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Search for characteristics of the enigmatic retinal relaxing factor

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List of abbreviations

20-HETE	20-hydroxyeicosatetraenoic acid
2-APB	2-aminoethyl diphenylborinate
4-AP	4-aminopyridine
A	adenosine receptor
AMPK	adenosine monophosphate-activated protein kinase
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BNP	brain natriuretic peptide
BP	blood pressure
CaCC	calcium-activated chloride channel
cAMP	cyclic adenosine monophosphate
CBS	cystathionine β-synthase
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CNP	C-type natriuretic peptide
CO	carbon monoxide
CPA	cyclopiazonic acid
CSE	cystathionine γ-lyase
Cx	connexin
DETCA	diethyldithiocarbamic acid sodium salt
DG	diacylglycerol
DL-APV	DL-2-amino-5-phosphonovaleric acid
EDTA	ethylenediaminetetraacetic acid
EET	epoxyeicosatrienoic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eNOS	endothelial nitric oxide synthase
EP	prostaglandin E
ET-1	endothelin-1
ET-2	endothelin-2

ET-3	endothelin-3
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
H_2S	hydrogen sulfide
HO-1	heme oxygenase-1
iNOS	inducible nitric oxide synthase
IOP	intraocular pressure
IP_3	inositol trisphosphate
K_{ATP}	adenosine triphosphate-sensitive potassium channel
K_{Ca}	calcium-actived potassium channel
K_{ir}	inward rectifier potassium channel
KRB	Krebs Ringer bicarbonate
K_V	voltage-dependent potassium channel
L-NA	Nω-nitro-L-arginine
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
MAPK/ERK	mitogen-activated protein kinases/extracellular signal-regulated kinases
MIO-M1	Moorfield/Institute of Ophthalmology-Müller 1 cells
MLC	myosin light chain
MOAP	mean blood pressure in the ophthalmic artery
MS-PPOH	N-(Methylsulfonyl)-6-(2-propoxyphenyl)hexanamide
mwco	molecular weight cut-off
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
ODQ	1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one
PDBu	phorbol 12,13-dibyturate
pGC	particulate guanylyl cyclase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP_2	phosphatidylinositol 4,5-bisphosphate

PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane Ca^{2+}-ATPase
RP-HPLC	reversed phase high pressure liquid chromatography
RRF	retinal relaxing factor
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
TEA	tetraethylammonium
TMEM16a	transmembrane protein 16A
TRP	transient receptor potential
TTX-R	tetrodotoxin-resistant
TTX-S	tetrodotoxin-sensitive
VEGF	vascular endothelial growth factor
ZnPP	zinc protoporphyrin IX

Chapter I

Introduction

Chapter I: introduction

I.1 Anatomy and physiology of the retina

The eye consists of three ocular layers: an outer fibrous layer, a middle vascular layer and an inner neural layer, which surround three transparent eye structures: the aqueous, the lens and the vitreous body (Fig.I.1). The outer fibrous layer is composed of the sclera and cornea. The cornea is located at the anterior side of eye, where it refracts and transmits the incoming light to the lens and subsequently to the retina. Moreover, the cornea protects the eye against infection and structural damage. At the limbus, the cornea is connected to the sclera, which protects also the eye and maintains its shape. The visible part of the sclera is covered by the conjunctiva, a transparent mucous membrane. The middle vascular layer consists of the iris, ciliary body and choroid. The iris regulates the size of the pupil and subsequently the amount of light reaching the retina. The ciliary body controls the power and shape of the lens and the choroid functions as vascular layer which provides oxygen and nutrients to the outer retina. The inner ocular layer is a light-sensitive layer called the retina, pivotal for the function of the eye. It converts namely the incoming light into neural activity, which is crucial to create vision (1, 2).

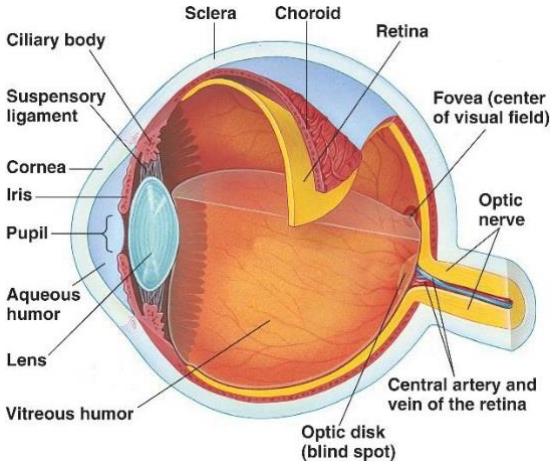

Figure I.1 Schematic overview of the tissues of the eye. Light passes through the transparent cornea and the opening in the center of the iris called the pupil. Then, it passes through the clear lens and the vitreous body, which fills the central cavity of the eye, and is captured by the retinal photoreceptor cells where the light signal is converted into an electrical signal. The electrical signal is processed by other retinal cells and transmitted down the optic nerve to specific brain areas. These areas do the final conversion of the signals into a visual image (3).

The layer of the retina adjacent to the choroid is the retinal pigment epithelium, which contains a single layer of cells. Next to this retinal pigment epithelium lay two types of

photoreceptors: cones and rods. Humans have a cones-to-rods ratio of about 1:20. Cones are only active at high light levels and responsible for color vision, whereas rods are also active at low light levels and not involved in color vision (1). Photoreceptors contain four main parts: outer segments, inner segments, somata and synaptic pedicles. The outer and inner segments lay in the photoreceptor layer, whereas the somata and synaptic pedicles lay in the outer nuclear layer and outer plexiform layer respectively. All these layers form together the outer retina, which thus contains the photoreceptors completely (Fig.I.2) (4).

Incoming light has to travel through the whole retina to reach and activate the outer segment of the photoreceptors, which are located in the photoreceptor layer. Activation of the photoreceptors transmits the information of the light stimuli to the bipolar cells, which synapses lay in the outer plexiform layer. The nuclei of the bipolar cells lay in the inner nuclear layer. They form synapses with ganglion cells in the inner plexiform layer, so the information about the light stimuli is transmitted from the bipolar cells to the ganglion cells. These ganglion cells, mainly located in the ganglion cell layer, will come together in the nerve fiber layer and form the optic nerve after leaving the eye (Fig.I.2) (4).

Horizontal and amacrine cells inhibit this direct pathway from photoreceptors via bipolar cells to ganglion cells laterally. Horizontal cells enhance contrast vision by sending inhibitory feedback to the activated and neighboring photoreceptors. Amacrine cells send inhibitory feedback to bipolar cells to control their output to the ganglion cells (5). The nuclei of the horizontal and amacrine cells are found in the inner nuclear layer. The synapses between photoreceptors and horizontal cells are located in the outer plexiform layer, and the synapses between amacrine cells and ganglion cells in the inner plexiform layer (Fig.I.2) (1).

Beside neuronal cells, the retina contains also glial cells which give structural and metabolic support to the neuronal cells. The most abundant glial cell type in the retina is the Müller cell, but astrocytes, microglial cells and rarely oligodendrocytes are also found in the retina (Fig.I.2) (6).

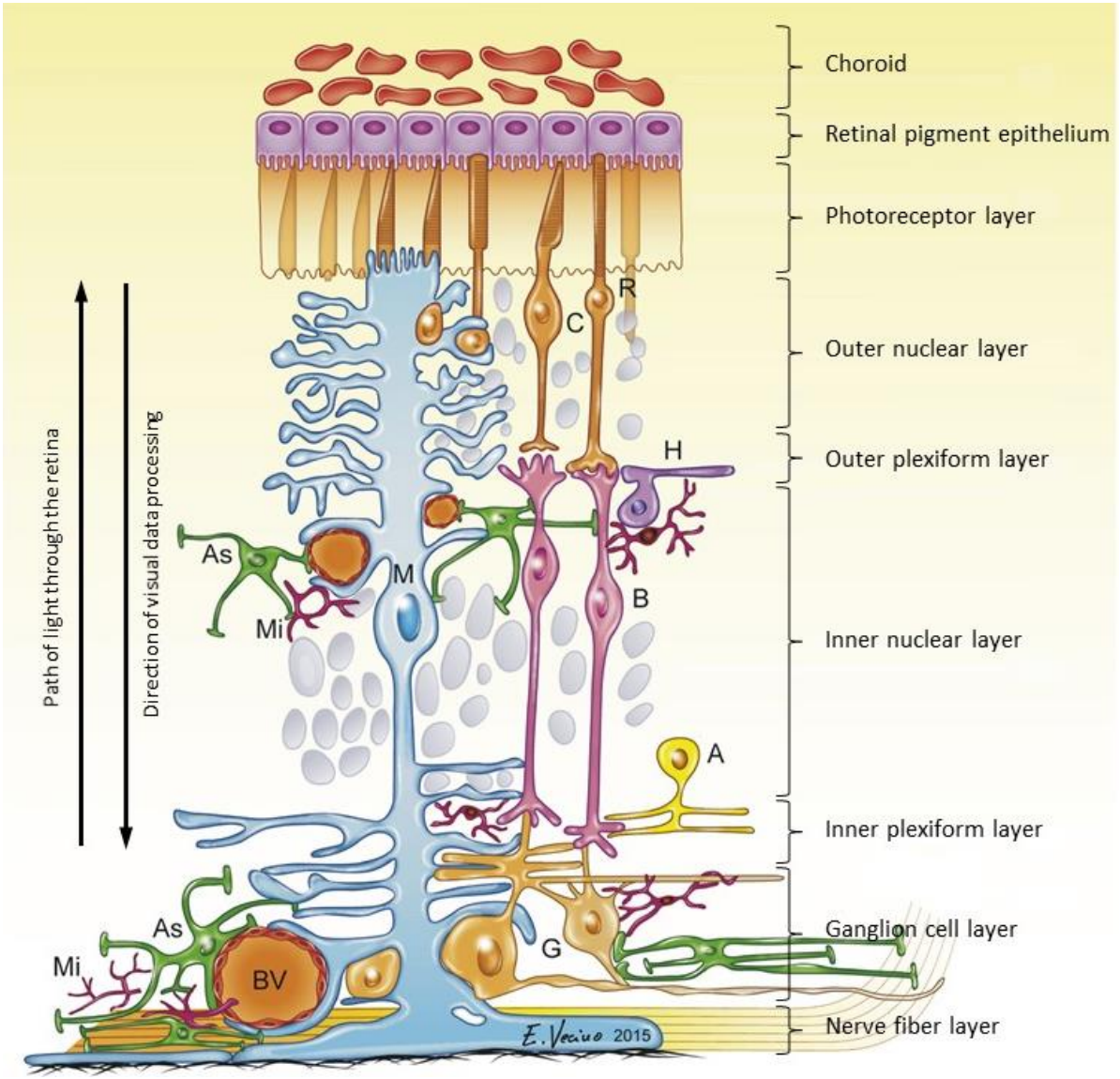

Figure I.2 Schematic overview of the retina: the retinal pigment epithelium and the outer retina (outer plexiform layer, outer nuclear layer and photoreceptor layer) are nourished by passive diffusion from the choroidal blood circulation. The inner retina (nerve fiber layer, ganglion cell layer, inner plexiform layer and inner nuclear layer) is nourished by the retinal blood circulation (BV). Incoming light crosses the retina to activate photoreceptors (cones (C) and rods (R)), which conduct their signal to bipolar cells (B) and subsequently to ganglion cells (G). Amacrine cells (A) and horizontal cells (H) inhibit this pathway laterally. Besides the retina contains following glial cells: Müller cells (M), astrocytes (As) and microglial cells (Mi) (based on (6)).

I.2 Blood supply of the retina

As in all other tissues, in the retina adequate blood supply is necessary to deliver nutrients and oxygen, and to remove waste products. The retina would even be one of the highest oxygen consuming tissues in the body (7). However, there is one additional difficulty in the retina, namely providing a sufficient blood supply without interfering too much with the visual function.

The blood circulation in the retina is arranged by two different circulations, the retinal and the choroidal circulation. Nevertheless both circulations are derived from the ophthalmic artery, a branch of the internal carotid artery (8), they differ substantially from each other, anatomically as well as functionally. Whereas the choroidal circulation has a high blood flow (9) and a low oxygen extraction (10-13), the retinal circulation has a low blood flow (9) and a high oxygen extraction (10, 14).

The choroidal blood circulation, which lays in the choroid next to the retinal pigment epithelium, provides nutrients to the outer layers of the retina (photoreceptor layer, outer nuclear layer and outer plexiform layer), the retinal pigment epithelium and the fovea, the part of the retina with a high density of cones to provide high acuity vision, by passive diffusion. In contrast, the other layers of the retina, the inner nuclear layer, inner plexiform layer, ganglion cell layer and nerve fiber layer, which all form the inner retina, are nourished by the retinal circulation (Fig.I.2) (15, 16). The retinal circulation is derived from the central retinal artery, which emerges and separates in two branches at the optic disc, the entry point of the optic nerve into the eye. These two branches divide then each in two arterioles, so each quadrant of the retina is supplied by one retinal arteriole. Retinal arteries and arterioles lay in the inner side of the retina, close to the inner limiting membrane, the boundary between the retina and vitreous body. They divide further into smaller arterioles and finally into terminal arterioles, which are connected to retinal capillary beds (8, 17). The retinal capillaries are organized in four retinal capillary plexuses. The first one, the superficial capillary plexus, is located in the nerve fiber layer and ganglion cell layer. The intermediate and deep capillary plexuses are located above and below the inner nuclear layer. The last plexus, called the radial peripapillary capillary plexus, is located in the nerve fiber layer, where it surrounds the optic disc (8, 18, 19). The capillary beds are connected to retinal venules, which leave the retina through the central retinal vein at the optic disc (8, 17).

Besides, the retina contains two blood-retinal barriers, an inner blood-retinal barrier and an outer blood-retinal barrier. These barriers are important to maintain the structural and functional integrity of the retina and to control the movement of fluid and molecules between blood and retina. The inner blood-retinal barrier exists of the endothelial cells of the retinal blood vessels connected to each other by tight junctions. Astrocytes, Müller cells and pericytes are thought to contribute to the efficiency of the inner blood-retinal barrier. The outer blood-

retinal barrier exists of the retinal pigment epithelium, also connected by tight junctions. The underlying Bruch's membrane, the inner layer of the choroid, is also part of the outer blood-retinal barrier. The outer blood-retinal barrier separates the neuronal retina from the blood vessels in the choroid, called the choriocapillaris (8, 20).

I.3 Regulation of the retinal blood circulation

The blood flow (F) through a vascular bed depends on the perfusion pressure gradient (ΔP), which is the pressure that drives blood into a vascular system, and the vascular resistance (R), which is generated by the blood vessels and the blood viscosity. The blood flow (F) is correlated to the perfusion pressure gradient (ΔP) and the vascular resistance (R) as follows: $F = \frac{\Delta P}{R}$.

The perfusion pressure gradient is determined as the difference between the arterial and the venous blood pressure. In the retinal blood circulation, this is the difference between the mean blood pressure in the ophthalmic artery (MOAP) and the blood pressure in the central retinal vein. The latter one is just slightly higher than the intraocular pressure (IOP), so that the veins do not tend to collapse. So, the perfusion pressure gradient (ΔP) in the retina can be defined as: $\Delta P = MOAP - IOP$. The MOAP is about 2/3 of the systemic arterial blood pressure which corresponds to $BP_{diastole} + \frac{1}{3}\,(\,BP_{systole} - \,BP_{diastole})$. Consequently, the perfusion pressure gradient (ΔP) in the retina is defined as follows:

$\Delta P = \frac{2}{3}\left[BP_{diastole} + \frac{1}{3}\,(\,BP_{systole} - \,BP_{diastole})\right] - IOP.$

The vascular resistance (R) depends on blood viscosity (η), the length (L) and diameter (2r) of the blood vessels, according to the law of Poisseuille: $R = \frac{8\eta L}{\pi r^4}$ or $F = \frac{\Delta P \pi r^4}{8\eta L}$. The length of the blood vessels does not play an important role in the acute regulation of the retinal blood flow as retinal capillaries are always perfused due to the absence of precapillary sphincters in the retinal arterioles, in contrast to capillaries in the pulmonary circulation. The viscosity of blood (η) is the inherent resistance of blood to flow and represents the thickness and stickiness of the blood (21). It depends, amongst others, on shear rate, the ratio of the blood velocity to the blood vessel diameter. The viscosity will be high at a low shear rate and it will decrease and become almost constant at a high shear rate. Besides, blood viscosity is mainly determined by hematocrit (e.g. anemia), but also by red blood cell deformation (e.g. sickle cell anemia) or red blood cell aggregation ('rouleaux' effect) (22, 23).

Finally, the diameter (2r) or radius (r) of the blood vessel is inversely related to the vascular resistance ($R \sim \frac{1}{r^4}$). A small change in the diameter will have a large effect on the vascular resistance and consequently on the blood flow. The retinal blood flow is thus largely determined by the diameter of the retinal blood vessels. This diameter depends on the tonus of the vascular smooth muscle cells, which can be altered by systemic factors and local factors causing smooth muscle contraction or relaxation (8, 15, 23).

Systemic factors include regulation by innervation and blood gases. However, in contrast to the choroid circulation which is autonomically innervated (15), the retinal circulation lacks any adrenergic, cholinergic or peptidergic innervation (24-28). Blood gases can modulate the retinal blood flow. Inhaling 100% oxygen (hyperoxia) induces vasoconstriction of the retinal blood vessels (29, 30). On the other hand, hypoxia induced vasodilation of the retinal blood vessels, which is significant when the oxygen partial pressure is below 65 mmHg (31). Finally hypercapnia, an increased carbon dioxide partial pressure, induces vasodilation and thus an increased retinal blood flow (8). Besides these systemic determinants, the retinal circulation depends on local vascular control mechanisms, such as autoregulation, metabolic factors and paracrine factors.

I.3.1 Smooth muscle tone regulation

I.3.1.1 Smooth muscle contraction

The vessel tone is determined by contraction or relaxation of its vascular smooth muscle cells. The contraction level is largely determined by the intracellular Ca^{2+} concentration, which is required to initiate contraction.

Intracellular Ca^{2+} can be increased by electromechanic and pharmacomechanic coupling. In the first case, the cell membrane is depolarized by stimuli like cell stretch or high K^+ levels. This depolarization causes subsequently the opening of voltage-dependent Ca^{2+} channels, which allow extracellular Ca^{2+} to flow into the cell. In the case of pharmacomechanic coupling, agonists bind to membrane receptors which are coupled to a heterotrimeric G protein. This G protein stimulates phospholipase C, an enzyme that catalyzes the formation of inositol trisphosphate (IP_3) and diacylglycerol (DG) out of phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 causes Ca^{2+} release from intracellular stores by binding to receptors on the sarcoplasmic

reticulum. DG activates protein kinase C (PKC) which phosphorylates specific target proteins, such as voltage-dependent Ca^{2+} channels or proteins that regulate cross-bridge cycling, resulting in a contraction (Fig.I.3).

Cross-bridge cycling between actin and myosin is necessary to develop force. This interaction between actin and myosin can only take place when the 20 kDa light chain of myosin is phosphorylated. So, the contractile activity in smooth muscle is primarily determined by the phosphorylation state of the light chain of myosin. When the intracellular Ca^{2+} concentration increases, Ca^{2+} interacts with calmodulin. This complex will activate myosin light chain (MLC) kinase, which is responsible for the phosphorylation of the light chain of myosin (Fig.I.3).

Besides this Ca^{2+}-dependent contraction, a Ca^{2+} sensitization mechanism can also determine the contraction. Whereas a rise in intracellular Ca^{2+} will rapidly initiate smooth muscle contraction, the Ca^{2+} sensitization pathway ensures a slow contraction and further force generation by regulating the phosphorylation state of the light chain of myosin independent of elevated intracellular Ca^{2+} levels. The Ca^{2+} sensitization pathway involves the activation of the small G protein RhoA and its downstream target Rho kinase. As a small guanidine triphosphate (GTP)ase, RhoA can switch between an active (GTP-bound) and an inactive (guanidine diphosphate (GDP)-bound) conformation. The active conformation is promoted by the binding of agonists to their G protein-coupled receptors, which will subsequently activate guanine nucleotide exchange factors (RhoGEFs), which will exchange GTP for GDP on RhoA. RhoA-GTP will then bind and activate Rho kinase. Rho kinase induces contraction through phosphorylation and subsequent inhibition of MLC phosphatase. Normally, MLC phosphatase dephosphorylates the light chain of myosin and induces smooth muscle relaxation. Thus, due to the inhibiting action of Rho kinase on MLC phosphatase, the light chain of myosin remains phosphorylated and the interaction between myosin and actin is promoted, hence contraction is maintained. (Fig.I.3) (17, 32-34).

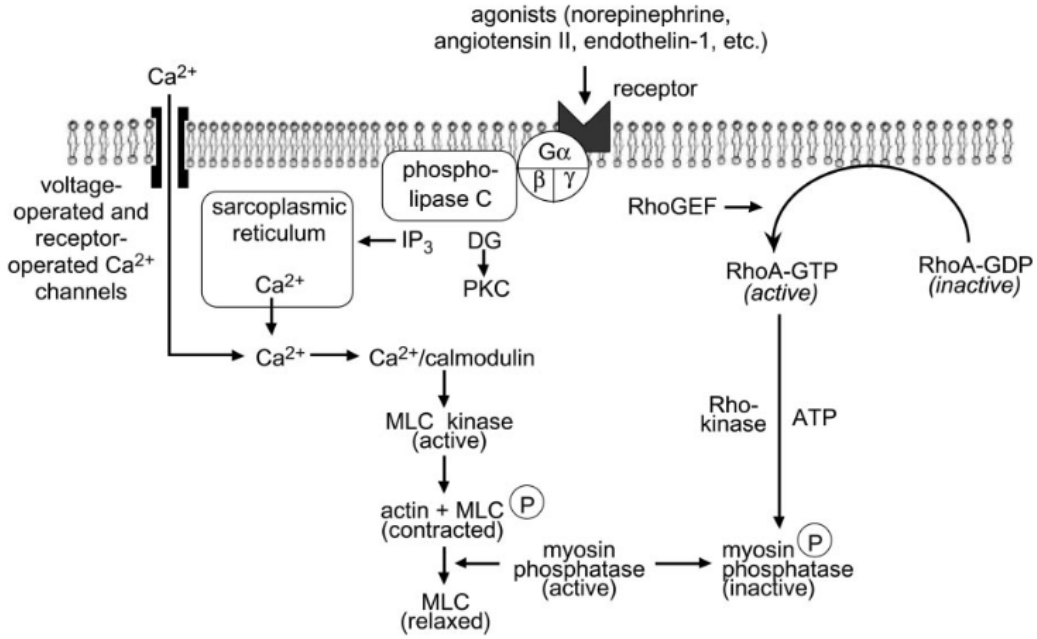

Figure I.3 Regulation of smooth muscle contraction. Various agonists bind to G protein-coupled receptors to activate smooth muscle contraction. The G protein increases the phospholipase C activity, which catalyzes then the formation of IP_3 and DG out of PIP_2. IP_3 binds to specific receptors on the sarcoplasmic reticulum to cause Ca^{2+} release. DG activates protein kinase C (PKC), which phosphorylates specific targets with contraction-promoting effects, such as phosphorylating voltage-dependent Ca^{2+} channels. Ca^{2+} binds to calmodulin, which causes then the activation of MLC kinase. Subsequently, MLC kinase phosphorylates the light chain of myosin, so cross-bridge cycling between actin and myosin can occur with shortening of the smooth muscle cell as result. The contractile response is maintained by the Ca^{2+} sensitization pathway. The G protein activates the GTP-binding protein RhoA via RhoGEFs. RhoA activates Rho kinase, which inhibits myosin phosphatase. As a result myosin phosphatase can no longer dephosphorylate the light chain of myosin (32).

I.3.1.2 Smooth muscle relaxation

Smooth muscle relaxation occurs by removing the contractile stimulus or by a direct action of a substance that stimulates inhibition of the contractile mechanism. Subsequently, this will result in a decrease of the intracellular Ca^{2+} concentration or an increased MLC phosphatase activity. Ca^{2+} can be decreased by uptake into the sarcoplasmic reticulum or transport through the cell membrane to the extracellular space, both mechanisms regulated by Ca^{2+},Mg^{2+}-adenosine triphosphate(ATP)ases. In addition, also Na^{+}-Ca^{2+} exchangers located in the cell membrane can decrease the intracellular Ca^{2+} by transporting one Ca^{2+} out of the cell for two Na^{+} ions into the cell. Furthermore, the inhibition of receptor-dependent or voltage-dependent Ca^{2+} channels will cause relaxation. For example, opening K^{+} channels in the cell membrane will cause hyperpolarization, which will close the voltage-dependent Ca^{2+} channels (Fig.I.4) (17, 32, 34).

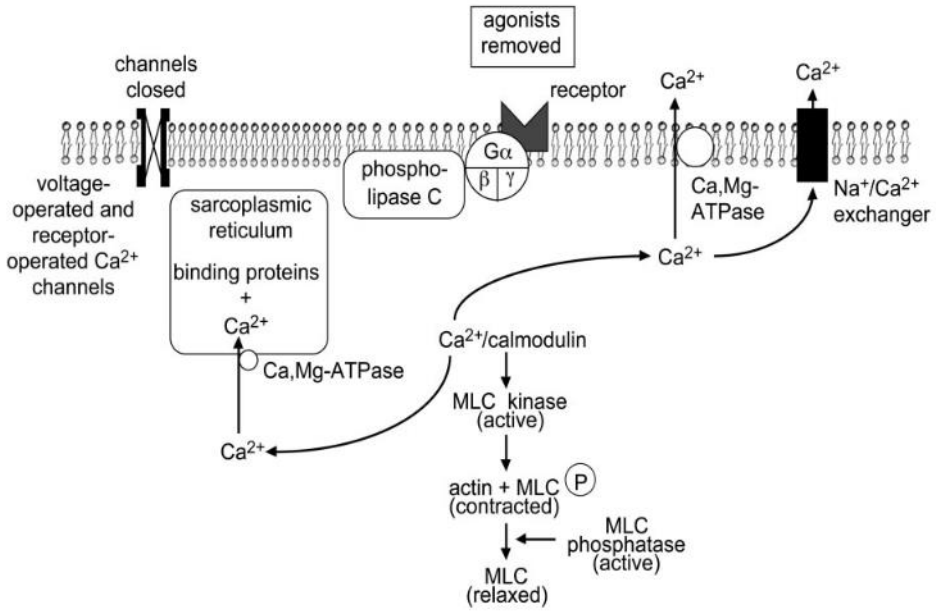

Figure I.4 Relaxation of smooth muscle. Removal of the contractile stimulus or direct action of a substance that stimulates inhibition of the contractile mechanism causes smooth muscle relaxation. A decrease in intracellular Ca^{2+} and an increase of MLC phosphatase activity is necessary to induce relaxation. Ca^{2+} can be removed by Ca^{2+},Mg^{2+}-ATPases on the sarcoplasmic reticulum, and Ca^{2+},Mg^{2+}-ATPases and Na^+-Ca^{2+} exchangers on the cell membrane. The Ca^{2+} entry is also decreased as receptor- and voltage-dependent Ca^{2+} channels in the cell membrane close during relaxation (32).

Another pathway by which smooth muscle relaxation can be induced, occurs via the intracellular increase of cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanidine monophosphate (cGMP). These second messengers are synthesized by adenylyl cyclases and guanylyl cyclases (particulate (pGC) and soluble (sGC)), respectively. Adenylyl cyclases are activated by agonists binding to a G protein-coupled receptor, pGC is activated by natriuretic peptides and sGC by nitric oxide (NO) and carbon monoxide (CO). cAMP and cGMP mainly activate protein kinase A and G, respectively. These protein kinases phosphorylate different target proteins, which will eventually result in vasorelaxation via decrease of intracellular Ca^{2+} levels, decrease of MLC kinase activity or increase of MLC phosphatase activity (33, 34).

I.3.2 Local control mechanisms

As mentioned before, the retinal blood circulation is regulated by local vascular control mechanisms, which are autoregulation, metabolic factors and paracrine factors. Autoregulation is defined in the strict sense as ‘the ability of a vascular bed to keep blood flow constant despite changes in perfusion pressure’ (35) or in the wide sense as ‘the ability of a

tissue to adapt blood flow to metabolic needs' (36) and is regulated by myogenic and metabolic mechanisms. Distension of the blood vessel wall, as result of an increased perfusion pressure, will result in a myogenic contraction of isolated retinal arteries (37). An increased concentration of retinal metabolites in the retinal blood vessels, caused by an diminished perfusion, will result in a vasodilation (15). The retinal circulation also adapts very well to changes in the retinal metabolism. An increased metabolism during adaptation to the dark or during 'flicker' stimulation (38, 39), will result in an increased retinal circulation (40, 41). At last, the retinal circulation is also regulated by factors released from cells surrounding the arteries. These factors are called paracrine factors.

I.3.2.1 Paracrine factors

The term 'paracrine' refers to a kind of hormone function in which the effects of the hormone are restricted to the local environment. Due to the close proximity of vascular smooth muscle cells and vascular endothelial as well as retinal cells, it is easy to understand that these neighboring cells can affect the vascular tone greatly by releasing paracrine factors. Some of the major vasoactive paracrine factors released by endothelial and/or retinal cells which can influence the vascular tone of retinal arteries and thus the retinal circulation are described below. These paracrine factors, accompanied by their cellular source and receptors, can also be found in Table I.1.

