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The effect of the renin-angiotensin system on contractility of retinal arterioles

Kristín Heba Gísladóttir

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A thesis for 90 ECTS credits towards a *Magister Scientiarum* degree in Natural Resource Sciences

Supervisors:
Ársæll Már Arnarsson
Kristinn P. Magnússon

Natural Resource Sciences
School of Science
University of Akureyri
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Auðlindadeild
Raunvísindasvið
Háskólinn á Akureyri
Sólborg, Norðurslóð 2
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Útdráttur

Í þessari in vivo rannsókn var samdráttur slagæðlinga í augnbotni rannsakaður með Myograph tækni. Markmiðið var að rannsaka ferli staðbundna renin-angiotensin kerfisins á samdrátt slagæðlinga í sjónhimnu.

RAS kerfið gegnir mikilvægu hlutverki í stjórnun blóðþrýstings, jafnvægi líkamsvessa og jónefna í líkamanum. Talið er að í augum sé staðbundið RAS kerfi sem og að allir þættir RAS kerfisins séu til staðar í sjónhimnu og augum, þar með talið Ang II myndunar-kerfi. RAS kerfið spilar mikilvægt hlutverk í þróun sykursýkissjónkvilla, sjónkvilla fyrirbura, aldursbundinni sjóndepilsrýrnunar og gláku. ACE hamlarar hindra umbreytingu Ang I í Ang II, sem leiðir til minni virkni AT_1R og AT_2R. ACE hamlarar, koma í veg fyrir samdrátt æða og ferli sem hækka blóðþrýsting auk þess að hamla nýmyndun æða, skertri starfssemi æðaþels, æðakölkun, bólgum og stýrðum frumudauða.

Æðabútarnir sem rannsakaðir voru, voru fjarlægðir úr sjónhimnu og slagæðlingarnir þræddir í Myograph 410a. Grunnmælingar voru teknar á hverjum æðabúti áður en viðkomandi efnum var seytt út í.

Staðbundið RAS kerfi er til staðar í slagæðlingum í sjónhimnu nautgripa. Adrenalín, Ang II og Ang I ullu samdrætti í slagæðlingum. DIZE hamlaði samdrætti Ang II marktækt en hamlaði ekki samdrætti Ang I marktækt. Captopril dró marktækt úr samdrætti Ang I.

Lykilorð:Renin-angiotensin kerfið, Angiotensin II, Angiotensin I, Captoril, Diminazeneaceturate (DIZE), blóðflæði um augnbotn.

Abstract

In this in vivo study the contractility of small vessels in bovine retina was examined using Myograph technology. The purpose was to investigate the effects of the local renin-angiotensin pathway on the contractility in those retinal arterioles.

RAS has an essential role throughout the body in controlling blood pressure, body fluid balance and electrolyte homoeostasis. Evidence suggests that a local tissue RAS system is present in the eyes. All components of the RAS are believed to be expressed in the retina and the eye contains an Ang II formation system. The RAS is known to play important role in the development of diabetic retinopathy, retinopathy of prematurity, age related macular degeneration and glaucoma. ACE inhibitors block the conversion of Ang I to Ang II, leading to decreased activation of both AT_1R and AT_2R. ACE inhibitors, inhibit vasoconstriction and other mechanisms that raise blood pressure as well as vascular hypertrophy, endothelial dysfunction, atherosclerosis, inflammation and apoptosis is reduced.

The vessel segment investigated was removed from the retina and an arteriole was threaded in a Myograph 410a. The contraction of the arteriole was recorded before and after adding the chemicals that was tested each time to the Myograph chamber.

Local RAS system is percent in bovine retinal arterioles. Epinephrine, Ang II and Ang I all caused constriction in those arterioles. DIZE significantly inhibited the contraction induced by Ang II but did not significantly inhibit the contraction induced by Ang I. Captopril significantly inhibited the contraction of Ang I

Keywords:Renin-angiotensin system, Angiotensin II, Angiotensin I, Captoril, Diminazeneaceturate (DIZE), ocular blood flow.

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Age-related macular degeneration	(AMD)
Angiotensin converting enzyme II	(ACE2)
Angiotensin converting enzyme inhibitor	(ACE inhibitor)
Angiotensin converting enzyme	(ACE1)
Angiotensin I	(Ang I)
Angiotensin II	(Ang II)
Angiotensin receptor blocker	(ARB)
Angiotensin receptor type 1	(AT_1R)
Angiotensin receptor type 2	(AT_2R)
Diabetic retinopathy	(DP)
Diminazeneaceturate	(DIZE)
Intra ocular pressure	(IOP)
Nitric oxide	(NO)
Renin-angiotensin system	(RAS)
Retina derived relaxing factor	(RRF)
Retinopathy of prematurity	(ROP)
Vascular endothelial growth factor	(VEGF)
(Pro)renin receptor	((P)RR)

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1 Introduction

In this study the contractility of small vessels in bovine retina was examined using Myograph technology. The aim was to investigate the effects of the local renin-angiotensin pathway on the contractility in those retinal arterioles. To this end, effect of Epinephrine, Angiotensin II (Ang II), Angiotensin I (Ang I), Captopril and Diminazeneaceturate (DIZE) on the contractility of bovine arterioles was studied.

1.1 Ocular blood flow

The circulatory system has important role, it transfers nutrients and chemicals necessary for cellular function (Dorrell, Friedlander & Smith, 2007). The majority of the ocular blood supply is provided through the ophthalmic artery. A branch of the ophthalmic artery is the central retinal artery which supplies blood to the the inner layers of the retina. The central retinal artery enters the optic disc through the lamina cribrosa. There it branches into four principal intra-retinal arteries. The arterioles bifurcate to form smaller arteriole branches and terminal arterioles, which feed into a capillary bed as they extend toward the peripheral retina. Retinal arterioles are typically surrounded by a capillary-free zone, because of the higher oxygen content of the blood they carry (Kur, Newman & Chan-ling, 2012; Kitaba, Martin, Gopalakrishnan & Tobias, 2013; Mackenzie & Cioffi, 2008; Anand-Apte & Hollyfield, 2010).

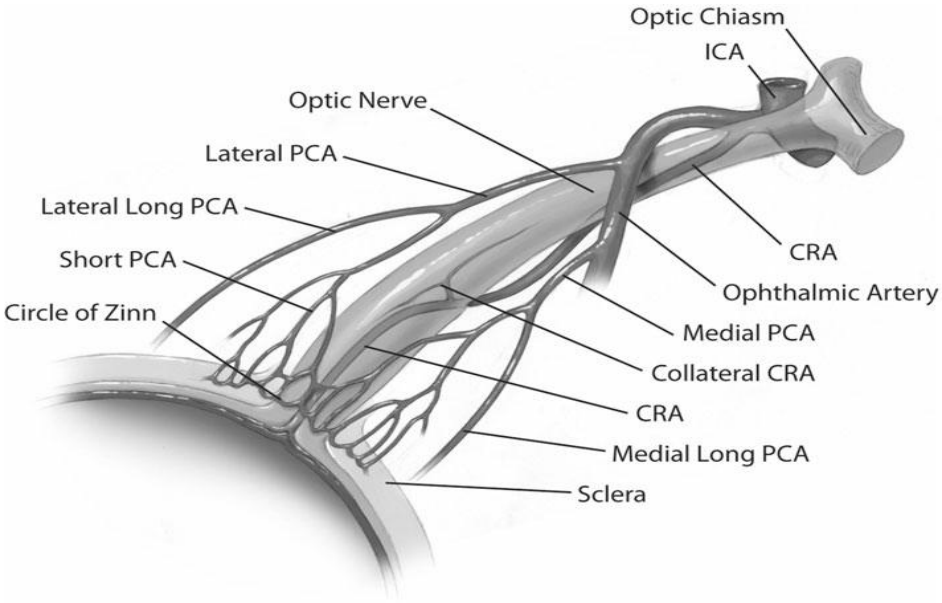

Figure 1 Blood flow to the eye (Kitaba et al. 2013).

Five to seven layers of smooth muscle cells (tunica media) make up the walls of the largest arterioles, near the optic disc. Circumferentially around the retinal arterioles are smooth muscle actin filaments. The number of layers diminishes to just one or two in the retinal periphery after several branching of the vascular network. Smooth muscle cells are orientated both circularly and longitudinally in retinal arterioles. Each of them is surrounded by a basal lamina that contains an increasing amount of collagen toward the cellular adventitia. The endothelial cells (part of the tunica interna) are orientated longitudinally along the axis of the vessel and share their basement membrane with adjacent smooth muscle cells and pericytes. The basement membrane acts as an important regulatory matrix for the passage and sequestration of vasoactive agents and pro-survival growth factors. The basement membrane is composed of collagen IV, fibronectin, laminin, matrix metalloproteinases (MMPs- 2, MMPs-9) and serine proteinase urokinase. Pre-capillary sphincters are not present in the human retina and therefor the retinal capillaries are continuously perfused (Kur et al. 2012).

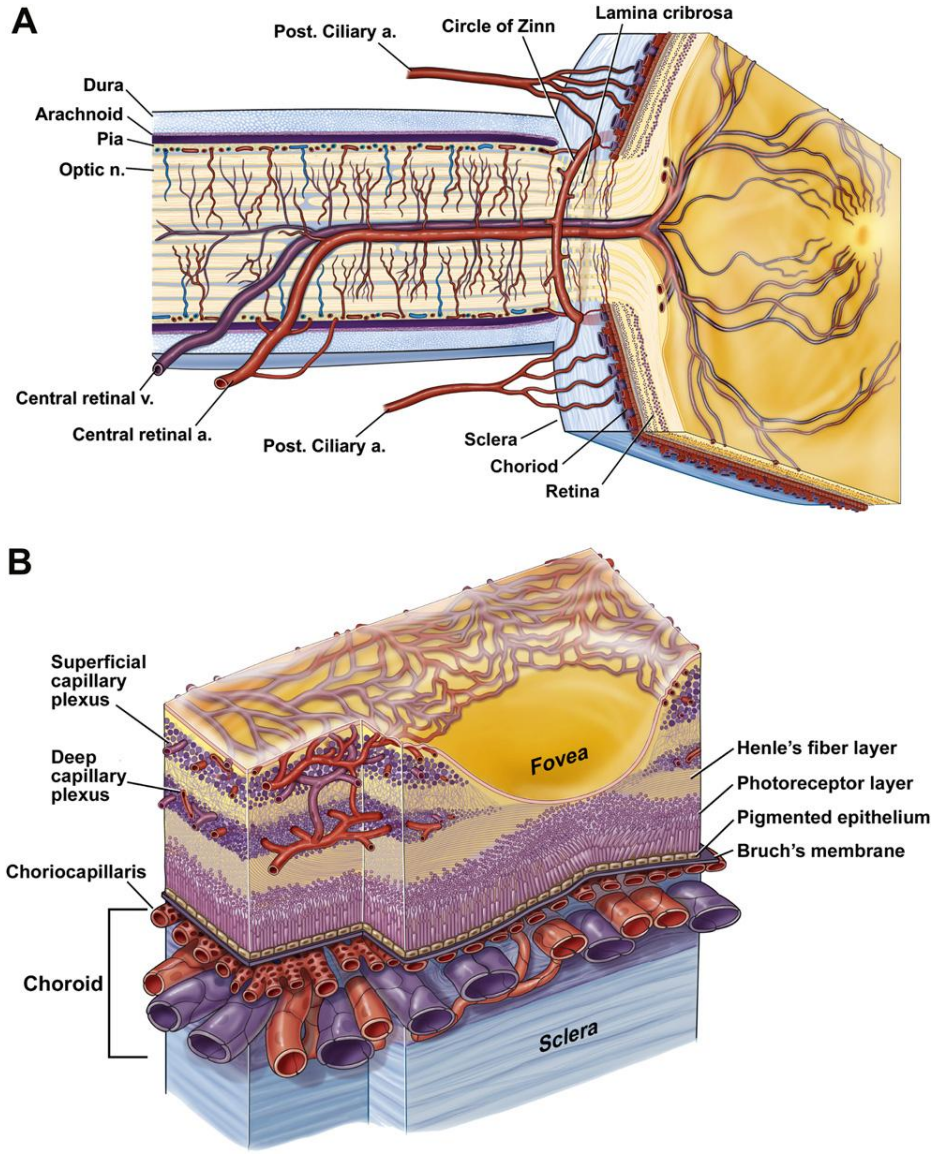

Figure 2 The ocular circulation. Part A shows the vascular supply of the retina and choroid. Part B shows the vasculature of the retina and choroid (Kur et al. 2012).

