Functional intraocular RAS

There is as yet only limited evidence regarding the role of the RAS in aqueous humor outflow, but *Ang II* has been reported to be able to induce cell proliferation in bovine trabecular meshwork cells and increase the synthesis of collagen *in vitro* (Shen et al. 2001). It has been reported that *Ang II* administered intracamerally diminishes uveoscleral outflow (Inoue et al. 2001b). On the other hand, synthetic and natural *Ang II* has been reported to reduce IOP in *in vivo* studies with anesthetized cats when administered intravenously (Macri et al. 1965). The same IOP-lowering effect has been seen in the enucleated, arterially perfused cat and human eye, the mechanism behind the effect being considered to consist in vasoconstriction of the iris artery. In recent human studies orally administerd losartan (ARB) (Costagliola et al. 2000) and captopril, an angiotensin-converting enzyme (*ACE*) inhibitor (Costagliola et al. 1995) have been shown to lower IOP even when administered orally. Topical application of olmesartan (ARB) (Inoue et al. 2001b; Wang et al. 2005), inhibitors of *ACE* (Watkins et al. 1987; Shah et al. 2000) and renin (Giardina 1990) has been reported to lower IOP in animal studies, the effect being more prominent in ocular-hypertensive animals (Inoue et al. 2001b; Wang et al. 2005a).

Taken together, expression of intraocular RAS has been demonstrated in a number of studies and it is involved in the regulation of IOP, being probably

more activated in glaucomatous eyes, the exact mechanism of action is remaining however unclear.

3 AIMS OF THE STUDY

Renin-angiotensin system (RAS) is an important regulator of blood pressure and body fluid homeostatic balance. Evidence is accumulating to indicate its importance also for local intraocular regulating systems. The aim of the present study was to investigate the expression of renin-angiotensin system in the different eye structures and its function in the regulation of IOP. Rabbits and rats were used as experimental animals for *in vivo* studies, and enucleated eyes of rats and pigs were used for *in vitro* studies.

The specific aims were:

1. To investigate RAS expression and function in the various ocular tissues (Studies I-III).

2. To compare the ocular hypotensive effects of different components of RAS using local (Study III) and systemic administration in ocular normotensive animals (Study IV).

3. To test whether the IOP-lowering mechanisms of angiotensins are related to changes in aqueous humor outflow facility (Study III).

4. To clarify the potential relationship between blood pressure and intraocular pressure in an experimental study (Study IV).

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS AND TISSUES

In Studies I and IV the experimental animal strains comprised male spontaneously hypertensive rats (SHR, n=16) together with their arterial normotensive Wistar Kyoto controls (WKY, n=16), and male double transgenic rats harboring human renin and human angiotensinogen genes (dTGR, n=35) together with their normotensive control animals (Sprague-Dawley, SD, n=12). The animals were kept at 22±1°C in a 12-h light-dark cycle (lights on 7 a.m. to 7 p.m.), relative humidity (40-70%) and ventilation (air volume change 20 times/h). The animals were kept four to a cage, and had free access to a standard laboratory diet and tap water. At the end of the *in vivo* study (Study IV) the rats were euthanized with CO_2/O_2 (AGA, Riihimäki, Finland) and decapitated. The eyes were immediately enucleated and snap-frozen for subsequent *in vitro* analyses (Study I).

Male New Zealand White rabbits (NZW, n=38) were used in Study III. Animals were housed in individual cages under a 12-h light-dark cycle (lights on 7 a.m. to 7 p.m.), and maintained conventionally during the study with regulated air temperature (15-21ºC), relative humidity (40-70%) and ventilation (air volume change 20 times/h). They had free access to a standard laboratory diet and tap water. At the end of the study the animals were euthanized by intravenous pentobarbital injection (100 mg/kg), (Mebunat®, Orion Ltd, Finland).

Fresh enucleated porcine eyes (n=56) were obtained from a local abattoir and from each eye three tissue samples were prepared for further enzyme activity analysis (Study II).

4.2 BIOCHEMICAL DETERMINATIONS

4.2.1 Real-time quantitative reverse transcriptase-polymerase chain reaction (RT- PCR) (I)

The rat eyes were enucleated immediately after sacrifice (Study IV), snap-frozen in isopentane at -40°C and stored at -70°C for subsequent RT-PCR analysis (Study I). The frozen eyes were embedded in OCT™ compound (Tissue-Tec®,Sakura, Japan), cornea upwards, and 150 µm *vertical* sections of the whole eye were cut using a cryostat (-19 ºC) (*Figure 5*.). The presence of

mRNA of AT_{1a}, AT_2 and Mas receptors was determined at the level of the ciliary body and the level of the anterior retina without ciliary tissue. The effect of oral antihypertensive treatment with *Ang II* receptor type 1 blocker on receptor mRNA expression was evaluated.

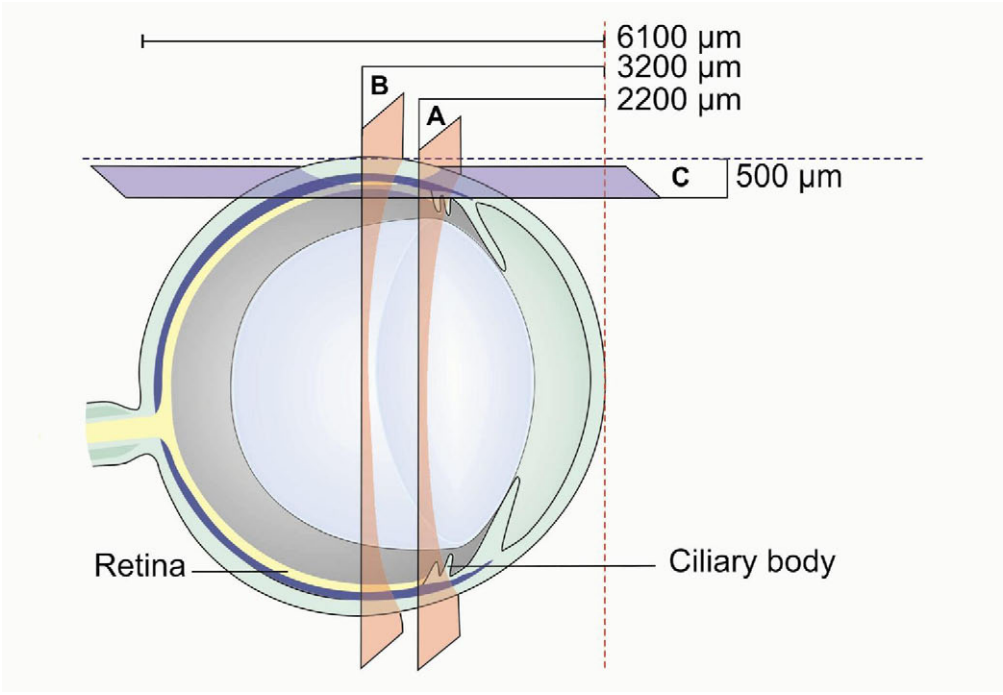


Figure 5. A schematic illustration of the rat eye. Two vertical sections were used for RT-PCR analysis: (A) level of ciliary body and (B) level of anterior retina. Horizontal sections were used for quantitative *in vitro* autoradiography analysis (C).

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue sections using Trizol reagent according to the manufacturer`s instructions. The RNA concentration was determined spectrophotometrically at 260 nm and the quality of RNA was checked by gel electrophoresis. Reverse transcription of RNA was performed by using M-MLV reverse transcriptase according to the manufacturer`s instructions.