Paracrine factor	Source	Receptor
NO	- Endothelium (42) - Retina (amacrine cells, ganglion cells photoreceptors, Müller cells and retinal pigment epithelium) (42)	- /
Cyclooxygenase metabolites	- Endothelium (43) - Retina (amacrine cells, astrocytes, ganglion cells, horizontal cells, photoreceptors and microglial cells) (44)	Different prostaglandin receptors (vascular smooth muscle)
Cytochrome P450 metabolites	- Endothelium (43) - Retina (astrocytes) (45)	- ?
Endothelin-1	- Endothelium (46,47) - Retina (astrocytes, ganglion cells, pericytes, photoreceptors and retinal pigment epithelium) (46,47)	- ET_{B1} receptor (endothelium) (48) - ET_A and ET_{B2} receptor (vascular smooth muscle) (48)
Histamine	- ?	- H_1 and H_2 receptor (endothelium) (49,50) - H_2 receptor (vascular smooth muscle) (50)
Adenosine	- Retina (amacrine cells, ganglion cells and photoreceptors) (51,52)	- A_{2A} receptor (retina) (53) - A_1 and A_{2B} receptor (vascular smooth muscle) (53)
Lactate	- Retina (ganglion cells, Müller cells, photoreceptors, retinal pigment epithelium) (54)	- ?
Dopamine	- Retina (amacrine cells) (55)	- A dopamine receptor (vascular smooth muscle) (56)
Calcitonin gene related peptide	- Retina (amacrine cells) (57)	- A CGRP receptor (vascular smooth muscle) (58)
Neuropeptide Y	- Endothelium (59) - Retina (amacrine cells, maybe bipolar cells, ganglion cells, horizontal cells, microglial cells and Müller cells) (59)	- Y_1 receptor (vascular smooth muscle) (60)
Substance P	- Retina (amacrine cells) (61)	- ?
Adrenomedullin	- Endothelium (62) - Retina (amacrine cells, ganglion cells, Müller cells, photoreceptors and retinal pigment epithelium) (62,63)	$CGRP_1$ receptor and AM receptor (vascular smooth muscle) (64)
VEGF	- Endothelium (65) - Retina (astrocytes, ganglion cells, Müller cells and retinal pigment epithelium) (66,67)	- VEGF receptor 2 (endothelium) (68)
Retinal relaxing factor	- Retina (69)	- ?

Tabel I.1 Overview of the retinal paracrine factors, accompanied by their cellular source and receptors.

I.3.2.1.1 Nitric oxide

Nitric oxide (NO), initially called the endothelium-derived relaxing factor, is formed out of L-arginine by NO synthase (70), of which there exists three isoforms: inducible (iNOS), endothelial (eNOS) and neuronal NO synthase (nNOS). The two latter ones are activated via the Ca^{2+}/calmodulin complex. In contrast, iNOS is activated Ca^{2+} independently and produces large amounts of NO in response to immunological and inflammatory stimuli. NO synthase catalyzes the oxidation of the guanidine group of L-arginine to form NO and L-citrulline. NO synthase has been found in the vascular endothelium of retinal vessels and in different retinal cells, such as Müller cells, photoreceptors, amacrine cells, ganglion cells and the retinal pigment endothelial cells (42). NO is a small molecule, which is soluble in tissues and diffuses across membranes. It has a vasorelaxing effect, but its role in the retinal blood flow regulation is unclear. Inhibiting NO synthase resulted in a reduction of the retinal blood flow or the retinal arterial diameter in pigs, cats and humans (71-75), but other studies in dogs and cats described no significant reduction or no reduction at all in the presence of an NO synthase inhibitor (76, 77). Moreover, contradictory results of the effect of NO donors on retinal arteries have been reported. Limited relaxations on bovine retinal arteries as well as substantial relaxations on porcine and human retinal arteries are described (78-80). On the other hand, relaxations of retinal arteries induced by numerous endogenous substances like acetylcholine, histamine, insulin, bradykinin and lactate are NO mediated (81-85). Also substance P and adrenomedullin might induce NO-mediated relaxations in canine retinal arteries, but these findings are contradicted on bovine retinal arteries (64, 86-88). In addition, NO may also be involved in hypoxia- and hypercapnia-induced relaxations (89-91), although the contrary has also been reported (72, 92).

I.3.2.1.2 Arachidonic acid metabolites

Arachidonic acid is a polyunsaturated fatty acid present in the cell membrane phospholipids. It is set free by phospholipase A_2, activated by physiological and pathophysiological stimuli, such as inflammation (93). Arachidonic acid can be converted by cyclooxygenase and cytochrome P450 enzymes, present in the endothelium and retina (43-45, 94, 95). There exists two types of cyclooxygenase: cyclooxygenase-1 and cyclooxygenase-2, the first one is predominantly expressed and the latter one is upregulated during pathologic conditions (93).

Cyclooxygenase-metabolized products, such as prostaglandins and thromboxans, can have vasoconstricting or vasorelaxing effects and may thus regulate the retinal blood flow (96-99). Prostaglandin E_1 has a vasorelaxing effect on retinal arteries and may also mediate hypercapnia-induced vasorelaxation in pigs (100). However, when administering prostaglandin E_1 intravenously in humans no effect on the retinal circulation is detected (101). Prostaglandin I_2, also called prostacyclin, also relaxes bovine retinal arteries. Prostaglandin E_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 cause vasoconstriction in bovine and porcine retinal arteries (102-105). However, when administered intravenously prostaglandin E_2 and $F_{2\alpha}$ induce retinal vasorelaxation in rabbits (106). Furthermore, prostaglandin E_1, E_2 and I_2 cause an increased retinal blood flow when administered intravenously in rats (107). Inhibiting cyclooxygenase by indomethacin reduces the retinal blood flow in rabbits, pigs and humans. However, it is unsure if this effect is due to a decreased prostaglandin synthesis or another effect of indomethacin (97, 108, 109). In pigs, the cyclooxygenase inhibitor ibuprofen was unable to reduce the retinal blood flow (109). In other words, prostaglandins and thromboxans can alter the retinal vascular tone, but their significance in the retinal blood flow regulation is uncertain. On the other hand, relaxations induced by NO donors have been described to be mediated by prostaglandin I_2 in vitro and in vivo (80, 110). Furthermore, the hypoxia-induced relaxation seems to be partially mediated by cyclooxygenase-derived products (90), but the contrary has also been reported (92, 100).

Cytochrome P450-derived products can be epoxyeicosatrienoic acids (EETs) or 20-hydroxyeicosatetraenoic acid (20-HETE), the first ones vasodilators and the latter one a vasoconstrictor. A role in the retinal blood flow regulation for these metabolites can be expected since they seem to be involved in retinal vasoconstrictions or –dilations (111). Furthermore, 20-HETE was shown to mediate the reduced retinal blood flow caused by hyperoxia and diabetic retinopathy, in pigs and mice respectively (112, 113).

I.3.2.1.3 Endothelin-1

Endothelins are vasoconstricting peptides containing 21 amino acids. There exists three isoforms of endothelin: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) (114). The retina contains predominantly ET-1, but also ET-3. ET-1, synthesized in the endothelium and the retina, may regulate the retinal blood flow (46, 47). ET-1 can bind on two receptor

subtypes ET_A and ET_B. The ET_A receptor is located in the vascular smooth muscle cells and mediates vasoconstriction. The ET_{B1} receptor is located on the endothelium and mediates vasodilation via NO. The ET_{B2} receptor mediates direct vasoconstriction (48). In vitro experiments on bovine retinal arteries proved the vasoconstricting effect of ET-1 (115). Also in vivo ET-1 exerts a vasoconstricting effect on rat, rabbit and human retinal arteries via the ET_A receptor (48, 116-118). Furthermore, retinal synthesized ET-1 mediates the decrease in retinal blood flow during hyperoxia in different animals and in humans (112, 116, 119, 120).

I.3.2.1.4 Histamine

Histamine is a small molecule formed out of L-histidine by histamine decarboxylase. It is mainly known for its important role in the immune system, but also for its neuromodulating role in the central nervous system. Although no histamine synthesizing cells in the retina has been detected yet (121), histamine is present in the retina. Therefore, it was suggested that histamine was involved in the regulation of retinal blood flow (122, 123). Indeed, histamine was able to relax bovine, porcine and canine retinal arteries in a NO-, cyclooxygenase- and cytochrome P450 enzyme-mediated way, which was thought to be an endothelium-dependent effect (49, 82). However, an endothelium-independent histamine-induced relaxation has also been described on canine retinal arteries (50). On the other hand, administering histamine intravenously to humans increased the retinal arterial diameter, but not the retinal blood flow (124, 125). This finding was confirmed in another study on histamine decarboxylase knockout mice, which retinal blood flow was not altered. Therefore, it is thought that histamine only plays in minor role in regulating the retinal blood flow (121).

I.3.2.1.5 Adenosine

Adenosine is a purine formed as a result of ATP hydrolysis and released from metabolic active cells. It is an important neurotransmitter, present in the retina (126). Increasing adenosine by adenosine uptake inhibitors, also increases the human retinal blood flow (127). Adenosine is also able to relax mouse retinal arteries in vitro as well as porcine retinal arteries in vivo (128, 129). However, intravenous administration of adenosine had no effect on the feline retinal blood flow (130). On the other hand, retinal arterial dilation in response to hypoxia, hypotension and hypoglycemia seems to be adenosine-mediated (92, 131, 132). Adenosine

seems to have a dual effect on porcine retinal arterioles in vitro, inducing a vasodilation as well as a vasoconstriction (53).

I.3.2.1.6 Lactate

Lactate is produced in neuronal and non-neuronal retinal cells by mainly the anaerobic metabolism of glucose but also the aerobic one (4, 54, 133). It may also regulate the retinal blood flow. L-lactate relaxes porcine retinal arteries, when administered in the vitreoretinal space but not when administered systemically. Apparently L-lactate has to reach the vascular smooth muscle cells from the extraluminal side (134). However, other studies reported that intravenous administration of lactate in rats and humans or hyperlactatemia in humans could increase the retinal blood flow (135-137). Also in pigs, in vitro vasodilations caused by L-lactate were demonstrated, which may be NO mediated (85). Furthermore, lactate may mediate the hypoxia-induced vasorelaxation, also in pigs (134).

I.3.2.1.7 Dopamine

Dopamine is a neurotransmitter present in the retina, which may also regulate the retinal blood flow. In vivo experiments on humans demonstrated that the intravenous administration of dopamine increased the retinal arterial diameter dose-dependently (138). The increasing retinal blood flow by dopamine was confirmed by another study on humans, which showed an increased retinal blood cell velocity after administering dopamine intravenously (56).

I.3.2.1.8 Neuropeptides

It was hypothesized that neuropeptides, such as calcitonin gene related peptide (CGRP), neuropeptide Y and substance P, all released from retinal neurons, might regulate the retinal blood flow (28). Indeed, CGRP is able to relax porcine, rabbit and bovine retinal arteries in vitro as in vivo (58, 87, 139). Neuropeptide Y contracts bovine retinal arteries in vitro (60). However, neuropeptide Y did not change the retinal blood flow when administered intravenously to rabbits (140). Substance P relaxes retinal arteries when administered intravenously to dogs, but no relaxation is seen on bovine retinal arteries in vitro (86, 87). Another neuropeptide produced in different retinal cell types, among which the retinal pigment epithelial cells, is adrenomedullin (62, 141). Its vasorelaxing effects are described on

bovine and canine retinal arteries in vitro and therefore a potential role in the retinal blood flow regulation is suggested (64, 88).

I.3.2.1.9 Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF) family consists of 7 proteins, amongst which VEGF-A (142). VEGF is produced and released by the endothelium, neurons and glial cells in the retina (68, 143). Its main known functions are promoting angiogenesis, hyperpermeability and hypotension (68). Indeed, VEGF (isoform not reported) and VEGF-A_{165}, one of the most predominant isoforms of VEGF-A, induce vasorelaxation in porcine retinal arterioles in vitro (68, 144). It occurs by activation of the VEGF receptor 2 in the endothelium of the retinal arterioles and is NO mediated (68). Also intravitreal injection of VEGF increased the retinal blood flow in rats, rabbits and monkeys (145-147). Furthermore, intravitreal injection of anti-VEGF antibodies reduce the retinal arteriolar diameter and retinal blood flow in humans with branch retinal vein occlusion or neovascular age-related macular degeneration (148, 149). In contrast, intravitreal administration of anti-VEGF antibodies could not significant alter the retinal arteriolar diameter in humans with diabetic macular edema (150).

I.3.2.1.10 Retinal relaxing factor

In 1998, Delaey and Van de Voorde (69) observed that isolated bovine retinal arteries could not be contracted when adherent retinal tissue was present. Although this observation was initially thought to be the result of technical problems, persistent research pointed towards the existence of (a) new substance(s) released from the retina which is able to relax retinal arteries. As it was shown that the proximity of a piece of bovine retina relaxed bovine retinal arteries, it was concluded that this retina-derived relaxing substance acts in a paracrine way (69). Moreover, the vasorelaxing effect of this paracrine substance was not restricted to bovine retinas, since similar vasorelaxing effects could be observed when using retinas from other species such as pigs, sheep, dogs, rats and mice (64, 151-155). In addition, even non-retinal arteries like rat renal, mesenteric, carotid arteries and aortas, and mouse aortas; and non-vascular smooth muscle cell preparations like rat main bronchi relaxed when bringing a piece of retina in their proximity (69, 152-154). This retina-derived relaxing factor appeared to be hydrophilic and thermostable but could not be identified as one of the previously mentioned and well-known paracrine factors or other common substances in the retina, like

neurotransmitters (69). Therefore, this unidentified factor was coined the name: retinal relaxing factor (RRF). In addition to discovering the identity of the RRF, several studies tried to unravel the mechanism by which the RRF relaxes the arteries. So far, the RRF-induced relaxation seems endothelium-independent and partially mediated by K^+ channels (69, 154-156). An extensive overview of the actual knowledge concerning the RRF can be found in chapter III. However until today, the identity and the mechanism underlying the vasorelaxing effect of the RRF remain largely elusive and so is its role in the retinal blood flow regulation.

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Chapter II

Aims

Chapter II: aims

The aim of this thesis is primarily to reveal more characteristics of the retinal relaxing factor (RRF), regarding its cellular source, releasing mechanism, vasorelaxing mechanism and identity, since these characteristics remain largely unknown. An overview about the actual knowledge of the RRF so far can be found in **chapter IV**.

The first study, described in **chapter V**, aims to identify the cellular source of the RRF as this is as yet unsettled. A previous study suggested that the RRF is probably not released by neuronal cells (1). Therefore, the potential release of the RRF from Müller cells, the most abundant glial cell type in the retina, will be investigated here. However, besides neuronal and glial cells most retinas contain also vascular cells, except for avascular retinas. In order to investigate whether the RRF is released from retinal vascular cells, the vasorelaxing effect of avascular chicken retinas will be studied. Furthermore, a recent study showed an enhanced vasorelaxing effect of veratridine, a voltage-dependent Na^+ channel inactivation inhibitor, in the presence of porcine retinal tissue (2). This suggests that veratridine causes the release of vasorelaxing substances from the retinal tissue by stimulating the neuronal activation as the result of a persistent voltage-dependent Na^+ channel activation. One of these vasorelaxing substances could be the RRF. Therefore, this hypothesis is tested here on mouse retinas. In addition, it will be evaluated whether the RRF is released in a Ca^{2+}-dependent or –independent manner.

The second study, described in **chapter VI**, contains several experiments investigating the mouse RRF-induced relaxation under different conditions. First, the influence of serotonin and glutamate, two common neurotransmitters in the retina (3, 4), on the RRF-induced relaxation will be investigated. Also the influence of L-cysteine, the precursor of hydrogen sulfide which might be involved in the bovine and porcine RRF-induced relaxation (5, 6), will be studied. Furthermore, the involvement of the cytochrome P450 pathway will be explored, since this pathway forms vasorelaxing epoxyeicosatrienoic acids (7). In addition, as the involvement of the cyclooxygenase pathway in the RRF-induced relaxation is not yet clear, this pathway will be further explored in this study. Inhibiting cyclooxygenase reduced the RRF-induced relaxation in pigs (8), but it did not in cows, rats and mice (1, 9, 10).

Oxidative stress in the retina induces cellular damage, which is related to retinal diseases, such as age-related macular degeneration or diabetic retinopathy (11, 12). This may lead to an

alteration in the effect or release of the RRF. Therefore, this study will also evaluate the effect of oxidative stress on the RRF. Finally, the sensitivity of retinal and non-retinal arteries towards to RRF will be studied.

The third study will attempt to identify the RRF chemically, which is described in **chapter VII**. One of the first steps in this study will be investigating the stability of the RRF and resistance to freezing and freeze drying. Furthermore, there will be investigated whether the RRF is a hydrophilic or hydrophobic molecule, since both have been proposed (1, 13). Furthermore, the molecular size of the RRF will tried to be determined. Taken together, this information may help to identify the RRF by mass spectrometry.

While investigating the effect of oxidative stress on the RRF-induced relaxation, a strong vasorelaxing effect of the antioxidant resveratrol on bovine retinal arteries was noticed. This suggests that resveratrol might be of interest for the treatment of eye diseases with an impaired blood flow, such as diabetic retinopathy or glaucoma (14, 15). Its vasorelaxing properties are already known on porcine retinal arterioles and other non-retinal arteries (16-18). Here, in **chapter VIII**, the vasorelaxing effect of resveratrol on bovine retinal arteries will be studied, including further elucidation of its vasorelaxing mechanism and its influence on the RRF-induced relaxation.

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Chapter III

Materials and methods

Chapter III: materials and methods

Regulation of the retinal blood flow is frequently studied in vivo, as the results of these studies represent the physiological situation the best. On other hand, these results are more difficult to interpret as different competing and compensating regulatory mechanisms take place in these in vivo experiments. In vitro experiments on isolated blood vessel segments are ideal to study the basic mechanisms of the local blood flow regulation without interference of the in vivo active compensating mechanisms. This chapter describes the technique of in vitro tension measurements of isolated blood vessel segments since this technique was the most used.

III.1. Experimental setup

Changes in vascular tone, which are related to changes in blood flow, were studied by in vitro isometric tension measurements. These measurements were performed on isolated arterial ring segments mounted in wire myograph baths.

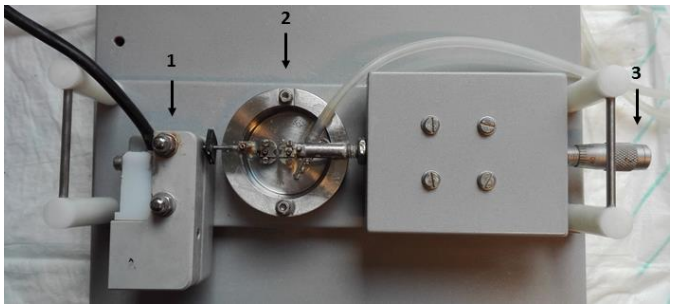

Figure III.1 Myograph bath: force transducer (1), organ bath (2) and micrometer (3)

Wire myograph baths (constructed by the technical department of our research unit) are 10 ml organ baths which contain two holders (Fig.III.1). An arterial segment can be mounted on these two holders by two wires put through the lumen of the artery. One holder is connected to a micrometer which adjusts the distance between the two holders and consequently the diameter of the arterial segment so a passive force on the arterial segment can be applied. The other holder is connected to a force transducer which measures the changes in isometric tension of the arterial segment. Subsequently, the force of this isometric tension change is translated into an electrical signal that is first amplified and then registered by a reading system (PowerLab and LabChart from ADInstruments, Dunedin, New Zealand). The myograph baths are placed on heating plates in order to obtain a constant temperature of 37°C in the

myograph baths, which are also continuously bubbled with carbogen (95% O_2-5% CO_2) gas (Fig.III.2).

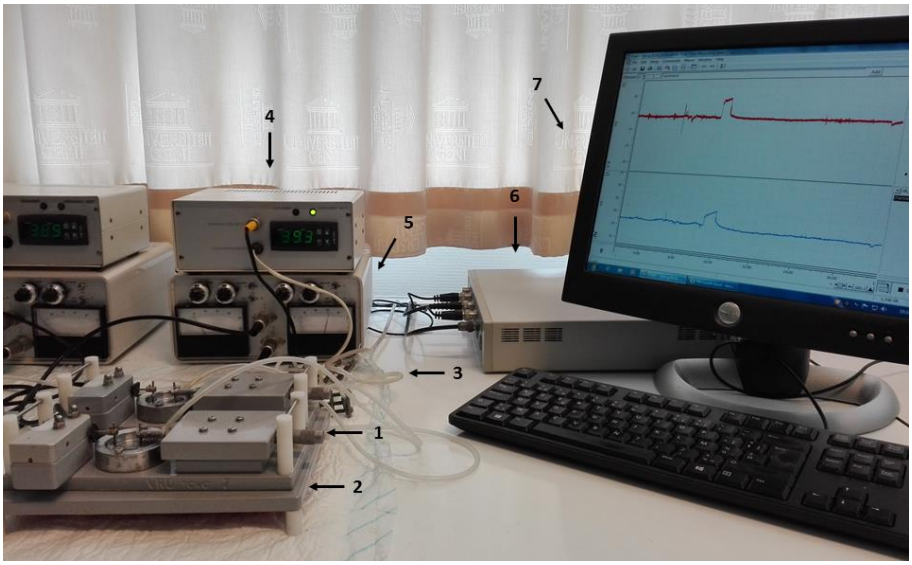

Figure III.2 Experimental setup: myograph bath (1), heating plate (2), carbogen gas supply (3), thermoregulator (4), amplifier (5), reading system (6), output displayed on a computer (7)

III.2 Tissue preparation

III.2.1 Mouse femoral arteries

Male Swiss mice (8-12 weeks, obtained from Janvier (France)) were sacrificed by cervical dislocation. Subsequently, the femoral artery was dissected by cutting it loose from the femoral vein and nerve and was transferred to cold Krebs Ringer bicarbonate (KRB) solution. The femoral artery was cut into segments and surrounding tissue was removed as much as possible without damaging the blood vessel.

III.2.2 Bovine retinal arteries

Fresh bovine eyes were obtained from the slaughterhouse and transported to the laboratory in cold KRB solution. Connective tissues and extraocular muscles at the outside of the bovine eye were removed, so that the eye could be cut open at the equator. Subsequently, the anterior segment of the eye and the vitreous were removed, in order to provide direct access to the retina. The part of retinal artery from the optic nerve to the first branch of the retinal artery was dissected and placed in cold KRB solution. After cutting into ring segments, the retinal vein and retinal tissue were removed, so the latter one could no longer exert a vasorelaxing influence on the artery.

III.2.3 Retinas

Mouse eyes were dissected from the previous mentioned Swiss mice and incised at the equator. The complete mouse retina was pushed out of the eye carefully by using forceps. Bovine retinas were used from previous mentioned bovine eyes. A random piece of retinal tissue was cut out, which size was about 230 mm^2 or 25 mm^2, as large as a mouse retina. All retinas or pieces of retinas were kept in warm (37°C) and carbogen gas bubbled KRB solution.

III.3 Tissue mounting

In order to perform in vitro tension measurements arterial segments have to be mounted in the myograph baths. This implies that 2 stainless steel wires of 40 µm diameter have to be put through the lumen of the arterial segment, which are then connected to the holders of the myograph bath. The procedure of mounting is the same for femoral arteries as for retinal arteries.

First, one wire is clamped between the 2 holders of the myograph bath by adjusting the micrometer and is attached to one side of the first holder with a screw (Fig.III.3). 10 ml KRB solution is added to the myograph bath and an arterial segment, about as long as the length of the gap in the middle of the holders, is placed in the myograph bath. This arterial segment is gently pulled over the wire. Then, the holders are set apart again, so the arterial segment can be pushed into the central gap of the holders. Next, the wire is also attached at the other side of the holder with a screw. Subsequently, a second wire is carefully pulled through the lumen of the arterial lumen. The holders are pushed together again and this second wire is connected to the second holder at both sides with screws. Lose ends of the wires were cut off to avoid interference with the experiments. Finally, the arterial segments could equilibrate for 30 minutes in the myograph baths at 37°C by continuously carbogen gas bubbling.

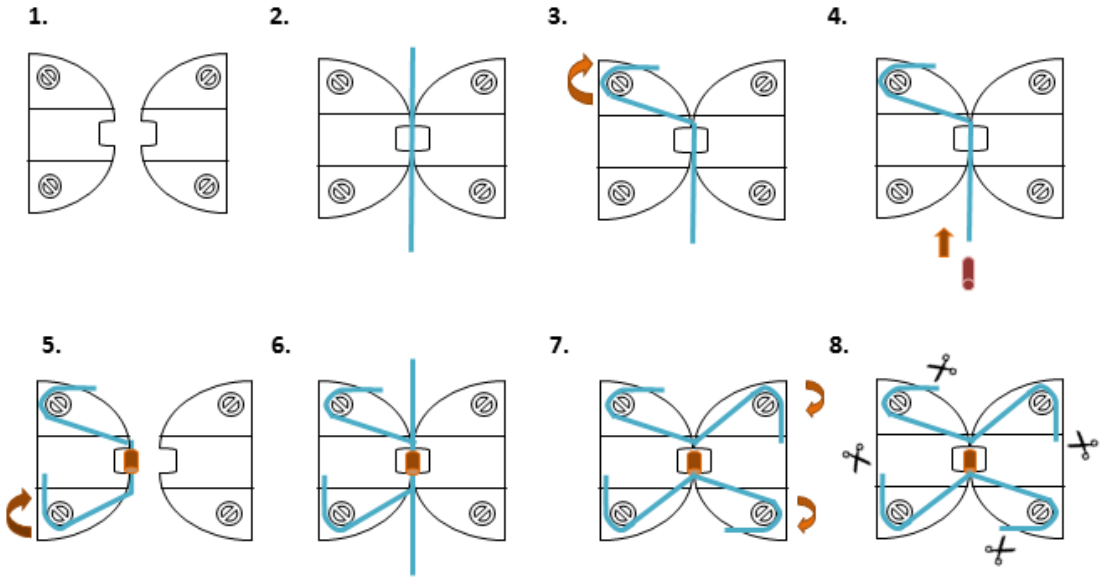

Figure III.3 Schematic overview of the mounting procedure of arterial ring segments

III.4 Normalization protocol

After equilibrating, the arterial segments are being normalized. This process implies that the arterial diameter or the internal circumference of the arterial segment is set at an value which provide optimal active force development in the vascular tissue. It is determined that optimal active force development occurs at an internal circumference which is 90% of the internal circumference at a transmural pressure of 100 mmHg. During the normalization process the transmural pressure of different internal circumferences will be determined in order to calculate the internal circumferences at a transmural pressure of 100 mm Hg. Subsequently, the internal circumference of the arterial segment will be set at 90% of this internal circumference.

At the start of the normalization procedure the arterial segment is brought to a resting position, which corresponds to a force of 0 mN and a micrometer setting x_0. This occurs when the holders of the myograph bath are just not touching each other. Next, the diameter, and consequently also the internal circumference, is enlarged in steps by adjusting the micrometer. One minute after each step, the micrometer setting (x_i) and the force (y_i) are registered. This stepwise enlargement of the diameter continues until the calculated transmural pressure exceeds 100 mmHg. The internal circumference (IC_i) can be calculated out of the distance between the two wires ($x_i - x_0$) and the radius of the wires (20 µm):

$IC_i = 2.\frac{2\pi\,.20\ \mu m}{2} + 4\,.\,20\ \mu m + 2\,.\,(x_i - x_0) = 250.66\ \mu m + 2\,.\,(x_i - x_0)$ (Fig.III.4)

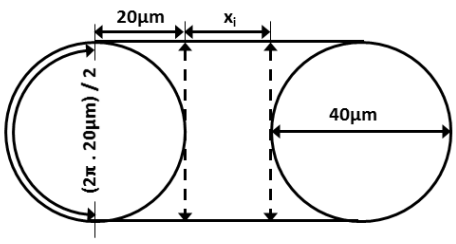

Figure III.4 Schematic representation of the arterial segment after a distention i.

The wall tension (T_i) can be calculated as the force (y_i) divided by the wall length. The wall length is two times the length of the vessel (l), since there is both an upper and lower wall. The length of the vessel was measured by using a calibrated eyepiece mounted on a dissecting microscope.

$$T_i = \frac{y_i}{2\,.\,l}$$

According to the law of Laplace, the wall tension (T_i) in a cylinder is proportional to the transmural pressure (P_i) times the radius of the cylinder, which can be calculated out of the internal circumference of the blood vessel $IC_i = 2\pi r$ or $r = \frac{IC_i}{2\pi}$.

$$T_i = P_i.\frac{IC_i}{2\pi} \text{ or } P_i = \frac{T_i}{(IC_i/2\pi)}$$

An exponential curve is constructed, which represents the relation between the internal circumference and the transmural pressure. In this way, the internal circumference at a transmural pressure of 100 mm Hg can be calculated. Subsequently, the diameter is adjusted by the micrometer in order that the internal circumference will be 0.9 times the internal circumference at a transmural pressure of 100 mm Hg. After the normalization procedure, the arterial segments could equilibrate again for 30 minutes.

III.5 Preliminary protocol

Before the actual experiments, mouse femoral and bovine retinal arteries were several times contracted to obtain maximal and stable contractions and relaxations during the experiment itself. The arterial segments were contracted by filling the myograph bath with 10 ml 120 mM K^+ KRB solution and 30 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Afterwards the arterial segment was

washed out until reaching the basic vascular tone. These contractions were performed 3 times before starting the experimental protocol.

III.6 Experimental protocols

III.6.1 Standard protocol

During most experiments, the standard protocol is used when performing in vitro tension measurements (Fig.III.5A). This means that the 10 ml myograph bath always contains a KRB solution. KRB solutions are changed by emptying the myograph bath with a syringe and pipetting 10 ml new KRB solution into the myograph bath. This new KRB solution is already carbogen gas bubbled and heated at 37°C in a warm water bath. $PGF_{2\alpha}$ and other drugs are administered to the myograph bath by adding a small volume of their stock solution to the myograph bath. An overview of the drugs used to study the RRF-induced relaxation or the resveratrol-induced relaxation can be found in table III.1.