The venous system of the retina leaves the eye through the optic disc and drains venous blood into the cavernous sinus. The terminal branches of the vessels, pre-capillary arterioles and post-capillary venules, are linked through anastomotic capillaries. Retinal capillaries are organized in an interconnecting two-layer network as seen in figure 2. First there is a

superficial layer that is located in the nerve fiber and ganglion cell layers and contains arterioles, venules and capillaries. Secondly there is a deep retinal plexus that lies deeper and is in the inner nuclear and outer plexiform layers that consists predominantly of capillary-sized vessels. Both layers reach almost to the edge of the retina (Kur et al. 2012).

Within the eye the tissues are highly variable, there is a tissue with dense vasculature (choroid) and there are also tissues without any vessels such as the vitreous body and the lens (Vaajanen & Vapaatalo, 2011). The fovea that contains the highest density of photoreceptors is avascular and is only found in primates. The thinness of the retina in this region permits adequate retinal oxygenation through the choroid circulation. The superior and inferior temporal vessels deviate in their paths to bypass the fovea and minimize their density in the temporal raphe region (Kur et al. 2012; Chan-Ling, 2010).

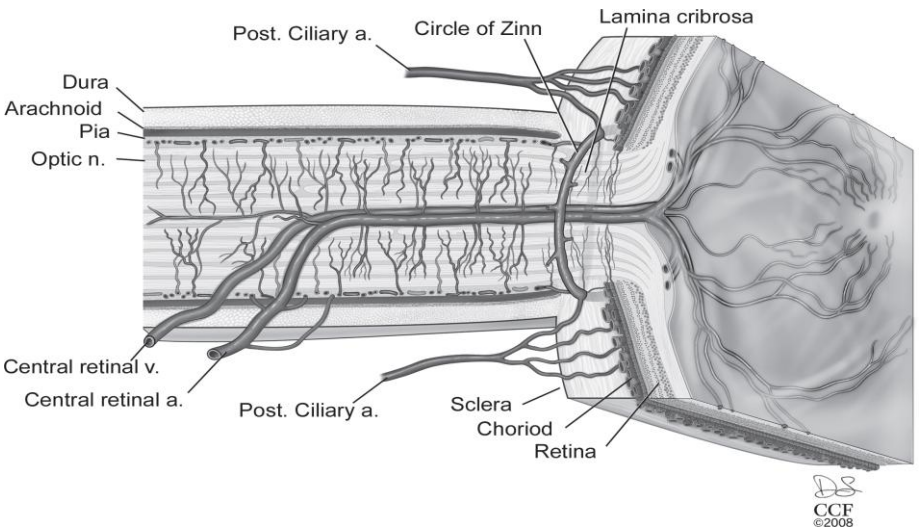

Figure 3 The vascular supply of the fovea (Anand-Apte & Hollyfield, 2010).

The eye has two blood–ocular barriers and its own auto-regulation in blood circulation that keeps the local tissue flow constant and counteracts with changes in the local metabolic environment (Vaajanen & Vapaatalo, 2011). These are the blood-aqueous barrier and the blood-retinal barrier. The blood-retinal barrier is fundamental for regulating the contents of the eyes inner fluids and for protecting the ocular tissues from variations that are present in the circulation. The blood-retinal barrier must provide suitable, highly regulated, chemical environment and serve as a drainage route. The blood-

retinal barrier is similar to the blood-brain barrier. The blood-retinal barrier consists of inner and outer components. The inner blood-retinal barrier is confined by tight junctions between neighboring retinal endothelial cells. The retinal endothelial layer functions as an epithelium and forms the main structure of the inner blood-retinal barrier. The endothelial cell layer rests on a basal lamina that is covered by astrocytes and Müller cells. Astrocytes, Müller cells and pericytes influence the inner blood-retinal barrier by transmitting regulatory signals to endothelial cells. The outer blood-retinal barrier plays a fundamental role in regulating access of nutrients from the blood to the photoreceptors and it also eliminates waste products (Cunha-Vaz, Bernardes & Lobo, 2011).

The blood-aqueous barrier prevents plasma derived proteins from entering the aqueous humor. The non-pigmented ciliary epithelium and the vascular endothelium and the vascular endothelium of the iris play a fundamental role in the blood-aqueous barrier. Its role is to separate the pristine environment behind the iris from the more permissive environment required to sustain the avascular tissues at the iris plane (Freddo, 2013). There are no major diffusion barriers between structures inside the eye. There for the blood-aqueous barrier and the blood-retinal barrier must influence each other and work in balance (Cunha-Vaz et al 2011).

Retinal blood flow

The retina has more energy demand per gram than the brain and requires precise regulation of blood flow because it is the most metabolically active tissue in the body. Well regulated blood flow in the retina is an important part of maintaining energy depended processes (Yu, Balaratnasingam, Morgan, Cringle, McAllister & Yu, 2010; Almasieh, Maclntyre, Pouliot, Casanova, Vaucher, Kelly & Di Polo, 2013; Osborne, Casson, Wood, Chidlow, Graham & Melena, 2004).

The role of the ocular circulation is to nourish the retina. Choroid and retinal blood vessels branch from the ophthalmic artery (Ikemura, Miyaji, Kashima, Yamaguchi & Hayashi, 2013). Two circulatory systems, the retinal and the choroid blood vessels supply the retina with blood. The retinal circulation provides the inner retina with blood except for the avascular fovea zone. The choroid vessels supply the outer avascular retina layers with nutrients through diffusion (Willoughby, Ponzin, Ferrari, Lobo, Landau & Omidi, 2010). The retinal arterial circulation in the human eye is a terminal system. In the retina there are two capillary beds, one in the ganglion cell layer and one in the inner nuclear layer (Anand-Apte & Hollyfield, 2010). The inherent intra-retinal vessels supply the inner two-thirds of the retina and the choroid vasculature supplies the outer third of the retina. Four main artery/veins

supply the retina. These are the superior nasal, inferior nasal, superior temporal and inferior temporal branches of the central retinal artery and vein. Smaller arterioles branch from these four main arteries. (Chan-Ling, 2010). The oxygen consumed by the retina is mostly delivered by the choroid circulation and this is indispensable for an adequate oxygenation of the retina. It is also believed that blood flow in the choroid is necessary to regulate the temperature of the eye (Geiser, Riva, Dorner, Diermann, Luksch & Schmetterer, 2000).

The blood flow of the eye is unique in many respects and the arrangements is because of the conflict between the requirements of sufficient blood supply on the one hand and the requirements of minimal interference with the light path on the other (Vaajanen & Vapaatalo, 2011; Geiser et al. 2000). For vision to be as clear as possible it is important to have a limited extent of retinal vasculature in the retina, so as much light as possible is transmitted to the photoreceptors. This anatomical structure of the retina makes it vulnerable. The outer retinal layers are avascular and are therefore dependent on metabolic support by diffusion from the retinal and choroid vascular beds. The fovea area, that is avascular, is almost completely dependent on choroid support. The choroid is highly vascularized and has very little regulatory ability to respond to changes. Many cellular elements such as the endothelium, smooth muscle cells, glia and neurons are believed to make up the metabolic and vascular regulation of the retina. The vascular endothelium synthesizes and releases various factors that modulate angiogenesis, inflammatory responses, homeostasis, vascular tone and is also dynamic, semi-selective barrier that regulates transport of fluid and macromolecules between blood and the interstitium (Yu et al. 2010).

The retinal circulation has auto-regulatory capacity that is controlled locally and is able to influence the autonomic innervations and it maintains the local tissue flow constant. The contribution of circulating hormones and neurotransmitters on retinal vascular resistance is generally assumed to be negligible due to the blood-retinal barrier (Yu et al. 2010; Kitaba et al. 2013; Vaajanen & Vapaatalo, 2011). The vascular bed capability to maintain a constant blood flow, despite variations in perfusion pressure is the definition of vascular auto-regulation (Almasieh et al. 2013). The retina maintains its circulation locally by releasing vasoactive substances from the retina and the endothelium of the retinal artery (Takir, Uydes-Dogan & Ozdemir, 2011). Cells that produce vasodilators, such as nitric oxide (NO) and prostacyclin, and vasoconstrictors, such as angiotensin and endothelins have been identified in the choroid, retina, and optic nerve (O'Brien & Harris, 2004). NO, prostacyclin and prostaglandin E_1 and E_2 play important part in controlling the vascular tone in the retina. Prostacyclin is a vasodilator and

prostaglandin produces relaxing effects. Retina derived relaxing factor (RRF) is a factor that causes relaxation in several vascular and nonvascular preparations beside the retinal artery ((Takir et al. 2011; Delaey & Van de Voorde, 1998). Palmitic acid methyl ester is also released from the retinal tissue and is considered to have similar properties as RRF. Ca^{++}ATPase and potassium channels are believed to affect the relaxing effect on the vasodilation of retinal arteries but the exact mechanism that mediates the relaxation of retinal vascular smooth muscle is not fully understood (Takir et al. 2011).

Changes in choroid and retinal blood flow are associated with changes in visual acuity and eye diseases like glaucoma, diabetes and age-related macular degeneration (AMD) (Ikemura et al. 2013; Boltz, Told, Napora, Palkovits, Werkmeister, Schmidl et al. 2013).

1.2 Renin-angiotensin system

The systemic Renin-angiotensin system (RAS) is one of the first recognized hormone system and it has an essential role throughout the body in controlling blood pressure, body fluid balance and electrolyte homoeostasis. The RAS may be the most important factor of the endocrine system that affects the control of blood pressure. The influence of the RAS extends to effects as diverse as proliferation, differentiation, regeneration and apoptosis (Kanda, Noda, Saito & Ishida, 2012; Vaajanen &Vapaatalo, 2011; Beevers et al. 2001; Rice, Thomas, Grant, Turner & Hooper, 2004; Fletcher, Phipps, Ward, Vessey & Wilkinson-Berka, 2010; McCarthy, Widdop, Deliyanti & Wilkinson-Berka, 2013). It is now believed that the RAS is more complex than it was considered to be at first (Wilkinson-Berka, Agrotis & Deliyanti, 2012). Although the RAS system has an important role in controlling blood pressure it is not believed that RAS is directly responsible for the rise in blood pressure, especially in essential hypertension in elderly people. It is believed that there are more important non-circulating local Renin-angiotensin paracrine systems, which have important roles in adjusting blood pressure and regional blood flow. There are angiotensin and enzymes such as Angiotensin converting enzyme 2 (ACE2) and angiotensin (1–7) that are involved in balancing blood pressure and work as counter-regulatory factors (Vaajanen & Vapaatalo, 2011; Beevers et al. 2001).