Real-time quantitative RT-PCR

PCR reactions were assayed either with SYBR Green I chemistry (AT1a and Mas) or Taqman chemistry (AT_2) using the ABI PRISM 7000 sequence detection system. The Taqman assay utilizes sequence-specific fluorogenic oligonucleotide probes and the 5'-nuclease activity of the DNA polymerase, whereas SYBR Green I is a fluorescent dye binding to double-stranded DNA. As SYBR Green I allows detection of any double-stranded DNA, both specific and non-specific, a melting curve analysis was included in each PCR run to confirm the amplification of the specific PCR product only.

The following primer sequences were used: AT_{1a} forward 5'-GGCAGCCTCTGACTAAATGGC-3' and reverse 5'-ACGGCTTTGCTTGGTTACTCC-3', AT_2 forward 5'-TGTCTGTCCTCATTGCCAACA-3', reverse 5'-TTCATTAAGGCAATCCCAGCA-3' and probe 5'FAM-TCAGAACCATTGAATACTT-MGB, and Mas forward 5'-TCATGTGTATTGACAGCGGAGAA-3' and reverse 5'-CACTAACATGAGCGGAGTGAAGA-3'. PCR reactions for AT_{1a} and Mas receptors were performed in duplicate in a 25 µl final volume containing 1X SYBR Green Master mix and 300 nM of primers. PCR reactions for AT_2 receptor and housekeeping control gene 18S were performed in duplicate in a 25 µl final volume containing 1X TaqMan Master mix and 300 nM of AT_2 receptor primers and 150 nM of AT_2 probe, or 1X 18S TaqMan Gene Expression Assay primer and probe mix. PCR cycling conditions were 10 min at +95ºC and 40 cycles of 20 seconds at +95ºC and 1 min at +60ºC. .

Data were analysed using the absolute standard curve method as described in the Applied Biosystems User Bulletin #2. Standard Curves were generated using a dilution series of corresponding purified PCR products. The amplification of a housekeeping gene18S was used for normalizing the inefficiencies in cDNA synthesis and in the amount of RNA applied. Briefly, the copy numbers of 18S were divided by the highest 18S value obtained in the experiment, resulting in a correction factor for every sample. These correction factors were then used to normalize the absolute copy numbers of each receptor. The normalized copy numbers were obtained by dividing the copy numbers of AT_{1a}, AT_2, and Mas receptors by the corresponding correction factors. The intra- and interassay coefficients of variation of the real-time PCR method were < 2% and < 3.3% respectively (Gibson et al. 1996; Heid et al.1996; Lakkisto et al. 2002).

4.2.2 Quantitative *in vitro* autoradiography (I)

Autoradiography was performed using enucleated snap-frozen rat eyes from Study IV as in RT-PCR analysis. Densities of AT_1 and AT_2 receptors were determined in the retina and in the ciliary body with the radioligand ^{125}I-[Sar^1-Ile^8]*Ang II*. The effect on receptor density of oral antihypertensive treatment with ARB was evaluated.

The whole frozen eyes were embedded in OCT™ compound, the cornea facing forward. Twenty-µm *horizontal* sections were cut through the ciliary body with a cryostat at –19 ºC (*Figure 5*.). Each eye section was thaw-mounted on Super Frost® Plus slides, dried, and stored at - 70 ºC. Iodination with ^{125}I of [Sar^1-Ile^8]*Ang II* was performed by the chloramine-T method, and the labelled peptides were purified on SP-Sephadex C-25 columns. For AT_1 receptor autoradiography eye slices were preincubated for 15 min at room temperature in 10 mM sodium phosphate buffer containing 150 mM NaCl, 5 mM Na_2- EDTA, 0.2% bovine serum albumin (BSA), at pH 7.4, followed by one hour incubation at 37 ºC in fresh volume of the same buffer containing 0.2 µCi/ml of ^{125}I-[Sar^1-Ile^8]*AngII*. Non-specific binding was determined in the presence of 1 µM unlabelled *Ang II*. The density of AT_1 receptors was determined from parallel incubations with AT_2 receptor antagonist *PD123319*, 10 µM final concentration, while the density of AT_2 receptors was measured in the presence of AT_1 receptor antagonist losartan in the 10 µM final concentration. After incubation, the sections were washed four times for one min in cold-buffer without BSA and radioligand and then dried under stream of cool air. The optical densities were quantified by Image Gauge analysis system coupled to the FUJIFILM BAS-5000 photoimager. Receptor density was measured as mean pixels/square area from five sections per eye; three squares from the retina and two squares from the ciliary body (Zhuo et al. 1999; Stewen et al. 2003).

4.2.3 Fluorometric assay (II)

Fresh enucleated porcine eyes were kept on ice during transfer to the laboratory and prepared under a microscope within five hours post mortem. Each eye was dissected into vitreous mass, retina and ciliary body samples. The respective tissue types from both eyes of the same animal were pooled in preweighed plastic tubes. Each pooled sample (except for vitreous body samples) was homogenized in 0.1 M disodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) with a Silent Crusher S homogenizer for 20-30 sec. After

homogenization the samples were deep-frozen at -80ºC for subsequent analysis. For measurement of enzyme activities, homogenates were thawed and centrifuged as instructed in the activity assay kits. Supernatants were separated and used in measurement of enzyme activity by a sensitive fluorometric assay (Friedland and Silverstein 1975).

ACE1 **activity** was determined from the amount of His-Leu formed by enzymatic breakdown of synthetic substrate N-Hippuryl-His-Leu. Ten µl of supernatant from the tissue sample or vitreous body as such was incubated for 60 min at +37ºC in a water bath with 125 µl of 10 mM substrate solution. In order to obtain a sufficient reaction volume for the measurement procedure, 115 µl of 0.1 M disodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) was added to the reaction mixture to give a final substrate concentration of 5 mM. After incubation the reaction was stopped by adding 1.5 ml 0.3 M NaOH. Thereafter 100 µl of OPA (20 mg/ml in methanol) was added to the reaction, followed by 10 min incubation, after which 200 µl of 3 M HCl was added to stop the reaction. Finally the reaction mixtures were centrifuged at 3000 rpm for 10 min. The amount of fluorescent His-Leu liberated in the reaction was measured fluorometrically, excitation wavelength 360 nm and emission wavelength 500 nm. Each sample was assayed in duplicate and the mean was used in calculations. Captopril, an *ACE1* inhibitor, was included as control at a final concentration of 1 µM in each measurement.

ACE2 **activity** was measured using the fluorescence-based commercial SensoLyte™390 *ACE2* Activity Assay Kit (AnaSpec), which is based on the use of Mc-Ala/Dnp fluorescence resonance energy transfer (FRET) peptide as substrate. *ACE2* cleaves the substrate into two separate fragments and fluorescence of Mc-Ala is monitored by fluorometer. The enzymatic activity of *ACE2* was measured according to manufacturer`s instructions. Each sample was assayed in duplicate and the mean used in calculations. DX600, an *ACE2* inhibitor, was included as control at a final concentration of 100 nM in each measurement.

Enzymatic activities were characterized as micromoles of His-Leu formed (for *ACE1*) and Mc-Ala (for *ACE2*) per minute. The enzyme activities in retina and ciliary body were expressed as units/mg protein measured by Lowry´s method (1951) and in the vitreous body as units per milliliter of sample. One unit (U) is defined as the amount of enzyme required to release one µmol of reaction product in one minute. The effects of the three tripeptides on *ACE1* activity were tested at concentrations from 1 to 330 µM, and *ACE1* inhibition by captopril was assayed at concentrations from 1 to 100 nM, while the effects of the three

tripeptides on *ACE2* activity were tested at concentrations from 1 to 10 mM, and the *ACE2* inhibition by DX600 was assayed at a final concentration of 100 nM. The IC_{50} values were determined by regression analysis of enzyme inhibition (%) *vs* inhibitor concentration.