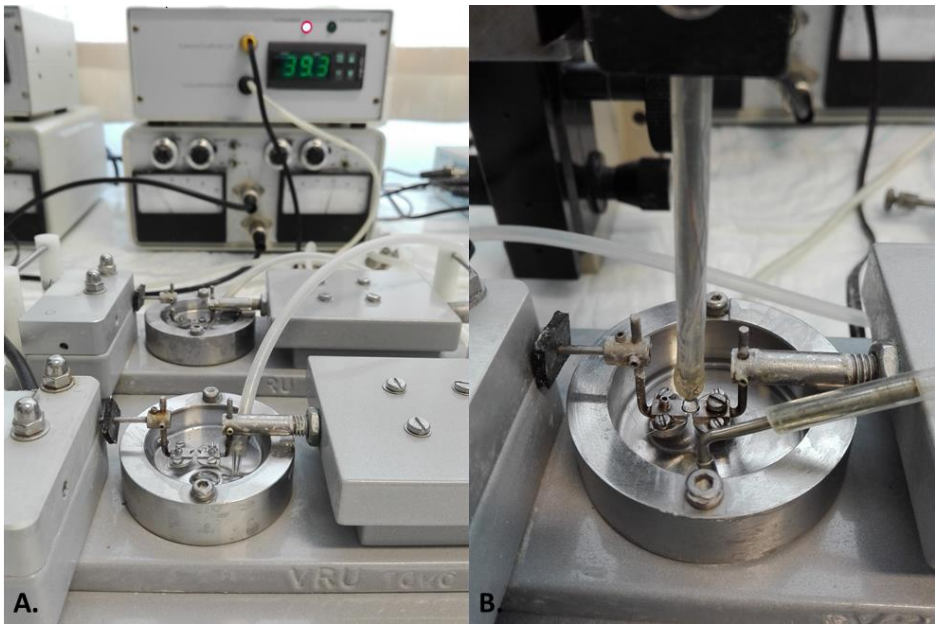

Figure III.5 Myograph baths during a standard protocol (A) or a bioassay protocol (B)

Abbreviation	Name	Effect	Conc.	Ref.
^{10}PanX1	^{10}PanX1	Pannexin 1 channel inhibitor	0.2 mM	(1)
2-APB	2-aminoethyl diphenylborinate	TRP channel and gap junction inhibitor	100 µM	(2)
4-AP	4-aminopyridine	K_V channel inhibitor	2 mM	(3)
A-803467	A-803467	TTX $Na_{V1.8}$ channel inhibitor	10 µM	(4)
Bicuculline	1(S),9(R)-(−)-Bicuculline	$GABA_A$ receptor antagonist	100 µM	(5)
CaCCInh-A01	CaCCInh-A01	CaCC inhibitor	30 µM	(6)
Carbenoxolone	Carbenoxolone disodium salt	Gap junction inhibitor + many other interactions	100 µM	(7)
Compound C	Compound C	AMPK inhibitor	10 µM	(8)
CPA	Cyclopiazonic acid	SERCA inhibitor	10 µM	(3,9)
Diclofenac	Diclofenac sodium salt	Cyclooxygenase inhibitor	10 µM	(10)
DL-APV	DL-2-Amino-5-phosphonovaleric acid	NMDA receptor antagonist	0.1 mM	(5)
DL-α-aminoadipic acid	DL-α-aminoadipic acid	Gliotoxin	4 mM	(11)
Eact	Eact	CaCC activator	10 µM	(12)
EX-527	EX-527	Sirtuin 1 inhibitor	5 µM	*
Flufenamic acid	Flufenamic acid	Cyclooxygenase inhibitor + other interactions	30 µM	**
Fulvestrant	Fulvestrant	Estrogen receptor antagonist	0.1 mM	(13)
Glibenclamide	Glibenclamide	K_{ATP} channel inhibitor	10 µM	(3,14)
Indomethacin	Indomethacin	Cyclooxygenase inhibitor	10 µM	(3)
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride	NO synthase inhibitor	0.1 mM	(15)
Minocycline	Minocyline	Microglia inhibitor	100 µM	(16,17)
MS-PPOH	N-(Methylsulfonyl)-6-(2-propoxyphenyl)hexanamide	Cytochrome P450 inhibitor	10 µM	(18,19)
Niacinamide	Niacinamide	Sirtuin 1 inhibitor	10 mM	(20)
ODQ	1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one	sGC inhibitor	10 µM	(3)
PD98059	PD98059	(MAPK/ERK) inhibitor	10 µM	(13,21)
PDBu	Phorbol 12,13-dibyturate	PKC activator	1 µM	(22)
PMA	Phorbol 12-myristate 13-acetate	PKC activator	10 µM	(22,23)
SQ 22,536	SQ 22,536	Adenylyl cyclase inhibitor	0.1 mM	(13,24)
T16AInh-A01	T16AInh-A01	TMEM16a inhibitor	10 µM	(25)
TEA	Tetraethylammonium	Nonselective K^+ channel inhibitor	10 mM	(3)
ZnPP	Zinc protoporphyrin IX	HO-1 inhibitor	10 µM	(15)

Tabel III.1 Overview of the drugs used to study the RRF-induced relaxation or the resveratrol-induced relaxation. * Concentration suggested by reviewer. ** Highest concentration which was soluble in DMSO.

III.6.2 Bioassay protocol

In the bioassay, the arterial segment is superfused by KRB solutions in an empty myograph bath (Fig.III.5B). KRB solutions, to which $PGF_{2\alpha}$ is added, are bubbled by carbogen gas and transported through a tube just above the arterial segment. This tube passes through a peristaltic pump which regulates the flow of the KRB solution, which drips on the arterial segment at a rate of 1 ml / 10 min. At the end of the tube, just before the solution drips on the arterial segment, a heating element surrounds the tube to heat the solution. The temperature is set in that way that the solution dripping out of the tube has a temperature of 37°C. Once the solution has dripped on the arterial segment, it flows into the empty myograph bath. The solution in the myograph bath is continuously emptied by another tube which passes also through the peristaltic pump to induce drainage (Fig.III.6).

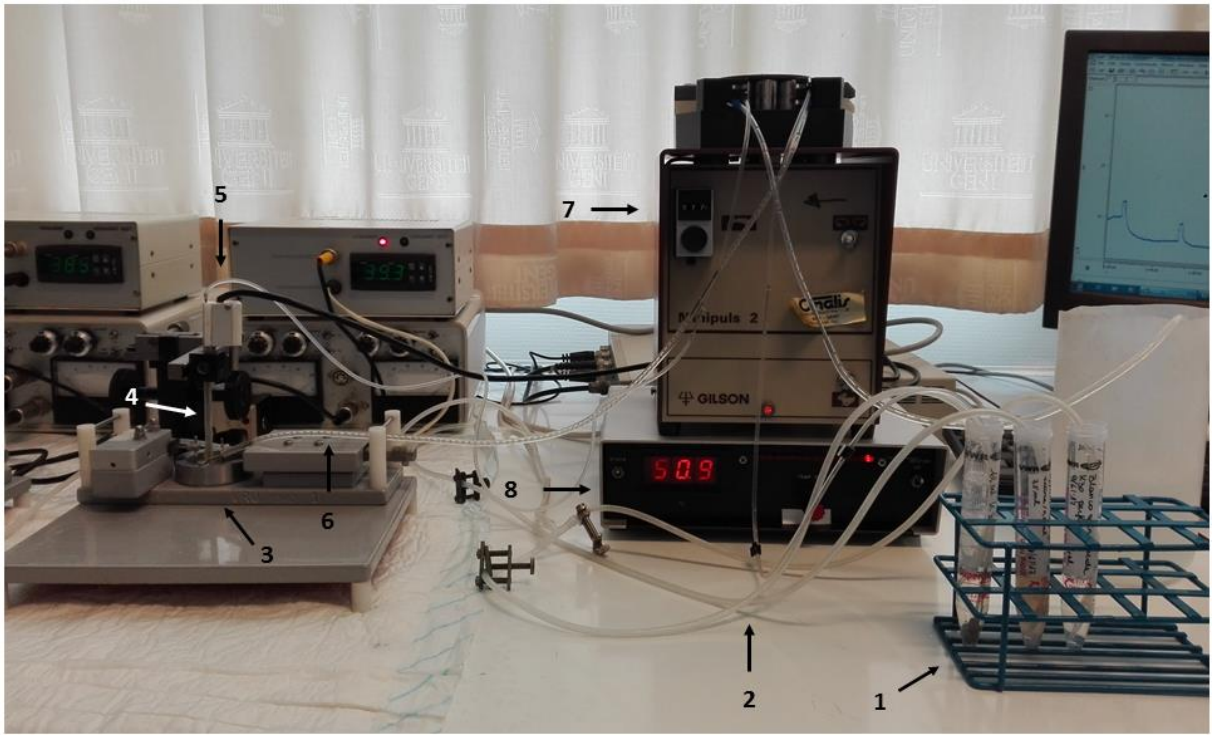

Figure III.6 Bioassay protocol: different KRB solutions (1), carbogen gas supply (2), myograph bath (3), heating element (4), solution supply tube (5), drainage tube (6), peristaltic pump (7) and thermoregulator (8)

III. 7 References

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Chapter IV

The retinal relaxing factor: update on an enigmatic regulator of the retinal circulation

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Chapter IV: the retinal relaxing factor: update on an enigmatic regulator of the retinal circulation

In 1998, a new paracrine factor was discovered during experiments on isolated retinal arteries. A huge variation in contractile response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was observed in a study on isolated bovine retinal arteries. Because damage of smooth muscle cells of the arteries during the isolation was thought to be the reason for this variation, efforts were made to reduce damage by cleaning them not too rigorously from adherent tissue. Surprisingly, the arteries then no longer contracted to $PGF_{2\alpha}$. In further experiments, it was found that the contraction level of bovine retinal arteries with adhering retinal tissue was significantly lower than of those completely cleaned of adhering retinal tissue. Similar results were obtained when contracting the bovine retinal arteries with other contractile substances than $PGF_{2\alpha}$, such as serotonin, endothelin-1 or the thromboxane A_2 mimetic U-46619. One explanation for this observation could be that the retina, like the endothelium releases nitric oxide (NO), releases continuously a relaxing factor. To test this hypothesis, a bioassay was set up in which a bovine retinal artery was mounted for isometric tension measurements and contracted with $PGF_{2\alpha}$. Once the contraction was stabilized, a piece of bovine retina was brought in proximity of the bovine retinal artery. This elicited a rapid, complete and stable relaxation. When the retina was removed, the relaxing influence completely disappeared. The relaxing influence of retinal tissue could be reproduced using retinal tissue of pigs, dogs and sheep (1). A relaxing effect was not seen when bringing bovine choroid tissue in proximity of the bovine retinal artery, illustrating the tissue specificity. On the other hand, retinal tissue also relaxes nonretinal arteries, such as rat renal and mesenteric arteries, and even nonvascular smooth muscle preparations, such as rat main bronchi. This more general relaxing factor was coined the name retinal relaxing factor (RRF) (1).

In the years following these initial observations a lot of efforts were made to reveal the secrets of the RRF. Several studies by different research groups have tried to find out the identity of RRF and the mechanism of the RRF-induced relaxation.

IV.1 Identity of the RRF

One of the first steps in the effort to discover the identity of the RRF was to incubate a bovine retina in a physiological solution and to test then whether this solution could elicit a

vasorelaxation. This solution could indeed induce vasorelaxation (1). Similar results were obtained by Lee et al. using rat retinas (2). These experiments prove that the RRF is a diffusible and stable chemical messenger. Hexane extraction, heating the solution up to 70°C or treatment with trypsin did not change the vasorelaxing effect of the RRF, suggesting that the RRF is hydrophilic, thermostable and not a polypeptide or protein (1).

Further research concentrated on the possibility that the RRF might be identified as NO, cyclooxygenase metabolites or other known vasorelaxants formed in the retina. Based on the characteristics, some molecules could already be excluded from being the RRF. NO can be excluded, because it is not a thermostable molecule. This finding is confirmed by the observation that Nω-nitro-L-arginine (L-NA), a blocker of NO synthase, does not reduce the RRF-induced relaxation (1, 3, 4). The RRF response was also observed in neuronal NO synthase (nNOS) knockout mice (4). Also the observation that NO as such has a much smaller relaxing influence than the RRF excludes NO as being the RRF (5).

Whether the RRF is a cylooxygenase product or not is unclear. In bovine retina indomethacin, a cyclooxygenase inhibitor, did not reduce the RRF effect, proving that cyclooxygenase products are not involved. This was confirmed on bovine, rat and mouse retina by using the cyclooxygenase inhibitors indomethacin and sodium diclofenac (1, 3, 4). However, on porcine tissues, the RRF-induced relaxation was found to be completely blocked by ibuprofen, another cyclooxygenase inhibitor (6). Epoxyeicosatrienoic acids (EETs) could be excluded from being the RRF, since blocking EET synthase with the inhibitors SKF-525A and miconazole had no effect (2).

The vasorelaxing capacity of different vasorelaxants, known to be released in the retina, were tested on bovine retinal arteries to see whether the RRF corresponded with one of them. Glutamate, glycine, γ-aminobutyric acid (GABA), melatonin, and dopamine all failed to relax the retinal arteries (1). Also aspartic acid and taurine failed to relax rat carotid arteries in contrast to the RRF (3). L-Lactate was able to relax retinal arteries, but the concentration needed for this relaxation was much higher than usually present in the retina (7). In addition, the relaxation induced by L-lactate is NO dependent, whereas the RRF-induced relaxation is not (8, 9). Acetylcholine, histamine and other endothelium-dependent vasodilators could also be excluded, because the RRF relaxation is endothelium independent. Adenosine did relax bovine retinal arteries (1, 10), but the adenosine receptor blocker 8-phenyltheophylline could

not reduce the RRF relaxation. In addition, adenosine could not relax rat main bronchi whereas the RRF did (1).

Also, adrenomedullin was excluded. Adrenomedullin relaxes bovine retinal arteries via an endothelium-dependent mechanism and is inhibited by calcitonin gene-related peptide (CGRP) 8-37, an antagonist of the $CGRP_1$ receptor. However, the vasorelaxing mechanism of the RRF is not endothelium dependent and is not inhibited by CGRP 8-37 (11). Also CGRP was studied, because it relaxes bovine retinal arteries. However, its mechanism is endothelial NO dependent, in contrast to the RRF. Natriuretic peptides ANP (atrial), BNP (brain) and CNP (C-type) have also been tested, but they were not able to elicit a substantial relaxation (12). It is also unlikely that proteins, such as vasoactive intestinal peptide, substance P or somatostatin, are the RRF, since the RRF is not destroyed by trypsin (1).

In 2010 Lee et al. reported evidence that the RRF might be identified as methyl palmitate. This was based on analysis by gas chromatography/mass spectrometry of a RRF-containing solution, obtained by incubation of rat retina. Further evidence was derived from the fact that the release of the RRF and methyl palmitate are both Ca^{2+} dependent, that their relaxations rely on activation of voltage-dependent K^+ (K_V) channels, and that their relaxations remain after being heated for 1 hour at 70°C (2). Furthermore, extraction with hexane reduces the relaxing effect of the RRF and methyl palmitate to the same degree. This result is in contrast to the findings of Delaey and Van de Voorde, who found that the RRF relaxation was similar after hexane extraction (1). As yet, the identification of the RRF as methyl palmitate has not been confirmed by other research groups. In contrast, Takir et al. could not detect a relaxing effect of methyl palmitate on bovine retinal arteries (13). Also, we were unable to confirm that methyl palmitate relaxes arteries and can be identified as the RRF (unpublished observation). Thus, the identity of the RRF remains unknown, which probably means that the right suspect has not been investigated yet. However, the possibility that the right one has been excluded wrongly should also be kept in mind. An overview of what is known about the identity of the RRF so far is summarized in table IV.1.

Identity of the RRF	
Excluded molecules	**Charactertistics**
- Nitric oxide (confirmed on bovine, rat and mouse retina) - Cyclooxygenase products (confirmed on bovine, rat and mice retina, but *not* on porcine retina) - Epoxyeicosatrienoic acids (confirmed on rat retina) - Glutamate, glycine, gamma-aminobutryic acid, melatonin, dopamine, L-lactate, adenosine, adrenomedulline, calcitonin gene-related peptide, natriuretic peptides ANP, BNP and CNP (confirmed on bovine retina) - Acetylcholine, histamine or other endothelium-dependent vasodilators (confirmed on bovine retina) - Aspartic acid and taurine (confirmed on rat retina)	- Diffusible, stable chemical messenger (confirmed on bovine and rat retina) - Hydrophilic (confirmed on bovine retina, but *not* on rat retina) - Thermostable (confirmed on bovine and rat retina) - Resistant to trypsin (confirmed on bovine retina)

Table IV.1 Overview of the knowledge about the identity of the RRF.

IV.2 Mechanisms mediating the RRF-induced relaxation

Adhering retinal tissue causes reduced contraction of arteries, and when a piece of retina is placed on top of an artery, a relaxation is seen. The percentage relaxation depends on the species and arteries being used. When a bovine retina is placed on a bovine retinal artery a complete relaxation is caused. Placing a rat retina on a rat carotid artery causes 33.24% relaxation and a mouse retina on a mouse aorta 21.57% relaxation. All relaxations in these studies seem to be monophasic (1, 3, 4). In the study of Takir et al., the relaxation seen by placing a bovine retina on a bovine retinal artery is biphasic, suggesting that there are two mechanisms or factors involved (13).

One of the first mechanisms to be tested was the involvement of the endothelium. Removal of the endothelium did not change the effect of the RRF on bovine retinal arteries (1, 13). This finding was confirmed for the rat RRF (3, 13). There is evidence that NO synthase and cyclooxygenase are not involved in the RRF-induced relaxation. However, Holmgaard et al. suggested the involvement of a cyclooxygenase product, because inhibition of cyclooxygenase

blocked the RRF-induced relaxation completely (6). Cyclic guanosine monophosphate (cGMP) seems not to be involved in the RRF-induced relaxation, because no differences in the RRF-induced relaxation were seen by using methylene blue, a blocker of guanylyl cyclase (1). 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ), a soluble guanidine cyclase (sGC) blocker, also had no effect. This is in accordance with the observation that NO donors, which activate sGC, elicit only a small relaxing effect on bovine retinal arteries (3-5). Furthermore, the RRF response is still present in $sGC_{\alpha 1}$ -knockout mice, while $sGC_{\alpha 1}$ is the most important isoform of sGC present in vascular smooth muscle (10). Also cyclic adenosine monophosphate (cAMP) is probably not involved, because its levels were not elevated in rat arteries that were relaxed by the RRF (10). Holmgaard et al. demonstrated that the RRF-induced relaxation is reduced by DL-2-amino-5-phosphonovaleric acid (DL-APV), a N-methyl-D-aspartate (NMDA) receptor antagonist, suggesting the involvement of NMDA receptors (6).

The Ca^{2+}-lowering mechanisms leading to smooth muscle relaxation were also studied. Blockade of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) with thapsigargin and cyclopiazonic acid did not influence the RRF-induced relaxation (3, 14). Also, blocking the Na^{+} - Ca^{2+} exchanger with dimethyl-2-thiourea (DMTU) or amiloride had no influence (3). L-type Ca^{2+} channels are also not involved, because blocking them with nifedipine did not change the RRF relaxation (14). Surprisingly, blocking the plasma membrane Ca^{2+}-ATPase (PMCA) with vanadate inhibited the RRF-induced relaxation (Fig.IV.1). However, it should be noted that vanadate is not a specific blocker of PMCA, so the conclusion that PMCA is involved in the RRF-induced relaxation requires further investigation (3).

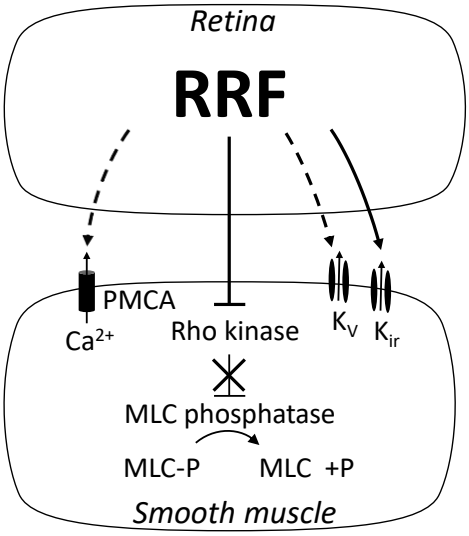

Figure IV.1 Overview of the mechanisms involved in the RRF-induced relaxation, uncertain connections are represented by *dotted lines*, more confirmed connections by *solid lines*. The RRF may induce relaxation via activation of PMCA (plasma membrane Ca^{2+}-ATPase), K_V (voltage-dependent K^+ channels), K_{ir} (inward rectifier K^+ channels) and by inhibition of Rho kinase, which then no longer inhibits MLC (myosin light chain) phosphatase, so MLC-P can be dephosphorylated resulting in relaxation.

Besides Ca^{2+}-lowering mechanisms, a Ca^{2+} sensitization mechanism could be involved in the relaxation caused by the RRF. Inhibition of myosin light chain phosphatase by calyculin A abolished the RRF-induced relaxation completely. There are two important Ca^{2+} sensitization pathways, one via Rho kinase and one via protein kinase C (PKC). Calphostin C, a PKC inhibitor, induced only a weak relaxation in $PGF_{2\alpha}$ or 120 mM K^+ Krebs Ringer bicarbonate (KRB) contracted arteries, indicating that the RRF does not act via PKC. On the other hand, Y-27632, a Rho kinase inhibitor, induced a complete relaxation in $PGF_{2\alpha}$-contracted arteries and a moderate relaxation in 120 mM K^+ KRB contracted arteries. This difference in the vasorelaxing effect of Y-27632 is comparable to the vasorelaxing effect of the RRF, indicating that the RRF might interfere with Rho kinase (Fig.IV.1). Interestingly, the retinal adhering tissue together with Y-27632 can cause an almost complete relaxation in 120 mM K^+ KRB-contracted arteries (14).

K^+ channels could be involved in the RRF-induced relaxation, because bovine retinal arteries relax less when contracted with a 120 mM K^+ KRB solution (1). It even changes the biphasic RRF-induced relaxation into a monophasic one (13). Lee et al. tested different K^+ channels inhibitors and observed that blocking K_V channels with 4-aminopyridine (4-AP) inhibited the RRF-induced relaxation on rat aortas (Fig.IV.1). A more general K^+ channel blocker,

tetraethylammonium (TEA), did the same (2). However, Takir et al. could not confirm these observations for bovine RRF, because 4-AP and TEA were unable to block the RRF-induced relaxation (13). The contradictory results about the involvement of K_V channels could be attributed to the use of different contractile substances (phenylephrine versus $PGF_{2\alpha}$) and different species or arteries (rat aorta versus bovine retinal artery) (13). Both studies also tested adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels with the blocker glibenclamide and concluded that it had no effect. Ca^{2+}-actived K^+ (K_{Ca}) channels were tested by using iberiotoxin on rat retina and charybdotoxin (big and intermediate K_{Ca} blocker) and apamin (small K_{Ca} blocker) on bovine retina. All these blockers did not reduce the RRF-induced relaxation. However, inward rectifier K^+ (K_{ir}) channels seem to be involved (Fig.IV.1). Their inhibition with $BaCl_2$ reduced the RRF-induced relaxation and changed the biphasic relaxation in a monophasic one (14).

IV.3 RRF in the hypoxia-induced relaxation

Development of several eye diseases, such as glaucoma or diabetic retinopathy, is associated with an impaired oxygen supply. Retinal blood vessels contract in response to hyperoxia and dilate in response to hypoxia. This hypoxic vasodilation can be regulated by direct effects from the blood vessel wall or indirect effects from the surrounding tissue (10, 15).

Winther et al. found that the hypoxia-induced vasorelaxation was present in porcine retinal arterioles with and without adhering retinal tissue (16). Delaey et al. provided evidence that the hypoxic vasodilation on bovine retinal arteries is largely regulated by an indirect effect from the retina. The induction of acute hypoxia in bovine retinal arteries without surrounding retinal tissue resulted in only a small relaxation, but when hypoxia was induced in the arteries with surrounding retinal tissue, it resulted in a large relaxation. Reoxygenation reversed this relaxation (7). This finding was confirmed on pigs, mice and rats (3, 4, 16, 17). Furthermore, it was discovered that direct contact between the retina and the blood vessel was not necessary for the hypoxic vasorelaxation, indicating the involvement of a diffusible factor (7).

The potential involvement of different hypoxia-induced vasorelaxants was tested. NO, cyclooxygenase metabolites, pH, K^+, adenosine, excitatory amino acids (GABA, glutamate, aspartic acid, glycine, and taurine) could all be excluded (3, 7). The hypoxic vasorelaxation was reduced when bovine retinal arteries were contracted with 120 mM K^+ KRB solution, which is

in line with the RRF being involved in the hypoxic vasorelaxation. In addition, ATP, or actually the reduction of ATP, could be involved. Blocking the ATP production with iodoacetate or sodium cyanide produced a complete relaxation, which indicated that a reduction of ATP, as occurs during hypoxia, causes vasodilation (7).

Kringelholt et al. reported that the hypoxia-induced relaxation by surrounding retinal tissue was mediated by prostaglandins and NO (Fig.IV.2) (17). Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a NO synthase inhibitor, could reduce the hypoxic relaxation, whereas Delaey et al. observed almost no inhibition by L-NA, also a NO synthase inhibitor (7, 17). Also, other studies confirmed the finding that NO is involved in the hypoxic relaxation of retinal arteries (18, 19). Furthermore, it was reported that this relaxation depends on inducible NO synthase (iNOS) rather than nNOS, because the iNOS inhibitor 1400W could also inhibit the relaxation, in contrast to the nNOS inhibitor 7-nitroindazole. The involvement of endothelial NO synthase (eNOS) could not be excluded (20, 21). It has even been suggested that lactate formed during hypoxia could stimulate NO synthase, and thus regulates the hypoxic relaxation (Fig.IV.2) (8). However, previous studies showed that L-lactate, which is released from retinal glial and neuronal cells during hypoxia, could only induce a relaxation at high concentrations (7, 9, 22). On the other hand, Hein et al. showed that L-lactate was able to produce a substantial relaxation at physiological relevant concentrations (8). Furthermore, this lactate-induced relaxation appeared to act via NO synthase, guanylyl cyclase and finally K_{ATP} channels activation (Fig.IV.2) (8, 9).

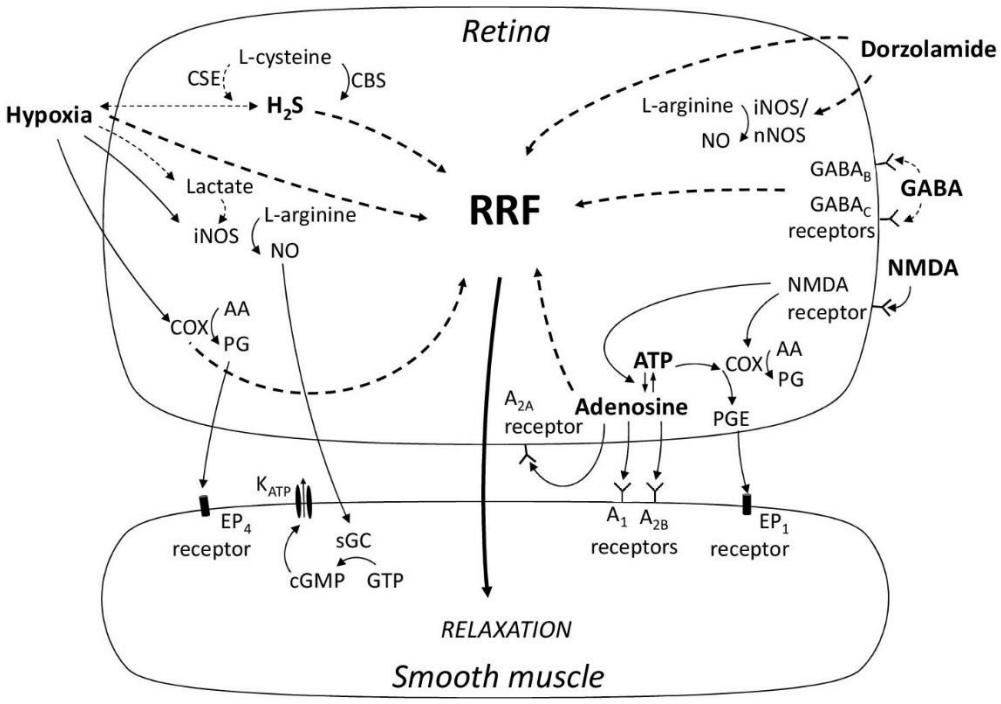

Figure IV.2 Overview of retina-dependent relaxation mechanisms of hypoxia, H_2S (hydrogen sulfide), adenosine, ATP (adenosine triphosphate), NMDA (N-methyl-D-aspartate), GABA (γ-aminobutyric acid) and dorzolamide, of which the relaxation mechanisms may also be related to the RRF. Uncertain connections are represented by *dotted lines*, more confirmed connections by *solid lines*.

Hypoxia induces relaxation by activating COX (cyclooxygenase) and iNOS (inducible NO synthase) (maybe via lactate) in the retina, which leads to the production of PG (prostaglandins) from AA (arachidonic acid) and NO (nitric oxide) from L-arginine. The PGs bind on the EP_4 receptor (prostaglandin E_2 receptor 4) and NO on sGC (soluble guanlylyl cyclase), which converts GTP (guanosine triphosphate) into cGMP (cyclic guanosine monophosphate), which then activates K_{ATP} (ATP-sensitive K^+ channels). Furthermore, hypoxia-induced relaxation mechanisms may include the RRF, like the H_2S-induced relaxation, which may be linked to the hypoxia-induced relaxation. H_2S is formed in the retina by CBS (cystathionin β-synthase) and maybe CSE (cystathionin γ-lyase).

Adenosine can induce relaxation by binding on A_1 and A_{2B} receptors on smooth muscle cells or on the A_{2A} receptor in the retina or by the RRF. Furthermore, adenosine can be transformed into ATP, which stimulates COX to produce PGE (prostaglandin E) that binds on the EP_1 receptor (prostaglandin E_2 receptor 1). NMDA (N-methyl-D-aspartate) also activates COX to produce PG, but not PGE, and it causes hydrolysis of ATP to adenosine.

GABA binds on the $GABA_B$ or $GABA_C$ receptor in the retina to induce relaxation, which may act via the RRF. The dorzolamide (a carbonic anhydrase inhibitor)-induced relaxation might also work via the RRF, but also stimulates iNOS or nNOS (neuronal NO-synthase) in the retina resulting in NO production.

Ibuprofen or diclofenac, cyclooxygenase inhibitors, also reduced the hypoxic relaxation, an observation that does not confirm the findings of Delaey et al. (7, 17, 20). Interestingly, the

lactate-induced relaxation could also be partially blocked by the cyclooxygenase inhibitor indomethacin (8). Further investigation showed that the prostaglandin E_2 receptor 4 (EP_4) was involved and not prostaglandin E_2 receptor 1 (EP_1) or prostaglandin E_2 receptor 2 (EP_2) receptors (Fig.IV.2). Inhibiting iNOS and the EP_4 receptor could not abolish the hypoxia-induced relaxation completely, implying the involvement of other mechanisms in the hypoxia-induced relaxation of retinal arteries (21).