The RAS system acts as a feedback system and has three important factors. First there is the Ang II that acts as the main regulatory peptide. Secondly there is the angiotensin converting enzyme (ACE1) that is the main regulatory enzyme. Thirdly there is the angiotensin receptor type 1 (AT_1R) that acts as the main regulatory receptor (Vaajanen & Vapaatalo, 2011).

In the RAS, prorenin is activated to form renin in juxtaglomerular cells in the kidney. Renin binds to renin substrate (angiotensinogen) to generate Ang I (Ang 1-10). Ang I is a physiologically inactive substance but is rapidly converted to Ang II (Ang 1-8) in the lungs by ACE1 (Beevers et al. 2001). Wilkinson-Berka et al. (2012) have reported that prorenin and renin may bind to the (pro)renin receptor ((P)RR) and binding to the (P)RR can elicit angiotensin independent actions. The (P)RR, seems to act in the kidney, the heart and in the retina. It is believed that the effect of binding to (P)RR can cause signal transduction mechanisms associated with proliferative and fibrotic effects of the RAS (Ferreira & Raizada, 2008; Robles, Cerezo & Hernandez-Gallego, 2014; Vaajanen & Vapaatalo, 2011). Figure 4 further descripes the system.

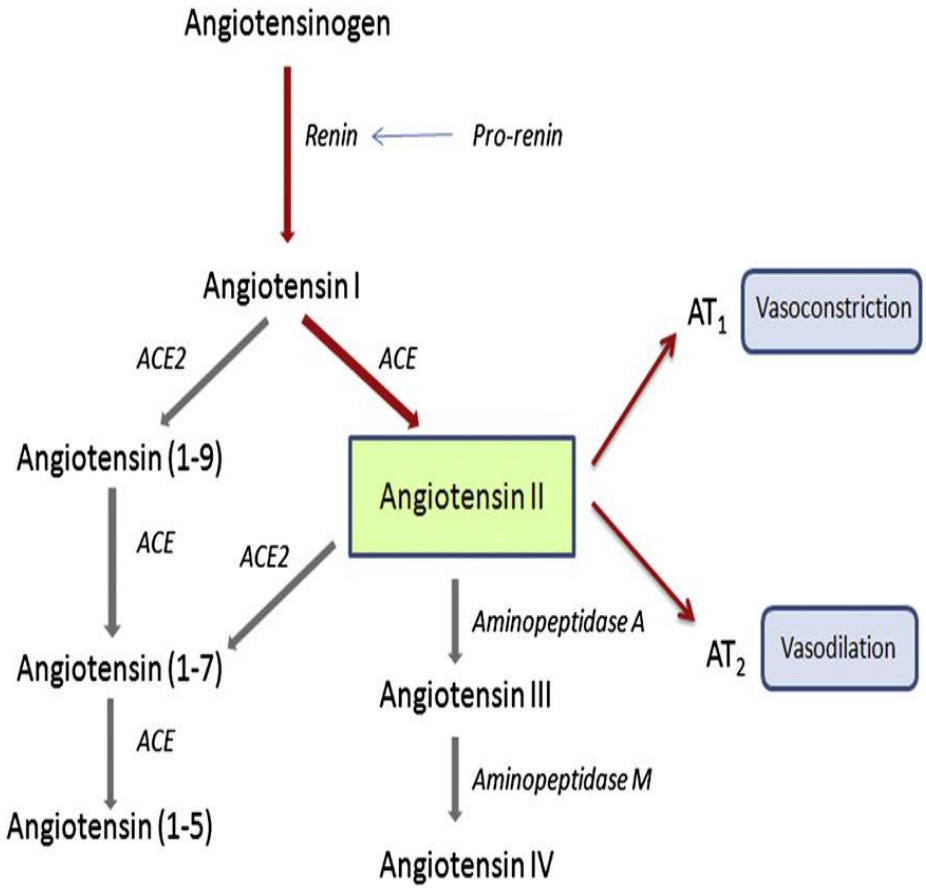

Figure 4 Schematric diagram of the main components of the RAS (Fletcher et al. 2010).

Angiotensin II

Ang II is the main effector hormone of the RAS. It is a potent vasoconstrictor and plays an active role during all RAS processed. It causes a rise in blood pressure and it stimulates the release of aldosterone from the zonaglomerulosa of the adrenal gland, which results in a further rise in blood pressure related to sodium and water retention. It is also involved in regulation of electrolyte homeostasis, fluid retention and vasoconstriction. It can activate numerous signaling cascades by binding to various cell surface receptors. In the central nervous system Ang II has a transmitter like function. Ang II acts on presynaptic and postsynaptic neural sites and modulates a number of transmitter systems. The two predominant receptors for Ang II are known as the AT_1R and the Angiotensin receptor type 2

(AT_2R) that both belong to the trans-membrane G-protein coupled receptors. Both of these receptors show similar binding affinity for Ang II. Ang II triggers the release of aldosterone from the adrenal cortex. Aldosterone that binds to the mineralocorticoid receptor influences electrolyte and water balance in the body (Jacobi, Osswald, Jurklies & Zrenner, 1994; McCarthy et al. 2013; Fletcher et al. 2010; Foureaux, Nogueira, Nogueira, Fulgencio, Menezes, Fernandes et al. 2013; Wilkinson-Berka et al. 2012).

When Ang II acts through the dominant AT_1R it has various effects. It promotes cellular pathology including apoptosis, hypertrophy, neovascularization, inflammation, fibrosis, induces vasoconstriction, raises aldosterone levels, mediates regional blood flow, hyperplastic, hypertrophic vascular smooth muscle cell proliferation and migration, the regulation of local sympathetic activity, pressure and cardiac responses. It is also believed that it is involved in platelet activation and aggregation and maintenance of cardiovascular structure and repair. AT_1R is associated with most of the biological and pathological actions of Ang II (Wilkinson-Berka et al. 2012; McCarthy et al. 2013; Fletcher et al. 2010; Rice et al. 2004; Robles et al. 2014).

The binding of Ang II to AT_2R is not fully understood but is believed to causes opposite actions to that of AT_1R. The AT_2R is highly expressed in fetal tissue and reduces after birth and is only found in low levels in adults (Wilkinson-Berka et al. 2012; McCarthy et al. 2013; Fletcher et al. 2010). Ang II causes different strength of contraction in vessels, depending on the luminar diameter and it causes a dose dependent response curve in bovine retinal arterioles (Kulkarni, Hamid, Barati & Butulija, 1999).

It has been reported that Ang II activates a Ca^{2+} signaling system that leads to increase in potassium ion channel activity and triggers aldosterone production. Ang II is also linked to cell volume loss that indicates that Ang II acts as an operated secretagogue in the non-pigmented ciliary epithelial cells. Ang II also causes increase in cytoplasmic sodium concentration owing to the activation of Na^+/H^+ exchange (Vaajanen & Vapaatalo, 2011).

Angiotensin converting enzyme

ACE1 is a rate limiting enzyme in the RAS (Jacobi et al. 1994). It is a component of the RAS and generates Ang II by a zinc metalloprotease that catalyzes cleavage of the C-terminal dipeptide from Ang I to produce the potent vasopressor octapeptide Ang II, a potent vasoconstrictor, and degrades bradykinin, a vasodilator. ACE1 mediates numerous systemic and local effects in the cardiovascular system (Donoghue, Hsieh, Baronas, Godbout,

Gosselin, Stagliano et al. 2000; Rice et al. 2004; Vickers, Hales, Kaushik, Dick, Gavin, Tang et al. 2002). It is believed that angiotensin converting enzyme inhibitor (ACE inhibitor) reduces systemic vascular resistance in patients with hypertension and it is also considered to be beneficial in preventing and treating cardiovascular, renal and retinal diseases in patients with normal blood pressure (Wang, Zheng, Jin & Xu, 2012).

Angiotensin converting enzyme II

Another important factor of the RAS system is ACE2 that is expressed in endothelial cells (Vickers et al. 2002). ACE2 can degrade Ang I to Ang (1-9) and Ang II to form active Ang (1-7), that in many aspects works opposits to Ang II. For ACE2 to generate Ang (1-7) it must degrade Ang I and Ang II. Ang II has higher affinity for ACE2 but the production of Ang (1-7) by ACE2 must go through two steps, first is a cleavage of Ang I to Ang (1-9) by ACE2 and then a conversion of Ang (1-9) to Ang (1-7) through ACE1 or neutral endopeptidase. There fore the production of Ang (1-7) by ACE2 is less efficient then the production of Ang II from ACE2. The main function of Ang (1-7) is through the Mas receptor. The Mas receptor is a novel angiotensin receptor type that is a G protein-coupled receptor encoded by the Mas proto-oncogene. The Mas activation causes vasodilation, anti-proliferation and anti-fibrosis and it plays a role in fluid volume homoeostasis. Its effects are the opposite of AT_1R activation. Ang (1-7) acts as a vasodilator with anti-proliferative effects. ACE2 also participates in the metabolism of important peptides like Apelin-13, neurotensin 1-8, dynorphin A^{1-13} and bradykinin, its diverse function may contribute to some of its beneficial effects that may not only be because of its function in the RAS system (Ferreira & Raizada, 2008; Rice et al. 2004; Vaajanen & Vapaatalo, 2011; Foureaux et al. 2013).

Angiotensin (1-7)

Ang (1-7) is a recently discovered key factor in the RAS system. It has beneficial effects in the cardiovascular system because of its counter-regulatory role. Ang I can be cleaved to Ang 1-7 through the ACE1 homolog enzyme ACE2 or neutral endipeptidase. ACE1 is the main Ang (1-7) forming enzyme. Ang (1-7) interacts with the G protein-coupled receptor Mas to mediate its vasoprotective effects and may stimulate the Mas receptor to antagonize the effects of the AT_1R by promoting vasodilation, the release of NO and phosphorylation (Ferreira & Raizada, 2008; Wilkinson-Berka et al. 2012).

Other components of the RAS

Another enzyme, a heart chymase that is secreted by mast cells, is capable of converting Ang I to Ang II. Ang I cleavage products have been found, for example, Ang 1-9, Ang1-7, Ang III and Ang IV (Vickers et al. 2002).

ACE2, Ang (1-7) and the Mas receptor have been identified and are believed to have beneficial effects that oppose the effects of the Ang II acting on the AT_1R (Foureaux et al. 2013). The effects of Angiotensin III, angiotensin IV and Ang (1-7) that are components of the RAS, have not been fully elucidated. Renin is both a rate limiting enzyme for RAS activation and the ligand for the protein renin/prorenin receptor that binds renin and prorenin. Prorenin induces an increase in the catalytic efficiency of angiotensinogen conversion to Ang I. That leads to local production of Ang II and binding of renin/prorenin to the renin/prorenin receptor (Robles et al. 2014).

Local RAS

There is a circulatory RAS and a localized tissue system. The local tissue RAS regulates long-term changes in organs such as the kidneys, adrenals, brain, reproductive organs, vasculature and the eyes (Vaajanen & Vapaatalo, 2011; Foureaux et al. 2013).

In peripheral organs, angiotensin peptides are mainly the result of kidney-derived renin acting on liver-derived angiotensinogen. In the local RAS angiotensinogen is synthesized and secreted by glia (McCarhy et al. 2013). Ang II is provided from the circulation or is produced in the tissue. The local Ang II production can be promoted by enzyme such as chymase and is a renin-independent pathway for Ang II production (Vaajanen & Vapaatalo, 2011). When Ang II is produced locally, the Ang II acts through the AT_1R and AT_2R. The effects of Ang II are activation of intracellular signal pathways, enhanced synthesis of DNA, stimulation of the release of plasminogen activator inhibitor 1, collagen 1, fibronectin and transforming growth factor b-1.When AT_1R is activated it causes vasoconstriction, aldosterone and vasopressin secretion, sodium retention and decreased renal perfusion. When AT_2R is activated it causes various anti-proliferative and anti-inflammatory effects and promotes tissue differentiation and regeneration and apoptosis. It is believed that AT_2R works oppose to AT_1R. The function of the AT_2R has not been clearly defined (Robles et al. 2014).