4.3 PHYSIOLOGICAL MEASUREMENTS

4.3.1 Aqueous humor outflow measurement (III)

For outflow studies anesthesia was induced and maintained by intramuscular injections of a combination of ketamine (Ketalar® 50 mg/ml, Parker-Davis Warner Lambert Nordic AB, Solna, Sweden) and xylazine (Rompun®Vet 20 mg/ml, Bayer AG, Leverkusen, Germany). Intravenous indomethacin was given at 10 mg/kg body weight (Confortid®, Dumex, Copenhagen, Denmark) before the eye cannulation in order to minimize the effect of endogenous prostaglandins. The test compounds and saline were injected into the vitreous 3 to 24 h before the outflow studies. When a specific antagonist was used, it was administered 1 h before the test compound except olmesartan which was given simultaneously with test compound. When the compounds were administered direct into the anterior chamber, outflow was registered 30 min after administration of the compounds.

The eyes of the animals were cannulated with three needles (27G) connected to polyethylene cannulas. One cannula was used for continous IOP monitoring, one for injection of the tes compound/vehicle and one for the infusion of fluid for outflow facility measurements. The aqueous humor outflow facility was measured with the constant pressure infusion method (Bárány 1964). IOP was measured using pressure transducer P-50 (Gould/Statham, Bilthoven, The Netherlands) and recorded by a Grass Model 79-D polygraph (Quincy, MA, USA). A mock solution of aqueous humor was infused to the anterior chamber from a reservoir until the IOP increased 5-7 mmHg above the preinfusion level and the level of the reservoir was adjusted to give a steady state IOP. The infusion rate (F_1) and the increase in IOP (ΔP_1) were registered over a 4-8 min period in steady-state conditions. Thereafter the reservoir was elevated to give a higher pressure (about 5-7 mmHg above the previous level and 10 to 14 mmHg above the preinfusion level), and the same procedure was repeated under steady state conditions to obtain the values of the infusion rate (F_2) and the increase in IOP (ΔP_2) above the preinfusion level. In order to constitute a technically successful experiment, IOP had to return to the preinfusion level

after the second infusion. The outflow facility could then be calculated from the formula C=F/ Δ P.

4.3.2 Intraocular pressure measurement (III,IV)

IOP of conscious rabbits (Study III) was measured with a pneumatonometer (Modular One Tonometer, Mentor, Cambridge, MA, USA) after topical anaesthesia of the cornea with 0.4 % oxybuprocain (Oftan Obucain®, Santen Oy, Tampere, Finland). The pneumatonometer was calibrated for IOP measurements in rabbits by simultaneous manometric and pneumatonometric pressure measurement in cannulated eyes using the closed stopcock method as described by Hammond and Bhattacherjee (1984). IOP was measured at 1h, 2h, 3h, 4h, 5 h and 6 h after administration of the test compounds except in the case of *Ang II* when IOP was measured also at 12 h and 24 h. The basal IOP for both eyes was always measured 1 h prior to the experiment.

The IOP of pre-trained conscious rats in Study IV was measured using a specific rebound tonometer (TonoLab®, Tiolat Oy, Helsinki, Finland) after topical anesthesia of the cornea with 0.4 % oxybuprocain (Oftan Obucain®, Santen Oy, Tampere, Finland). The effect of general anesthesia on IOP was determined using intraperitoneal injection (0.2 ml/100 g) of a 1:3 combination of ketamine 50 mg/ml and medetomidine hydrochloride (Domitor® 1 mg/ml, Orion Pharma, Espoo, Finland).

4.3.3 Blood pressure measurement (III,IV)

Blood pressure in anesthetized rabbits (Study III) was measured by cannulating a femoral artery with PE-50 polyethylene tubing containing heparinized isotonic saline. The pressure was measured with a Statham P-50 pressure transducer and recorded with a Grass Model 79 D polygraph. The blood pressure in pre-trained conscious and anesthetised rats (Study IV) was measured using a tail-cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179–2AB, IITC Life Science, Woodland Hills, CA, USA). The rats were prewarmed for 15-20 min at +32ºC to improve detection of the pulsation of the tail artery. The arithmetic mean of three successive measurements without disturbances of the signal indicated the systolic blood pressure.

4.4 TEST COMPOUNDS

Studies I and IV. The test compounds were *Ang II* receptor type 1 antagonists: olmesartan medoximil (10 mg/kg/day, Olmetec®, Leiras, Turku, Finland) and valsartan (30 mg/kg/day, Diovan®, Novartis, Basel, Switzerland). Tablets were ground and mixed in the food.

Study II. The test compounds were: IPP (isoleucyl-prolyl-proline), VPP (valyl-prolyl-proline) and LPP (leucyl-prolyl-proline) purchased from Bachem, Weil am Rhein, Germany. Positive control captopril, o-phthaldialdehyde (OPA), His-Leu, purified *ACE1* enzyme and N-Hippuryl-His-Leu hydrate (substrate) were provided by Sigma-Aldrich (Schnelldorf**,** Germany). All compounds were dissolved in disodium tetraborate buffer containing NaCl (pH 8.3), except that OPA was dissolved in methanol. *ACE2* activity assay kits, including all necessary reagents, were purchased from AnaSpec (San Jose, CA, USA).

Study III. The compounds used were: human *Ang II* acetate (Sigma-Aldrich, Schnelldorf, Germany), olmesartan (Daiichi Sankyo Co., Ltd., Japan), A-779 (GenScript Corporation, Piscataway, NJ, USA), and [Sar^1Ile^8] *Ang II* human, *CGP 42112A, PD 123319* and *Ang (1-7)* (purchased from NeoMPS, Strassbourg, France). All compounds were dissolved in isotonic saline. The fellow eye was treated with saline and served as a control in all experiments. Dipivefrin hydrochloride (Propine®, Allergan, Mayo, Ireland) was used as a positive control in topical administration.

Test compounds were given topically (1 to 3 gtt), intravitreally (50 µl) or intracamerally (5µl).

4.5 STATISTICAL ANALYSES

The results are expressed as mean ± standard error of mean (SEM) in Studies I-IV, and 95% confidence intervals (CI) in Studies III-IV. T-tests (Sigma Plot Version 10.0) in Studies I-II or one-way analysis of variance (ANOVA) followed by Dunnett´s post-hoc test (Study II) were used for statistical comparisons for paired or unpaired data. Relationship between BP and IOP (Study IV) was expressed using area under the curve (AUC), and statistical analysis between and within groups by using t-test or permutation test (Stata Version 10.0).
$P \leq 0.05$ was considered statistically significant in all experiments.

4.6 ETHICS

All animal procedures were in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and with the Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the National Animal Ethics Committee in Finland.

5 RESULTS

5.1 RAS EXPRESSION

5.1.1 Angiotensin receptors in the eye tissue (I)

RT-PCR

The novel Mas receptor was found for the first time in the eye tissue in addition to traditional AT_{1a} and AT_2 receptors. The mRNA expression of the Mas receptor was lower than that of AT_{1a}, but markedly higher than that of AT_2 receptors in every rat strain except in WKY rats. Angiotensin receptor expression was overall more obvious in retina than in ciliary body. No direct differences were seen between four rat strains, but ocular angiotensin receptor expression was higher in dTGR compared to SHR. For receptor expressions, see *Table 4.*

Oral treatment with ARB did not systematically influence the expression of mRNA for Mas receptors, nor that of AT_{1a} or AT_2 receptors. Only the treated SHR animals evinced slightly lowered expression of AT_{1a} receptors *vs.* normotensive controls in both tissues evaluated.