IV.4 RRF and adenosine and ATP

Besides hypoxia, adenosine-induced relaxations are stronger in the presence of perivascular retinal tissue (23, 24). This suggests that adenosine might promote the release of the RRF (24). Adenosine is formed by the hydrolysis of ATP, which also causes a relaxation of retinal arteries that is enhanced by the retinal tissue (23). Maenhaut et al. reported that the enhancement of the adenosine-induced relaxation by retinal tissue was larger than that of the ATP-induced one on bovine retinal arteries (24), whereas Holmgaard et al. saw the opposite on porcine retinal arterioles, explaining this by ATP-induced ATP release in the retina (23). This difference might be species related, because Maenhaut et al. reported that the vasorelaxing effect of adenosine was enlarged by using rat retina instead of bovine retina, but reduced by using porcine retina instead of bovine retina (24). The adenosine-induced relaxation was independent of NO synthase, cyclooxygenase and EET synthesis (23, 24). It was reduced in presence of the 120 mM K^+ KRB solution and by using rat carotid arteries instead of bovine retinal arteries, which is in accordance with the characteristics of the RRF, suggesting an involvement of the RRF in the adenosine-induced relaxation (24). It has been reported that adenosine has both vasocontractile and vasorelaxing effects on porcine retinal arteries with perivascular tissue. It induces vasoconstriction by acting on A_3 receptors on the retinal artery and A_1 receptors on the perivascular tissue, whereas it induces relaxation by acting on the A_1 and A_{2B} receptors on the retinal artery and the A_{2A} receptors on the retinal tissue (Fig.IV.2) (25).

The ATP-induced relaxation in the presence of the perivascular retina is, in contrast to the adenosine-induced one, mediated by cyclooxygenase, which produces prostaglandin E that acts on EP_1 receptors on the retinal artery. Prostaglandin E is produced by ATP but not by adenosine (26).

NMDA can induce the hydrolysis of ATP to adenosine in the retina by binding on its NMDA receptor (27). Besides inducing vasorelaxation via adenosine, NMDA also produces cyclooxygenase products but not prostaglandin E. It is suggested that the RRF is related to these pathways (Fig.IV.2) (26).

IV.5 RRF and GABA

The GABA-induced relaxation depends on the presence of perivascular retinal tissue (6, 28, 29). Hinds et al. proved that GABA was able to relax rat retinal arteries in presence but not in the absence of perivascular retinal tissue (29). Bek et al. showed similar results, but remarkably GABA was only able to induce relaxation of porcine retinal arterioles when the NMDA relaxation pathway was inhibited. Furthermore, the GABA-induced relaxation is mediated via $GABA_C$ receptors in porcine retina (28), whereas the $GABA_B$ receptor is involved in rat retina (Fig.IV.2) (29). This difference may be due to the use of different contractile substances (U46619 and DL-APV versus endothelin-1) or the use of different species (porcine versus rat retinal arterioles). However, the rat retinal arterioles could not contract with U46619 and DL-APV, in contrast to the porcine retinal arterioles, indicating that the contradictory results are due to species differences (29).

IV.6 RRF and hydrogen sulfide

Hydrogen sulfide (H_2S) is endogenously synthesized from L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in a lot of tissues, including bovine eyes (30). The H_2S donor GYY4137 is able to induce a substantial vasorelaxation in porcine retinal arterioles and the H_2S donor NaHS in porcine and bovine retinal arteries (16, 31). The NaHS-induced relaxation is reduced in the presence of 100 mM K^+, just like the RRF-induced relaxation. Further research revealed that the H_2S-induced relaxation was endothelium, NO, cyclooxygenase, guanylyl cyclase and adenylyl cyclase independent, but that K_{ir} and K_V channels were involved. H_2S is probably formed by CBS and not by CSE in bovine retina, because the relaxation induced by L-cysteine could be blocked by a CBS inhibitor, but not by a CSE inhibitor (31). This is in contrast to the findings on porcine retinal arterioles where the CBS and CSE blockers both could inhibit the vasorelaxing influence of L-cysteine. The effect of the CBS and CSE blockers was larger in the presence of retinal tissue and during hypoxia.

Therefore, it is suggested that H_2S partially mediates the RRF-induced and hypoxia-induced relaxation on retinal arteries (Fig.IV.2) (16).

IV.7 RRF and carbonic anhydrase inhibitors

Carbonic anhydrases catalyze the association of carbon dioxide and water to carbonic acid, which dissociates spontaneously into bicarbonate and hydrogen ions. This reaction can be catalyzed by carbonic anhydrase in both directions (32). Some inhibitors of carbonic anhydrases are known to relax bovine retinal arteries or porcine retinal arterioles (32, 33). The relaxing effect of dorzolamide and acetazolamide, in contrast to methyl bromopyruvate and ethyl bromopyruvate, is reduced in the absence of perivascular retinal tissue, indicating that the retina plays a role in their relaxation (32, 34). The relaxation is not due to inhibition of carbonic anhydrase and the dorzolamide-induced relaxation is independent of the acidification in the extracellular and intracellular space of the retinal vascular smooth muscle cells. The dorzolamide-induced relaxation acts partially via NO. The origin of NO is as yet unknown, but it might be produced in the retina by nNOS or iNOS or in the vascular endothelium by eNOS. However, other factors of the retina must also be involved in this relaxation, and this might include the RRF (Fig.IV.2) (35).

IV.8 Cellular source of the RRF

Flickering light increases the retinal blood flow indicating that, as in the brain, there exists a neurovascular coupling in the retina. This neurovascular coupling means that when the neural activity increases, in this case by flickering light, the higher demand for nutrients and oxygen is compensated by an increased blood flow through release of retinal vasodilators (36-39). The RRF could be one of these retinal vasodilators. Vasorelaxing substances can be released from two cell types of the retina: neurons and glial cells (40). Incubating bovine retina with tetrodotoxin, which inhibits neuronal activity, had no effect on the vasorelaxing effect of the retinal tissue, indicating that the RRF is rather released from retinal glial cells than from retinal neurons (1). Misfeldt et al. showed that the ATP-induced relaxation of porcine retinal arterioles, which is retina-dependent, is associated with increased Ca^{2+} activity in perivascular cells that are located external to the smooth muscle cells but internal to the glial cells (41, 42). These perivascular cells are similar to pericytes and it is known that pericytes, which are only present on smaller arteries without vascular smooth muscle cells, regulate the blood flow (43).

Further research is needed to find out if these perivascular cells might be the source of the RRF.

IV.9 RRF in retinal pathology

There is not much known about the RRF in retinal pathology. Hyperglycemia, the hallmark of diabetes, causes macrovascular and microvascular damage, also in retinal vessels (44). Agus et al. tested the retina-derived relaxation on carotid and mesenteric arteries of streptozotocin-induced diabetic rats. However, the retina-induced relaxations were similar when using tissues from diabetic and control animals (45). The retina-derived relaxations were also evaluated in rats with hypertension induced by L-NAME, since hypertension causes also vascular damage in retinal arteries (46, 47). The relaxing effect of the RRF was unaltered by hypertension (46). Furthermore, the influence of glaucoma, which is characterized by an elevated intraocular pressure and damage of the optic nerve and surrounding retinal tissue, was studied on the RRF response (48, 49). Glaucoma conditions were induced by elevating the intraocular pressure by cauterization of the episcleral veins. The RRF-induced relaxation was unaltered by glaucoma (48). Thus far, no retinal pathology has been associated with an altered RRF response.

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Chapter V

Search for the source of the retinal relaxing factor

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Chapter V: search for the source of the retinal relaxing factor

V.1 Abstract

Purpose: the retinal relaxing factor (RRF) is an unidentified paracrine factor, which is continuously released from retinal tissue and causes smooth muscle cell relaxation. This study tried to identify the cellular source of the RRF. Furthermore, the possible RRF release by voltage-dependent Na^+ channel activation and the Ca^{2+} dependency of the RRF release was investigated.

Materials and methods: mouse femoral arteries were mounted in myograph baths for in vitro isometric tension measurements. The vasorelaxing effect of chicken retinas, which contain no vascular cells, and of solutions incubated with MIO-M1 or primary Müller cell cultures were evaluated. The RRF release of other retinal cells was investigated by using cell type inhibitors. Concentration-response curves of veratridine, a voltage-dependent Na^+ channel activator, were constructed in presence or absence of mouse retinal tissue to evaluate the RRF release. The Ca^{2+} dependency of the RRF release was investigated by evaluating the vasorelaxing effect of RRF-containing solutions made out of chicken retinas in absence or presence of Ca^{2+}.

Results: Chicken retinas induced vasorelaxation, whereas solutions incubated with Müller cell cultures did not. Moreover, the gliotoxin DL-α-aminoadipic acid, the microglia inhibitor minocycline and the tetrodotoxin-resistant voltage-dependent Na^+ channel 1.8 inhibitor A-803467 could not reduce the RRF-induced relaxation. Concentration-response curves of veratridine were not enlarged in the presence of retinal tissue, and RRF-containing solutions made in absence of Ca^{2+} induced a substantial, but reduced vasorelaxation.

Conclusions: the RRF is not released from vascular cells and probably neither from glial cells. The retinal cell type which does release the RRF remains unclear. Veratridine does not stimulate the RRF release in mice, and the RRF release in chickens is Ca^{2+} dependent as well as Ca^{2+} independent.

V.2 Introduction

Since the original observation in 1998 (1), showing that isolated retinal tissue exerts a strong vasorelaxing influence on the blood vessel tone, several research groups have confirmed the existence of the so-called retinal relaxing factor(s) (RRF(s)) (2-4). Despite many efforts to characterize this RRF, the nature and potential role in physiology and pathophysiology remain largely unknown. In addition, although some previous efforts were made to characterize the cellular source of the RRF, it remains unsettled which cell type(s) release(s) the RRF (1). Therefore, the present study aimed to elucidate the cellular source of the RRF. Previously, it was reported that the neurotoxin tetrodotoxin, an inhibitor of tetrodotoxin-sensitive (TTX-S) voltage-dependent Na^+ (Na_V) channels (5), did not affect the vasorelaxing influence of the retinal tissue. From this, it was concluded that the RRF is more likely released by glial cells than by neuronal cells (1). However, besides neuronal and glial cells, one has to keep in mind that most retinas contain a third cell type namely vascular cells, except for avascular retinas such as avian retinas (6). As in all previous studies on the RRF retinas from cows, pigs, dogs, sheep, rats or mice were used, all containing vascular cells (1, 2, 7, 8), the RRF may still be released from a retinal vascular cell. Therefore, in the present study, the vasorelaxing effect of chicken retinas, not containing blood vessels, was examined to exclude vascular cells as the cellular source of the RRF.

Furthermore, our study evaluated whether the RRF could be released from glial cells and in particular from Müller cells. Müller cells are the most abundant glial cell type in retina, which function is to support neuronal cells structurally and metabolically (9, 10). By evaluating the vasorelaxing effect of solutions containing Müller cell-derived substances, the RRF release from Müller cells was examined.

In addition to the cellular source of the RRF, little is known about the processes involved in the RRF release. A recent study described an enhanced vasorelaxing effect of veratridine, a Na_V channel inactivation inhibitor, in the presence of porcine retinal tissue (11). This suggests that veratridine causes the release of vasorelaxing substances by stimulating the neuronal activity as a result of persistent Na_V channel activation (11, 12). One of these released vasorelaxing substances might have been the RRF. Therefore, to confirm this finding, this experiment was repeated on mouse retinas. Besides, it was also investigated whether the RRF is released in a Ca^{2+}-dependent manner.

V.3 Materials and methods

V.3.1 RRF-containing solution preparation

Chicken retinas were dissected from chicken heads (obtained from a local slaughterhouse) and incubated in a 30 mM K^+ Krebs Ringer bicarbonate (KRB) solution (0.5 ml 30 mM K^+ KRB solution per retina) during 6 hours at 37°C and bubbled with carbogen (95% O_2 – 5% CO_2) gas. After this incubation the solutions were centrifuged (2000 rpm, 5 min) and the supernatants, which are the RRF-containing solutions, were stored at -20°C. Blank solutions were obtained in the same way without adding chicken retinas to the 30 mM K^+ KRB solutions.

V.3.2 Müller cell cultures preparation

MIO-M1 (Moorfield/Institute of Ophthalmology-Müller 1) cells were obtained from the UCL Institute of Ophthalmology (London, UK) and cultivated in Dulbecco's Modified Eagle's Medium with GlutaMAX, pyruvate and glucose (1 g/l), supplemented with 1% L-glutamin, 2% penicillin/streptomycin and 10% fetal bovine serum (FBS).

Primary Müller cell cultures were isolated from fresh bovine eyes (obtained from the local slaughterhouse). Bovine eyes were carefully bisected and the vitrous was removed, exposing the posterior eye cup, which was cut in four flaps. The retinal tissue was peeled off and transferred into a tissue grinder, containing separation medium (advanced Dulbecco's Modified Eagle's Medium with 1% penicillin/streptomycin and 1% GlutaMAX). After thoroughly grinding, the retinal cells' suspensions were poured through a 40 µM filter and centrifuged (1200 rpm, 5 min). Subsequently, the supernatant was discarded and the cell pellets were washed with separation medium. After washing and centrifuging the cells three times, the cell pellet was resuspended in Müller cell growth medium (separation medium with 10% heat inactivated FBS and 4 ng/ml epidermal growth factor) and the retinal cells from two eye flaps were transferred into one T75 Cellbind flask. The cells were cultured in a 5% CO_2 incubator at 37°C, and the medium was renewed once a week.

V.3.3 Müller cell culture-incubated solution preparation

Confluent immortal MIO-M1 cell cultures in cell culture flasks of 75 cm^2 were incubated by replacing the cell culture medium by 10 ml of 30 mM K^+ KRB solution. These cell cultures were placed in a 5% CO_2 incubator at 37°C during 6 hours and thereafter the supernatants, which

are then the MIO-M1 cell culture-incubated solutions, were collected and stored at -20°C. Primary Müller cell culture-incubated solutions were collected in the same way by using confluent primary Müller cell cultures isolated from bovine eyes instead. Blank-incubated solutions were obtained by incubating 10 ml 30 mM K^+ KRB solution in an empty T75 Cellbind flask.

V.3.4 Tissue preparation

Mouse femoral arteries and retinas were dissected from male Swiss mice (8-12 weeks old, obtained from Janvier, Saint-Berthevin, France) and chicken retinas from chicken heads (obtained from a local slaughterhouse). Femoral arteries were carefully cleaned from their surrounding tissue and mounted in a myograph bath (constructed by the technical department of the research unit) by guiding two stainless steel wires of 40 µm diameter through the lumen of the femoral artery, one wire connected to a force-displacement transducer and the other one to a micrometer. The myograph bath was filled with 10 ml KRB solution (37°C, pH 7.4 and bubbled with carbogen gas). In addition, mouse and chicken retinas were kept in KRB solutions.

The femoral arterial segments could equilibrate for 30 min before normalization. This process implied determination of the passive wall tension-internal circumference characteristics of the arterial segment, to set the internal circumference of the arterial segment at 90% of its internal circumference at a transmural pressure of 100 mmHg. Thereafter, the femoral arteries were contracted three times by adding 120 mM K^+ KRB solution and 30 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the myograph bath.

During most experimental protocols, the myograph baths contained different KRB solutions during the whole experiment and substances, like $PGF_{2\alpha}$, were added to the myograph. In contrast, during the bioassay, myograph baths were emptied and the arteries were continuously superfused by different warmed and carbogen bubbled KRB solutions. Those solutions dripped on the artery at a rate of 1 ml / 10 min and at the same time the solution in the myograph was continuously removed.

V.3.5 Experimental protocols

V.3.5.1 RRF release from avascular retinas

Chicken retinas were used to evaluate the RRF release from avascular retinas. A chicken retina was placed on top of a mouse femoral artery, contracted with 30 mM K^+ KRB solution and 30 µM $PGF_{2\alpha}$, and afterwards removed. This procedure was repeated twice, and the average of the relaxations was calculated.

V.3.5.2 RRF release from Müller cells

The release of the RRF from Müller cells was investigated in a bioassay. A mouse femoral artery, mounted for isometric tension recording, was superfused by a warmed (37°C) and carbogen bubbled 30 mM K^+ KRB with 30 µM $PGF_{2\alpha}$ control solution. Subsequently, the artery was superfused with the blank-incubated solution to which 30 µM $PGF_{2\alpha}$ was also added. After shifting again to the control solution, the Müller cell culture-incubated solution with 30 µM $PGF_{2\alpha}$ was also tested. This protocol was performed three times and the average of the relaxations was calculated.

V.3.5.3 RRF release from glial cells, microglia and TTX-R $Na_{V1.8}$ channels-containing cells

The inhibiting effect of the gliotoxin DL-α-aminoadipic acid, the microglia inhibitor minocycline or the tetrodotoxin-resistant (TTX-R) $Na_{V1.8}$ channel inhibitor A-803467 on the RRF release was tested by placing a mouse retina on top of a 30 mM K^+ KRB with 30 µM $PGF_{2\alpha}$ contracted mouse femoral artery to induce relaxation. The retina was then removed. This procedure was repeated twice. Afterwards, this protocol was repeated on the same femoral artery using the same retina, but after incubating the retina with DL-α-aminoadipic acid (4 mM) or the retina and artery with minocycline (100 µM) or A-803467 (10 µM). Averages of the 3 relaxations of each condition were used in the data analysis.

V.3.5.4 RRF release by veratridine

The RRF release by veratridine was tested by performing a concentration-response curve of veratridine (1-100 µM) on contracted mouse femoral arteries either in presence or absence of a piece of mouse retinal tissue on top of the femoral artery.

V.3.5.5 Ca^{2+} dependency of the RRF release

The Ca^{2+} dependency of the RRF release was tested by the previous described bioassay in which a femoral artery was superfused by different solutions. RRF-containing solutions made in a Ca^{2+}-containing or a Ca^{2+}-free 30 mM K^+ KRB solution were compared. Before performing the bioassay, the Ca^{2+}-free RRF-containing solutions were supplemented with Ca^{2+}.

V.3.6 Drugs and chemicals

The KRB solution contained the following components (mM): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. 120 mM K^+ and 30 mM K^+ KRB solutions were made by equimolar replacement of NaCl by KCl and Ca^{2+}-free 30 mM K^+ KRB solutions were made by removing $CaCl_2$ without substitution. Veratridine was obtained from Alomone labs (Jerusalem, Israel), A-803467, DL-α-Aminoadipic acid and minocycline hydrochloride from Sigma-Aldrich (St. Louis, MO, USA) and $PGF_{2\alpha}$ from Zoetis (Florham Park, NJ, USA). All stock solutions were made in DMSO, except for minocycline that was made in water and DL-α-aminoadipic acid that was directly solved in KRB solution. The final concentrations of DMSO in the solutions containing arteries or retinas did never exceeded 0.1%.

V.3.7 Data analysis

The data were computed as means ± (standard error of the mean) S.E.M. and evaluated statistically using a Wilcoxon test. Two groups of data were considered significantly different if $P < 0.05$. Relaxations are expressed in percent decrease of the pre-existing tone elicited by $PGF_{2\alpha}$. N = number of preparations tested all from different animals.

V.4 Results

V.4.1 RRF release from avascular retinas

Avascular chicken retinas were placed on contracted mouse femoral arteries to investigate whether avascular retinal tissue also releases the RRF (Fig.V.1). This elicited a vasorelaxation of 24.12 +/- 2.95% (n = 8).

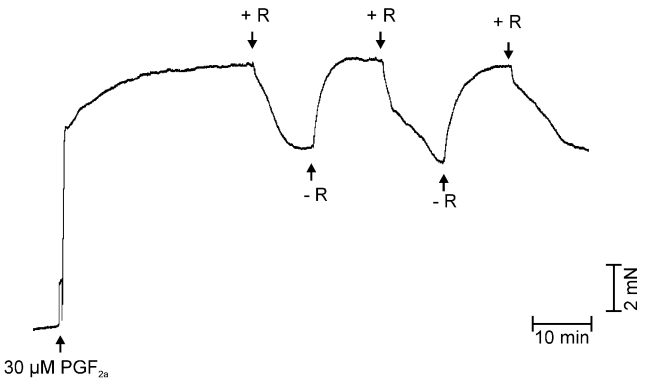

Figure V.1 Original tracing of an experiment in which a chicken retina was placed 3 times on a 30 mM K^+ KRB solution and 30 µM $PGF_{2\alpha}$ contracted mouse femoral artery (+R) and removed again (-R).

V.4.2 RRF release from Müller cells

The vasorelaxing effect of 30 mM K^+ KRB solutions incubated with different Müller cell cultures was evaluated to investigate whether the RRF is released from Müller cells. The MIO-M1 cell culture-incubated solution caused no relaxation in comparison to the blank-incubated solution (5.98 +/- 1.40% versus 4.05 +/- 0.80%) (Fig.V.2A). In addition, the primary Müller cell culture-incubated solution induced no relaxation (2.42 +/- 0.37%), relative to the blank-incubated solution (2.99 +/- 1.00%) (Fig.V.2B).

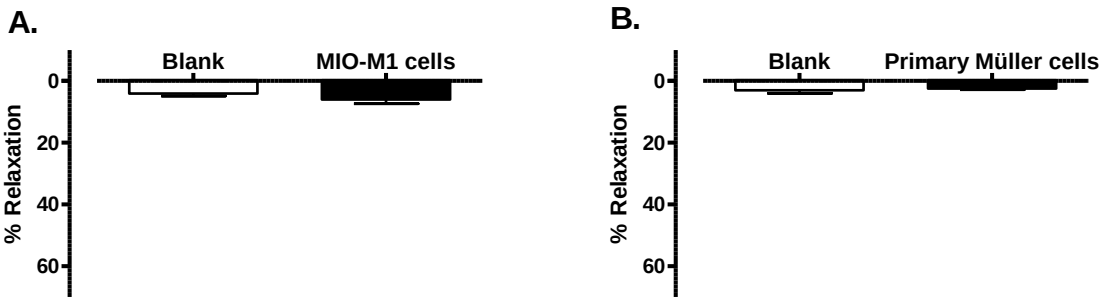

Figure V.2 Percentage relaxation of mouse femoral arteries induced by a blank-incubated (blank) or a MIO-M1 cell culture-incubated (MIO-M1 cells) 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ solution (A), n=3, or by a blank-incubated (blank) or a primary Müller cell culture-incubated (Primary Müller cells) 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ solution (B), n=4.

V.4.3 RRF release from glial cells, microglia and TTX-R $Na_{V1.8}$ channels-containing cells

The RRF release from glial cells was tested by comparing RRF-induced relaxations without or with incubating mouse retinas with the gliotoxin DL-α-aminoadipic acid, but there was no difference detected (21.45 +/- 1.80% versus 24.37 +/- 6.36%) (Fig.V.3A). The RRF-induced relaxation was also not altered by incubating mouse retinas and femoral arteries with the

microglia inhibitor minocycline (20.90 +/- 2.85% versus 18.84 +/- 0.88%) (Fig.V.3B). In addition, the RRF release from retinal cells with TTX-R $Na_{V1.8}$ channels was investigated by incubating mouse retinas and femoral arteries with the specific inhibitor A-803467. No difference was detected between the RRF-induced relaxations without and with A-803467 incubation (38.14 +/- 4.19% versus 34.64 +/- 3.08%) (Fig.V.3C).

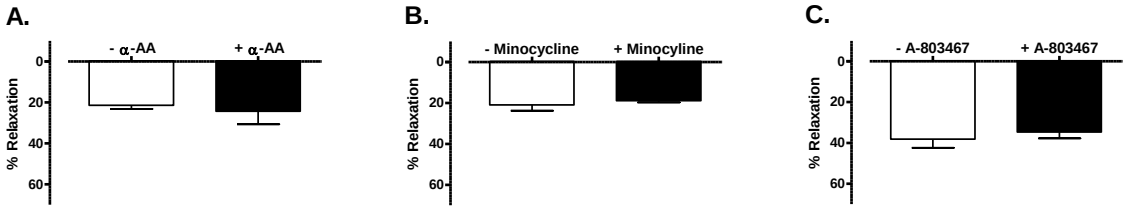

Figure V.3 Percentage relaxation of 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ contracted mouse femoral arteries induced by mouse retinas without or with incubating the retina with DL-α-aminoadipic acid (4 mM), n=4 (A), without or with incubating the retina and artery with minocycline (100 µM), n=4 (B), or without or with incubating the retina and artery with A-803467 (10 µM), n=6 (C).

V.4.4 RRF release by veratridine

The stimulation of the RRF release by veratridine was evaluated by concentration-response curves of veratridine in the presence or absence of retinal tissue. A piece of mouse retina was used so that the contraction levels of the femoral arteries were not significantly changed and both concentration-response curves could be compared. Veratridine relaxed the femoral arteries concentration dependently. Only at the concentration of 0.1 mM veratridine, the femoral arteries in the absence of retinal tissue relaxed significantly more than the ones in the presence of retinal tissue (56.50 +/- 4.95 % versus 36.84 +/- 4.69 %). There was no significant difference detected at all other concentrations (Fig.V.4).

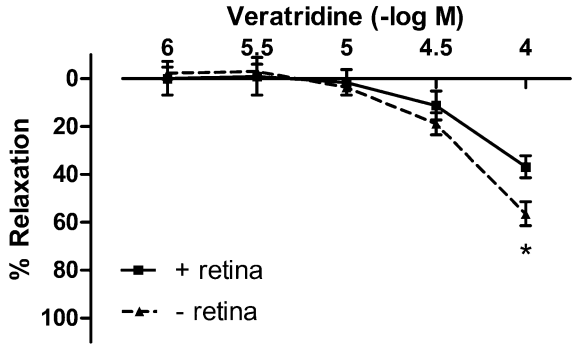

Figure V.4 Percentage relaxation of 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ contracted mouse femoral arteries induced by veratridine (1-100 µM) in presence (+ retina) or absence (- retina) of a mouse retina, n=4; *p<0.05.

V.4.5 Ca^{2+} dependency of the RRF release

The Ca^{2+}-dependent release of the RRF was tested by superfusing mouse femoral arteries by RRF-containing solutions, made by incubating chicken retinas in a 30 mM K^+ KRB solution with or without Ca^{2+}. Both solutions induced a substantial vasorelaxation, but the vasorelaxation induced by the RRF-containing solution made in absence of Ca^{2+} was significantly smaller (34.23 +/- 3.82% versus 44.21 +/- 3.32%) (Fig.V.5).

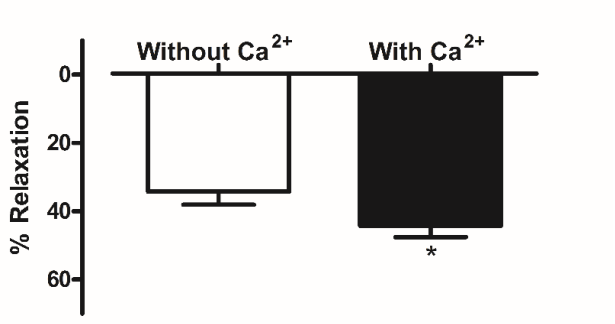

Figure V.5 Percentage relaxation of mouse femoral arteries caused by a RRF-containing solutions, made by incubating chicken retinas in a Ca^{2+}-free (without Ca^{2+}) or a Ca^{2+}-containing (with Ca^{2+}) 30 mM K^+ KRB solution and to which 30 µM $PGF_{2\alpha}$ was added, n=6; *p<0.05.

V.5 Discussion

Our study demonstrates for the first time that the RRF is also released from avascular chicken retinas. As all retinas used in previous RRF studies contained vascular cells, one could wonder whether the RRF is released from retinal vascular cells. The vasorelaxing effect of chicken retinas (24.13%) is comparable to those elicited by rat (33.24%) and mouse (21.57%) retinas (7, 8). The observation that even avascular chicken retinas induce a vasorelaxing effect, exclude retinal vascular cells as the cellular source of the RRF. Thus, the RRF will probably be released from neuronal or glial cells.

In this perspective Müller cells seem to be the most likely source of the RRF since their abundant presence and multiple functions in the retina. Müller cells are the principal glial cells in the retina and are extended in all layers of the retina. They are important in maintaining the retinal structure and function. Major functions of Müller cells are providing neurons with nutrients and removing metabolic waste products, regulating the extracellular volume and ion and water homeostasis, regulating neuronal activity by the uptake of neurotransmitters and release of neuroactive substances, and regulating the retinal blood flow by the synthesis and release of vasoactive substances (10, 13-16). In previous studies RRF-containing solutions

were made by incubating whole retinas in a KRB solution (1, 3). In the present study a solution was made by incubating a MIO-M1 Müller cell culture with a solution that was then tested for the presence of vasorelaxing substances. MIO-M1 cells are a spontaneously immortalized human cell line which seems to retain the characteristics of Müller cells (17). Unfortunately, the incubated solution was unable to induce any relaxation. As cell characteristics might still have been altered in this immortal cell line, this experiment was repeated by using a primary Müller cell culture made out of bovine retinas. Unfortunately, also the incubated solution of this primary Müller cell culture was unable to induce vasorelaxation. The whole of these results does not support the hypothesis that Müller cells are the source of the RRF.

Other glial cell types in the retina, and thus still possible RRF-releasing cell types, are astrocytes, microglia and oligodendrocytes (9). The vasorelaxing effect of a retina incubated with the gliotoxin DL-α-aminoadipic acid was investigated, but it did not decrease the retina-induced relaxation. Therefore, astrocytes could be excluded as the RRF-releasing cell type. Moreover, astrocytes can also be excluded as they are not present in avascular retinas (18), while avascular chicken retinas also release the RRF. It should be noted that microglia and oligodendrocytes are not influenced by DL-α-aminoadipic acid and can still be the RRF source (19-21). However, oligodendrocytes can be excluded as being the RRF releasing cell type since they are not present in mammalian retinas except those of rabbits (22). They are also present in chicken retinas (23, 24). Taken together, only microglia remain as glial cells which might release the RRF. Therefore, the effect of the inhibitor of microglia activity minocycline on the RRF-induced relaxation was investigated (25, 26). As it did not have any effect, microglia do probably not release the RRF.