Local RAS in the retina

Components of the RAS are expressed in many organs (Fletcher et al. 2010). Most of the components of the RAS system have been identified in the human eye (Vaajanen & Vapaatalo, 2011) and there is evidence that suggest all components of the RAS are expressed in the retina (Fletcher et al. 2010). Ferrari-Dileo, Davis and Anderson (1987) have reported that binding sited for Ang II are present in bovine and human retinal vascular trees.

There is a local RAS in the retina that has components that are expressed in retinal micro vessels, glia and neurons. Components of the RAS have also been identified in other ocular structures like the choroid and ciliary body. Prorenin and renin are expressed in almost the entire retina and it interacts with the inner retinal microvasculature. Prorenin and renin are expressed in Müller cells but other components of the RAS like ACE1, ACE2. Ang 1-7, Ang II and AT_1R have also been identified in the retinal Müller cells in variety of species. In ganglion cells almost all components of the RAS have been identified. Some components of the RAS have been identified in astrocytes, microglia, amacrine cells, bipolar cells, photoreceptors, endothelial cells and pericytes of the retinal microvasculature. It is believed that a local aldosterone system exists in the retina (Wilkinson-Berka et al. 2012).

Because various parts of the eye express components of the RAS it is believed that the eye contains Ang II formation system. What further supports this hypothesis is a large difference between the concentration of Ang II, prorenin and renin in the retina compared to the plasma and Ang II does not cross the blood retinal barrier. All components of the RAS are expressed in the cells of the retina. It is believed that formation of Ang I and Ang II occurs within retinal glial cells and that Ang II regulates retinal function (Fletcher et al. 2010).

Ang II and aldosterone are believed to influence endothelial cells and pericytes in retinal microvasculature. Ang II is involved in both the survival and proliferation in retinal endothelial cells (Wilkinson-Berka et al. 2012).

AT_1R is expressed by retinal glial cells. AT_2R is localized in cohorts of retinal neurons. Ang II causes vasoconstriction of retinal arterioles, capillaries and venules when acting through AT_2R receptor. These effects occur because Ang II induces an increase in intracellular calcium through mechanisms that involve release of calcium from intracellular stores and through influx of calcium through L-type voltage gated calcium channels. Influx of calcium into pericytes is an important step in the effects of Ang II.

Ang II also uncouples pericytes from their micro-vascular neighbors through a Protein Kinase C dependent mechanism. Ang II regulates pericytes contractility and their cell dynamics and gene function (Fletcher et al. 2010).

Regarding the Mas receptor for Ang (1-7) researchers do not fully agree about the existence of Mas receptor in the eyes. Vaajanen and Vapaatalo (2011) state that the Mas receptor does not exist in the eyes while Foureaux et al. (2013) report the contrary.

1.3 RAS and ophthalmic diseases

The eye is one of the most complex organs in the human body (Willoughby et al. 2010) and numerous factors need to work in perfect harmony in order for vision to function normally. Optimum intraocular pressure (IOP), adequate blood and oxygen supply, with efficient venous blood drainage combined with normal functioning of the cornea, pupil, lens, retina, optic nerve, and cerebral cortex are examples of those factors that are necessary for normal vision (Kitaba et al. 2013).

With the anticipated rise in mean age in populations in many countries it is predicted that the number of individuals suffering from age-related ocular diseases will increase, resulting in increasing health costs (Kompella, Amrite, Ravi & Durazo, 2013). Retinal diseases with vascular components and angiogenic ocular conditions are the leading cause of irreversible vision loss in the developed countries (Yu et al. 2010). The RAS is known to play important role in the development of diabetic retinopathy (DP), retinopathy of prematurity (ROP), AMD (McCarthy et al. 2013) and glaucoma (Fletcher et al. 2010) (see figure 5).

In patients with retinal pathology prorenin, renin, ACE1 and Ang II are elevated in plasma and the eyes (Wilkinson-Berka et al. 2012). Blocking AT_1R and ACE1 may improve the pathology of these diseases (McCarthy et al. 2013).

Progressive damage of arterial vessels is caused by systemic arterial hypertension. The responses of the microvasculature to increased blood pressure is endothelial dysfunction, impaired vessel relaxation, enhanced contractile response, intima-media thickening, decreased lumen diameter and increased vessel resistance. Reduction of blood pressure reduces the progression of small arteries damage (Madej, Gierek-Ciaciura, Haberka, Lekston-Madej, Basiak, Domanska et.al, 2010).

Retinal ischemia is when blood flow to the retina is inadequate and does not meet the cellular energy demand of the tissue. Ischemia deprives the tissue of oxygen, metabolic substrates and removal of waste. That leads to lower homeostatic responses that cause death in the tissue if withheld for long. Evidence suggests that if circulatory disturbance lasts for more than about 100 minutes then vision is affected because of irreversible structural changes (Osborne, Casson, Wood, Chidlow, Graham & Melena, 2004). Retinal ischemia can have two causes. Firstly from a general circulatory failure such as severe left ventricular failure and hypovolaemic shock. Secondly from local circulatory failure that is a more common cause. The effects of

occlusion are different after its position. Occlusion of a non-terminal artery causes no serious damage, but occlusion of a terminal artery causes ischemia and infarction (Osborne et al. 2004).

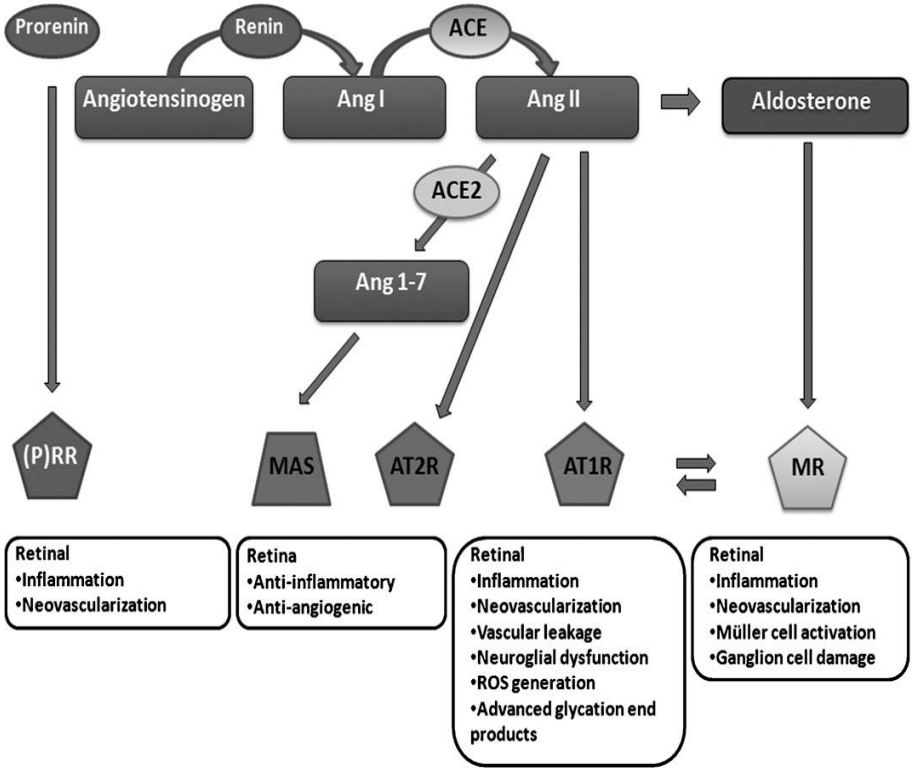

Figure 5 The RAS components and their effects on ROP and DP (Wilkinson-Berka, Agrotis & Deliyanti, 2012).

Diabetic retinopathy

DP is also one of the most common cause of blindness in the western world (Wang et al. 2012; Osborne et al. 2004) and is in most cases either directly or indirectly a result of retinal ischemia (Osborne et al. 2004). The vision loss is caused by breakdown in the blood-retinal barrier that leads to macular oedema, tractional retinal detachment and inner retinal and vitreous hemorrhage (Wilkonson-Berka, Rana, Armani & Agrotis, 2013).

DP is largely a disease of retinal microvasculature (Wilkonson-Berka et al. 2013) and leads to dysfunction in metabolic pathway, non-enzymatic-

glycation of proteins and advanced glycation end products, increased production of sorbitol, activation of protein kinase C, reactive oxygen species, and inducible form of NO synthase. Inhibition of the RAS system may be very beneficial in treating DP. Inhibiting Ang II can reduce the generation of vascular endothelial growth factor (VEGF). ACE inhibitor leads to decreased expression of VEGF in the retina (Wang et al. 2012). Inhabitation of the RAS has been shown to prevent the development of pathological effects in animal models and patients with DP (Fletcher et al. 2010). More specifically blockade of AT_1R has been shown to have beneficial effects on incidence and progression of DP (Kanda et al. 2012). Directly or indirectly, the local RAS may play a crucial role in the development of DP (Vaajanen & Vapaatalo., 2011).

Retinopathy of prematurity

ROP is a retinal vascular disease in premature infants (Raghuveer & Bloom, 2011). In infants born prematurely the retinal vasculature is not fully established (Kaciok, Krohne, Poulaki & Joussen, 2007).

ROP is caused by pathological angiogenesis due to breakdown in the blood-retinal barrier. The incidence of ROP is estimated to be 68% among infants lighter than 1251 g and 98% among infants lighter than 750 g. ROP leads to damage in retinal microvasculature that can cause pathological angiogenesis, vascular leakage and retinal detachment. Therefore ROP is a major cause of vision loss and blindness in infants and is a result of traction arising from the formation of a fibrovascular scar that can detach from the retina. The treatment of ROP aims to control the oxygen saturation to prevent abnormal retinal neovascularization and laser photocoagulation is used to cause regression of abnormal vessels (Wilkinson-Berka et al. 2013).

ACE inhibitors given on the second or third trimester of pregnancy can cause oligohydramniosis, foetal growth restriction, pulmonary hypoplasia, neonatal hypotension, renal failure with oligo/anuria, renal tubular abnormalities, calvarian hypoplasia and patent ductusarteriosus. AT_1R blockage has shown similar effects (Hard. Wennerholm, Niklasson & Hellstrom, 2008).

Age-related macular degeneration

The most common cause of legal blindness among patients who are over 65 years old in the developed countries is AMD (Ucer, Kayikcioglu, Seymenoglu, Var & Cam, 2011). AMD affects the retina and impairs vision to the extent that it causes blindness (Alcazar, Cousins, Striker, Marin-Castano, 2009). AMD is a disease in the retinal pigment endothelium, Bruch´s membrane and choriod capillaris and is complicated by choroidal neovascularization that leads to central visual loss. The disease is progressive and leads to irreversible vision loss. It is believed that genetic and environmental factors have impact on the pathogenesis of the disease (Ucer et al. 2011; Nagai, Oike, Izumi-Nagi, Urano, Kubota, Noda et al. 2006). The activation of the RAS system is believed to contribute to the progression of AMD but the mechanism by which the RAS activation may affect AMD is not fully understood (Alcazar et al. 2009). Choroidal neovascularization in AMD develops after chronic inflammation in the retinal epithelium (Nagai et al. 2006). The RAS is believed to influence the development of AMD in several ways. Systemic hypertension is a risk factor for the development of AMD, Ang II is believed to modulate retinal pigment epithelium function and Ang II has also been implicated in the cause of choroid neovascularization because of its involvement in retinal angiogenesis (Fletcher et al. 2010). It is believed that prorenin may play a part in the pathology of the disease after binding to the (P)RR. Prorenin is considered to have biological activity in the RAS system. The plasma levels of prorenin can be 10 fold higher than renin and therefore researchers are interested in its role. Prorenin synthesis, have been identified in the eye and the activation of prorein is believed to play a role in pathological neovascularization (Alcazar et al. 2009).