Autoradiography

The densities of AT_1 and AT_2 receptors in the retina were more pronounced compared to that in the ciliaris in every rat group in keeping with the results of the RT-PCR analysis. The density of AT_1 receptors was higher than that of AT_2 receptors. There was seen no clear difference in receptor densities between different rat strains *(Table 5).* Mas receptor could not been identified due to the lacking methodology.

In contrast to the result obtained in RT-PCR analysis a clear difference was seen between ARB-treated and non-treated animals in autoradiography analysis. The apparent density of AT_1 receptors in the retina in treated animals was approximately 35% lower in SHR and SD rats and about 60% lower in dTGR and WKY as compared to non-treated animals. This tendency was also seen in the ciliary body. Medication with ARB did not systematically influence the density of AT_2 receptors, but medicated dTGR had more AT_2 receptors especially in the retina.

Table 4. Expression of Mas, AT_{1a} and AT_2 receptors in arterial hypertensive rats and their controls (for abbreviations, see text) in ocular tissues measured by RT-PCR. Activity of *ACE1* and *ACE2* in porcine ocular tissues measured by fluorometric assay. For RT-PCR: values (mRNA copies x 10^3/ng total RNA) are expressed as mean±SEM, n=4. NA= not assayed. For fluorometric assay: values in retina and ciliary body are expressed as mean ± SEM mU/mg protein and in the vitreous body mean ± SEM nmol/min/ml of sample, n=5-6.

RAS component		*Retina*	*Ciliary body*	*Vitreous body*
RT-PCR				
Mas	***SHR***	**4.7±2.3**	**1.2±0.3**	**NA**
	WKY	**6.5±3.6**	**3.8±1.6**	**NA**
	dTGR	**11.1±1.3**	**1.3±0.1**	**NA**
	SD	**9.6±3.3**	**2.5±1.1**	**NA**
AT_{1a}	***SHR***	**6.6±3.2**	**2.9±1.0**	**NA**
	WKY	**10.2±6.2**	**6.4±2.2**	**NA**
	dTGR	**16.5±1.9**	**2.1±0.4**	**NA**
	SD	**15.8±5.6**	**3.2±0.4**	**NA**
AT_2	***SHR***	**3.2±1.2**	**0.5±0.1**	**NA**
	WKY	**7.3±3.3**	**2.1±1.0**	**NA**
	dTGR	**6.4±0.5**	**0.3±0.0**	**NA**
	SD	**6.0±1.9**	**0.4±0.2**	**NA**
FLUOROMETRIC ASSAY				
ACE1		**0.2±0.02**	**3.7±0.70**	**8.2±0.31**
ACE2		**0.2±0.01**	**0.2±0.02**	**0.1±0.02**

The autoradiography results suggest that the density of AT_1 receptors in the eye is independent of the blood pressure level of the animals, but is influenced by oral ARB treatment. Densities of AT_1 and AT_2 receptors and the respective blood pressure values in individual animals are presented in *Figure 6.*

Table 5. Density of AT_1 and AT_2 receptors in the ocular tissues of arterial hypertensive rats and their normotensive controls expressed as mean±SEM, n=4. Medicated= oral treatment with ARB. For abbreviations, see text.

RAS-component		*Retina*		*Ciliary body*	
		Non-medicated	*Medicated*	*Non-medicated*	*Medicated*
AT1	*SHR*	3.6±1.1	2.5±0.5	2.3±0.6	1.2±0.2
	WKY	4.3±0.2	1.7±0.6	3.1±1.3	1.4±0.5
	dTGR	5.0±0.9	1.7±0.4	2.4±0.9	1.9±0.8
	SD	4.4±0.7	2.5±0.5	4.6±0.0	2.5±1.3
AT2	*SHR*	2.8±1.8	1.0±0.5	0.9±0.6	0.4±0.2
	WKY	2.0±0.3	1.4±0.3	0.7±0.2	0.8±0.3
	dTGR	0.8±0.3	2.3±0.3	0.2±0.1	0.5±0.3
	SD	1.5±0.9	2.6±0.1	1.1±0.4	2.6±0.1

5.1.2 Angiotensin enzymes in the eye tissue (II)

Fluorometric assay

ACE1 activity was significantly higher in ciliary body compared to retina. The activities of *ACE1* and *ACE2* were at the same level in the retina, whereas the *ACE1* activity in the ciliary and vitreous bodies was manifold compared to *ACE2*. *ACE2* activity was for the first time found to be existed in vitreous and ciliary bodies in addition to its known retinal activity *(Table 4).*

Different concentrations of the bioactive tripeptides were tested, the aim being to compare the inhibitory activity of *ACE1 vs.* that of *ACE2*. The tripeptides IPP, VPP and LPP showed inhibition of *ACE1* activity at micromolar concentrations in all tissues studied, i.e. porcine retina, ciliary and vitreous bodies (IC_{50} 9-25 µM), while for *ACE2* inhibition was obtained first at millimolar concentrations (IC_{50} 4-22 mM). For the inhibitory effects of IPP, VPP and LPP on *ACE1* activity in the ciliary body, higher peptide concentrations were needed than in the two other tissues. The same effect was seen with the positive control, captopril. Captopril effectively inhibited *ACE1* but not *ACE2* activity in all tissues (IC_{50} 5-10 nM).