So far, this study points out that the RRF-releasing cell type is rather a neuronal cell than a glial cell type, although previous experiments with tetrodotoxin suggested the opposite (1). However, it should be taken into account that tetrodotoxin does not inhibit all Na_V channels. There are nine different subtypes of voltage-dependent Na^+ (Na_V) channels, which can be divided in two classes: TTX-S and TTX-R Na_V channels. $Na_{V1.1}$, $Na_{V1.2}$, $Na_{V1.3}$, $Na_{V1.4}$, $Na_{V1.6}$ and $Na_{V1.7}$ are TTX-S Na_V channels and $Na_{V1.5}$, $Na_{V1.8}$ and $Na_{V1.9}$ are TTX-R Na_V channels (27). TTX-R $Na_{V1.8}$ and $Na_{V1.9}$ channels were detected in mouse retinas (28). $Na_{V1.9}$ channels are present in Müller cells and photoreceptors. Since Müller cells are likely not the RRF-releasing cell type and photoreceptors may have reacted nonselectively to $Na_{V1.9}$ channel antibodies, $Na_{V1.9}$

channels were not further investigated. TTX-R $Na_{V1.8}$ channels are present on starburst amacrine cells and α-like ganglion cell types. To find out whether one of these two cell types is the cellular source of the RRF, the effect of a specific $Na_{V1.8}$ channel inhibitor A-803467 (29) on the RRF-induced relaxation was investigated. However again, no alteration in the RRF response was seen, which suggests that also starburst amacrine cells and α-like ganglion cells are not the cellular source of the RRF.

Nevertheless, the finding that the veratridine-induced relaxation was enhanced in the presence of porcine retinal tissue by Kratholm et al. (11) was still interesting for our study. Veratridine stimulates the neuronal activity by inhibiting Na_V channel inactivation and subsequently preventing restoration of the membrane potential (30). The extra vasorelaxing effect in the presence of retinal tissue can be explained by the release of vasorelaxing compounds out of the retina (11). One of these compounds could be the RRF and therefore worth investigating. Unfortunately, no enhancement of the veratridine-induced relaxation on mouse femoral arteries in the presence of a mouse retina was seen. On the contrary, an even larger percentage relaxation in absence of retinal tissue was seen at the concentration of 0.1 mM veratridine. This contradictory result in comparison to the previous study can be explained by species differences (mouse versus pig) or different contractile substances (30 mM K^+ KRB solution with $PGF_{2\alpha}$ versus low K^+ physiological saline solution with U46619) (11). It should be noted that veratridine induces a direct vasorelaxing effect independent of the presence of retinal tissue. This can be explained by the presence of perivascular sensory nerves, which release vasorelaxing substances (31). However, the most important conclusion of these experiments is that veratridine does not stimulate the RRF release in mouse retinas as the presence of retina does not enhance the relaxing effect of veratridine.

Finally, the Ca^{2+} dependency of the RRF release was investigated in this study. Molecules can be released out of the cell Ca^{2+} dependently or independently. Passive diffusion through the cell membrane and Ca^{2+}-independent transporters are examples of Ca^{2+}-independent release (32). Exocytosis of neurotransmitters by depolarization of the presynaptic axon terminal and subsequently Ca^{2+} influx is an example of a Ca^{2+}-dependent release (33). The RRF-containing solutions, made in absence or presence of Ca^{2+}, induced both substantial relaxations. However, the relaxation percentage induced by the RRF-containing solution made in absence of Ca^{2+} was significant lower than the one made in presence of Ca^{2+}, which suggests that the

RRF is released in both a Ca^{2+}-independent way and a Ca^{2+}-dependent way. These findings are not fully in line with a previous study which reported that the RRF release was completely Ca^{2+} dependent in rat (3).

In conclusion, our study demonstrates that the RRF is not released from vascular cells and most likely not from glial cells either. The retinal cells which do release the RRF still remain elusive. In addition, veratridine does not stimulate the RRF release from mouse retina and the RRF release from chicken retina seems to occur partially in a Ca^{2+}-independent and partially in a Ca^{2+}-dependent way.

V.6 Acknowledgements

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Chapter VI

Characterization of the retina-induced relaxation in mice

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Chapter VI: characterization of the retina-induced relaxation in mice

VI.1 Abstract

Purpose: the retinal relaxing factor (RRF) is a continuously released factor from the retina that causes vasorelaxation, the identity and potential role in physiology of which remain largely unknown. Experiments were performed to find out whether the RRF-induced relaxation is influenced by serotonin, glutamate, L-cysteine, the cytochrome P450 pathway, the cyclooxygenase pathway or oxidative stress. In addition, the sensitivity of retinal and non-retinal arteries towards the RRF was compared.

Methods: in vitro tension measurements were performed on isolated mouse femoral or bovine retinal arteries to study the vasorelaxing effect of the RRF, induced by mouse or bovine retinas.

Results: the presence of serotonin, glutamate or L-cysteine did not alter the RRF-induced relaxation. Increasing oxidative stress by hydroquinone and diethyldithiocarbamic acid sodium salt enhanced the RRF response. Inhibition of the cytochrome P450 or the cyclooxygenase pathway did not cause any alteration. Surprisingly, the RRF-induced relaxation was enhanced by the presence of flufenamic acid or carbenoxolone. Furthermore, bringing retinal tissue in close contact with retinal or non-retinal arteries induced comparable relaxations.

Conclusions: serotonin, glutamate, L-cysteine, the cytochrome P450 and the cyclooxygenase pathway do not influence the RRF-induced relaxation and the RRF-induced relaxation seems to be resistant to oxidative stress. The mechanism responsible for the enhanced RRF-induced relaxation in the presence of flufenamic acid or carbenoxolone remains elusive and the RRF does not show more effectivity on retinal arteries.

VI.2 Introduction

About 20 years, ago the so-called retinal relaxing factor (RRF) was discovered, which is a continuously released factor from the retinal tissue causing smooth muscle cell relaxation (1). This RRF may be an important paracrine factor for the regulation of the retinal blood flow. Until today, its identity and vasorelaxing mechanism remain largely unknown (2). Research on the RRF is essential to understand its potential role in the retinal blood flow regulation and to discover potential treatments for diseases with an impaired retinal blood flow. Although common retinal neurotransmitters, such as glutamate, glycine, γ-aminobutyric acid (GABA), dopamine, melatonin and adenosine, have been excluded as being the RRF (1), the influence of neurotransmitters on the release of the RRF and/or the effect of the RRF on the vascular tone has never been investigated. Therefore, the influence of serotonin and glutamate, both found in substantial amounts in the retina (3-5), was investigated on the RRF-induced relaxation.

Besides serotonin and glutamate, the release and/or the effect of the RRF could also be affected by hydrogen sulfide (H_2S) as it has been suggested that H_2S is partially involved in the bovine and porcine RRF-induced relaxation (6,7). Since H_2S is synthesized from L-cysteine in the retina, also the influence of L-cysteine on the RRF-induced relaxation was evaluated in this study.

Furthermore, it is still a matter of debate whether cytochrome P450 and cyclooxygenase, both present in the retina (8), are involved in the RRF-induced relaxation. In pigs, inhibition of cyclooxygenase reduced the RRF-induced relaxation (9), while in cows, rats and mice no effect of cyclooxygenase inhibition was seen (1,10,11). In addition, it was reported that the cytochrome P450 pathway, which forms epoxyeicosatrienoic acids (EETs) from arachidonic acid (8), is not involved in the RRF-induced relaxation in rat (12). In the present study, the involvement of cytochrome P450 and cyclooxygenase products in the RRF-induced relaxation was further evaluated in mouse femoral arteries.

It is also known that reactive oxygen species induce cellular damage in the retina leading to retinal diseases such as age-related macular degeneration or diabetic retinopathy (13,14). Therefore, the influence of oxidative stress on the vasorelaxing effect of the RRF was investigated.

The RRF-induced relaxation was first demonstrated using bovine retinal arteries and bovine retinas. This was later confirmed on different arteries and retinas of other species (1,9,10,11). However, the strongest RRF-induced relaxation was reported when bringing bovine retinal tissue in close contact with a bovine retinal artery (1,15). Weaker RRF-induced relaxations were reported when testing rat retinas on rat carotid arteries or mouse retinas on mouse aortas (10,11). This could be due to a different effectivity of RRF on retinal arteries compared to non-retinal arteries. Therefore, the sensitivity of retinal and non-retinal arteries to the RRF was compared.

VI.3 Materials and methods

VI.3.1 Tissue preparation

Femoral arteries and retinas were dissected from male Swiss mice (8-12 weeks old, obtained from Janvier, Saint-Berthevin, France), retinal arteries and retinas from bovine eyes (obtained from the local slaughterhouse). The retinas were kept in Krebs Ringer bicarbonate (KRB) solution (37°C, pH 7.4 and bubbled with carbogen (95% O_2 – 5% CO_2) gas). The arteries were cleaned from their surrounding tissue and mounted in 10 ml organ baths (constructed by the technical department of the research unit), for isometric tension measurements by guiding two stainless steel wires of 40 µm diameter through the lumen of the artery. One wire was connected to a force-displacement transducer and the other to a micrometer.

After mounting, the arterial segments were allowed to equilibrate for 30 min before normalization. During normalization, the passive wall tension-internal circumference characteristics of the arterial segment were determined, so the internal circumference of the arterial segment could be set at 90% of its internal circumference at a transmural pressure of 100 mmHg. Thereafter, the arteries were contracted three times by adding 120 mM K^+ KRB solution and 30 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the organ bath. All precontractions in the protocols were elicited using 30 mM K^+ KRB solutions and 30 µM $PGF_{2\alpha}$.

VI.3.2 Experimental protocols

VI.3.2.1 Influence of serotonin, glutamate, L-cysteine, oxidative stress, cytochrome P450 and cyclooxygenase on the RRF-induced relaxation

The influence of serotonin, glutamate, L-cysteine, oxidative stress, the cytochrome P450 pathway and the cyclooxygenase pathway on the RRF-induced relaxation was investigated using mouse retinas and mouse femoral arteries. Oxidative stress was induced by hydroquinone and diethyldithiocarbamic acid sodium salt (DETCA), the cytochrome P450 pathway was inhibited by N-(Methylsulfonyl)-6-(2-propoxyphenyl)hexanamide (MS-PPOH) and the cyclooxygenase pathway by diclofenac or flufenamic acid. The influence of these substances on the RRF-induced relaxation was tested as follows. A mouse retina was placed on top of a contracted mouse femoral artery to induce relaxation. When maximal relaxation was obtained, the retina was removed and the relaxation disappeared. This procedure was repeated twice. Thereafter, this protocol was repeated on the same femoral artery using the same retina, but after incubating retina and artery with the test substance for 15 min. Averages of the three relaxations in the absence or presence of the test substance were used in the data analysis. Precontraction levels of the arteries were comparable before and after incubation with the test substances.

VI.3.2.2 Sensitivity of retinal versus non-retinal arteries to the RRF

The sensitivity of retinal and non-retinal arteries to the vasorelaxing effect of the RRF was investigated on a mouse femoral artery and a bovine retinal artery, used in parallel. When the precontraction levels were stable, a mouse retina was placed on top of a mouse femoral artery and a piece of bovine retina, with similar size as a mouse retina (about 25 mm^2), on top of a bovine retinal artery eliciting a RRF relaxation. Thereafter, the retinas were removed and the relaxing effect disappeared. This procedure was repeated twice. Thereafter, the same protocol was performed again, now placing the mouse retina on top of the bovine retinal artery and the bovine retina on top of the mouse femoral artery. The averages of the three relaxations of each condition were used in the data analysis.

VI.3.3 Drugs and chemicals

The KRB solution contained the following components (mM): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. 120 mM K^+ and 30 mM K^+ KRB solutions were made by equimolar replacement of NaCl by KCl. 1(S),9(R)-(−)-Bicuculline methiodide (bicuculline), 2-aminoethyl diphenylborinate (2-APB), CaCCinh-A01, carbenoxolone disodium salt (carbenoxolone), compound C, diclofenac sodium salt (diclofenac), diethyldithiocarbamic acid sodium salt (DETCA), flufenamic acid, hydroquinone, L-cysteine, L-glutamic acid (glutamate), serotonin and T16Ainh-A01 were obtained from Sigma-Aldrich (St. Louis, MO, USA), Eact, DL-2-amino-5-phosphonovaleric acid (DL-APV) from Tocris (Bristol, United Kingdom), MS-PPOH from Cayman Chemical (Michigan, USA), sodium nitroprusside (SNP) from Merck (Darmstadt, Germany) and $PGF_{2\alpha}$ from Zoetis (Florham Park, NJ, USA). WRQAAFVDSY (^{10}PanX1) was synthesized by Covalab (Villeurbanne, France). All stock solutions were made in water, except those of 2-APB, CaCCinh-A01, compound C, Eact, flufenamic acid, MS-PPOH and T16Ainh-A01 were made in DMSO and that of probenecid was made in 0.2 M NaOH. ^{10}PanX1 was immediately solved in KRB solution.

VI.3.4 Data analysis

The data were computed as means ± S.E.M and evaluated statistically using a Mann-Whitney U test or a Wilcoxon test. Two groups of data were considered significantly different if $P<0.05$. Relaxations are expressed in percent decrease of the pre-existing tone elicited by $PGF_{2\alpha}$. N = number of preparations tested all from different animals.

VI.4 Results

VI.4.1 Influence of serotonin, glutamate and L-cysteine on the RRF-induced relaxation

The effect of serotonin, glutamate and L-cysteine on the RRF-induced relaxation was investigated before and during incubation of mouse femoral arteries and retinas with 0.5 mM serotonin (Fig.VI.1A), 0.1 mM glutamate (Fig.VI.1B) or 1 mM L-cysteine (Fig.VI.1C). None of these incubations had a significant influence on the RRF-induced relaxations.

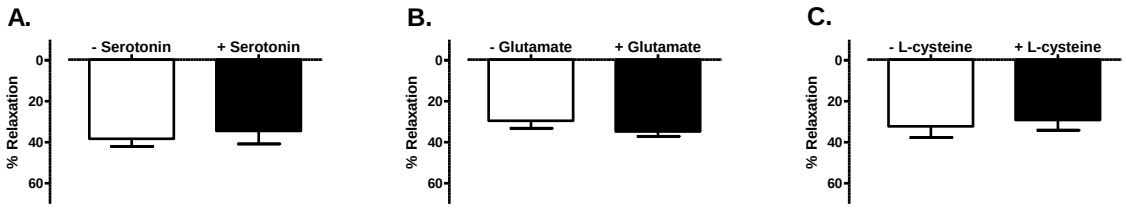

Figure VI.1 Percentage relaxation of mouse femoral arteries elicited by mouse retinas before and after incubation of the retina and femoral artery with serotonin (0.5 mM), n=4 (A), glutamate (0.1 mM), n=5 (B) or L-cysteine (1 mM), n=5 (c)

VI.4.2 Influence of oxidative stress on the RRF-induced relaxation

Induction of oxidative stress in mouse retinas and femoral arteries by hydroquinone (10 µM) and DETCA (8 mM) enhanced the RRF response significantly (Fig.VI.2).

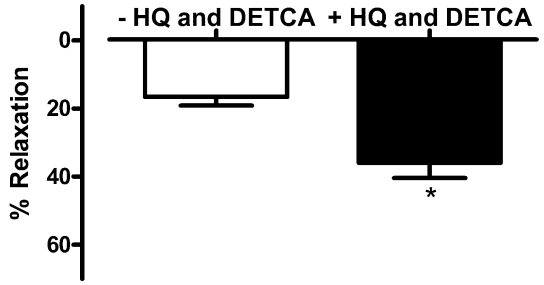

Figure VI.2 Percentage relaxation of mouse femoral arteries elicited by mouse retinas before and after incubation of the retina and femoral artery with hydroquinone (HQ) (10 µM) and DETCA (8 mM), n=5 ; *p<0.05

VI.4.3 Influence of cytochrome P450 and cyclooxygenase inhibition on the RRF-induced relaxation

In order to evaluate the potential role of the cytochrome P450 pathway, mouse retinas and femoral arteries were incubated with its inhibitor MS-PPOH. However, the presence of 10 µM MS-PPOH did not alter the RRF response (Fig.VI.3A). Moreover, the involvement of the cyclooxygenase pathway in the RRF-induced relaxation was investigated using a cyclooxygenase inhibitor, diclofenac. Incubation with 10 µM diclofenac resulted in a small but not-significant reduction of the RRF response (Fig.VI.3B). On the other hand, incubation with 30 µM flufenamic acid, also known to inhibit cyclooxygenase, enhanced the RRF-induced relaxation significantly (23.62 +/- 4.09% versus 57.07 +/- 8.77%) (Fig.VI.3C,4).

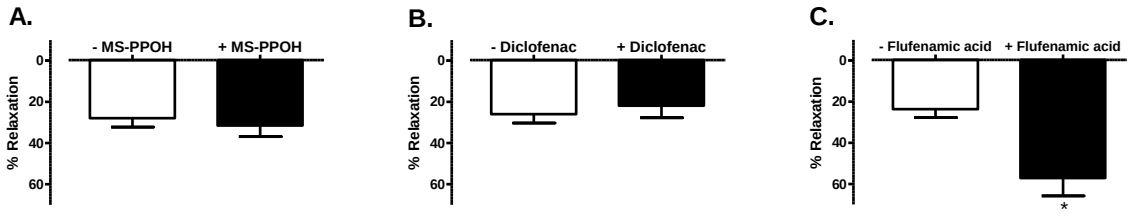

Figure VI.3 Percentage relaxation of mouse femoral arteries elicited by mouse retinas before and after incubation of the retina and femoral artery with MS-PPOH (10 µM) (A), with diclofenac (10 µM) (B) or with flufenamic acid (30 µM) (C); n=6; *p<0.05

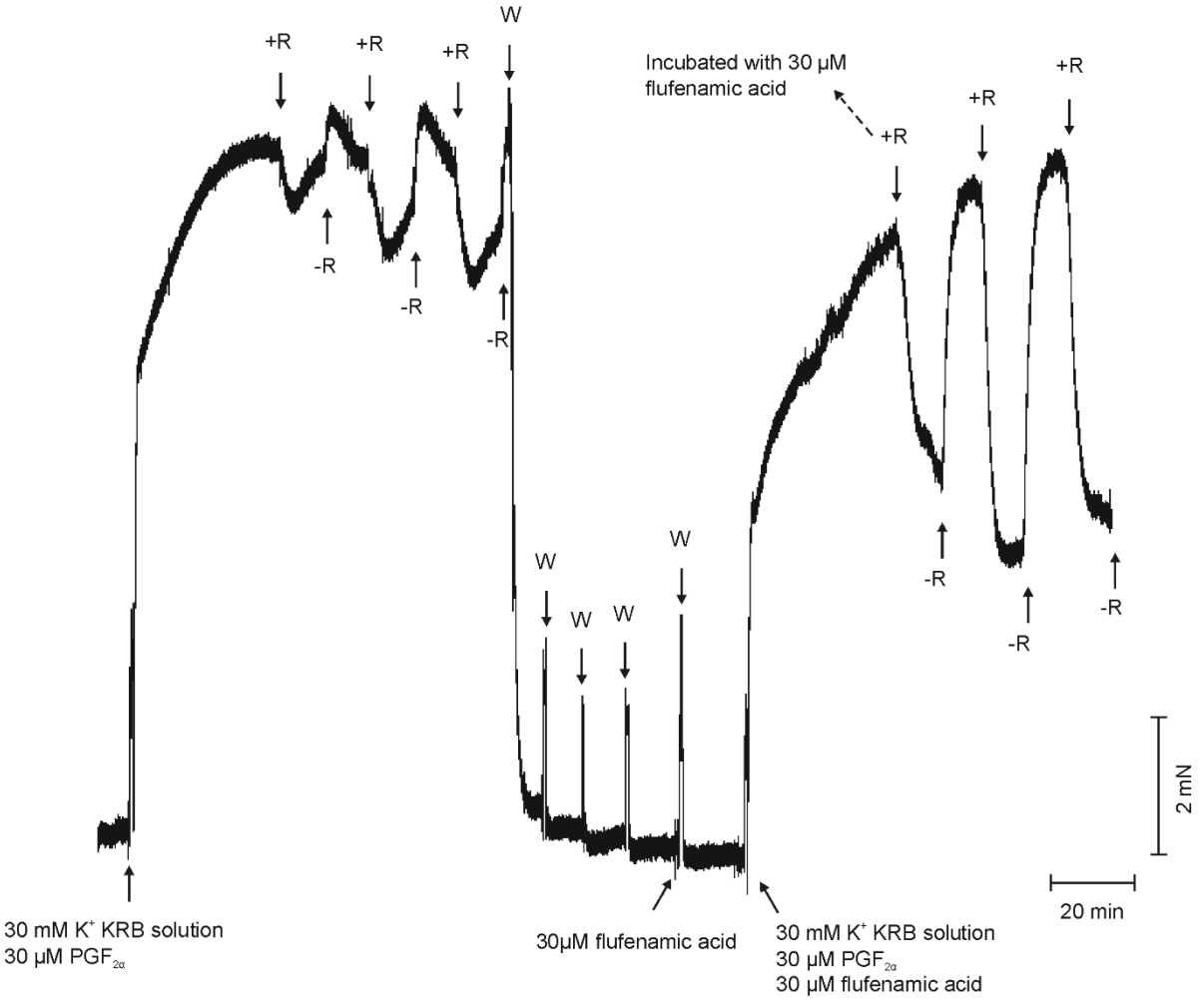

Figure VI.4 Original tracing of an experiment which compared the relaxation of precontracted mouse femoral arteries induced by mouse retinas (R), before and after incubating the retina and femoral artery with flufenamic acid (30 µM) (W= wash with KRB solution).

VI.4.4 Role of non-cyclooxygenase targets of flufenamic acid in the RRF-induced relaxation

Since flufenamic acid is known to influence many other targets besides cyclooxygenase, the potential role of these targets was further explored. Inhibition of the transient receptor potential (TRP) channel with 2-aminoethoxydiphenylborane (2-APB) (100 µM) resulted in a small, non-significant enhancement of the RRF-induced relaxation (Fig.VI.5A). Compound C (10 µM), an inhibitor of AMP-activated protein kinase (AMPK) (whereas flufenamic acid is an activator), caused only a small reduction of the RRF-induced relaxation (Fig.VI.5B). Also, bicuculline (100 µM), a $GABA_A$ receptor antagonist, caused a small non-significant reduction of the RRF-induced relaxation (Fig.VI.5C).

T16Ainh-A01 is an inhibitor of the transmembrane protein 16A (TMEM16a), which forms a Ca^{2+}-activated chloride channels (CaCC) subunit (16). The incubation with T16Ainh-A01 (10 µM) enhanced the RRF-induced relaxation significantly (Fig.VI.5D). On the other hand, another CaCC inhibitor CaCCinh-A01 (30 µM) did not alter the RRF-induced relaxation (Fig.VI.5E). Also, the CaCC activator Eact had no significant influence on the RRF-induced relaxation (Fig.VI.5F). In the experiments with CaCCinh-A01 or Eact, only the retina but not the femoral artery was incubated with because these substances significantly reduced the precontraction level.

In the presence of the gap junction inhibitor carbenoxolone (100 µM) the RRF-induced relaxation was significantly enhanced (Fig.VI.5G). In contrast, the pannexin 1 channel mimetic inhibiting peptide ^{10}PanX1 had a rather small but significantly inhibitory effect on the RRF-induced relaxation (Fig.VI.5H).

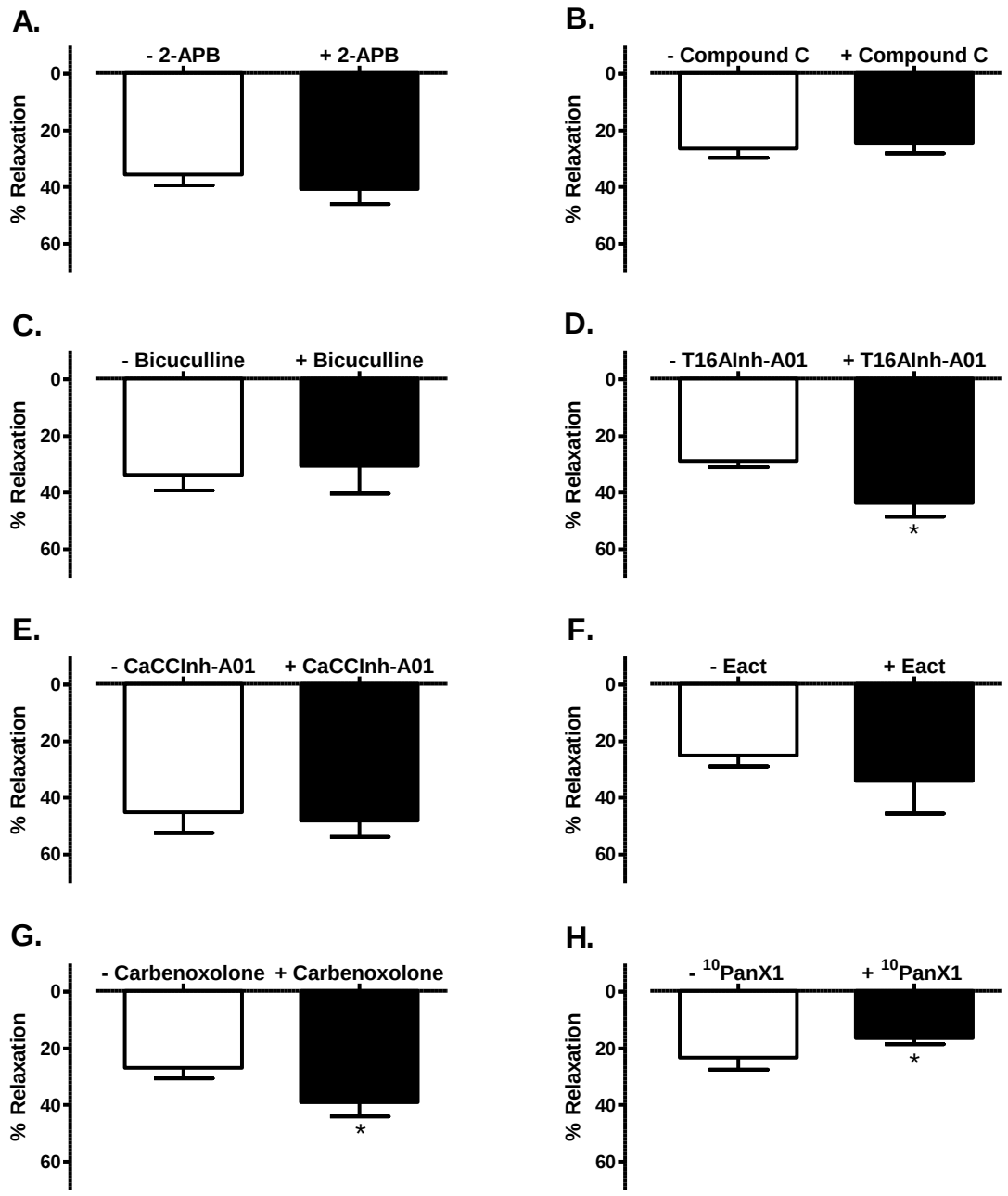

Figure VI.5 Percentage relaxation of mouse femoral arteries elicited by mouse retinas before and after incubation of the retina and femoral artery with 2-APB (100 µM), n=6 (A), compound C (10 µM), n=4 (B), bicuculline (100 µM), n=4 (C), T16AInh-A01 (10 µM), n=7 (D), incubation of the retina with CaCCInh-A01 (30 µM), n=5 (E), incubation of the retina with Eact (10 µM), n=5 (F), incubation of the retina and femoral artery with carbenoxolone (100 µM), n=6 (G), or ^{10}PanX1 (0.2mM), n=6 (H); *p<0.05

VI.4.5 Sensitivity of retinal versus non-retinal arteries to the RRF

Placing mouse or bovine retinas on mouse femoral or bovine retinal arteries elicited similar RRF-induced relaxations of about 30% (Fig.VI.6A). The presence or absence of 30 mM K^+ did not alter the relaxation response, elicited by placing a small piece (about 25 mm², similar size

as a mouse retina) bovine retina on a bovine retinal artery (Fig.VI.6B). However, when using a large piece of bovine retina (about 230 mm²) instead of a small piece (about 25 mm²) the RRF-induced relaxation was significantly larger both in presence and absence of 30 mM K^+ (Fig.VI.6C,D).

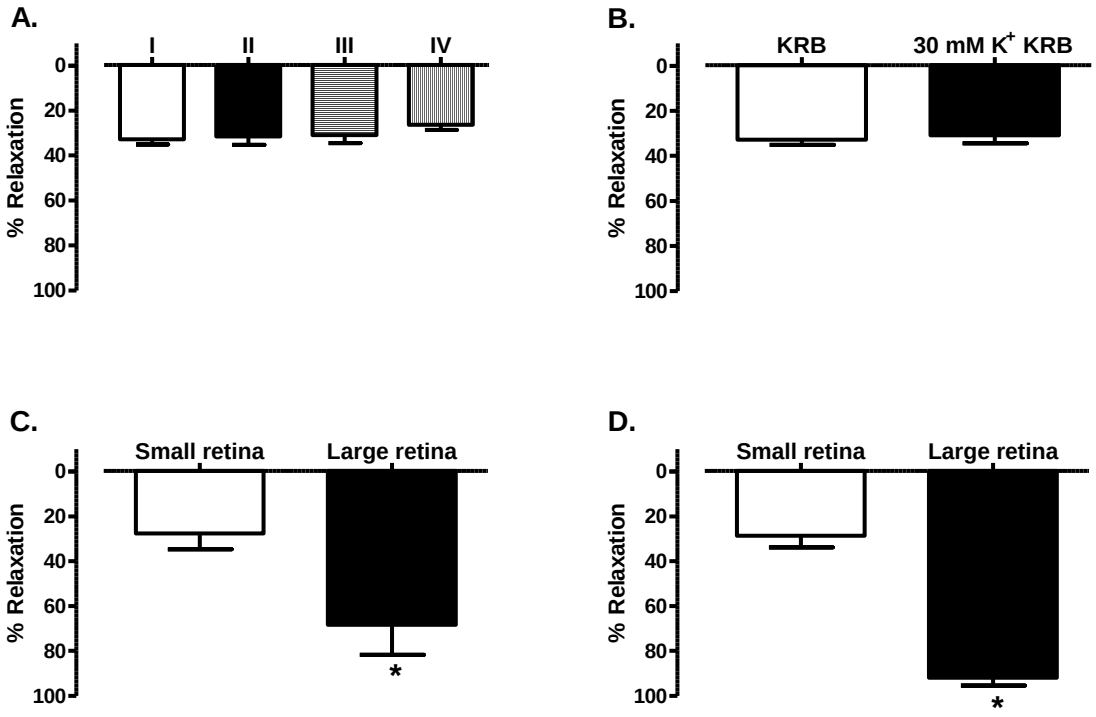

Figure VI.5 Percentage relaxation of (I) mouse femoral arteries elicited by mouse retinas, (II) mouse femoral arteries elicited by bovine retinas, (III) bovine retinal arteries elicited by bovine retinas and (IV) bovine retinal arteries elicited by mouse retinas, n=7 (A), bovine retinal arteries elicited by bovine retinas in absence or presence of 30 mM K^+ KRB solution, n=6 (B), bovine retinal arteries elicited by small (25 mm²) and large (230 mm²) bovine retinas in presence of 30 mM K^+ KRB solution, n=6 (C), or in absence of 30 mM K^+ KRB solution, n=6 (D); *p<0.05

VI.5 Discussion

In order to reveal more characteristics of the RRF, this study investigated the effect of serotonin, glutamate, L-cysteine, oxidative stress, the cytochrome P450 pathway, and the cyclooxygenase pathway on the RRF-induced relaxation. In addition, the activity of the RRF on retinal and non-retinal arteries was compared.