Glaucoma

Population based surveys indicate that 80 million people in the world are affected by glaucoma. 11.2 million people are estimated to have bilateral blindness because of the disease. It is now estimated that in 2020 glaucoma will be the second most common cause of blindness (Mayama & Araie, 2013). Progressive optic nerve degeneration that leads to visual field loss and in some cases irreversible blindness is what defines a group of diseases that fall into the category of glaucoma (Almasieh et al. 2013).

Glaucoma leads to progressive loss of the visual field, retinal nerve fiber structural abnormalities and optic disc changes. Risk factors for glaucoma are for example elevated IOP, age, myopia, exfoliation syndrome, positive family history, belonging to a race other than Caucasian, diabetes, systemic hypertension, migraine ⁄ vasospasms and vascular dysfunction. Vascular

deficits and ischemia are also believed to contribute to the progression of glaucoma. When the RAS is not balanced it is believed to be a risk factor for glaucoma (Vaajanen & Vapaatalo, 2011; Almasieh et al. 2013; Foureaux et al. 2013). The loss of retinal ganglion cells that leads to subsequent visual field deterioration is the final end point in the pathogenesis in glaucoma (Hommer, Sperl, Resch, Popa-Cherecheanu, Qiao, Schmetterer et al. 2012).

Inhibition of the RAS has been proposed to be beneficial both in reducing IOP and to prevent neuronal death (Fletcher et al. 2010).

1.4 Drugs and the RAS system

The RAS system has been the target of a large number of drugs for the last decades because of its involvement in cardiovascular diseases. ACE inhibitors and angiotensin receptor blockers (ARB) are the two major classes of drugs that target the RAS by effecting Ang II. Both of these classes of drugs are commonly used to treat hypertension (Robles et al. 2014). Ang II can be blocked by competitive antagonists and by preventing ACE1 from converting Ang I into Ang II (Rockwood, Fantes, Davis & Anderson, 1987).

ACE1 inhibitors were initially applied as therapeutic agents for the treatment of hypertension, but several additional clinical indications have been identified and approved since. In the eye, ACE1 inhibitors have been shown to lower intraocular pressure in patients with ocular hypertension or primary open-angle glaucoma (Wheeler-Schilling, Sautter, Guenther & Kohler, 2001). Both ACE1 inhibitors and AT_2R blockers can reduce IOP in both normotensive and glaucomatous patients. Furthermore, RAS agents can also be neuroprotective against ganglion cell loss (Vaajamem & Vapaatalo, 2011). Angiotensin receptor inhibitors, for example losartan, have been shown to reduce the development of retinal neovascularization in newborn rats (Wang et al. 2012).

ACE2 is believed to be a promising therapeutic target because it degrades Ang II to generate Ang (1-7). Diminazeneaceturate (DIZE) in eye drops was used to treat glaucomatous rats. DIZE increased the expression of ACE2 in the eyes and decreased IOP in rats with glaucoma. DIZE did not affect the blood pressure in the rats (Fouraux et al. 2013). DIZE treatment has been shown to increase the vaso-protective axis of the lungs RAS, improved pulmonary vaso-reactivity and enhance cardiac function in experimental studies (Rigatto, Casali, Shenoy, Katovich & Raizada, 2013).

Angiotensin converting enzyme inhibitors

ACE inhibitors are effective in treating essential hypertension. The reduce mortality from congestive heart failure and asymptomatic left-ventricular systolic dysfunction after myocardial infarction (Rice et al. 2004).

ACE inhibitors block the conversion of Ang I to Ang II, leading to decreased activation of both AT_1R and AT_2R. The effects of decreasing the activation of AT_2R, inhibits vasoconstriction and other mechanisms that raise blood pressure as well as vascular hypertrophy, endothelial dysfunction, atherosclerosis, inflammation and apoptosis is reduced. Conseqences of

decreasing the activation of AT_2R is loss of positive effects such as promoting anti-proliferation, differentiation, regeneration, anti-inflammation, and apoptosis. ACE inhibitors also block the enzymatic degradation of bradykinin. Bradykinin has positive effect as it activates β-2 receptor that encourages release of NO that contributes to vasodilation and tissue protective results. Known side effects of ACE inhibitors are angioedema and cough. The negative impact of ACE inhibitors is that they reduce the activity of AT_2R and the AT_1R (Robles et al. 2014). ACE inhibitors are for example Lisinopril. The ACE inhibitor perindopril has been shown to have many beneficial effects. It prevents increased VEGF, decreases apoptosis of retinal vascular cells and the formation of acellular capillaries, reduces the thickness of retinal vascular basement membrane and alleviated the retinal vascular damage (Wang et al. 2012). The research of Jacobi et.al (1994) where cats were treated with the ACE inhibitor quinpiril showed that neurons in the inner retina are likely modulated by the RAS. The effect of ACE inhibitors, on individuals with hypertension has also been studied and the results indicate that ACE inhibitors such as cilazapril slightly improve the blood flow in retinal vessels (Madej et al. 2010).

ACE inhibitors and AT_1R antagonists have been shown to lower intraocular pressure following systemic or ocular administration. These effects are believed to involve both actions on the ciliary body aqueous humour production as well on uvealsceral outflow (Fletcher et al. 2010). ACE inhibitors are reported to slow the progression of proliferative diabetic and may have a role in preventing the proliferative phase of ROP (Hard et al. 2008).

The ACE inhibitor captopril has also been shown to be beneficial in slowing down the progression of DP in type 2 diabetic patients (Wang et al. 2012). It is likely that development of DP is influenced by RAS activation in the retina and blocking the AT_1R in patients with DP has been shown to be beneficial in clinical trials (Kanda et al. 2012). Captopril has been shown to significantly reduce retinal blood pressure in rats (Suzuma, Hata, Clermont, Pokras, Rook, Suzuma, 2001) and can also inhibit ET-1 release in isolated bovine retinal endothelial cells at 10^{-4} M and 10^{-6} M (Higgins, Hendricks-Munoz, Caines, Gerrets & Rifkin, 1998).

Angiotensin receptor blockers

ARB antagonize the binding of Ang II to the AT_1R. ARB is more efficient in inhibiting RAS than ACE. The pharmacological advantage of ARB is that it has only a minor effect on AT_2R and therefore does not reduce their possible positive role in the RAS system. ARB has no effect on bradkinin. These drugs have highly favorable tolerability profile (Robles et al. 2014).

AT_1R blockade can have dual benefits on the retinal vasculature, by restoring astrocyte survival resulting in revascularization of the peripheral retina and reducing pre-retinal neovascularization at the retinal surface. In studies, lisinopril has been shown to reduce the progression of DP by 50% and progression to proliferative DP by 80% (Wilkinson-Berka et al. 2012). However, lisinopril failed to affect the diameter of arterioles in normotensive patients with type 1 diabetes, in a study by Mehlsen, Jeppesen, Erlandsen, Poulsen & Bek (2011). It was concluded that lisinopril had no significant effect on retinal auto-regulation that might potentially affect retinal blood flow significantly. ARB has been reported to affect several retinal pathologies like ischemia induced retinal neovascularization and endotoxin induced retinal inflammation. Losartan has been reported to suppress choroidal neovascularization but the underlying mechanisms are not understood (Nagai et al. 2006).

Both ACE1 inhibitors and AT_1R blockade have been shown to be able to prevent neovascularization in rats (Moravski, Kelly, Cooper, Gilbert, Bertram, Shahinfar et al. 2000). Studies on diabetic rodents indicate that ACE1 inhibitors and AT_1R blockers can lessen retinal micro-vascular damage with reductions in several factors such as vascular leakage, formation of acellular capillaries, and expression of angiogenic factors. Similar reductions have also been seen in rodents with ROP (Wilkinson-Berka et al. 2012).

In diabetic animals, ACE1 inhibition and AT_1R blockade has been reported to attenuate the deficits in retinal function, indicating that the benefits of RAS blockade can also extend to non-vascular cells. AT_1R inhibition has also been reported to attenuate nuclear factor-κβ and microglia accumulation in animals with both diabetes and hypertension and studies on hypertensive Ren-2 rats indicate that AT_1R blockade normalizes not only blood pressure but also improves retinal vascular pathology (Wilkinson-Berka, 2012).

It has been suggested that RAS blockage can be an important anti-angiogenic strategy in preventing diabetes-induced neovascularization in the retina whereby ACE1 inhibitors reduce vessel growth in diabetic retina

(Ebrahimian, Tamarat, Clergue, Duriez, Levy & Silvestre, 2005). A study of normotensive and normo-albuminuric individuals with type 1 diabetes, showed a significant reduction in retinopathy progression with either ACE1 inhibition or AT_1R blockade (Wilkinson-Berka et al. 2012). Ang II receptor antagonists may be clinically superior to ACE1 inhibitors because they target Ang II synthesized by both ACE-dependent and -independent pathways, whereas ACE1 inhibitors target only ACE-dependent Ang II (Senanayake, Drazba, Shadrach, Milsted, Rungger-Brandle, Nishiyama et al. 2007).

2 Methods

2.1 Myograph

The Myograph technology was first described by Mulvany and Halpern in 1976. Prior to that, most information about the properties of vascular smooth muscle was confined to large vessels. Bevan and Osher first suggested a technique for investigating small vessels with internal diameters down to around 100 μm. The Myograph technique makes it possible to mount a segment of these small vessels as ring preparation and provides measurements of highly isometric responses (Mulvany, 2004).

Myograph technique is only used for vessels that have the function of directing blood to various organs and are therefore proximal resistance vessels or small arteries. The technique is not suitable for distal resistance vessels. It is important that the Myograph is only used for vessels with the internal diameter of 100-400 μm, but not smaller or larger vessels (Mulvany, 2004).

This kind of in vivo research provides information about vascular tone when isolated from the surrounding retinal tissue (Holmgaard, Aalkjær, Lambert, Hessellund & Bek, 2008). The limitation of this research method is that the process of removing the arteriole from the retina and placing it in the Myograph damages some smooth muscle cells and causes reduction in contractibility. The peri-vascular tissue will also be damaged to some extent. Another factor is that there is no blood flow in the arterioles when being examined, the influence that has on the endothelium is unknown.

2.2 Solutions and chemicals

Krebs solution

The tissue was stored in Krebs solution prepared as shown in table 2.1. When the vessel segment had been threaded in the Myogaph an oxygenated (95% O_2, 5% CO_2), 36.5 °***C*** Krebs was put in the Myograph chamber. The oxygenated Krebs solution has the pH value of 7.4.

Table 1 Krebs solution.

Krebs	**M**	**g per L**
Nacl	58.44	6.58
KCl	74.55	0.22
NaHCO3	84.01	2.09
MgCl2	95.21	0.11
NaH2PO4	119.98	0.14
CaCl2	110.98	0.22
Glucose	180.16	2.07

Table 2 Chemicals used in the research.

	Ang I	Ang II	Captopril	DIZE	Epinephrine
g/mol	1.285.45	1046.18	217.29	515.52	333.29
Concentration	10^{-4}-10^{-10} M	10^{-5}-10^{-12} M	10^{-4}M	10^{-4}- 10^{-7} M	10^{-4} M
Product	A9402	A9525	C4042	D7770	E4375
Manufact-urer	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich
Disolved in	Distilled water	Distilled water	Distilled water	Krebs solution	Distilled water

Table 2 shows the chemicals used in this research. Ang I was used separately and in combination with Captopril. Ang II was used on its own and with DIZE. Epinephrine was used for comparison of the effects of Ang I and Ang II.