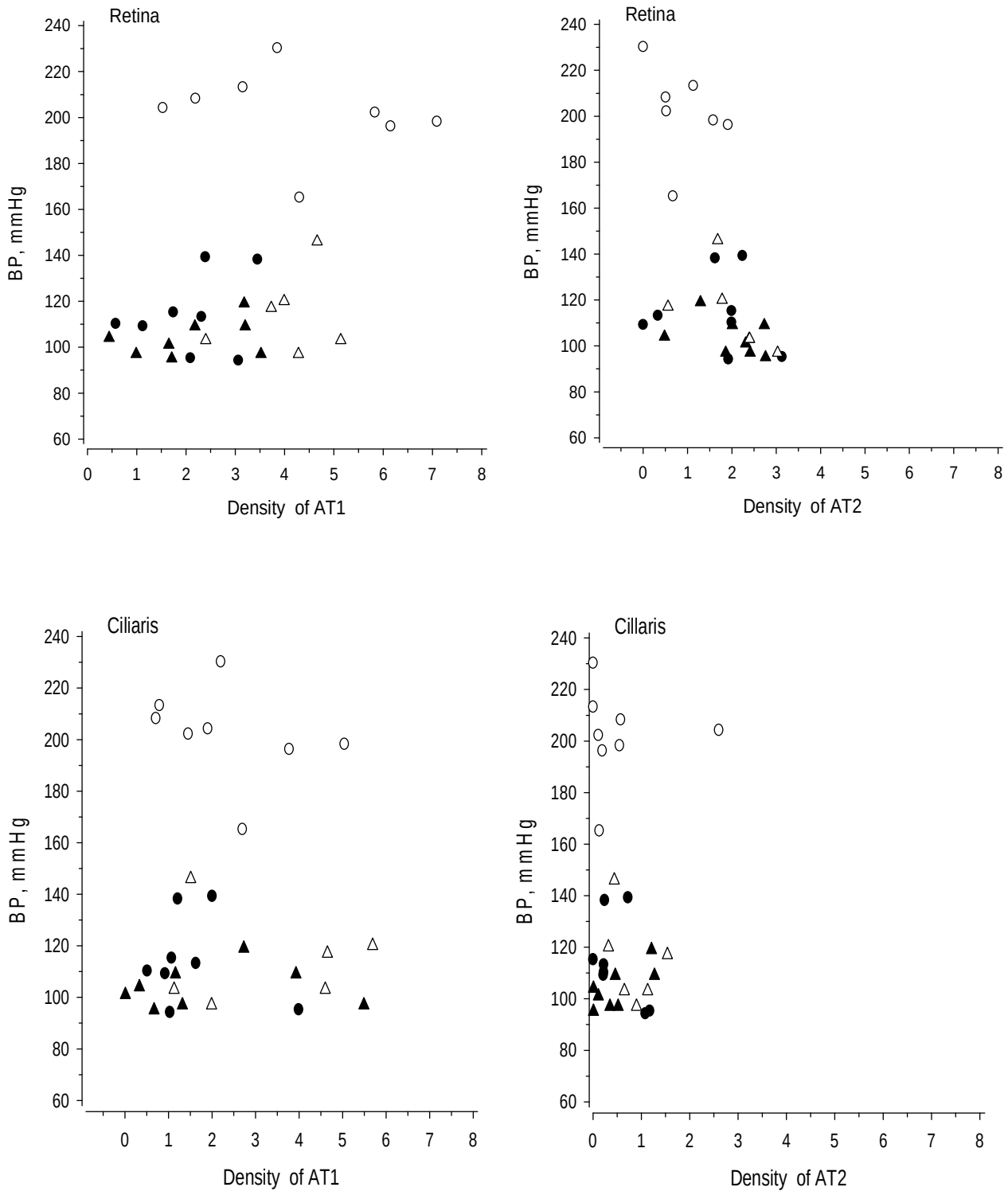


Figure 6. Blood pressure (BP) and angiotensin receptor densities in individual animals. Open circle = non-treated hypertensive rat, filled circle = ARB-treated hypertensive rat, open triangle = non-treated normotensive control, filled triangle = ARB- treated normotensive control.

5.2 FUNCTIONAL RAS

The ocular effects of RAS components are summarized in *Table 6.*

5.2.1 Topically administered RAS components (III)

Topical administration of the test compounds had no effect on normal IOP in rabbits during 6-h follow-up (24-h for *Ang II)*, and they had no adverse effects on the surface of the eye. Dipivefrin was used as positive control and it reduced IOP maximally at 3 h from baseline, 19.5 mmHg to 12.2 mmHg.

5.2.2 Intraocularly administered RAS components (III)

Intravitreal administration

The major finding in Study III was the oculohypotensive effect of intravitreally administered 1 mM *Ang (1-7)* (p=0.008) in ocular normotensive rabbits. *Ang (1-7)* reduced IOP maximally at 2 to 3 h after its intravitreal injection. The IOP-reducing effect of *Ang (1-7)* was inhibited by *A-779* (a specific Mas receptor antagonist) and partially by *PD123,319* (an AT_2 receptor antagonist). When administered alone, these two receptor antagonists had no effect on IOP. Intravitreally administered *Ang (1-7)* had no effect on outflow facility.

Olmesartan (an AT_1 receptor antagonist) administered intravitreally did not antagonize the *Ang (1-7)* lowering effect (p=0.03). It reduced IOP in both eyes; there was no difference between the test and the saline-injected fellow eyes. To study the effects of intravitreal injection *per se* eight rabbits received isotonic saline 50 µl in both eyes without significant changes in IOP.

Ang II did not influence IOP at the various concentrations tested.

[Sar^1Ile^8]*Ang II* (an unspecific *Ang II* receptor ligand) tended to lower IOP between 2 to 4 h *vs.* the control eye (p=0.12).

CGP42112A (an AT_2 receptor agonist) had no significant effect on IOP at the various concentrations tested. Intravitreally administered *CGP42112A* had no effect on outflow facility.

Table 6. The effects of RAS compounds on IOP and outflow facility in rabbits after intravitreal, topical and intracameral administration of test agents and the effects of oral ARB on IOP and BP in rats. Different concentrations were tested in rabbits, the main founding being oculohypotensive 1 mM *Ang (1-7)* when administered intravitreally (50µl). For details, see text. Intravitreally administered *Ang II* caused dose- dependent rise in IOP (↑/-). ↑ = enhance, ↓ = diminish, - = no effect, NA = not assayed, olme = olmesartan. * p≤0.05, ** p<0.005, *** p<0.001.

Compound	*Mechanism of action*	*IOP* *intravitreal*	*topical*	*Outflow facility* *intravitreal*	*intracameral*
Ang II	*AT1 agonist*	↑/-	-	NA	↓***
Ang (1-7)	*Mas receptor agonist*	↓***	-	-	-
+olme	*AT1 antagonist*	↓*	-	NA	NA
+ A-779	*Mas receptor antagonist*	-	-	NA	NA
+PD123,319	*AT2 antagonist*	↓	-	NA	NA
CGP42112A	*AT2 agonist*	↓	-	-	-
+olme topic.	*AT1 antagonist*	↓	NA	NA	NA
PD123,319	*AT2 antagonist*	-	-	NA	NA
A-779	*Mas receptor antagonist*	-	-	NA	NA
Sar[1]Ile[8] Ang II	*Unspecific AT receptor ligand*	↓***	-	NA	NA
Olmesartan	*AT1 antagonist*	↓	-	NA	NA
+CGP42112A	*AT2 agonist*	NA	-	NA	NA
NaCl	*Control*	-	-	NA	NA
Dipivefrin	*Control: adrenergic agonist*	NA	↓**	NA	NA
Oral treatment (rat):		*IOP*		*BP*	
Valsartan 30 mg/kg	*AT1 antagonist*	-		↓***	
Olmesartan 10 mg/kg	*AT1 antagonist*	-		↓***	

Intracameral administration

Neither intracamerally administered *Ang (1-7)* nor *CGP42112A* had any effect on the outflow facility, while *Ang II* reduced it dose-dependently.

5.2.3 Orally administered RAS components (IV)

IOP

Oral treatment with ARBs seemed to have a slight effect on normal IOP in growing animals, while it abolished the development of hypertension in SHR and dTGR. The mean baseline IOP in SHR (age 5 weeks) was 18.4 mmHg and in dTGR (age 4 weeks) 30.7 mmHg. Baseline IOP in their age-matched control groups were 15.8 mmHg (WKY) and 13.1 mmHg (SD). IOP in non-treated adult SHR, WKY (age 13 weeks), dTGR and SD (age 7 weeks) was 22.4 mmHg, 18.3 mmHg, 19.4 mmHg and 13.5 mmHg. IOP in oral ARB-medicated adult animals was 19.1 mmHg (SHR-M), 20.3 mmHg (WKY-M), 18.2 mmHg (dTGR-M) and 12.0 mmHg (SD-M), respectively.

Blood pressure

Oral treatment with ARBs effectively abolished the development of hypertension in SHR and dTGR. Systolic BP in non-treated adult SHR, WKY, dTGR and SD was 183 mmHg, 102 mmHg, 205 mmHg and 106 mmHg, and that in ARB-treated adult animals 100 mmHg, 59 mmHg, 107 mmHg and 95 mmHg, respectively.

5.2.4 Relationship between IOP and BP (IV)

A slight positive relationship between IOP and developing BP was seen in SHR: non-treated hypertensive rats had higher mean IOP *vs.* normotensive animals (P=0.048). However, in dTGR, IOP was not directly associated with high BP. The high baseline IOP was reduced during the two weeks` follow-up in non-medicated and medicated rats, while BP remained high in untreated animals but was well controlled in treated rats.