VI.5.1 Influence of serotonin, glutamate and L-cysteine on the RRF-induced relaxation

The retina is a source of many neurotransmitters and metabolites that could influence the release and/or the effect of the RRF. Serotonin and glutamate are common neurotransmitters

in the retina (3-5). However, neither the presence of serotonin nor the presence of glutamate influenced the RRF-induced relaxation. Previously, a similar lack of effect of glutamate has been reported in cow and rat (1,10). It should however be noted that this lack of effect could be due to the fact that glutamate is only able to relax cerebral arteries via the N-methyl-D-aspartate (NMDA) type glutamate receptor if D-serine is present, which is not the case in our experiments (17). Therefore, also the influence of the NMDA receptor antagonist DL-APV (0.1 mM) was investigated, but no inhibition of the RRF-induced relaxation was detected (results not shown). This confirms our results with glutamate, but this is not in line with a study on pigs reporting that inhibition of the NMDA receptor inhibited the relaxing effect of the RRF (9).

Recently, it was suggested that H_2S, which is able to relax retinal arteries, may also partially mediate the RRF-induced relaxation (6,7). Since H_2S is synthesized from L-cysteine (18), the effect of L-cysteine on the RRF response was studied. No influence on the RRF-induced relaxation was observed, so the involvement of H_2S in the RRF response in mice could not be confirmed.

VI.5.2 Influence of oxidative stress on the RRF-induced relaxation

Oxidative stress is present in the retina and is associated with retinal diseases. It can be hypothesized that oxidative stress influences the effect or release of the RRF and consequently the retinal blood flow. Therefore, the influence of oxidative stress on the RRF-induced relaxation was evaluated. Inducing oxidative stress by the superoxide generator hydroquinone and superoxide dismutase inhibitor DETCA (19,20), did not reduce but rather enhanced the RRF response. This indicates that the RRF is chemically resistant to oxidative stress which is in line with a previous study demonstrating that the antioxidant resveratrol has no influence on the RRF response (21).

VI.5.3 Influence of cytochrome P450 and cyclooxygenase on the RRF-induced relaxation

EETs are found in the retina and elicit a vasorelaxation (22). Their role in the RRF-induced relaxation in mice was studied by using the selective cytochrome P450 epoxygenase inhibitor MS-PPOH, which inhibits the EET synthesis from arachidonic acid (23). As it did not have any effect, EETs are unlikely to be involved in the RRF response in mice. Similar results were previously reported in rats (12).

The potential involvement of cyclooxygenase products in the RRF-induced relaxation is controversial. Studies reported no reduction of the RRF response when cyclooxygenase is inhibited in cow, rat and mouse (1,10,11), while in pigs a reduction was reported (9). The presence of the cyclooxygenase inhibitor diclofenac had no significant inhibitory influence on the RRF response. However, very surprisingly, another cyclooxygenase inhibitor, flufenamic acid, substantially enhanced the RRF-induced relaxation. This enhancement was specific for the RRF-induced relaxation, since flufenamic acid did not enhance the sodium nitroprusside (SNP)-induced relaxation (results not shown).

VI.5.4 Mechanisms involved in the flufenamic acid-induced enhancement of the RRF response

Flufenamic acid is known to interfere with many pathways besides cyclooxygenase (24). For instance, flufenamic acid is known to inhibit TRP channels (25,24), which are also expressed in mouse retinas (26). However, as the broad-range TRP channel inhibitor 2-APB did not significantly enhance the RRF-induced relaxation, it seems that flufenamic acid does not enhance the RRF-induced relaxation by interference with TRP channels. Besides inhibition of TRP channels, flufenamic acid is also known to activate AMPK (27,28). In this study, the AMPK inhibitor compound C had no influence on the RRF-induced relaxation, suggesting that flufenamic acid does not exert its effect on the RRF through interference with AMPK. Although it is reported that flufenamic acid can inhibit the $GABA_A$ receptor (24,29), bicuculline, a $GABA_A$ antagonist, did not alter the RRF response. This result excludes the role of the $GABA_A$ receptor, however it does not exclude that the RRF-induced relaxation might be related to the GABA pathway (30).

Flufenamic acid is also known to inhibit CaCCs (24), and more specifically the TMEM16A subunit (24,31,32), which is expressed in the retina (33,34). The TMEM16A inhibitor T16Ainh-A01 augmented the RRF-induced relaxation significantly. On the other hand, the CaCC inhibitor CaCCinh-A01 did not influence the RRF-induced relaxation. In addition, the CaCC activator Eact did not reduce the RRF-induced relaxation. Taken together, the whole of these experiments indicate that CaCCs are probably not involved in the enhanced RRF-induced relaxation in the presence of flufenamic acid.

Flufenamic acid also inhibits gap junctions (24,35,36). Therefore, the effect of the gap junction inhibitor carbenoxolone was investigated. Surprisingly, it had the same effect as flufenamic acid, indicating that inhibition of gap junctions might be responsible for the enhancement of the RRF-induced relaxation. Since carbenoxolone did not alter the concentration-response curve of SNP (results not shown), its effect seems to be specific for the RRF-induced relaxation. It should be noted that 2-APB, which we used to assess the involvement of TRP channels, also inhibits gap junctions (35,37,38) and did not enhance the RRF-induced relaxation. This can be explained by the selectivity of the gap junction inhibitors for the different types of connexins, which form the gap junctions (39,40). Flufenamic acid and carbenoxolone display no selectivity for the different types of connexins, whereas 2-APB is more selective for Cx36 and Cx50 than for Cx23, Cx32, Cx43 and Cx46 (35,39). Moreover, carbenoxolone and flufenamic acid, in contrast to 2-APB, can inhibit pannexin 1, although this requires relatively high concentrations of flufenamic acid (24,41,42). Pannexins are membrane channels opening into the extracellular space while gap junctions are intercellular channels (43,44). However, when the retina and artery were incubated with ^{10}PanX1, a specific inhibitor of pannexin 1 and Cx46 (45,46), the RRF-induced relaxation was significantly decreased. This demonstrates that inhibition of pannexin 1 channels is certainly not responsible for the enhanced RRF-induced relaxation by flufenamic acid and carbenoxolone. This observation rather suggests that pannexin channels contribute in part to the relaxation induced by retinal tissue in mice.

VI.5.5 Sensitivity of retinal versus non-retinal arteries to the RRF

The RRF-induced relaxation seems to be independent of the type of arteries or retinas used. There is no indication that bovine retinas produce more RRF/mm² than mouse retinas or vice versa. More importantly, the RRF is not more active on retinal arteries since a similar relaxation is seen in mouse femoral arteries as in bovine retinal arteries. Previous studies reported that bovine retinal arteries relaxed completely when covered by bovine retinas (1,15). This was not seen in the present study, which can be explained by differences in the size of the retina and the amount of K^+ used to contract the artery. Using larger bovine retinas and 5 mM K^+ instead of 30 mM K^+, similar to previous studies, RRF-induced relaxation of almost 100% were observed.

VI.5.6 Conclusions

The main findings of this study are that (i) the presence of serotonin, glutamate or L-cysteine has no influence on the RRF relaxing pathway; (ii) the RRF response is resistant to oxidative stress; (iii) the RRF response is not influenced by the blockade of the cytochrome P450 and the cyclooxygenase pathway; (iv) the RRF relaxing pathway is enhanced in the presence of flufenamic acid or carbenoxolone; and (v) the RRF is not more effective on retinal arteries. Taken together, although the present study could exclude involvement of some substances and pathways, the mechanism of action of the RRF could not be elucidated. Therefore, further research is necessary to reveal the mechanism of the RRF, and thus the role of the RRF in physiology and pathophysiology.

VI.6 Acknowledgements

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Chapter VII

Stability, solubility and molecular size of the retinal relaxing factor from chicken retina

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Article in preparation

Chapter VII: stability, solubility and molecular size of the retinal relaxing factor from chicken retina

VII.1 Abstract

Purpose: the retinal relaxing factor (RRF) is a paracrine factor released from the retinal tissue, which causes smooth muscle cell relaxation. Despite all research on the RRF in the past years, the identity of the RRF remains unknown. Therefore, this study tried to identify characteristics of the RRF that may help to lead to chemical identification.

Materials and methods: the vasorelaxing effect of RRF-containing solutions, obtained by incubation of chicken retinas, was evaluated on isolated mouse femoral arteries, which were mounted in myograph baths. First of all, the stability of the RRF-containing solutions after freezing and freeze drying was investigated. Hexane and ethyl acetate extractions were performed on RRF-containing solutions to study the hydrophobicity of the RRF. Furthermore, the molecular size of the RRF was estimated by dialyzing RRF-containing solutions with dialysis membranes with different molecular weight cut-offs.

Results: the RRF-containing solutions still induced vasorelaxation when stored at 4°C, at -20°C or freeze dried. Furthermore, the RRF-containing solutions still caused vasorelaxation after hexane and ethyl acetate extractions. No vasorelaxing effect was observed when dialyzing the RRF-containing solutions through dialysis membranes with molecular weight cut-offs of 3.5-5 kDa and 0.1-0.5 kDa.

Conclusions: freezing and freeze drying do not affect the vasorelaxing effect of the chicken RRF. Although the identity of the RRF remains still unknown, it is suggested that the chicken RRF exists of one or more hydrophilic molecules smaller than 0.1-0.5 kDa.

VII.2 Introduction

In 1998 a new vasorelaxing factor released from the retina was discovered. This factor could not be identified as one of the already known vasorelaxants released from the retina such as nitric oxide, prostaglandins, adenosine or acetylcholine. Therefore, this unknown factor was given the name retinal relaxing factor (RRF) (1). Despite many research efforts in the following years, even nowadays the identity of the RRF remains still unknown (2). Initially, the characteristics of the RRF were explored by testing the vasorelaxing effect of potential RRF candidates on retinal arteries or by studying the vasorelaxing effect of the retina in presence of inhibitors of specific vasorelaxing pathways (1, 3-7). However, these methods failed at identifying the RRF. Therefore, there was need to use a different approach and to identify the RRF in a chemical manner. Earlier research demonstrated already that the RRF, released from bovine retinas, was thermostable, resistant to trypsin and hydrophilic (1). Another study on the RRF, this time released from rat retinas, confirmed that the RRF was thermostable, but contradicted that the RRF was hydrophilic (7).

Important aspects for the chemical analysis of a molecule in a biological solution are its stability, the possibility to concentrate the molecule, the water solubility and the molecular size. Therefore, we studied the vasorelaxing capacity of RRF-containing solutions after freezing and freeze drying, after extraction of hydrophobic substances and after dialyzing with membranes with different molecular weight cut-offs (mwcos).

VII.3 Materials and methods

VII.3.1 RRF-containing solution preparation

Chicken retinas were dissected from chicken heads (obtained from the local slaughterhouse) and incubated in 30 mM K^+ Krebs Ringer bicarbonate (KRB) solution (0.75 ml or 0.5 ml 30 mM K^+ KRB solution per retina) at 37°C and bubbled with carbogen (95% O_2 – 5% CO_2) gas. After 6 hours the solutions were centrifuged (2000 rpm, 5 min) and the supernatants, which are the RRF-containing solutions, were collected and stored at 4°C or -20°C. Blank solutions were obtained in the same way without adding chicken retinas to the 30 mM K^+ KRB solutions.

In some experiments, RRF-containing and blank solutions were freeze dried after storage at -20°C. After freeze drying, the original volume was restored with distilled water and tested for its vasorelaxing effect.

VII.3.2 Hexane and ethyl acetate extractions

Hydrophobic compounds were removed from the RRF-containing solutions (0.5 ml 30 mM K^+ KRB solution per retina and stored at -20°C) by hexane and ethyl acetate extractions. Hexane was added to the RRF-containing solutions (2:1 ratio) and vortexed during 20 min. Next, the solutions were centrifuged (2000 rpm, 5 min) and the hexane fractions were discarded. This procedure was carried out 3 times. Thereafter, ethyl acetate was added to the remaining solutions (2:1 ratio) and also vortexed during 20 min. The solutions were centrifuged (2000 rpm, 5 min) and the ethyl acetate fractions were discarded. After performing the ethyl acetate extractions 3 times, the remaining solutions were evaporated to dryness by nitrogen gas. Finally, distilled water was added to these samples to restore the volume to their original volume before evaporation. The same hexane and ethyl acetate extraction protocols were carried out on blank solutions.

VII.3.3 Dialysis experiments

The RRF-containing solutions (0.5 ml 30 mM K^+ KRB solution per retina and stored at -20°C) were first freeze dried and diluted in an appropriate volume distilled water, which fitted inside the dialysis membrane tube (Float-A-Lyzer G2). Next, these concentrated RRF-containing solutions were loaded inside a dialysis membrane tube, which was placed in a beaker containing distilled water (100 times the volume of the RRF-containing solution inside the dialysis membrane tube). The dialysis process was stopped after 12 hours and the solutions from inside the dialysis device were collected. These were freeze dried again in order to perform the same dialysis procedure again. The solutions inside the dialysis device were collected and freeze dried again, and finally diluted in 30mM K^+ KRB solution to their original volume. Blank solutions were dialyzed in the same way. The dialysis procedure was carried out twice, because besides diffusion also osmosis took place through the dialysis membrane. As a result, molecules which could pass through the dialysis membrane might have been present inside the dialysis membrane tube in a concentration still high enough to cause vasorelaxation.

VII.3.4 Tissue preparation

Mouse femoral arteries were dissected from male Swiss mice (8-12 weeks old, obtained from Janvier, Saint-Berthevin, France) and cleaned from their surrounding tissue. A piece of femoral artery was then mounted in a myograph bath (constructed by the technical department of the research unit) by guiding two stainless steel wires of 40 µm diameter through the lumen of the femoral artery. One wire is connected to a force-displacement transducer and the other wire to a micrometer. The myograph bath contained 10 ml KRB solution at 37°C, pH 7.4 and bubbled with carbogen gas.

The femoral arterial segments could equilibrate during 30 min. Thereafter, the passive wall tension-internal circumference characteristics of the femoral arterial segments were determined, so the internal circumference of the arterial segment could be set at 90% of its internal circumference at a transmural pressure of 100 mmHg. Subsequently, the femoral arteries were contracted 3 times by adding 120 mM K^+ KRB solution and 30 µM $PGF_{2\alpha}$ to the organ bath. Thereafter, the myograph baths were emptied in order the perform a bioassay in which the arteries were continuously superfused by different warmed and carbogen bubbled KRB solutions. Those solutions dripped on the artery at a rate of 1 ml / 10 min and at the same time the solution in the myograph bath was continuously removed.

VII.3.5 Experimental protocol

The vasorelaxing effect of blank and RRF-containing solutions, on which also hexane and ethyl acetate extractions or dialyses may have been performed, were tested in a bioassay. In this bioassay a mouse femoral artery was superfused by a warmed and carbogen bubbled 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ solution at a rate of 1 ml / 10 min. Subsequently, the superperfusion solution was changed to a blank solution, to which also 30 µM $PGF_{2\alpha}$ was added. Thereafter, the superperfusion solution was changed again to the 30 mM K^+ KRB and 30 µM $PGF_{2\alpha}$ solution. Finally, the femoral artery was superfused by a RRF-containing solution, to which 30 µM $PGF_{2\alpha}$ was added. This protocol was carried out three times and the averages of the relaxations induced by the blank and RRF-containing solutions were calculated.

VII.3.6 Chemicals and devices

The KRB solution contained the following components (mM): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. 120 mM K^+ and 30 mM K^+ KRB solutions were made by equimolar replacement of NaCl by KCl. Hexane was obtained from Thermo Fisher Scientific (Waltham, MA, USA), ethyl acetate from Sigma (St. Louis, MO, USA), $PGF_{2\alpha}$ from Zoetis (Florham park, NJ, USA) and Float-A-Lyzer G2 from SpectrumLabs (Rancho Dominguez, CA, USA).

VII.3.7 Data analysis

The data were computed as means ± S.E.M. and evaluated statistically using a Wilcoxon test. Two groups of data were considered significantly different if $P<0.05$. Relaxations are expressed in percent decrease of the pre-existing tone elicited by $PGF_{2\alpha}$. N = number of preparations tested all from different animals.

VII.4 Results

VII.4.1 Vasorelaxing effect of RRF-containing solutions

Superfusing mouse femoral arteries by RRF-containing solutions (0.75 ml 30 mM K^+ KRB solution per retina) stored at 4°C induced a significant stronger relaxation compared to the blank solutions (41.33 +/- 6.86% versus 5.72 +/- 1.34%) (Fig.VII.1A). Also the RRF-containing solutions (0.75 ml 30 mM K^+ KRB solution per retina) stored at -20°C were still able to induce vasorelaxations, compared to blank solutions (28.50 +/- 4.17% versus 2.58 +/- 2.64%) (Fig.VII.1B). Moreover, freeze dried RRF-containing solutions (0.75 ml 30 mM K^+ KRB solution per retina), dissolved in distilled water, could still induce vasorelaxations, which were larger than the ones induced by blank solutions (26.99 +/- 1.98% versus 6.49 +/- 2.54%) (Fig.VII.1C). To make sure that the concentration of RRF was high enough during all steps of the identification process, it was decided to make more concentrated RRF-containing solutions to use in further experiments (incubation in 0.5 ml instead of 0.75ml 30 mM K^+ KRB solution per retina).

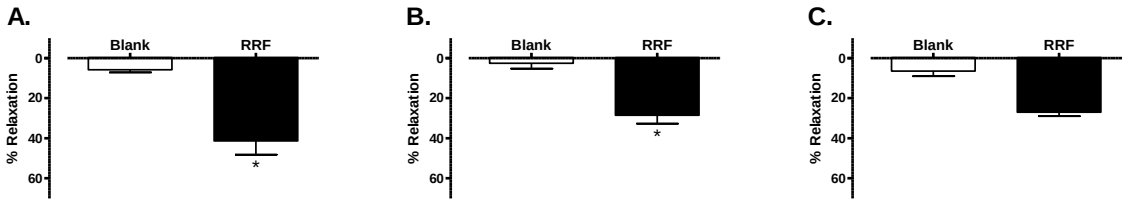

Figure VII.1 Percentage relaxation of mouse femoral arteries induced by a blank solution (Blank) or a RRF-containing solution (RRF) stored at 4°C, n=6 (A), stored at -20°C, n=6 (B), stored at -20°C and freeze dried, n=4 (C); *p<0.05

VII.4.2 Hydrophobicity of the RRF

The water fractions of RRF-containing solutions (0.5 ml 30 mM K^+ KRB solution per retina) after hexane and ethyl acetate extractions induced vasorelaxations (33.69 +/- 2.88%), significant larger than the vasorelaxations induced by the blank solutions (7.13 +/- 0.61%) (Fig.VII.2).

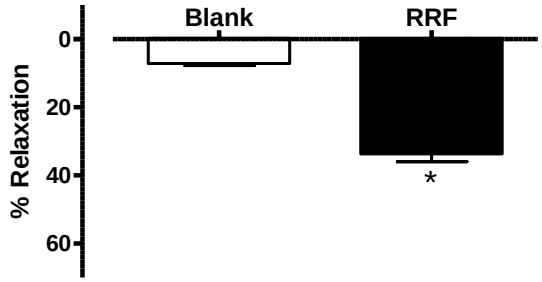

Figure VII.2 Percentage relaxation of mouse femoral arteries induced by the water fraction of a blank solution (Blank) or a RRF-containing solution (RRF) after hexane and ethyl acetate extractions, n=6; *p<0.05

VII.4.3 Molecular size of the RRF

After dialysis through membranes with mwco of 3.5-5 kDa or 0.1-0.5 kDa the RRF-containing (0.5 ml 30 mM K^+ KRB solution per retina) and blank solutions did not induce any significant relaxation on mouse femoral arteries, 4.17 +/- 1.27% versus 4.21 +/- 0.79% and 6.82 +/- 1.20% versus 5.08 +/- 0.96%, respectively (Fig.VII.3A,B).

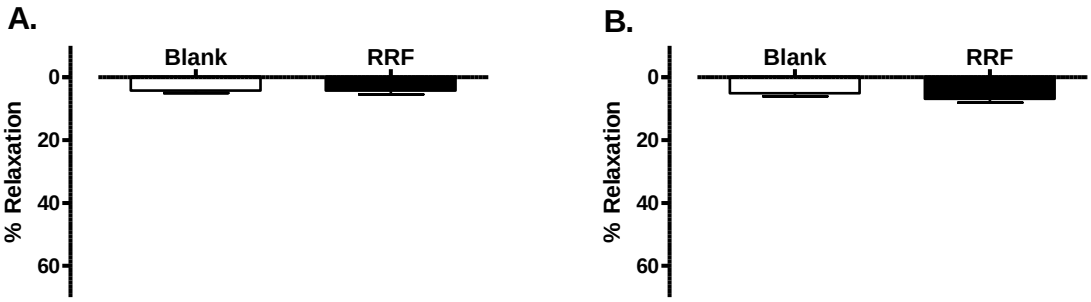

Figure VII.3 Percentage relaxation of mouse femoral arteries induced by the remaining blank solution (Blank) or a RRF-containing solution (RRF) after filtration through a dialysis membrane with mwco of 3.5-5 kDa, n=6 (A), 0.1-0.5 kDa, n=6 (B)

VII.4.4 Vasorelaxing effect of RRF-containing solutions after long-term storage at -20°C

RRF-containing solutions (0.5 ml 30 mM K^+ KRB solution per retina), stored for about 10 months at -20°C, still induced strong vasorelaxations, significantly larger than the blank solutions, which were also stored at -20°C for the same period (41.05 +/- 2.60% versus 6.11 +/- 1.27%) (Fig.VII.4A). Also after freeze drying, these long-term stored RRF-containing solutions were able to induce vasorelaxations, which were significantly larger than the vasorelaxations induced by long-term stored freeze dried blank solutions (37.78 +/- 4.57% versus 5.73 +/- 1.09%) (Fig.VII.4B).

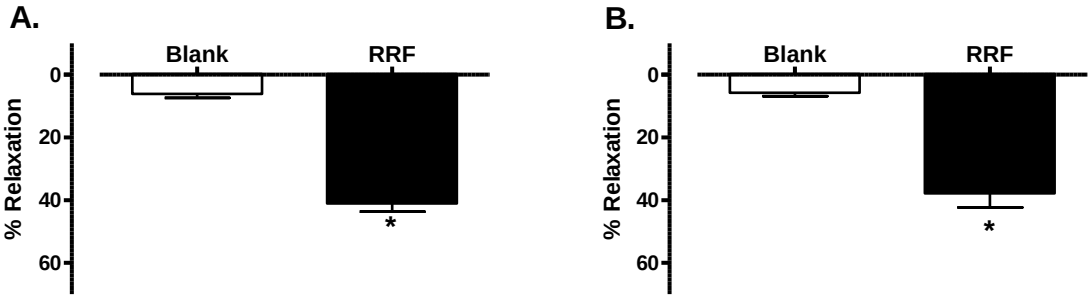

Figure VII.4 Percentage relaxation of mouse femoral arteries induced by a blank solution (Blank) or a RRF-containing solution (RRF) stored for 10 months at -20°C, n=6 (A), stored for 10 months at -20°C and freeze dried, n=6 (B); *p<0.05

VII.5 Discussion

This study aimed to obtain basic information on the chemical characteristics of the RRF in order to be able to design more sophisticated methods to identify the RRF. In order to do so, it was crucial to create a solution containing a high concentration of the RRF. In previous studies RRF-containing solutions were made out of bovine or rat retinas (1, 7). Here, a RRF-

containing solution was made out of chicken retinas, of which it has been proved in our previous study that these retinas release also the RRF. Indeed, 30 mM K^+ KRB solutions incubated with chicken retinas were able to induce a strong vasorelaxation. These RRF-containing solutions were stored at 4°C and were used as fresh as possible in the experiments.

However, research aiming to identify the RRF chemically will require longer storage of large quantities of concentrated RRF-containing solutions. Therefore, the vasorelaxing effect of the RRF-containing solutions after storage at -20°C was tested. As the RRF-containing solutions still induced a significant vasorelaxation, the RRF seems to be resistant to storage at -20°C. Furthermore, the influence of freeze drying on the effect of the RRF was evaluated as concentrating the solution by freeze drying would be useful for the RRF identification. Also after freeze drying, the RRF-containing solutions could still induce substantial vasorelaxations.

Some contradictory results exist about the hydrophobicity of the RRF. According to one study on bovine retina, the RRF is hydrophilic (1); according to another study on rat retina, the RRF is hydrophobic (7). In both studies hexane extractions were used to determine the hydrophobicity. Here, we used hexane and ethyl acetate extractions to determine the hydrophobicity of the RRF. Hexane is a strong hydrophobic solvent in which only strong hydrophobic molecules will dissolve. In contrast, ethyl acetate is a weak hydrophobic solvent in which also less strong hydrophobic molecules, which have remained after the hexane extractions, can dissolve. The finding that RRF-containing solutions were still able to induce strong vasorelaxations after combined hexane and ethyl acetate extractions indicates that the RRF is a hydrophilic molecule. This is in accordance with the study on bovine retinas (1).

The next step in the identification process of the RRF was determining the molecular size. Depending on the molecular size of the RRF different strategies for the chemical identification will have to be used. RRF-containing solutions were dialyzed using membranes with a mwco of 3.5-5 kDa. The RRF-containing solution inside the dialysis device, containing only molecules larger than 3.5-5 kDa, could not induce any vasorelaxation. From which there could be concluded that the RRF contains no molecules larger than 3.5-5 kDa. Next, a similar experiment was performed, but by using dialysis membranes with a lower mwco of 0.1- 0.5 kDa to evaluate if the RRF contains any molecules larger than 0.1-0.5 kDa. These dialyzed RRF-containing solutions, now containing only molecules larger than 0.1-0.5 kDa, could also not induce vasorelaxations.

So far, it seems that the RRF exists of no molecules larger than 0.1-0.5 kDa. However, it may also be possible that the absence of any vasorelaxation in the last experiments was due to the fact that in these experiments RRF-containing solutions were used already stored for 10 months. Too long storage of the RRF-containing solutions could results in a not properly working RRF. To exclude this possibility, the vasorelaxing effect of RRF-containing solutions stored for about 10 months at -20°C was evaluated, after defrosting and after freeze drying. After both procedures, the RRF-containing solutions induced still a strong vasorelaxation, which means that the RRF still works after long-term storage at -20°C and freeze drying. Taken together as (i) long-term storage at -20°C and subsequently freeze drying does not affect the vasorelaxing influence of the RRF-containing solutions and (ii) no vasorelaxing effect could be observed after dialyzing the RRF-containing solutions by dialysis membranes with a mwco of 3.5-5 kDa or 0.1-0.5 kDa, it thus seems that the molecular size of the RRF is not larger than 0.1-0.5 kDa.

To conclude, although the identity of the RRF itself remains unknown for now, this study demonstrated that the chicken RRF contains one or more hydrophilic molecules smaller than 0.1-0.5 kDa. Moreover, the chicken RRF is resistant to long-term freezing and freeze drying. This characteristic might be of great importance for the success of the chemical identification of the RRF.

VII.6 Acknowledgements

This work was supported by the Fund of Research in Ophthalmology (FRO). The authors would like to thank Lies Vancraeynest and Tom Vanthuyne for the excellent technical assistance, and the slaughterhouse Nollens in Kruishoutem to provide us with chicken heads.

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Chapter VIII

Vasorelaxing effect of resveratrol on bovine retinal arteries

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Chapter VIII: vasorelaxing effect of resveratrol on bovine retinal arteries

VIII.1 Abstract

Purpose: resveratrol is a red wine polyphenol that causes vasorelaxation, which could be of interest in the treatment or prevention of eye diseases with an impaired blood flow. In this study, the vasorelaxant capacity of resveratrol (cis and trans) on bovine retinal arteries, its mechanism, and its influence on the relaxation induced by the retinal relaxing factor (RRF) were examined.

Materials and methods: isolated bovine retinal arteries were mounted in wire myographs for isometric tension measurements. Concentration-response curves of cis- and trans-resveratrol and concentration-response curves of resveratrol in absence or presence of the endothelium or different inhibitors were constructed. Relaxations elicited by the RRF with and without resveratrol incubation were also compared.

Results: both resveratrol isomers caused a similar strong concentration-dependent relaxation. Removal of the endothelium or blocking endothelium-dependent pathways did not change the relaxation. Also K^+ channel blockers did not reduce the relaxation, except the 120 mM K^+ Krebs Ringer bicarbonate solution. Phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate blocked the relaxation partially, and so did the inhibition of heme oxygenase-1. Blocking adenylyl cyclase, AMP-activated protein kinase, estrogen receptors, sirtuin 1 or sarco/endoplasmic reticulum Ca^{2+}-ATPase did not have an effect. The relaxation caused by the RRF was not altered by resveratrol incubation.

Conclusions: cis- and trans-resveratrol relax bovine retinal arteries similarly and concentration-dependently. The main relaxation mechanism remains unclear, but K^+ channels, carbon monoxide and the myosin phosphatase pathway may be involved. Resveratrol does not have an influence on the RRF.