2.3 Experimental procedure

In this in vivo experiment, bovine eyes from the local abattoir B. Jensen were used. The eyes were available to researchers once a week. The butcher removed the eyes from the freshly slaughtered cattle when the researchers arrived to the abattoir. The eyes were immediately put into 4°C Krebs solution and brought to the laboratory within 10 minutes. In January 2014 researchers also started getting bovine eyes from another local abattoir, Norðlenska. The same procedure was used in handling eyes form both locations.

The experiments were conducted at the Neuroscience Laboratory at Borgir, University of Akureyri. The samples obtained from the abattoir were immediately dissected on arrival to the laboratory as the samples were only usable within a 24 hours period.

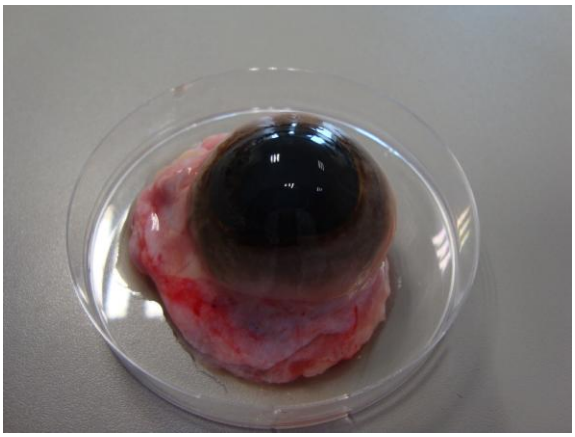

Figure 6 Bovine eye before dissection.

The orbital muscles and optic nerve were cut from the external of the eye. Scissors were used to cut around the cornea which was then lifted from the eye.

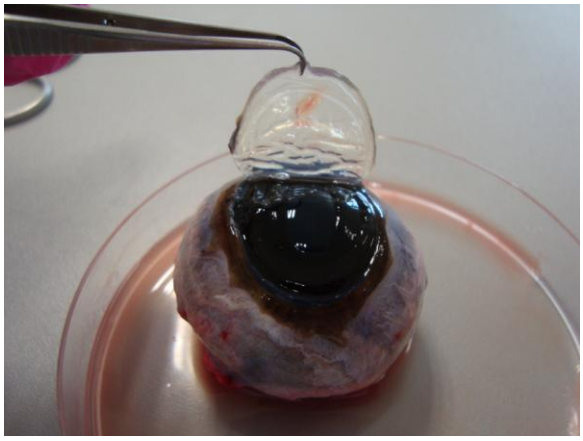

Figure 7 Scissors were used to cut around the cornea which was then lifted from the eye.

The iris and the lens were removed from the eye and the anterior part of the eye along with the vitreous body was evacuated, leaving only the posterior part of the eye.

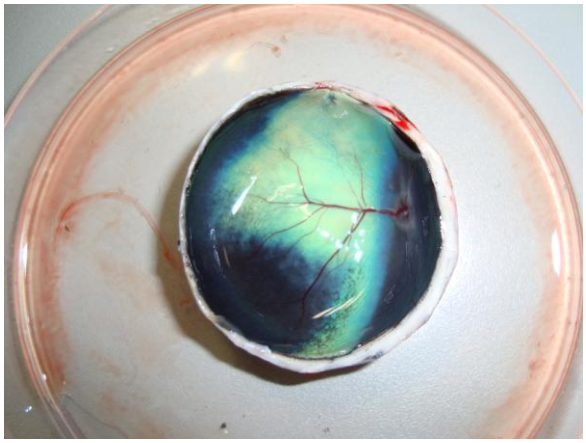

Figure 8 The posterior part of the eye.

For convenience, all of the remaining part of the posterior part was removed except for approximately 2 cm part around the optic nerve. A vessel segment from top of the choroid was removed gently from the retina.

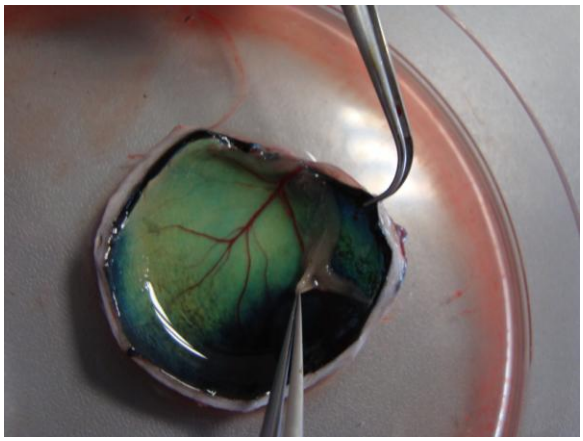

Figure 9 The vessel segment from the top of the choroid being removed.

The vessel segment was put on a Petri dish that contained 5 mm thick layer of Sylgard and 4°C Krebs solution. The segment was pinned down to the Sylgard with fixing pins without stretching.

Using a binocular microscope, arteriole near the optic disk was selected and ocular dissection scissors used to cut across the arteriole. Another cut was made 2 mm away from the first cut and a 2.2 cm long Tungstein wire threaded through the vascular lumen of the arteriole. After the vessel had been threaded, dissection succors were used to cut the artery from the surrounding tissue without touching the vessel

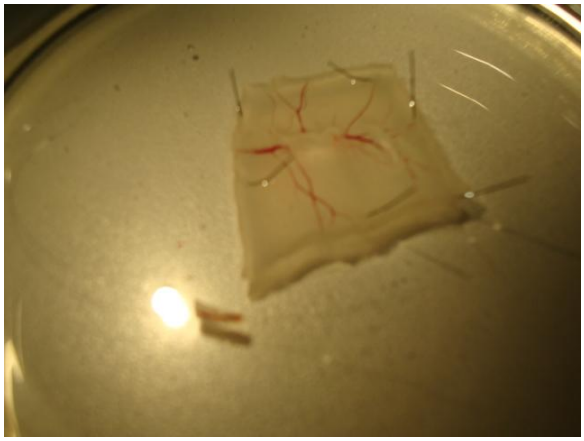

Figure 10 The vessel segment has been pined down and arteriole has been threaded.

The vessel segments from the bovine eyes were placed in small vessel Myograph 410a containing 4°C Krebs solution.

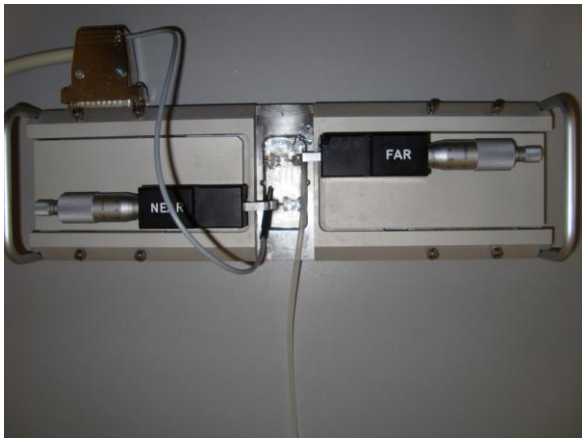

Figure 11 Small vessel Myograph 410a.

The Tungsten wire with the vessel was placed between the jaws and the wire wrapped around the fixing screws that fastened it to the force transducer. Another Tungsten wire was threaded through the vessel lumen, along the first wire and attached to the micrometer. The micrometer measures the contraction of the vessel but at the same time it is possible to control the

diameter of the vessel and therefore the vascular tone. The Myograph chamber was filled with 10 mL of 37°C Krebs solution that had been oxygenated for at least 30 minutes with 5% carbon dioxide and 95% oxygen. The temperature was kept constant by a heating element in the Myograph chamber (Mulvany, 2004; Edvinsson, Ahnstedt, Larsen & Sheykhzade, 2014).

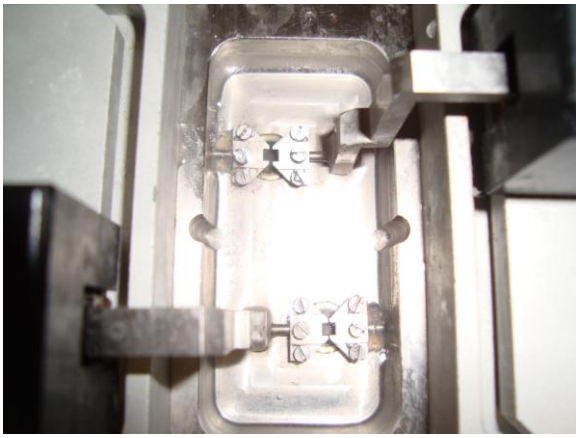

Figure 12 The Myograph chamber.

When the arteriole had been attached to the force transducer and micrometer it was left to rest for approximately 30 minuets. Each arteriole was stretched 1.5 mN. That is the force shown to be optimal for maximum contractility of vessel segment with the diameter of 200 μm in a study by Gísladóttir (2006). When the arteriole had been stretched it was left to rest for few minuets or until the reading on the Myograph converter unit was stable. 10 μl of the drug being tested each time was added to the Myograph bath and its effect recorded. In all measurements, adding the solution of each chemical to the Myograph bath caused contraction according to the Myograph output. The effects of each chemical was the amount of contractility that took place after the chemical was added to the bath and for each measurement the strongest contractility of the arteriole was recorded.

Researchers emptied the Myograph bath quickly after the recording of the drug effects on retinal arteriole contraction and immediately added oxygenated 37 °C Krebs solution to the bath. The arteriole was left to rest until the reading on the converter unit was stable and a drug being tested added to the bath. This made it possible for researchers to test the contraction

of each arteriole more than once. When recording a cumulative response curve each dosage was added on top of the other, the researchers did not empty the Myograph chamber between measurements.

2.4 Myograph maintenance

Myograph calibration

The Myograph was weight calibrated monthly. The weight calibration is based on simple physics of net torque acting on a balance when applying a certain amount of weight. When executing the calibration the Myograph chamber is filled with double distilled water and the Tungsten wire threaded to the jaw of the force transducer. The calibration kit is placed on the Myograph for the kit to be warmed up with the equipment. After 30 minutes, when the temperature was 37°C, the calibration bridge is placed in the gap between the wire and the jaw without touching either. The calibration is then started and a 2 gram weight is put on the bridge. The force reading should be very close to 9,81mN after the calibration (Danish Myo Technology, n.d).

Force transducer

The Myograph force transducer is very fragile. In every session the greasing that insures that the buffer does not get in touch with the force transducer was inspected (Danish Myo Technology, n.d). The force transducer was replaced once during the experiment.

2.5 Data collection and analysis

The p-values were set at $p<0.05$ and results presented graphically as mean ± standard deviation. The data is collected with Myodaq Data Acquisition and analyzed with Myodaq Analysis software. In statistical analysis paired t-tests were used (Misfeldt, Pedersen & Bek, 2013).

3 Results

3.1 Epinephrine

The contractilliy of bovine retinal arterioles were observed with the Myograph after addition of Epinephrine. The effects of Epinephrine on bovine retinal arterioles are well known and were used to test the reliability of our model, and to serve as comparison for the expected constriction induced by Ang I and Ang II. Figure 13 shows the effect of Epinephrine in the strength 10^{-4} on five preparations. One measurement was excluded as outlier (2.54 mN). The outlier was defined as two standard deviation low or higher then mean, 0.31±0.20 mN (*n*=5). As both the figure as well as the standard deviation show, results from the model are quite varied.