The baseline IOP in young SD rats declined age-dependently, being 23.2 mmHg at the age of 4 weeks, 21.5 mmHg at 5 weeks, 17.8 mmHg at 6 weeks

and 14.0 mmHg at 7 weeks, indicating the lowering tendency accompanying ageing. BP according to our experience does not change markedly during such a short follow-up time.

5.2.5 The effect of general anesthesia on IOP

We show here that intraperitoneally induced ketamine/medetomidine hydrochloride anesthesia reduces IOP by at least 30 % (WKY) or even more in hypertensive (SHR) rats (Study IV). As similar findings were observed in ARB-treated animals it is evident that IOP measurements should be made without general anesthesia.

6 DISCUSSION

A functional RAS has recently been demonstrated in the human eye in several studies, and there is accumulating evidence that the widely used RAS antagonizing antihypertensive drugs are also able to reduce IOP. Until now no drugs acting on RAS are in use in ophthalmology, but they may emerge as potential antiglaucomatous drugs in the future. The present study determined the expression and density of angiotensin receptors in the various eye structures using RT-PCR and *in vitro* autoradiography analysis. The most important RAS enzyme activities and their inhibition by bioactive tripeptides were described using a fluorometric assay method. In addition, the present study clarified the effects of different exogenous compounds acting on RAS, on normal IOP and aqueous humor outflow, as well as the relationship between IOP and developing high BP in *in vivo* experiments.

The main finding in the present study was the oculohypotensive effect of heptapeptide *Ang (1-7).* The result, obtained with ocular normotensive rabbits, indicated that *Ang (1-7)* acts via its own receptor type. A specific *Ang (1-7)* receptor, the Mas- receptor, was then described in the eye structures of rats, for the first time in the literature. A third finding in the context of intraocular RAS was the existence of *ACE2* in vitreous and ciliary bodies in addition to the previous finding of its retinal activity (Tikellis et al. 2004; Senanayake et al. 2007). *ACE2* is an enzyme responsible for degrading angiotensins, e.g. *Ang II* to vasorelaxing products (Tipnis et al. 2000; Vickers et al. 2002).

6.1 METHODOLOGICAL ASPECTS

This experimental study was carried out to obtain new knowledge regarding intraocular RAS and thus possibly a basis for the development of antiglaucomatous drugs. Preclinical *in vitro* as well as *in vivo* animal studies are indispensable before any tests on humans are possible. Rabbit and rat eyes are both well proven models for the human eye in many biological respects, although these animal eyes are designed for long-sighted use in the dark and have limited near vision (Meyer at al. 1972; Artal et al. 1998).

Experimental animals

Like many laboratory animals, rabbits are albinos with non-pigmented eye structures, which property may have an effect on drug responses. In the rabbit

eye the trabecular endothelial layer transports aqueous humor direct to the trabecular meshwork, rendering the uveoscleral outflow a less significant pathway (Bergmanson 1985). Rabbits are overall ideal for ophthalmological studies, in that they are easy to handle and have large eyes easy to manipulate. The rat eye differs anatomically more from the human eye in having a larger lens and a minor ciliary system, but rats are also largely used experimental animals. The porcine eye is phylogenetically close to the human eye after simians (Prince et al. 1960; McMenamin and Steptoe 1991) and many previous studies have shown that the domestic pig is a suitable subject for a variety of ophthalmological studies (Wagner et al. 2004; Ruiz-Ederra et al. 2005). Porcine eyes are cheap and easily available from an abattoir. The average anterior chamber volume of the porcine eye is 300 µl and globe size almost equal to the human. In addition, the pig eye contains a shallow scleral sulcus with a wedge-shaped mass of corneoscleral tissue comparable in size to the human trabecular meshwork (McMenamin and Steptoe 1991). Furthermore, ultrastructural investigations indicate that the cribriform and subendothelial regions of the porcine trabecular meshwork have an architecture similar to that of the primates (Bachmann et al. 2006).

Pressure measurements

Experimental animals used in the present study have a circadian rhythm both in IOP and BP in relation to their awake/sleep cycle. BP altrerations are more investigated in rats (Janssen et al. 1992; Lemmer 2006) but also in rabbits (van den Buuse and Malpas 1997). The circadian rhythm of normal IOP with peaks occurs in the evening in nocturnal animals (Moore et al. 1996; Sugimoto et al. 2006) and early in the morning in humans (Liu et al. 1998; Liu et al. 1999), i.e. the IOP diurnal rhythms of most laboratory animals are approximately 180º out of phase with these rhythms in humans. To minimize the effect of diurnal variation in IOP and BP on the results, measurements were made at the same time of day in all animal experiments. Furthermore, BP as well as IOP may be influenced by environmental factors or acute stress. To minimize such sources of error, the animals were pretrained before the beginning of the experiments and measurements were made by the same person.

The IOP values obtained in this study are in good agreement with earlier reports, where the same kind of equipment has been used (Kontiola et al. 2001; Goldblum et al. 2002; Kotikoski et al. 2002; Wang et al. 2005b). The normal IOP of the awake rabbit is about 20 mmHg (Seidehamel and Dungan 1974; Gregory

1990), but it varies by even 10 mmHg being highest in the dark hours when the animal is normally awake (Rowland et al. 1981). The IOP of the conscious rat is about 18 mmHg (measured by TonoLab) (Wang et al. 2005) showing fluctuation between mean peak and mean trough 8 mmHg (measured by TonoPen 1) (Krishna et al. 1995). In the present series, the effect of general anesthesia on IOP was investigated in normotensive and hypertensive rats. General anesthesia reduced IOP markedly both in non-treated and in ARB-treated rats, in agreement with previous findings (Jia et al 2000; Wang et al. 2005).

The animals used in IOP studies here were ocular normotensive. The optimal situation to evaluate the IOP-lowering effect of different test compounds would involve preferably congenitally glaucomatous animals, or those with artificially high IOP, e.g. in animals with laser-induced or alpha chymotrypsin-induced glaucoma (Shah et al. 2000; Kalesnykas et al. 2007; Nissirios et al. 2008). However, the availability of naturally occurring glaucomatous animals is limited and the mechanism of increased IOP in glaucoma is not understood. According to the literature, oculohypotensive agents involving RAS seem to be more efficient in glaucomatous than in normotensive eyes: topical application of olmesartan (ARB) has been reported to lower IOP in *in vivo* studies, the effect being more prominent in ocular-hypertensive animals (Inoue et al. 2001b; Wang et al. 2005a). Very preliminary data obtained by our study group also indicate that in rabbits with congenitally elevated IOP, the oculohypotensive effect of *Ang (1-7)* is more pronounced than in normotensive animals (unpublished data, Vaajanen et al. 2007).

The blood pressure measurement methods used in this study were all of high quality. Our research group has e.g. long experience with the tail-cuff method used for blood pressure measurements in awaken animals and administration of RAS blocking antihypertensive drugs mixed in the feed in these hypertensive rat strains (Mervaala et al. 2001; Pilvi et al. 2006).

Outflow measurements

Outflow facility was measured by the two-level constant pressure infusion method (Bárány 1964) in order to test the mechanism whereby *Ang (1-7)* reduces IOP: via aqueous humor formation or drainage. This system is accurate but laborious and needs practised workers. It determines the whole aqueous humor drainage, including trabecular and uveoscleral outflow at two artificial pressure levels. A disadvantage of this method is that the two different drainage systems cannot be distinguised from each other, but on the other hand test

compounds such as large and rapid degrading angiotensin system molecules / peptides can be administered direct into the anterior chamber in addition to other dosing systems.