VIII.2 Introduction

In an era with huge interest in healthy food and nutraceuticals, much attention is given to resveratrol, a polyphenolic compound present in the skin of grapes and thus in red wine (1-3). Resveratrol is also found in other food, such as cranberries and peanuts. However, it is best absorbed from the wine matrix. The interest in resveratrol has exploded since resveratrol was advanced as being responsible for the cardiovascular benefit of moderate red wine consumption (2). This hypothesis originates from the so-called “French paradox”, the lower incidence of cardiovascular diseases in the French population despite their higher intake of dietary saturated fat (1, 4). The potential beneficial effects of resveratrol are explained by many studies showing that resveratrol has vasorelaxant, antioxidant, antiapoptotic, antitumorigenic, antiangiogenic and anti-inflammatory properties (5).

These properties are also of interest for diseases of the eye. Many studies have already focused on the potential effect of resveratrol on eye diseases. It has been proven that resveratrol causes a decrease in the expression of glaucoma markers due to its antioxidant, anti-inflammatory and antiapoptotic properties (6). Resveratrol also reduces diabetic retinopathy (7) and apoptosis and/or oxidative stress in models of (diabetic) cataract (8-10). Furthermore, resveratrol protects retinal pigment epithelial cells against oxidative stress, which is involved in the pathogenesis of age-related macular degeneration (11). Resveratrol also inhibits the tumor growth of uveal melanoma and retinoblastoma and the neovascularization in animal models of retinopathy of prematurity and macula telangiectasia (12-15).

Resveratrol is also well known for its vasorelaxing influence. However, studies on its effect on the retinal circulation are scarce despite the fact that the vasorelaxant activity of resveratrol would be beneficial to treat or prevent eye diseases associated with an impaired blood flow, such as glaucoma, age-related macular degeneration and diabetic retinopathy (5). As yet only one study reported that resveratrol relaxes retinal arteries; porcine retinal arterioles are relaxed by resveratrol and this occurs through both an endothelium-dependent (nitric oxide (NO)-mediated) and endothelium-independent (large-conductance Ca^{2+}-activated K^{+} channels-mediated) mechanism (16).

The aim of the present study was to investigate whether resveratrol also relaxes retinal arteries from another species, namely bovine retinal arteries; potential differences between cis- and trans-resveratrol; potential mechanisms involved in the vasorelaxing action of resveratrol; and a potential influence of resveratrol on the continuously released retinal relaxing factor (RRF) (17-19).

VIII.3 Methods

VIII.3.1 Tissue preparation

Bovine eyes were obtained from the local slaughterhouse and were transported and stored in cold Krebs Ringer bicarbonate (KRB) solution. Extraocular muscles and connective tissue were cut off the eye. The eyecup was then cut open and the anterior segment and the vitreous were removed. Under a dissection microscope, the part of the retinal artery between the optic disc and the first branching of the artery was gently detached with its surrounding retinal tissue from the choroid.

The retinal arterial segments were mounted in wire myographs (constructed by the technical department of the research unit) for isometric tension measurements, containing 10 ml KRB solution (37°C, pH 7.4 and bubbled with 95% O_2 - 5% CO_2). Two stainless steel wires of 40 µm diameter were guided through the lumen of the vessel. One wire was connected to a force-displacement transducer and the other to a micrometer. Before guiding the wires through the lumen of the artery, the adhering retinal tissue was carefully removed. The femoral arteries were dissected from male Swiss mice (8-12 weeks, from Janvier (Saint-Berthevin, France)) and cleaned from its surrounding tissue after guiding the first wire through the lumen of the artery.

The arterial segments were allowed to equilibrate for 30 min in the KRB solution before their normalization. During the normalization process the passive wall tension-internal circumference characteristics of the vessels were determined. The internal circumference of the vessels was then set to 90% of the internal circumference of the vessels at a transmural pressure of 100 mmHg.

At the start of each experiment the arteries were three times contracted by adding 120 mM K^+ KRB solution and 30 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the organ bath. Next, 0.1 mM

acetylcholine was added to a retinal artery contracted with 30 µM $PGF_{2\alpha}$ to test the functionality of the endothelium (17, 19).

VIII.3.2 Experimental protocols

To test the potential difference in relaxation between cis- and trans-resveratrol, concentration-response curves of cis- and trans-resveratrol (1-100 µM) were performed on the same $PGF_{2\alpha}$–contracted bovine retinal arteries. To investigate the relaxation mechanism the concentration-response curves of resveratrol were performed on arteries with or without endothelium or in presence or absence of blockers of certain pathways. The influence of resveratrol on the RRF response was tested by comparing the relaxing effect, elicited by the placing retinal tissue on the precontracted artery, with and without incubation of the retina and artery with resveratrol, performed on the same artery with the same retina. Because placing a piece of bovine retina on bovine retinal arteries caused 100% relaxation and so a possible enlargement of the relaxing effect could not be detected, mouse retinas and mouse femoral arteries were used instead, as described before (20).

VIII.3.3 Drugs and chemicals

The KRB solution contained the following components (mM): NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O. The 120 mM K^+ and 30 mM K^+ KRB solutions were made by equimolar replacement of NaCl by KCl. (Trans-) Resveratrol, acetylcholine, tetraethylammonium (TEA), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, 4-aminopyridine (4-AP), glibenclamide, 1H-[1,2, 4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), phorbol 12-myristate 13-acetate (PMA), compound C, zinc protoporphyrin IX (ZnPP), fulvestrant (ICI 182780), cyclopiazonic acid (CPA), niacinamide, PD98059, SQ 22,536 and EX-527 were obtained from Sigma-Aldrich (St. Louis, MO, USA), $PGF_{2\alpha}$ from Zoetis (Florham Park, NJ, USA), phorbol 12,13-dibyturate (PDBu) from Axon MedChem (Groningen, The Netherlands), cis-resveratrol from Cayman (Ann Harbor, MI, USA) and papaverine from Sterop (Brussels, Belgium). All stock solutions were made in DMSO, except those of $PGF_{2\alpha}$, acetylcholine, TEA, 4-AP, L-NAME, niacinamide and papaverine, which were made in water, indomethacin was made in ethanol and ZnPP was made in 0.1 M NaOH.

VIII.3.4 Data analysis

The data were computed as means ± S.E.M. and evaluated statistically using a Wilcoxon test. Two groups of data were considered significantly different if $P<0.05$. Relaxations are expressed in percent decrease of the pre-existing tone elicited by $PGF_{2\alpha}$ (n = number of preparations tested all from different animals).

VIII.4 Results

VIII.4.1 Effect of trans- and cis-resveratrol

Resveratrol relaxed bovine retinal arteries clearly concentration-dependently (Fig.VIII.1). A rapid and almost complete relaxation was reached at the concentration of 0.1 mM (trans-resveratrol: 102.27 ± 7.09%; cis-resveratrol: 90.44 ± 8.08%). The EC_{50} of trans- and cis-resveratrol was, respectively, 31.67 ± 9.64 µM and 39.54 ± 8.19 µM (Fig.VIII.2). Because there was no significant difference detected, further experiments were performed using only trans-resveratrol.

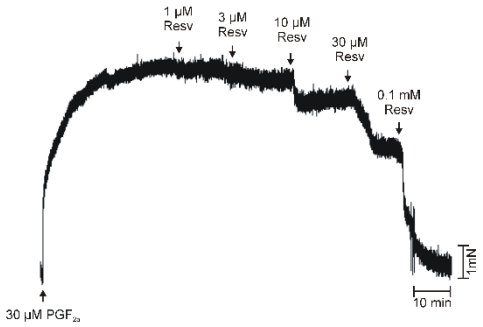

Figure VIII.1 Original tracing showing the relaxation curve of (trans-)resveratrol (Resv) on 30 µM $PGF_{2\alpha}$ contracted bovine retinal arteries

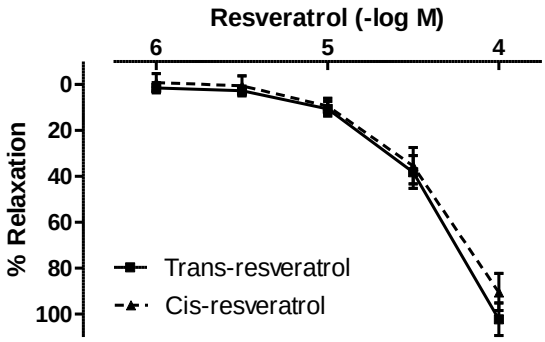

Figure VIII.2 Percentage relaxation of bovine retinal arteries caused by trans- and cis-resveratrol (1-100 µM); n=4

VIII.4.2 Role of endothelium and NO

Removal of the endothelium, performed by rubbing a hair through the lumen and considered successful if 0.1 mM acetylcholine did not induce a relaxation, did not reduce the relaxation (Fig.VIII.3A). Also, the incubation with the NO synthase inhibitor L-NAME (0.1 mM, 10 min), the cyclooxygenase inhibitor indomethacin (10 µM, 20 min), the soluble guanylyl cyclase (sGC) inhibitor ODQ (10 µM, 20 min) or the mitogen-activated protein kinases/extracullular signal-regulated kinases (MAPK/ERK) inhibitor PD98059 (10 µM, 20 min) did not change the relaxation (Fig.VIII.3B,C,D,E).

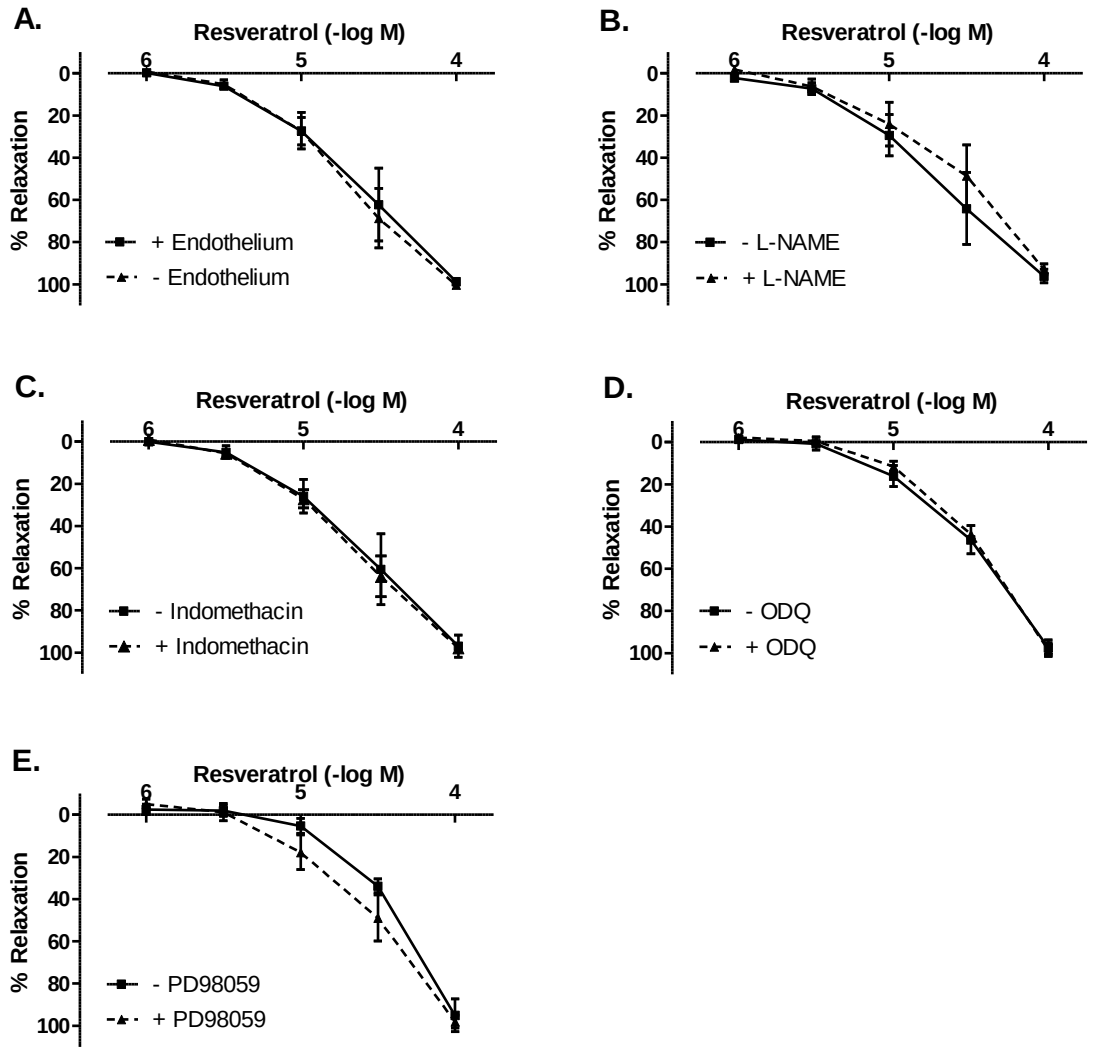

Figure VIII.3 Percentage relaxation of bovine retinal arteries caused by resveratrol (1-100 µM) in presence or absence of the endothelium (A), L-NAME (0.1 mM) (B), indomethacin (10 µM) (C), ODQ (10 µM) (D), PD98059 (10 µM) (E); n=4

VIII.4.3 Role of K^+ channels

The 120 mM K^+ KRB solution (20 min) inhibited the resveratrol relaxation slightly. However, a significant difference was detected at the concentration of 0.1 mM resveratrol (97.02 ± 3.08% versus 82.88% ± 4.74%) (Fig.VIII.4A). The nonselective K^+ channel blocker TEA (10 mM, 20 min), the voltage-gated K^+ channel blocker 4-AP (2 mM, 20 min) and the ATP-sensitive K^+ channel blocker glibenclamide (10 µM, 20 min) did not reduce the relaxation (Fig.VIII.4B,C,D). The relaxation in the presence of glibenclamide was even somewhat increased, probably a result of the impaired precontractile tone.

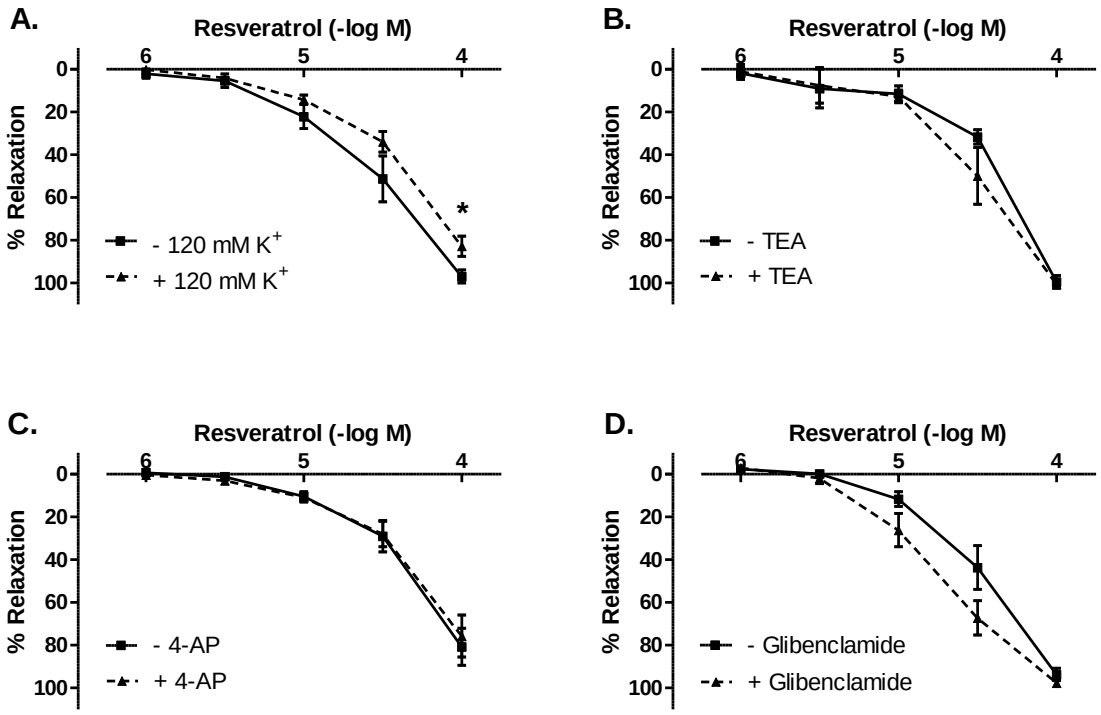

Figure VIII.4 Percentage relaxation of bovine retinal arteries caused by resveratrol (1-100 µM) in absence or presence of 120 mM K^+ KRB solution (A), TEA (10 mM) (B), 4-AP (2 mM) (C), glibenclamide (10 µM) (D); n=4-6; *P<0.05

VIII.4.4 Role of protein kinase C

Both protein kinase C (PKC) activators PMA (10 µM, 20 min) and PDBu (1 µM, 20 min) decreased the relaxation caused by resveratrol (Fig.VIII.5A,B). The presence of PMA caused a small but significant increased relaxation in response to 3 µM of resveratrol, but a large significant decreased relaxation in response to 100 µM of resveratrol (89.51 ± 5.40% versus 60.45 ± 5.03%). PDBu reduced the relaxation significant at the resveratrol concentrations of 1 µM, 30 µM and 100 µM (81.31 ± 7.16% versus 48.23 ± 7.21% at 100 µM resveratrol).

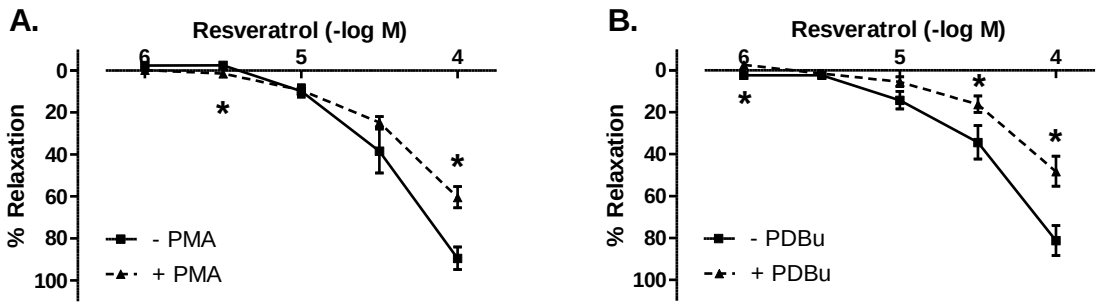

Figure VIII.5 Percentage relaxation of bovine retinal arteries caused by resveratrol (1-100 µM) in absence or presence of PMA (10 µM) (A), PDBu (1 µM) (B); n=5-6; *P<0.05

VIII.4.5 Role of other mediators

The adenylyl cyclase blocker SQ 22,536 (0.1 mM, 20 min) and the AMP-activated protein kinase (AMPK) blocker compound C (10 µM, 20 min) did not reduce the resveratrol-induced relaxation (Fig.VIII.6A,B). In addition, neither did the estrogen receptor blocker fulvestrant (0.1 mM, 20 min), nor the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) blocker CPA (10 µM, 20 min) and nor the sirtuin 1 blockers EX-527 (5 µM, 15 min) and niacinamide (10 mM, 20min) (results not shown) reduce the resveratrol relaxation (Fig.VIII.6C,D,E). The incubation with fulvestrant reduced the contractile tone, explaining the larger relaxation percentages. ZnPP (10 µM, 60 min), a blocker of heme oxygenase-1 (HO-1), did cause a significant reduction at resveratrol concentrations of 3 µM, 10 µM and 100 µM (95.50 ± 1.22% versus 78.37 ± 8.48% at 100 µM resveratrol) (Fig.VIII.6F).

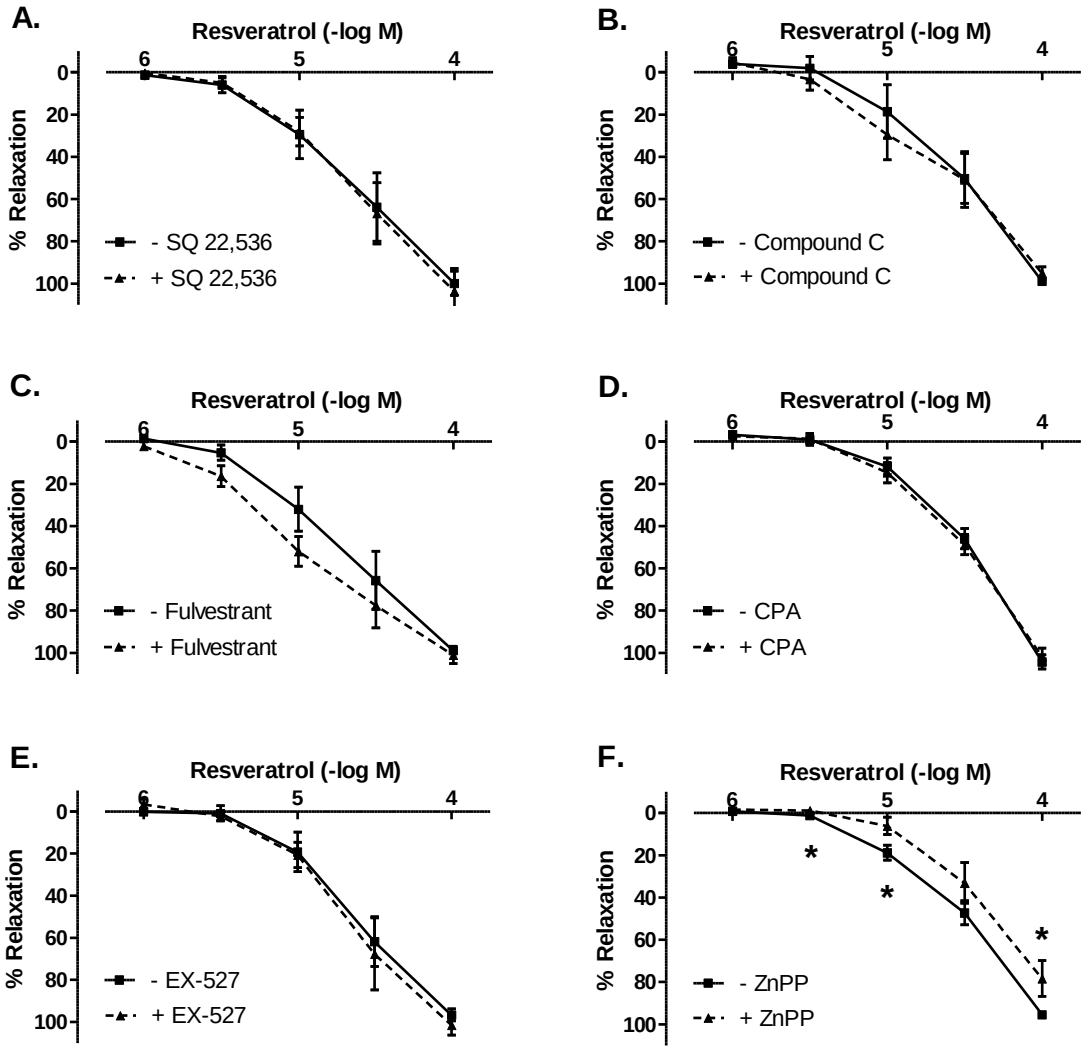

Figure VIII.6 Percentage relaxation of bovine retinal arteries caused by resveratrol (1-100 µM) in absence or presence of SQ 22,536 (0.1 mM) (A), compound C (10 µM) (B), fulvestrant (0.1 mM) (C), CPA (10 µM) (D), EX-527 (5 µM) (E), ZnPP (10 µM) (F); n=4-6; *P<0.05

VIII.4.6 Influence of resveratrol on the RRF

The relaxation of mouse femoral arteries caused by placing a mouse retina on top of the femoral artery (as previously described (20)) was similar before and after incubating the retina and femoral artery with resveratrol (0.1 mM, 15 min) (24.91 ± 2.35% and 24.40 ± 4.91% respectively) (Fig.VIII.7).

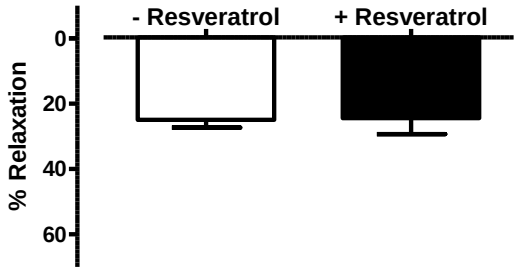

Figure VIII.7 Percentage relaxation of mouse femoral arteries caused by the RRF in absence or presence of resveratrol (0.1 mM); n=4

VIII.5 Discussion

Our study demonstrates for the first time that resveratrol has a very prominent relaxing influence on bovine retinal arteries. Relaxation starts at a concentration of 3 µM and is almost complete at a concentration of 0.1 mM, similar to the relaxation on porcine retinal arterioles (16). Retinal arteries have approximately the same sensitivity to resveratrol as porcine and sheep coronary arteries and rat aortas because their EC_{50} is similar (21-23). The EC_{50} turns out to be higher for human internal mammary arteries and lower for rat abdominal aortas and rat mesenteric arteries (24-26).

Resveratrol exists as both cis- and trans-isomers. Cis-resveratrol is found only in small amounts in grapes because trans-resveratrol is the more stable natural form. However, trans-resveratrol can be transformed into the cis-isomer by ultraviolet irradiation or yeast isomerases during fermentation, with the result that cis-resveratrol is present in wine at variable concentrations (5, 27-29). Differences in effectivity of the isomers have been reported. Several studies provided evidence that the antioxidant capacity of trans-resveratrol is the strongest, whereas other studies showed a similar effect (27, 30, 31). Furthermore, trans-resveratrol has been shown to be a stronger inhibitor of platelet aggregation, angiogenesis and tumor growth in vivo than cis-resveratrol, and only trans-resveratrol is able to inhibit vascular inflammation (32-34). The present study shows that both isomers have a similar vasorelaxing effect, at least in bovine retinal arteries. To the best of our knowledge, isomer sensitivity has not yet been studied on other blood vessels.

Many studies have shown that the vasorelaxing effect of resveratrol depends at least partially on the presence of endothelium and the formation of NO (16, 21, 25). On porcine retinal arteries, the relaxation of resveratrol is partially mediated by NO released from endothelium

(16). However, our study provides no evidence for that in bovine retinal arteries. Removal of the endothelium, blocking NO synthase with L-NAME or blocking cyclooxygenase with indomethacin does not alter the relaxation in response to resveratrol. However, it should also be noted that on some other blood vessels L-NAME failed to block the resveratrol relaxation (35, 36) and cyclooxygenase has not yet been reported to be involved in the relaxation caused by resveratrol (16, 21, 25, 35, 37). It has also been revealed that the release of NO in porcine retinal arteries is activated by the MAPK/ERK pathway and that NO activates then sGC (16). Also, these mediators do not seem to be involved in the relaxation on bovine retinal arteries.

The endothelium-independent pathway of resveratrol on porcine retinal arterioles is mediated by large-conductance Ca^{2+}-activated K^+ channels, the nonselective K^+ channel blocker TEA blocked the relaxation significantly (16). This is not seen in bovine retinal arteries. Incubation with 120 mM K^+ KRB solution reduced the relaxation caused by 0.1 mM resveratrol minimal, indicating only a small involvement of K^+ channels. In other tissues, resveratrol relaxations could be partially blocked by glibenclamide or 4-AP, even when TEA failed to block the relaxation (26, 38). However, this was not the case with bovine retinal arteries. All used K^+ blockers failed to block the relaxation of resveratrol. This is in line with what has been reported on mouse corpus cavernosum (35).

Resveratrol has also been reported to form cyclic guanosine monophosphate (cGMP) through particulate or membrane-bound guanylyl cyclase (pGC) and not through sGC (22). Because pGC is blocked by PKC, the PKC activators PMA and PDBu were used to inhibit pGC (39). Both reduced the relaxation of resveratrol significantly, with PDBu having the strongest effect. From this could be concluded that resveratrol induces relaxation partially via pGC. However, earlier research on bovine retinal arteries showed very little relaxation in response to cGMP (40). Therefore, it is unlikely that pGC plays a substantial role in the strong resveratrol relaxation. It should be mentioned that the activation of PKC has also other effects besides blocking pGC, such as inactivating myosin phosphatase (as does Rho kinase), which then can no longer dephosphorylate the light chain of myosin to induce relaxation (41). Therefore, an interaction of resveratrol with the myosin phosphatase pathway could be involved in the relaxation effect of resveratrol. Rho kinase inhibition has previously already been reported to make a major contribution to the resveratrol-induced vasorelaxation (42). We tried to test this influence by blocking Rho kinase with Y-27632 dihydrochloride, but the incubation caused loss of

contractile tone, which could be explained by the fact that Y-27632 dihydrochoride is a strong dilator of $PGF_{2\alpha}$ contracted retinal arteries (43). To be sure that the inhibition of the resveratrol relaxation by PMA or PDBu is not aspecific, experiments were carried out with papaverine, a strong dilator of bovine retinal arteries (40). Relaxations in response to papaverine were not affected by PDBu (Fig.VIII.8), proving that PDBu blocks the resveratrol relaxation in a specific way.

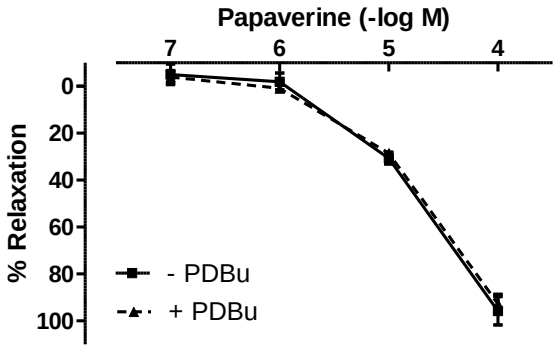

Figure VIII.8 Percentage relaxation of bovine retinal arteries caused by papaverine (0.1-100 µM) in absence or presence of PDBu (1 µM), n=4

The potential involvement of cyclic adenosine monophosphate (cAMP) was also verified, although resveratrol activates rather cGMP than cAMP (22). Indeed, the resveratrol relaxation was not changed by inhibiting adenylyl cyclase with SQ 22,536. Furthermore, it has been described that resveratrol interacts with SERCA, AMPK, sirtuin 1 and the estrogen receptor (44-48), but none of these seem to be involved in the relaxation as respectively CPA, compound C, EX-527, niacinamide and fulvestrant did not alter the resveratrol relaxation. It has been described that resveratrol activates HO-1 (49), which can be confirmed because the relaxation was a little, but significantly, reduced by ZnPP, a HO-1 blocker. HO-1 catalyzes the degradation of heme, which results in the production of carbon monoxide (CO) (50).