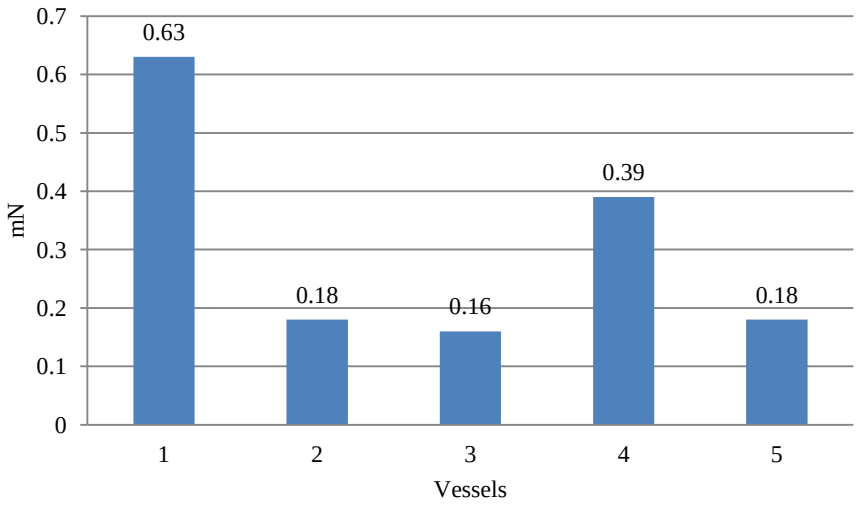

Figure 13 The contraction in mN on wall tension in bovine retinal arterioles, elicited by Epinephrine in the strength 10^{-4}. 0.31±0.20 mN (n=5).

3.2 Angiotensin II

The concentration of Ang II elicited different strength of response in the bovine retinal arterioles. Figure 14 shows the average contractility of Ang II in mN in the concentrations 10^{-6}(*n*=13), 10^{-7}(*n*=6), 10^{-8}(*n*=2), 10^{-9}(*n*=5), 10^{-10}(*n*=4) and 10^{-11}(*n*=5). Ang II in 10^{-8} caused the strongest contractility. Only one outlier was excluded from these measurements for the strength 10^{-6} (1.15 mN). The outlier was defined as two standard deviation low or higher then mean.

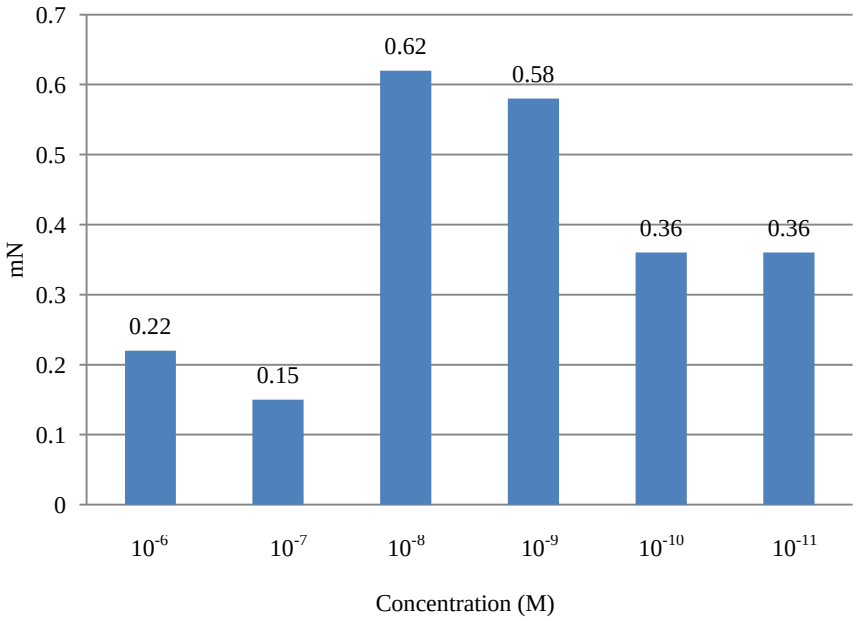

Figure 14 The average contraction in mN on wall tension in bovine retinal arterioles, elicited by Ang II.

The effect of Ang II was also tested in a cumulative manner. In the cumulative dose response each dosage is added on top of the previous one, starting with 10^{-12} and ending with 10^{-6} (*n*=1). The contraction rises with the weaker concentration and reaches the strongest contraction in the concentration 10^{-8}.

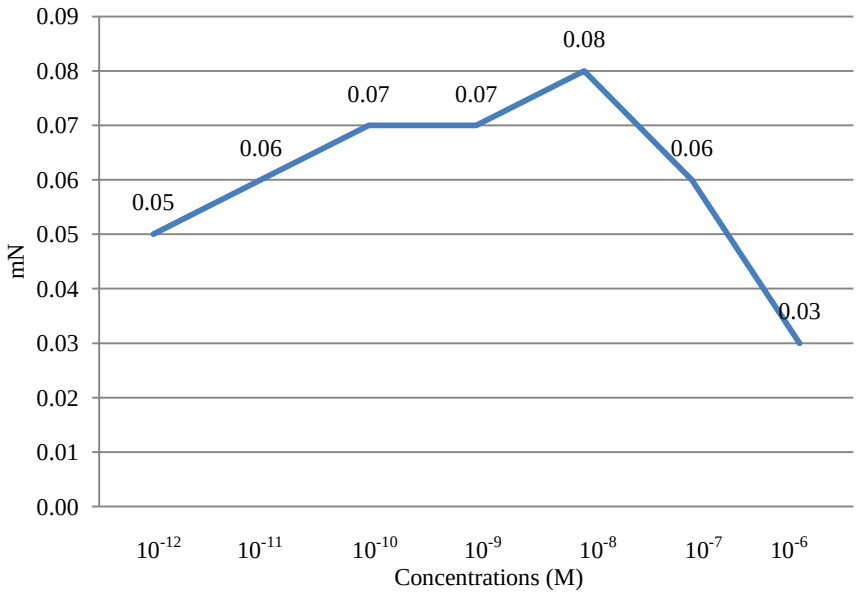

Figure 15 Ang II cumulative response curve.

3.3 Angiotensin I

The contraction of bovine retinal arterioles produced with Ang I in the strength 10^{-4} is shown in figure 16. The average contraction caused by Ang I in the 11 experiments done was 0.27 mN, but as the standard deviation (0.18 mN) and the figure show, there was a great variability in measurements.

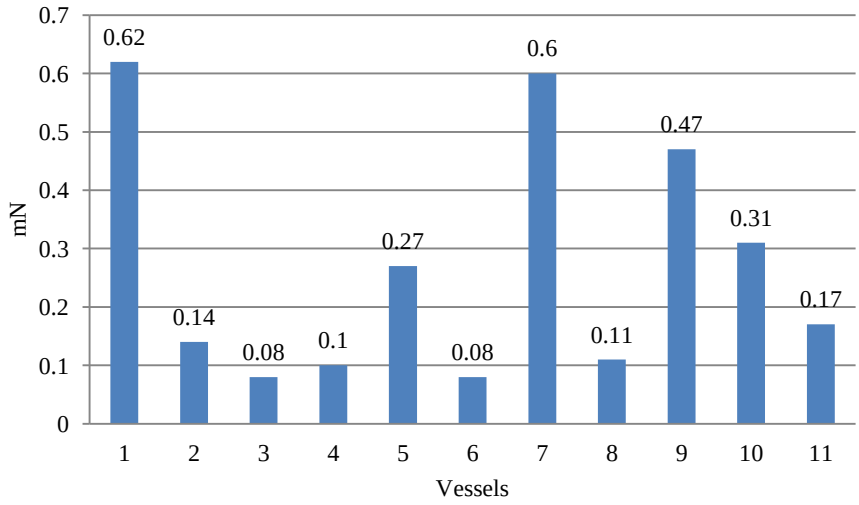

Figure 16 The contraction in mN on wall tension in bovine retinal arterioles, elicited by Ang I in the strength 10^{-4}.

The effect of Ang I was tested in a cumulative manner. In the cumulative dose response each dosage is added on top of the previous one, starting with 10^{-10} and ending with 10^{-4} (n=1). The effects are shown in figure 17. The results show that the concentration of Ang I 10^{-5} caused the strongest contraction. The weakest concentration caused the weakest response but the results are not in a cumuluative matter.

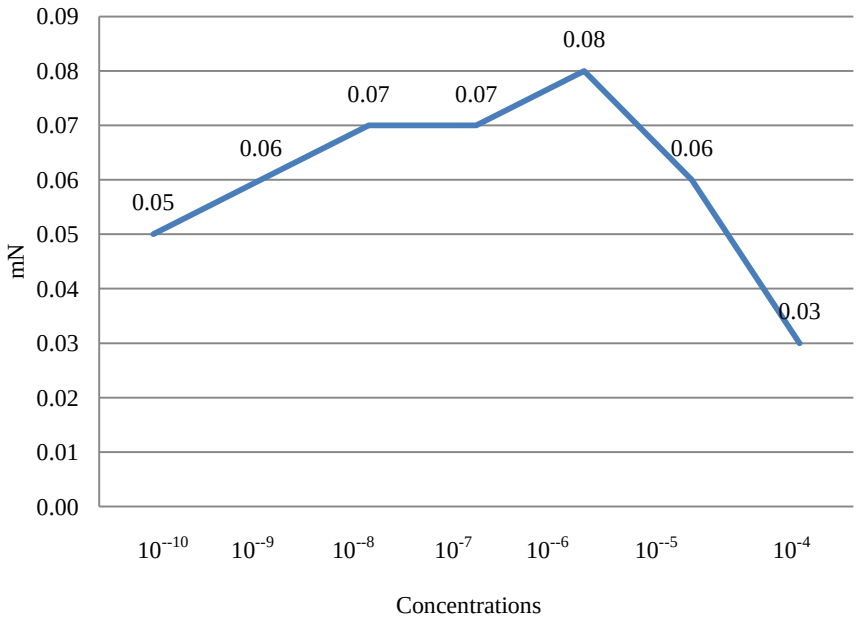

Figure 17 Ang I cumulative response curve. Each dose was added on the top of the other.

3.4 Diminazeneaceturate

Ang II and Ang I was tested with the ACE2 activator DIZE. The purpose was to examine if DIZE would inhibit the contraction induced by Ang II and Ang I. During the testing Ang II or Ang I were added to the Myograph chamber and the effect recorded to see if the vessel segment was responding to those chemicals that do induce contraction. Only if the Ang II or Ang I caused contraction, DIZE was then added to the chamber, to the same arteriole. After that Ang II or Ang I was added for the second time to the arteriole.

Figure 18 shows the effect of Ang II in the strengths 10^{-5}(n=1), 10^{-6}(n=1), 10^{-7} (n=1), 10^{-8} (n=1), 10^{-9} (n=1), 10^{-10} (n=1), 10^{-11} (n=1) and the contraction of Ang II after DIZE in the same concentrations, had been added to the Myograph chamber. One retinal arteriole was tested for each concentration and the same arteriole was measured with Ang II and with Ang II after DIZE.When each concentration had been tested the Myograph bath was emptied.

A paired t-test ($t(6)=5.091$, $p= 0.002$) was conducted to compare the contraction of Ang II and the contraction of Ang II after DIZE had been added to the chamber. There was a significant difference between the contraction for Ang II (0.44±0.17 mN) and Ang II after DIZE had been added (0.06±0.06 mN).