In vitro measurements

RT-PCR methodology measures the expression of mRNA in tissues (Heid et al. 1996; Bustin and Müller 2005). It does not indicate that real protein, i.e. receptor, is expressed in the tissue but it is more an indication that "tools" for receptors exist in the tissues studied. Immunohistochemical studies would have confirmed this finding of mRNA for Mas receptor, but due to the very novel finding, no immunohistochemical reagents were available to us. mRNA here was determined from slices cut from a whole eye-ball at the level of the ciliary body and at the level of the retina without ciliary tissue. It was thus not possible to establish more specifically the type of cells in which each receptor was localized. It may be debated whether this receptor expression is only derived from the vascular bed around the retina and ciliary tissue or even from behind the blood ocular barriers.

Autoradiography is a semiquantitative method which measures the ^{125}I-[Sar^{1}-Ile^{8}] *AngII* binding in receptors, whereby the receptor densities in different tissues can be determined (Mendelsohn et al. 1987; Stewen et al. 2003). In the present study, the sections of the whole eye-ball were cut horizontally and the densities of AT_1 and AT_2 receptors were measured as mean pixels/square area from five sections per eye; three squares from the retina and two from the ciliary body, i.e. more tissue specifically than in RT-PCR. The results obtained by this analysis were in agreement with those of RT-PCR.

Fluorometric assay. *ACE1* activity in ocular tissue was determined using a fluorometric assay modified from Friedland and Silverstein (1975); many concentrations of reagent materials were tested before the final set were accepted. In the evaluation of *ACE2* activity the manufacture's instructions in the widely used kit were utilized. One advantage of all *in vitro* methods is ease of repetition. The assay of Friedland and Silverstein has been used in enzyme activity measurements during the past years, the latter method being a more novel one. The inhibition of both enzyme activations was tested using several concentrations of bioactive tripeptides, which are known to reduce blood pressure mainly by *ACE* inhibition. Captopril, the first developed and now clinically used *ACE1* inhibitor, served as positive control in these tests.

6.2 OCULAR EFFECTS OF LOCALLY AND SYSTEMICALLY ADMINISTERED RAS COMPONENTS

The main finding in this study was an oculohypotensive effect of heptapeptide *Ang (1-7),* when administered intravitreally into the normotensive rabbit eye. The most potent concentration of this peptide was 1 mM in an injection volume of 50 µl and the effect set in as early as two to three hours after application of the compound. Using specific receptor antagonists it was shown that only *A-779* (a specific Mas antagonist) was able to abolish the IOP-lowering effect of *Ang (1-7).* This would indicate for the first time that *Ang (1-7)* acts via its own receptor type also in the eye tissue in addition to other effects in different organs recently studied, e.g. heart, vasculature and brains (Santos et al. 2003; Iwata et al. 2005).

The oculohypotensive effect of *Ang (1-7)* at a concentration of 1 mM (in an injection volume of 50 µl) was the basis for the concentrations for other test compounds used. The approximate final concentration of *Ang (1-7)* in the vitreous space of rabbit was about 20 µM, but potentially much smaller in the anterior chamber and ciliary body of the animal; i.e at the point of action. Endogenous *Ang II* concentrations in the aqueous humor have been reported to range from 5 to 16 fmol/mg protein in the rabbit (Ramirez et al. 1996) or 0.5 pM in normal human subjects (Danser et al.1994). The half-life of test compound is an important factor, and peptides are known to degrade fast. The half-life of *Ang (1-7)* in the vitreous space is not known. According to the literature, *Ang (1-7)* degrades after systemic administration within 30 min in the canine lung, which is known to have very high ACE activity (Chappell et al. 1998). However, the ACE activity in the vitreous is known to be much lower (Ramirez et al. 1996), which bespeaks a longer half-life in the eye. On the other hand, the effect of a compound can last far beyond its half-life.

In outflow studies *Ang (1-7)* had no effect on outflow, while *Ang II* administered intracamerally reduced it dose-dependently. Probably *Ang (1-7)* reduces IOP via reduction of aqueous humor formation. According to literature *Ang (1-7)* promotes release of prostanoids from endothelial and smooth muscle cells, release of nitric oxide, vasorelaxation, inhibition of vascular cell growth and fibrosis and, less frequently, vasoconstriction (Muthalif et al. 1998; Santos et al. 2000). It is also known to stimulate the kallikrein-kinin system, having a vasodilatatory effect (Brosnihan et al. 1996). Finally, it may be that nitric oxide has a key role at cell level in IOP reduction. The outflow reducing effect of *AngII* was not likely to be due to solely an increase in systemic blood pressure, since

the first elevated pressure had returned to normal by the time of outflow registration.There are a few reports on the effects of *Ang II* on aqueous humor dynamics (Kaufman and Barany 1981; Inoue et al. 2001b). These findings are in accord with results obtained from monkey studies (Kaufman and Bárány 1981). *Ang II* has been reported to have effects on uveoscleral outflow in ocular normotensive rabbits (Inoue et al. 2001b). In the study in question, *Ang II* was administered in perfusion fluid at a concentration of 0.5 µM and it diminished the uveoscleral outflow. It may be that ocular *Ang II* has only a minor influence on aqueous humor outflow in eyes under physiological conditions.

Interestingly olmesartan (an AT_1 receptor antagonist) administered intravitreally into one eye lowered IOP in both, the experimental and the contralateral saline injected eye. To verify that intravitreal injections *per se* do not cause any marked decrease in IOP, saline was injected bilaterally and no significant change in IOP was observed. In addition, in experiments conducted with AT_2 and Mas receptor antagonists alone, no significant change in IOP could be observed neither in experimental nor in the control eyes. The mechanism of IOP reduction of olmesartan is potentially AT_1 receptor blockade, but the reason for the lowering of pressure in the fellow eye remained obscure.

Topical administration of angiotensins did not influence IOP in rabbits. Angiotensins are peptides easily degraded to inactive forms and as large molecules do not readily penetrate through the cornea (Ghate and Edelhauser 2008).

Oral administration of *Ang II* receptor type 1 blockers (ARBs), olmesartan medoximil and valsartan, only tended to lower normal IOP in hypertensive rats, while they reduced blood pressure in SHR and dTGR and even in normotensive animals. According to autoradiography analysis ARB medication blocked AT_1 receptors especially in the retina but also in the ciliaris, the latter being involved in the regulation of IOP. Here again, presumably intraocular RAS is more markedly activated in pathological conditions such as glaucoma, when ARBs may also have a significant lowering effect on IOP. In human studies, orally administered losartan and captopril have lowered IOP both in normotensive and glaucomatous subjects, although blood pressure was lowered only in arterial hypertensive patients (Costagliola et al. 1995; Costagliola et al. 2000).

In Study IV the relationship between the development of hypertension and IOP was evaluated in arterial hypertensive rats and their controls with/without oral antihypertensive treatment. The slight but statistically significant relationship

between IOP and the development of high BP in SHR was apparent in repeated measurements during eight weeks' follow-up. On the other hand, double transgenic RAS "overexpressed" rats showed no relationship between blood pressure and IOP, which initially high, fell drastically in both non-treated and ARB-treated rats.

6.3 OCULAR RAS ENZYME ACTIVITY

ACE1 as well *ACE2* activity were found in ocular structures; in the retina, ciliaris and vitreous body. The activity of *ACE1* was manifold compared to that of *ACE2*, especially in the ciliary body, which is responsible for the formation of aqueous humor. On the other hand, the inhibition effected by tripeptides IPP, VPP and LPP, was achieved in much lower concentrations on *ACE1* than *ACE2*. This means that the negative effects of *ACE1* are blocked by tripeptides known to also have positive cardiovascular effects (Möller et al. 2008; Erdmann et al. 2008) at concentrations which do not affect the activity of *ACE2*. These tripeptides are composed of only three amino acids, which may offer better penetration through the cornea if applied topically. *ACE* inhibitors have generally been reported to have positive ocular effects.