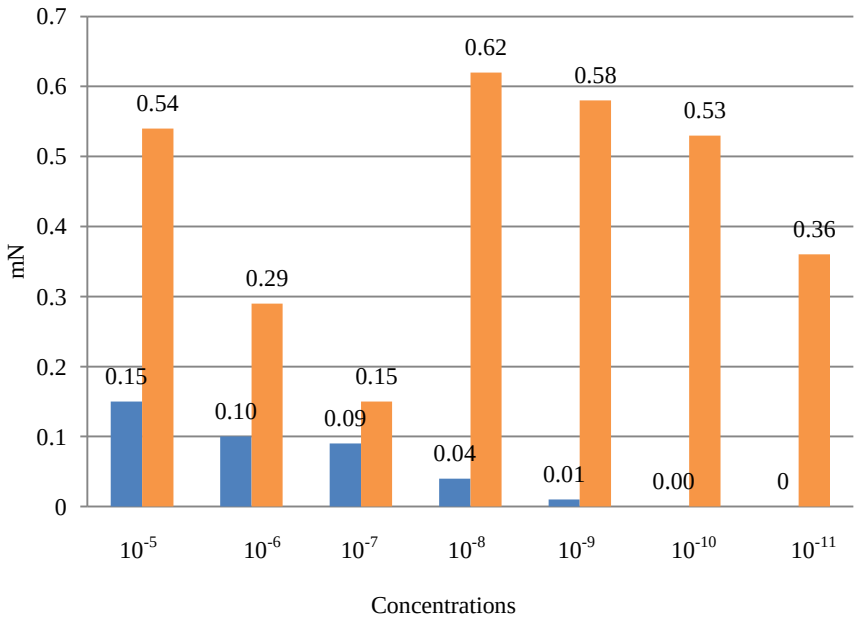

Figure 18 The contraction of Ang II in different strengths (orange) and the contraction induced by Ang II after DIZE had been added (blue).

In figure 19 the effect of Ang I is compared to the contraction of Ang I after DIZE had been added, in the strengths 10^{-4} (n=1), 10^{-6} (n=1), 10^{-7} (n=1), 10^{-8} (n=1), 10^{-9} (n=1) and 10^{-10} (n=1). Each arteriole was tested with Ang I then DIZE, in the same concentrations, was added to the bath and at last Ang I was added to the bath for the second time. When each concentration had been tested the Myograph bath was emptied.

Paired t-test was conducted to compare the contraction of only Ang I and the contraction of Ang I after DIZE had been added to the chamber. There was not a significant difference ($t(6)=1.214$, $p= 0.270$) between the contraction for Ang I (0.07±0.04 mN) and Ang I after DIZE (0.04±0.02 mN). One measurement of Ang I in the strength 10-^{5} (1.2 mN) was excluded as outlier.

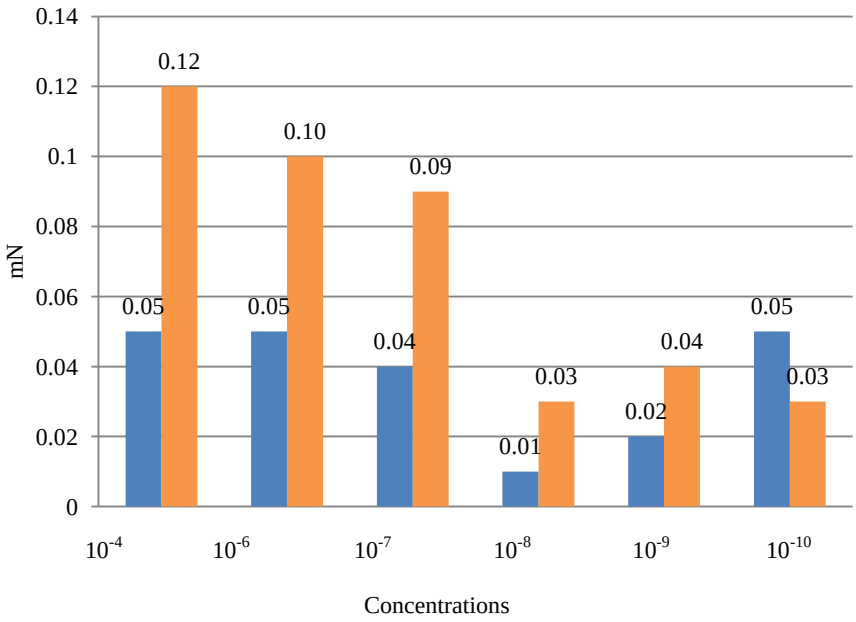

Figure 19 The contraction of Ang I in different strengths (orange) and the contraction of Ang II after DIZE had been admitted (blue).

3.5 Captopril

Captopril was used with Ang I to test if it could reduce the contraction of the Ang I. The effects- of Captopril were tested in the same manner as DIZE. Ang I was added and if it caused vasoconstriction Captopril was added also in the concentration 10^{-4}. Then Ang I was added again. The difference in the response of Ang I in the first and second time represent the effects of Captopril, that is, how strongly it inhibits the contraction of Ang I.

Figure 20 shows the effect of Ang I in the strength 10^{-4} and the effects of the same strength of Ang I after Captopril had been added to the Myograph chamber (n=6). Each arteriole was tested with Ang I, than Captopril was added to the chamber and at last Ang I was added for the second time. A paired t-test was conducted to compare the contraction of only Ang I and the contraction of Ang I after Captopril and it revealed that the difference was significant ($t(5)=7.258$, $p= 0.001$). There was a significant difference between the contraction for Ang I (0.12±0.04 mN) and Ang I after Captopril (0.01±0.01 mN).

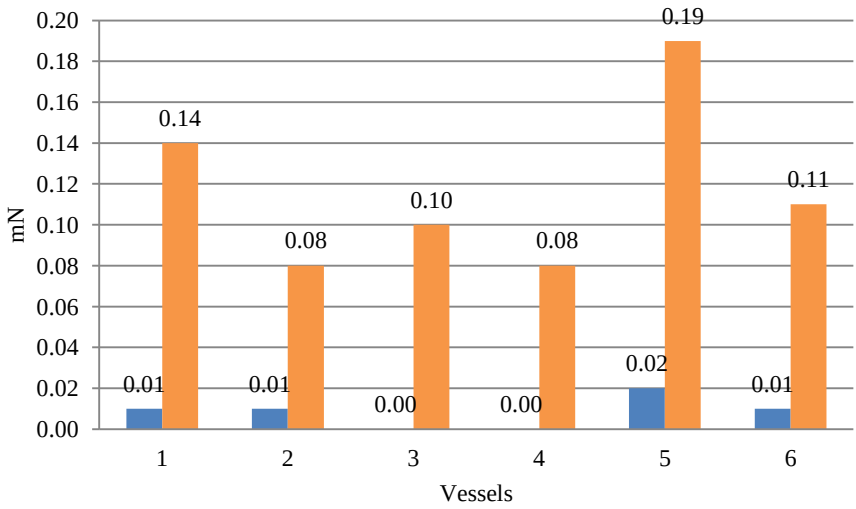

Figure 20 Ang I in the strength 10^{-4} (orange) and Ang I after Captopril (blue).

4 Discussions

This study showed that receptors for Ang II are present in bovine retinal arterioles, which indicates that a local ocular RAS system is present. The results are in accordance with the research of Ferrari-Dileo, Davis and Anderson (1987) that reported that binding sites for Ang II were present in bovine and human retinal vascular trees. Vaajanen & Vapaatali (2011) and Fletcher et al (2010) have also reported that a local RAS system is present in the human retina.

Epinephrine was studied because it effects on retinal vasculature are known and useful to test the integrity of our model. The effect of Epinephrine had also been studied in the research of Gísladóttir (2006) who used the same equipment as used in this study, giving valuable comparison. As in the study of Gísladóttir (2006) Epinephrine caused contraction in bovine retinal arterioles, but the results were quite varied.

Ang II is the main effector hormone of the RAS (Jacobi, Osswald, Jurklies & Zrenner, 1994), and in the current study it caused contraction in bovine retinal arterioles in all the strengths tested. Ang II in the strength 10^{-8} caused the strongest contraction but the strength 10^{-9}caused very similar strength of contraction, as shown in figure 14. Ang II was also tested in a cumulative manner were each dosage was added on top of the previous one. In the cumulative dose response curve as shown in figure 15, the strength 10^{-8} caused the maximum contraction. It was expected that the strongest concentration of Ang II would cause the greatest contractility but our results showed that the strongest concentration caused the weakest contraction.

The results in this study are comparable to previous research. Ang II has been shown to cause vasoconstriction in retinal arterioles (Wilkinson-Berka, 2012) as was the case in this study. The Ang II caused almost immediate contraction in bovine retinal arterioles as expected. That is in accordance with previous studies, Ang II does not have to go through any conversion for it to trigger its response. The results of Kulkarni et al. (1999) showed a cumulative dose dependent response in the small retinal arterioles.

Ang I is a physiologically inactive substance that is rapidly converted to Ang II (Wilkinson-Berka et al. 2012). In previous studies the effects of Ang I on retinal blood flow have not been precisely described. Ang I caused

constriction in bovine retinal arterioles in this research, as shown in figure 16. Ang I was also tested in a cumulative manner and in figure 17 a cumulative dose response curve is shown. Each dosage was added on top of the previous one and the strength 10^{-5} caused the maximal constriction in bovine retinal arterioles. The strength of the contraction of the strengths 10^{-4} and 10^{-5} were very similar but the strengths 10^{-8}, 10^{-9} and 10^{-10} caused less contraction.

Ang I is believed be converted to Ang II and therefore it causes vaso-constriction. In this study the contraction elicited by Ang I did not occur immediately after the chemical was added to the Myograph chamber as it did with Ang II. Therefore Ang I elicited a response after longer time period then Ang II. These results are in accordance with previous studies, it is likely that because Ang I has to be converted to Ang II before it can cause contraction in the retinal arterioles, the process is are more time consuming than with Ang II.

DIZE is an ACE2 activator that is believed to play a protective role. ACE2 degrades Ang II to generate Ang(1-7) and it has been reported in the research of Fouraux et al. (2013) that DIZE did not affect blood pressure in rats. The positive effect of DIZE had been reported in the research of Rigatto et al. (2013) were DIZE increased the vaso-protective axis of the lungs RAS, improved pulmonary vaso-reactivity and enhanced cardiac function in experimental studies.

This study showed that DIZE inhibited the contraction of Ang II as shown in figure 18. The strength of the concentration of only Ang II compared to Ang II after DIZE had been added to the Myograph chamber was statistically significant. DIZE completely inhibited the contraction of Ang II in the concentrations 10^{-10} and 10^{-11} but in greater concentration of Ang II were used, DIZE could not totally inhibit the contraction but it reduced them.

DIZE was also tested with Ang I. The results are shown in figure 19 and shows that DIZE did not inhibit the contraction of Ang I after DIZE had been admitted to the Myograph chamber to the same extend as it did with Ang II. The difference was not statistically significant. It is possible that the inhibition of DIZE had diminished when the Ang I had been converted to Ang II and therefore the contraction is almost the same with or without DIZE.

ACE inhibitors block the conversion of Ang I to Ang II, leading to decreased activation of both AT_1R and AT_2R. The effects of decreasing the activation of Ang II type 1 receptors inhibits vasoconstriction and other mechanisms that raise blood pressure as well as vascular hypertrophy, endothelial

dysfunction, atherosclerosis, inflammation and apoptosis is reduced (Wang et al. 2012). The ACE inhibitor Captopril blocks the Ang II type 1 receptor and treatment with Captopril has been shown to be beneficial in treating diabetic retinopathy (Suzuma, Hata, Clermont, Pokras, Rook, Suzuma, 2001).

The effect of Captopril was studied with Ang I. Figure 20 shows the difference in contraction when only Ang I was used compared to when Ang I was used when Captorpil had been added before. The results are significantly different and therefore the study showed that Captopril effectively inhibited the contraction caused by Ang I.

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