ACE-inhibitors lower *Ang II* levels in aqueous humor (Osusky et al. 1994). They might reduce the production of aqueous humor by reducing blood flow in the ciliary body (Reitsamer and Kiel 2003). In addition, they promote synthesis of prostaglandins by preventing the breakdown of bradykinin, which could in turn lower IOP by increasing the uveoscleral outflow (Nilsson et al. 1989; Lotti et al. 1990). The precise mechanism underlying increased uveoscleral outflow is not known, but there would appear to be associations with increased biosynthesis of certain matrix metalloproteinases. This would lead to relaxation of the ciliary muscle and reduction and compaction of extracellular matrix components within the ciliary muscle, the iris and the sclera and within the tissues of the uveoscleral outflow pathway. All these effects might facilitate aqueous humor outflow and thus lower IOP (Weinreb et al. 2002). By preventing bradykinin breakdown, ACE-inhibitors activate the nitric oxide pathway and the vasodilatatory effects of prostanoids, and reduce the formation of the vasoconstrictive peptide endothelin- 1. Endothelin- 1 has been shown to elicit contraction e.g. in porcine ophthalmic and ciliary arteries and in the human ophthalmic artery (Yao et al. 1991; Haefliger et al. 1992).

6.4 SIGNIFICANCE OF RAS EXPRESSION IN OCULAR TISSUES

Although intraocular RAS has already been identified in many eye tissues, the precise function and significance of local RAS has not yet been established. Local RAS may have a significant role in the formation of aqueous humor, but also in its drainage, or intraocular RAS may be involved in both of these mechanisms. Many RAS components have been shown to be present in cultured human NPEC, in cells especially responsible for aqueous humor formation (Lin et al. 1990; Culliane et al. 2002). *Ang II* has been reported to activate a Ca^{2+} signalling system which increases potassium ion channel activity and triggers aldosterone production (Capponi et al. 1984). These effects are accompanied by cell volume loss, indicating that *Ang II* acts as an operated secretagogue in the NPEC (Culliane et al. 2002). *Ang II* has also been found to cause an increase in cytoplasmic sodium concentration due to activation of Na^+/H^+ exchange (Hoy and Delamere 2002). In point of fact, mechanisms related to sodium handling are common pathogenetic factors in both ciliary and renal tubular epithelia, which may explain the coexistence of glaucoma and systemic hypertension (Langman et al. 2005). On the other hand, RAS expression has also been demonstrated in the trabecular meshwork, which is involved in aqueous humor outflow. *Ang II* has been reported to be able to induce cell proliferation in bovine trabecular meshwork cells and increase the synthesis of collagen *in vitro* (Shen et al. 2001). It has also been reported that *Ang II* administered intracamerally diminishes uveoscleral outflow (Inoue et al. 2001b), as also showed in the present study. Synthetic and natural *Ang II* have been reported to reduce IOP in *in vivo* studies with anesthetized cats when administered intravenously. The same IOP-lowering effect was seen in the enucleated, arterially perfused cat and human eye. The mechanism behind the effect was considered to consist in vasoconstriction of the iris artery (Macri 1965).

There has also been debate as to the origin of RAS; does intraocular angiotensin originate from local production or from the blood compartment (Danser et al. 1994). It has been shown that neither *Ang I, Ang II* nor angiotensinogen are able to pass the blood-brain barrier (Schelling et al. 1980; Danser et al. 1994), and the barriers between the eye and circulating blood are comparable to this (Cuncha-Vaz 1979). If the barrier is intact, circulating angiotensin cannot reach the vitreous fluid (Danser et al. 1994), whereas if the barrier is disrupted this becomes possible (Danser et al. 1989). It is therefore obvious that the levels of angiotensins and other RAS molecules in the eye are too high to have originated from blood-borne peptides. In porcine ocular tissues *Ang I* and *II* levels have proved to be 5-to 100-fold higher than could be

accounted for by admixture with blood or diffusion from blood (Danser et al. 1994). Also ACE activity has been shown to be lower in plasma than in ocular tissues in the rabbit and pig (Ramirez et al. 1996; Geng et al. 2003).

6.5 INTRAOCULAR RAS AND DRUG DEVELOPMENT IN THE FUTURE

Evidence is now accumulating to indicate that antihypertensive drugs acting on RAS can also reduce intraocular pressure, and compounds blocking RAS may eventually prove to be potential antiglaucomatous drugs. Especially agents increasing ACE2 activity and the formation of *Ang (1-7)* or activating Mas receptors are new options, in addition to the classical ACE inhibitors and *Ang II* receptor type 1 blockers. An alternative *Ang II*-generating enzyme, chymase, may also importantly influence the regulation of IOP. Intraocular chymase injection has resulted in an increase in IOP in rabbits, which effect was attenuated by a specific chymase inhibitor (Konno et al. 2005). Other interesting and potential agents are renin-inhibitors which can block the whole renin-angiotensin system by inhibiting the action of renin.

However, the nature of the present experimental compounds constitutes a significant pharmacokinetic challenge in penetration to the inner parts of the eye. In addition to their oculohypotensive effect, blockade of ocular RAS may also exert a neuroprotective effect in glaucoma, and angiotensin-induced vasoconstriction of ocular blood vessels has been considered a pathogenic mechanism in optic nerve damage (Mabuchi et al. 2004). Drug intervention may also have a positive effect on certain ocular diseases like sarcoidosis, in which intraocular RAS is known to be activated (Immonen et al. 1986). Compounds acting on RAS may also have a potential in the treatment (Zhang et al. 2007; Zheng et al. 2007) and prevention of diabetic retinopathy, a leading cause (Sjølie and Chaturvedi 2002) of blindness in people of working age.

7 SUMMARY AND CONCLUSIONS

The present study investigated the effects of different exogenous RAS compounds on IOP and aqueous humor outflow dynamics as well as the relationship between IOP and development of high BP in *in vivo* experiments. The RAS expression in the different eye structures was determined by *in vitro* methods using enucleated eyes of experimental animals and fresh enucleated eyes of domestic pigs.

The main findings are as follows:

1. A degrading product of angiotensins, heptapeptide *Ang (1-7),* was found to be the most potent oculohypotensive agent when administered intravitreally into animals with normal IOP. Topical application of different RAS compounds did not affect IOP. Systemically administered antihypertensive agents lowered blood pressure but did not have any significant lowering effect on normal IOP.

2. A novel angiotensin system receptor type, the Mas receptor for *Ang (1-7),* was described for the first time in the eye tissues. An active *ACE2*, an enzyme degrading angiotensins to vasorelaxing forms, was also found for the first time in the ciliary body and vitreous. These findings strongly indicate the existence of local active intraocular RAS.

3. *Ang II* given intravitreally decreased dose-dependently aqueous humor outflow facility while *Ang (1-7)* had no effect on it suggesting that *Ang (1-7)* reduces IOP via inhibition of formation of aqueous humor.

4. There was a significant relationship between development of high blood pressure and intraocular pressure in spontaneously hypertensive rats (SHR), but this relationship was not seen in double transgenic rats (dTGR) with very high basal IOP lowering fast to the level of IOP of SHR.

In conclusion, the function and expression of intraocular RAS in the normotensive eyes has been in focus in this study. Intraocular RAS seems to be involved in the regulation of IOP, but probably the local RAS is even more activated under pathological conditions like in glaucoma. Present findings suggest the potentials of agents which increase ACE2 activity and formation of

angiotensin (1-7) or activate Mas receptors as antiglaucomatous drugs in the future.

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