So the relaxation mechanisms of resveratrol on porcine retinal arterioles seem to differ from those on bovine retinal arteries (16). However, it should be mentioned that besides species and caliber differences, the experimental setup was also different, which might have influenced the results. In our study, the arteries were mounted into a wire myograph for isometric force measurements, in the other study the arteries were cannulated for isobaric diameter measurements. The arteries in the wire myograph show only a passive tension, so a vasoconstrictor has to be added first in order to see a vasorelaxing effect. The cannulated

arteries in the pressure myograph have already a certain tone, probably due to myogenic autoregulatory mechanisms (51), so a preconstrictor is not needed to detect vasorelaxing effects.

It has been shown that the presence of retinal tissue influences the tone of retinal arteries by releasing a relaxing factor (so-called retinal relaxing factor, RRF) (17, 18). The identity of the RRF is still a matter of debate, but Takir et al. investigated the vasorelaxing mechanism of the RRF. An induction of relaxation by activating inward rectifier K^+ channels and an inhibition of contraction by inhibiting the Ca^{2+} sensitization pathway probably via the Rho kinase pathway was suggested (43). Changes in the RRF pathway could affect the retinal blood flow. Therefore, in bio-assay experiments we also investigated whether resveratrol influences the relaxing effect of RRF elicited by placing mouse retinal tissue on a precontracted mouse femoral artery. However, the RRF-induced relaxing effect was not influenced by incubation with resveratrol.

In conclusion, trans- and cis-resveratrol relax bovine retinal arteries similarly and clearly concentration dependently. The relaxation mechanism of resveratrol on these arteries is still unclear and differs from that on porcine retinal arteries. There might be a small involvement of K^+ channels, CO and the myosin phosphatase pathway. Further mechanisms explaining the complete relaxation caused by resveratrol remain to be discovered. In addition, resveratrol does not seem to influence the relaxing influence of the RRF.

VIII.6 Acknowledgements

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Chapter IX

Discussion and future perspectives

Chapter IX: discussion and future perspectives

The retinal blood flow, which lacks any adrenergic, cholinergic or peptidergic innervation, is regulated by local vascular control mechanisms. Besides myogenic and metabolic mechanisms, paracrine factors released from vascular endothelial cells and retinal cells play an important role in the retinal blood flow regulation. In 1998, it was discovered that retinal tissue releases a factor which has a strong vasorelaxing effect on smooth muscle cells, which could not be identified as one of the already known paracrine factors (1). This vasorelaxing factor was called the RRF and may be a new paracrine factor. Moreover, research about the identity and working mechanism of this RRF could be useful in the long run for treating diseases with an impaired retinal blood flow.

An example of these diseases may be diabetic retinopathy, an important complication of diabetes mellitus, characterized by microvascular damage in the retina. A reduced retinal blood flow velocity or retinal blood flow has been described in this disease, though an increased or unaltered retinal blood flow has also been demonstrated (2-4). Also glaucoma may be associated with a reduced retinal blood flow. The loss of visual field in glaucoma is the result of damage to the optic nerve, which is generally thought to be caused by an elevated intraocular pressure, but also an insufficient ocular blood supply, or a reduced retinal blood flow may be a risk factor. However, the reduced retinal blood flow may also be due to the loss of neuronal tissue (5-7). A last example may be Alzheimer's disease, which has also visual complications. A reduced retinal blood flow is reported in patients with mild cognitive impairment or Alzheimer's disease, but it is unclear whether this is the cause or the result of retinal damage (8-10).

A reduced retinal blood flow will hamper sufficient blood supply to the retina, which is crucial for its optimal working. Modulating the release of the RRF or the vasorelaxing pathway of the RRF pharmacologically, could help in normalizing the retinal blood flow again. Moreover, identification of the RRF can lead to the use of this vasorelaxant molecule or related compounds in the treatment of these diseases with a reduced retinal blood flow.

However, despite all research on the RRF since its discovery, the RRF stays largely unknown and so is its role in the retinal blood flow regulation. Consequently, further research on the RRF is needed to reveal its role in physiology and to use this knowledge for the treatment of

diseases with an impaired retinal blood flow. Therefore, the general aim of this thesis was revealing more characteristics of the RRF, regarding its cellular source, releasing mechanism, vasorelaxing mechanism and identity. As we found, while studying the influence of resveratrol on the RRF, that resveratrol had a strong relaxing effect on retinal arteries, this interesting finding of this polyphenol with potential beneficial vascular effects was further analyzed.

IX.1 The cellular source of the RRF

Until now, the retinal cell type releasing the RRF remained unknown. Earlier research suggested that RRF is rather released from glial cells than neuronal cells (1). In addition, as vascular cells were present in retinas used in all previous studies about the RRF, retinal vascular cells could also be the RRF releasing cell type (1, 11-13). However, our first study, described in chapter V, demonstrated that even avascular chicken retinas induced smooth muscle cell relaxation. This finding led to the exclusion of retinal vascular cells as the RRF-releasing cell type. Moreover, astrocytes and oligodendrocytes, two types of glial cells, could also be excluded since astrocytes are not present in RRF-releasing chicken retinas and oligodendrocytes are not present in the mammalian retinas used in previous studies (14, 15). Consequently, the remaining glial cell types which could release the RRF were Müller cells and microglial cells. However, also in chapter V, it was shown that neither immortal MIO-M1 Müller cell cultures nor primary Müller cell cultures seemed to release the RRF. This finding was confirmed by the incompetence of the gliotoxin DL-α-aminoadipic acid to inhibit the RRF-induced relaxation. Also the microglia inhibitor minocycline was unable to inhibit or reduce the RRF-induced relaxation. Taken together, our experiments indicated that the RRF is rather released by neuronal cells than glial cells or vascular cells.

In contrast to our findings, a previous study suggested that non-neuronal cells are the source of the RRF based on the incompetence of the neurotoxin tetrodotoxin to inhibit the RRF-induced relaxation (1). This contradiction may be explained by the existence of tetrodotoxin-resistant Na^+ channels, besides tetrodotoxin-sensitive channels, in neuronal cells which can not be inhibited by tetrodotoxin (16). However, inhibiting the tetrodotoxin-resistant voltage-dependent $Na_{1.8}$ channel, present in a subtype of amacrine cells and a subtype of ganglion cells, could also not inhibit the RRF-induced relaxation. As a result, the cellular source of the RRF remains elusive.

In this study different strategies were used to reveal the cellular source of the RRF. The first approach, testing the vasorelaxing effect of different types of retina lacking specific cell types, is straightforward. As all retinas exerted a vasorelaxing effect, the absent cell types are not the RRF releasing cell types. This led to the exclusion of vascular cells, astrocytes and oligodendrocytes. A second approach used general or more specific cell type inhibitors to exclude these cells as the cellular source of the RRF. This method is less reliable as there is no evidence that the inhibitor, used in a particular concentration, led to the effective inhibition of certain cell types. Consequently, the exclusion of Müller cells and microglial cells based on pharmacological inhibition has to be handled with caution. A last approach implied testing the vasorelaxing effect of one single retinal cell type cell cultures. This approach seems straightforward, but the characteristics of the cultivated Müller cells in immortalized cell cultures as well as in primary cell cultures may have been altered. In other words, it can be that those cultivated cells no longer release the same molecules as they do when freshly harvested. However, this strategy can still be useful in further research for the cellular source of the RRF. When a single retinal cell type cell culture exerts no vasorelaxing effect, this specific retinal cell type can not be excluded as the RRF releasing cell type with certainty due to possible altered characteristics. However, when a specific retinal cell type cell culture does exert a vasorelaxing effect, this will be a strong indication that this cell type releases the RRF. In literature, the existence of cell cultures of different single retinal cell types is described, such as retinal ganglion cells or microglial cells (17, 18), which could be used in further research.

IX.2 The RRF-releasing mechanism

A recent study demonstrated an enhanced vasorelaxing effect of veratridine, a voltage-dependent Na^+ channel activator, in the presence of porcine retinal tissue (19). The underlying mechanism could represent the neurovascular coupling, which implies that the blood flow increases as result of an increased neuronal activity (20, 21). This is regulated by the release of a vasorelaxing factor(s), which may be amongst others also the RRF. Unfortunately, in chapter V, we could not confirm the enhanced vasorelaxing effect of veratridine in the presence of mouse retinal tissue, and consequently it could not be concluded that an increased neuronal activity caused the release of the RRF. Species differences and protocol differences (a piece of retina was placed on a mouse femoral artery versus a porcine retinal

artery with adherent retinal tissue) may explain the contradictory results. Therefore, additional experiments were performed using bovine retinal arteries with adhering retinal tissue. Unfortunately, contraction levels between bovine retinal arteries with and without adherent retinal tissue differed too much to obtain reproducible results. The development of a valid method to study bovine retinal arteries with adhering retinal tissue, could therefore be a helpful strategy to investigate the RRF releasing mechanism in bovine retinal arteries.

Besides by veratridine, the neuronal activity can also be stimulated by electrical field stimulation. Experiments were performed where electrical field stimulation was applied via two electrodes, placed at each side of a bovine retinal artery surrounded by adherent retinal tissue. Like in porcine retinal arterioles (19), electrical field stimulation failed to induce any vasorelaxation in our set-up with bovine retinal arteries (unpublished results). Taken together, we did not succeed in creating a reliable model for the neurovascular coupling in the retina and consequently could not investigate whether the RRF is involved in this mechanism.

Earlier research claimed that RRF release from rat retina is completely Ca^{2+} dependent (22). In contrast, our results showed that the RRF release from chicken retina is partly Ca^{2+} dependent and partly Ca^{2+} independent. Before concluding that the RRF is released in two different ways, we have to be sure that the Ca^{2+} in our experiments was completely removed. We used the Ca^{2+} chelator ethylenediaminetetraacetic acid (EDTA) to capture all Ca^{2+}. Which Ca^{2+} chelator or if a Ca^{2+} chelator was used in the other study is not mentioned (22). Using a strong Ca^{2+} chelator, such as ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), could ensure a more complete Ca^{2+}-free environment. However, it has to be taken into account that the presence of EGTA can cause problems in the further experimental protocol as the RRF-containing solution finally needs to be supplemented with Ca^{2+} for the bio-assay and EGTA can capture part of this added Ca^{2+}. Though according to Takir et al., the $PGF_{2\alpha}$-induced contraction on bovine retinal arteries still occurs in a Ca^{2+}-free and EGTA-supplemented medium. Moreover, the RRF-induced relaxation is unaltered in this Ca^{2+}-free medium, supporting our finding that the RRF release is at least partly Ca^{2+} independent (23).

IX.3 Releasing and vasorelaxing mechanism of the RRF

Further research, described in chapter VI, focused on the RRF-releasing mechanism as well as on the vasorelaxing mechanism of the RRF. Neurotransmitters serotonin and glutamate, and

L-cysteine, the precursor of hydrogen sulfide, did not influence both mechanisms. Also the cytochrome P450 pathway and the cyclooxygenase pathway did not seem to be involved, all tested in mice. Although cyclooxygenase seemed to be involved in the RRF-induced relaxation in pigs (13), our result confirms the finding that the RRF-induced relaxation is independent of cyclooxygenase in cows, rats and mice (1, 11, 12).

While investigating the involvement of the cyclooxygenase pathway, flufenamic acid appeared to enlarge the RRF-induced relaxation. Flufenamic acid has besides inhibiting cyclooxygenase a lot of other interactions (24). We did not succeed in finding the mechanism responsible for the enlargement of the RRF-induced relaxation by flufenamic acid. However, we did find that carbenoxolone had the same effect as flufenamic acid on the RRF-induced relaxation. Carbenoxolone and flufenamic acid both inhibit gap junctions, but also 2-APB is a gap junction inhibitor (25), which was also tested and did not alter the RRF-induced relaxation. These contradictory results could be explained by the selectivity of the gap junction inhibitors for the different types of gap junctions (26). However, also carbonoxolone is known to have a lot of other interactions (27). Consequently, it could not be concluded that the enlargement of the RRF-induced relaxation by flufenamic acid and carbenoxolone is due to the inhibition of gap junctions.

Additional experiments, in which the retina or artery were separately incubated with flufenamic acid or carbenoxolone, suggested that flufenamic acid and carbenoxolone influence the RRF-releasing mechanism as well as the mechanism of RRF-induced vasorelaxation. Besides, it is important to note that the effect of flufenamic acid and carbenoxolone on the RRF-induced relaxation is not necessarily due to the interaction with the release or effect of the RRF, but it can also be due to an interaction with the release or effect of another retina-derived molecule. If the latter option is true, this would mean that flufenamic acid and carbenoloxone are no good candidates to use for investigating and trying to understand the mechanism of action of the RRF.

In chapter VI also, the effect of oxidative stress, associated with retinal diseases such as diabetic retinopathy or age-related macular degeneration (28, 29), was investigated on the release and effect of the RRF. The RRF appeared to be resistant to oxidative stress, which may indicate that the RRF is not altered in the previously mentioned retinal diseases.

Based on previous studies about the RRF, retinal arteries seemed the most sensitive to the vasorelaxing effect of the RRF (1, 30). However, our study demonstrated that the assumed higher sensitivity of retinal arteries is due to differences in protocols, more specific the different sizes of retinal tissue and different amounts of K^+ in the KRB solution. The RRF has the same activity on retinal as on non-retinal arteries and retinas of different species produce the same amount of RRF, proportional to the size of the retina.

IX.4 Identity of the RRF

The next part of this thesis, described in chapter VII, focused on the possibility to identify the RRF in a chemical manner. Earlier research failed to identify the RRF by testing the vasorelaxing effect of possible RRF candidates or inhibiting certain vasorelaxing pathways (1, 11, 12, 22, 23, 30). The achievement to produce RRF-containing solutions out of chicken retinas in larger amounts and store them for a longer period is a huge step forwards in this research.

The hydrophobicity of the RRF has been studied before, resulting in contradictory results (1, 22). Here, it was demonstrated that the RRF is a hydrophilic molecule, whereas another study suggested otherwise (22). Our finding was based on hexane and ethyl acetate extractions, in contrast to the previous studies which used only hexane extractions. The additional extraction with ethyl acetate, a less hydrophobic solvent, makes sure that all hydrophobic compounds are removed, which makes these results more reliable.

Until now, no research has been performed to determine the molecular size of the RRF. By dialyzing RRF-containing solutions through membranes with different molecular weight cut offs, it could be concluded that the RRF from chickens does not exist of any molecule larger than 0.1-0.5 kDa. This complicates the analysis of the RRF by mass spectrometry, as planned originally. The RRF-containing solution contains beside the RRF also other small retina-derived molecules and Krebs Ringer bicarbonate components, which need to be present to make the RRF-containing solution. As these KRB components are also smaller than 0.1-0.5 kDa and hydrophilic, it is difficult to isolate the RRF from these interfering molecules, which is necessary to analyze the RRF-containing solution by mass spectrometry. Consequently, another strategy will need to be figured out to identify the RRF.

Future experiments aim to identify the RRF by separating the RRF-containing solution in different fractions by chromatography and analyzing the RRF-containing fraction by mass spectrometry. Before performing chromatography, hydrophobic molecules will be already removed from the RRF-containing solution by performing hexane and ethyl acetate extractions. Then, the RRF-containing solution will be separated in 4 fractions by reversed phase high pressure liquid chromatography (RP-HPLC), using a polar embedded column. The first fraction, which will interact the least with the RP-HPLC column, will contain the most hydrophilic and smallest compounds, such as KRB components. The second fraction, will contain small peptides, whereas the last fraction will contain the most hydrophobic and largest molecules. The fraction which is still able to induce vasorelaxation is the fraction that contains the RRF. If the second, third or fourth fraction contains the RRF, the RRF-containing solution can be separated in more fractions by RP-HPLC to obtain a fraction which contains almost only the RRF, so this fraction can be analyzed by mass spectrometry. However, if the first fraction contains the RRF, the RRF will be a small hydrophilic molecule which is difficult to separate from other small hydrophilic molecules, such as the KRB components, as all these molecules have almost no interaction with the polar embedded column. Therefore, if the RRF is found in the first fraction, the RRF-containing solution needs to be separated by another type of column, a hilic (hydrophilic interaction liquid chromatography) column. Hydrophobic molecules will have almost no interaction with this type of column and will be found in the first fraction. Small hydrophilic molecules will appear in later fractions, of which one will contain the RRF and will be analyzed by mass spectrometry. The analysis by mass spectrometry will, hopefully, reveal the identity of the RRF.

IX.5 The effect of resveratrol on the RRF and bovine retinal arteries

The last part of this thesis, described in chapter VIII, investigated the potential vasorelaxing effect of resveratrol on bovine retinal arteries. Resveratrol is a red wine polyphenol of which anti-oxidant, anti-angiogenic, anti-inflammatory, anti-tumorigenic, anti-apoptic and vasorelaxing effects are well known (31). At first, we were interested whether the anti-oxidant action of resveratrol could alter the effect of the RRF. Resveratrol did not influence the RRF, but it exerted a significant vasorelaxing effect on bovine retinal arteries. This strong vasorelaxing effect of resveratrol, makes it a potential molecule for the treatment of ocular diseases with an impaired retinal blood flow such diabetic retinopathy or glaucoma (31). Until

now, the vasorelaxing effect of resveratrol has been only demonstrated on retinal arterioles in pigs (32). Here, both isomers of resveratrol, trans-resveratrol and cis-resveratrol, relaxed bovine retinal arteries concentration-dependently.

Resveratrol relaxed porcine retinal arterioles endothelium-dependently via nitric oxide and endothelium-independently via Ca^{2+}-activated K^+ channels (32). In contrast, the resveratrol-induced relaxation on bovine retinal arteries did not seem to be endothelium dependent. Moreover, we did not succeed to inhibit the resveratrol-induced relaxation pharmacologically and consequently discover its vasorelaxing mechanism. K^+ channels, carbon monoxide and the myosin phosphatase pathway may be partially involved. Species differences may explain the failure to discover the vasorelaxing mechanism of resveratrol on bovine retinal arteries, which did not resemble the one on porcine retinal arterioles. Another explanation may be the many interactions of resveratrol (32-39). As resveratrol may activate different vasorelaxing mechanisms, the resveratrol-induced relaxation can never be completely inhibited by using only one inhibitor. Combining different inhibitors might solve this problem.

IX.6 Conclusions

To conclude, this thesis proved that the RRF from chickens exists of one or more hydrophilic molecules of molecular size smaller than 0.1-0.5 kDa, which shows the same vasorelaxing activity on retinal as on non-retinal arteries. The RRF is not released from vascular cells, astrocytes and oligodendrocytes. Also Müller cells are unlikely the cellular source of the RRF, but it can not be ruled out.

The RRF release from mouse retinas can not be stimulated by veratridine. In chicken retinas the RRF release seems to be Ca^{2+} dependent and independent. Furthermore, the RRF-induced relaxation is not altered by glutamate, serotonin, L-cysteine or inhibition of the cytochrome P450 pathway or the cyclooxygenase pathway. Only flufenamic acid and carbenoxolone enlarge the RRF-induced relaxation, but the underlying mechanism remains unknown.

In order to evaluate the potential role of the RRF in the (patho)physiology of the retinal circulation, future research concerning the RRF should focus on unravelling the identity and the mechanism of action of the RRF.

Furthermore, the RRF seems resistant to oxidative stress and is not influenced by the antioxidant resveratrol. The vasorelaxing effect of resveratrol on bovine retinal arteries makes resveratrol a potential molecule in the treatment of eye diseases with an impaired retinal blood flow. However, the mechanism of the resveratrol-induced mechanism remains largely unknown.

IX.7 References

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Chapter X

Summary

Chapter X: summary

Regulation of the retinal blood flow is crucial to provide sufficient blood supply to retina under any circumstances. The retinal blood flow seems to be largely regulated by paracrine factors released by endothelial cells and retinal cells. One of these paracrine factors may be the so-called retinal relaxing factor (RRF), a factor released from the retina which exerts a strong vasorelaxing effect on smooth muscle cells. This thesis aimed to gain more knowledge about the cellular source, releasing mechanism, vasorelaxing mechanism and identity of the RRF, as these characteristics of the RRF still remain largely unknown. This could clarify the role of the RRF in the regulation of the retinal blood flow and create opportunities to use the RRF or related substances for the treatment of diseases with an impaired retinal blood flow. The characteristics of the RRF were mainly studied using in vitro tension measurements on isolated arteries, described more detailed in **chapter III**.

The first study investigated the cellular source of the RRF (**chapter V**). Vascular cells, oligodendrocytes and astrocytes could be excluded due to their absence in retinas of some species which do release the RRF. Müller cells, the most abundant glial cell type in the retina, could not be excluded, although solutions incubated with immortal MIO-M1 or primary Müller cell cultures could not induce any vasorelaxation. Based on pharmacological inhibition, microglial cells did not seem to release the RRF either. So far, our experiments indicated that the RRF is rather released by neuronal cells than glial cells, yet this could not be confirmed experimentally.

Further research in this chapter focused on the releasing mechanism of the RRF. A previous study demonstrated that the vasorelaxing effect of veratridine was enhanced in the presence of porcine retinal tissue. It might have been possible that veratridine caused the release of RRF amongst other vasorelaxing substances out of the retina. However, this could not be concluded as the vasorelaxing effect of veratridine was not enhanced in the presence of mouse retinal tissue. In addition, it was found that the RRF seems to be released Ca^{2+} dependently and Ca^{2+} independently.

The next study investigated the influence of different substances and pathways on the releasing and vasorelaxing mechanism of the RRF by studying their effect on the RRF-induced relaxation (**chapter VI**). The neurotransmitters serotonin and glutamate, and L-cysteine, the

precursor of hydrogen sulfide, did not influence the RRF-induced relaxation. Neither did the inhibition of the cytochrome P450 pathway or the cyclooxygenase pathway. Flufenamic acid, a substance with a wide range of interactions beside inhibiting cyclooxygenase, did enlarge the RRF-induced relaxation. Carbenoxolone, which displays also many interactions, had the same effect on the RRF-induced relaxation. Both substances are known to inhibit gap junctions, but it is uncertain whether gap junction inhibition is responsible for the enlargement of the RRF-induced relaxation by flufenamic acid or carbenoxolone. Moreover, it is unknown whether the RRF or another retina-derived substance is influenced by flufenamic acid or carbenoxolone.

Furthermore, the effect of oxidative stress on the RRF was studied. As the RRF seems to be resistant to oxidative stress, the RRF is probably not altered in retinal diseases with oxidative stress. Finally, the sensitivity of the RRF towards retinal and non-retinal arteries was studied, but the RRF seems to have the same activity on both types of arteries. The RRF activity seems also independent of species, but depends on the size of the retina.

The following study aimed to find characteristics of the RRF allowing to identify the RRF in a chemical manner (**chapter VII**). RRF-containing solutions, obtained by incubation of chicken retinas, were used. These RRF-containing solutions were resistant to long-term freezing and freeze drying. Based on combined hexane and ethyl acetate extractions, which removed all hydrophobic compounds, it could be concluded that the RRF is hydrophilic. Dialysis experiments, carried out with membranes with different molecular weight cut offs, indicated that the RRF contains only molecules smaller than 0.1-0.5 kDa.

The last part of this thesis focused on the vasorelaxing effect of resveratrol on bovine retinal arteries (**chapter VIII**). While investigating the effect of oxidative stress on the RRF, the antioxidant resveratrol appeared to have a strong vasorelaxing effect. Resveratrol had no influence on the RRF, but both isomers of resveratrol strongly relaxed bovine retinal arteries similarly and concentration-dependently. Due to this strong vasorelaxing capacity, resveratrol could be useful for the treatment of diseases with an impaired retinal blood flow. The vasorelaxing mechanism of resveratrol remained largely unclear, but it was found that the resveratrol-induced relaxation was endothelium independent. Furthermore, K^+ channels, carbon monoxide and the myosin phosphatase pathway seemed to be partially involved.

In conclusion, the RRF exists of one of more hydrophilic molecules smaller than 0.1-0.5 kDa, which are not released by vascular cells, astrocytes and oligodendrocytes. Müller cells and microglial cells are also not likely to be the cellular source of the RRF. The RRF displayed the same activity on retinal as on non-retinal arteries. Veratridine does not release the RRF and the RRF release is Ca^{2+} dependent and independent. Furthermore, serotonin, glutamate, L-cysteine, inhibition of the cytochrome P450 pathway or the cyclooxygenase pathway could not alter the RRF-induced relaxation. Flufenamic acid and carbenoxolone enlarged the RRF-induced relaxation, but the underlying mechanism remained unknown. The RRF seemed resistant to oxidative stress and was not altered by the antioxidant resveratrol, but resveratrol could relax bovine retinal arteries strongly. By the research in this thesis some characteristics of the RRF could be further elucidated, but more research is still necessary to unravel the identity and mechanism of the RRF. This will make the role of the RRF in the regulation of the retinal blood flow clear and may create a role for the RRF in the treatment of diseases with an impaired retinal blood flow.

Chapter XI

Samenvatting

Chapter XI: samenvatting

Aanpassing van de retinale doorbloeding is van cruciaal belang om in alle omstandigheden een optimale doorbloeding te voorzien. Paracriene factoren, dit zijn factoren vrijgesteld door naburige cellen zoals endotheelcellen en retinale cellen, lijken hierbij een grote rol te spelen. Eén van deze paracriene factoren zou de zogenaamde retinale relaxerende factor, of kortweg RRF, kunnen zijn. Dit is een factor vrijgesteld uit de retina die gladde spiercellen en bijgevolg ook bloedvaten relaxeert. Aangezien de bron, het vrijstellingsmechanisme, het relaxatiemechanisme en de identiteit van de RRF toe nu toe grotendeels onbekend zijn, was het hoofddoel van deze thesis om deze eigenschappen verder te onderzoeken. Hierdoor kan de rol van de RRF in de regeling van de retinale doorbloeding worden ontrafeld. Daarnaast zou de kennis over de RRF kunnen worden toegepast in de behandeling van ziektes met een verminderde retinale doorbloeding. De eigenschappen werden grotendeels bestudeerd aan de hand van in vitro spanningsmetingen in ringsegmenten van bloedvaten, meer gedetailleerd beschreven in **hoofdstuk III**.

De eerste studie trachtte de retinale cel die de RRF vrijstelt te identificeren (**hoofdstuk V**). Bloedvat-gerelateerde cellen, oligodendrocyten en astrocyten konden worden uitgesloten op basis van hun afwezigheid in retina's van bepaalde diersoorten die wel de RRF vrijstelden. Müller cellen, de meeste voorkomende gliacel in de retina, konden niet met zekerheid worden uitgesloten, alhoewel vloeistoffen geïncubeerd met onsterfelijke MIO-M1 of primaire Müller celculturen geen relaxatie veroorzaakten. Ook microgliacellen lijken, op basis van farmacologische inhibitie, de RRF niet vrij te stellen. Deze bevindingen doen vermoeden dat de RRF eerder door neuronale cellen wordt vrijgesteld in plaats van gliacellen. Dit kon echter experimenteel niet worden bevestigd.

Daarnaast werd in deze studie ook het vrijstellingsmechanisme van de RRF bestudeerd. Uit een eerdere studie bleek dat veratridine retinale bloedvaten sterker relaxeerde in de aanwezigheid van retinaal weefsel van varkens. Deze bevinding kon helaas niet worden bevestigd met retinaal weefsel van muizen waardoor er ook niet kon worden besloten dat veratridine voor de vrijstelling van de RRF of andere relaxerende stoffen uit de retina zorgde. Daarnaast bleek uit deze studie dat de RRF deels Ca^{2+}-afhankelijk en deels Ca^{2+}-onafhankelijk wordt vrijgesteld.

In de volgende studie werd de invloed van verschillende stoffen en signalisatiewegen op het vrijstellingsmechanisme en relaxatiemechanisme van de RRF bestudeerd aan de hand van hun effect op de RRF-geïnduceerde relaxatie (**hoofdstuk VI**). Serotonine, glutamaat, L-cysteïne, inhibitie van de cytochroom P450 signalisatieweg en inhibitie van de cyclooxygenase signalisatieweg hadden geen invloed op de RRF-geïnduceerde relaxatie. Enkel flufenaminezuur en carbenoxolon konden de RRF-geïnduceerde relaxatie versterken. Beide stoffen hebben een breed gamma aan interacties waaronder het inhiberen van gap junctions, maar het is onzeker of dit mechanisme verantwoordelijk is voor de versterkte RRF-geïnduceerde relaxatie in aanwezigheid van flufenaminezuur of carbenoxolon. Daarnaast is het ook onduidelijk of flufenaminezuur en carbenoxolon een effect hebben op de RRF of op mogelijks een andere stof vrijgesteld uit de retina.

Vervolgens werd ook de invloed van oxidatieve stress op de RRF onderzocht. De RRF bleek resistent te zijn tegen oxidatieve stress, waardoor de RRF vermoedelijk onveranderd is bij retinale ziekten die gepaard gaan met verhoogde hoeveelheden aan oxidatieve stress. Ten slotte werd de activiteit van de RRF op retinale en niet-retinale arteries vergeleken, welke gelijkaardig bleek te zijn ongeacht welk bloedvat er werd gebruikt of van welke diersoort de retina afkomstig was. De RRF activiteit bleek wel afhankelijk te zijn van de grootte van de retina.

De volgende studie trachtte karakteristieken van de RRF te leren kennen die belangrijk zijn om de RRF op een chemische manier te identificeren (**hoofdstuk VII**). RRF-bevattende oplossingen gemaakt uit het incuberen van kippenretina's werden hiervoor gebruikt. Deze RRF-bevattende oplossingen bleken resistent te zijn tegen langdurige bewaring bij -20°C en tegen vriesdrogen. Daarnaast kon aan de hand van hexaan en ethyl acetaat extracties, die waterafstotende stoffen elimineerden, aangetoond worden dat de RRF wateroplosbaar is. Op basis van dialyse experimenten met verschillende dialyse membranen die moleculen tot een bepaald moleculair gewicht konden doorlaten, bleek dat de RRF enkel moleculen kleiner dan 0.1 – 0.5 kDa bevat.

In de laatste studie van deze thesis werd het relaxerend effect van resveratrol op retinale arteries van runderen geanalyseerd (**hoofdstuk VIII**). Tijdens experimenten over het effect van oxidatieve stress op de RRF bleek namelijk dat het antioxidant resveratrol geen effect had op de RRF, maar beide isomeren van resveratrol retinale arteries van runderen wel gelijkaardig

en concentratie-afhankelijk te relaxeren. Door dit sterk relaxerend effect zou resveratrol gebruikt kunnen worden in de behandeling van ziekten met een verminderde retinale doorbloeding. Het relaxatiemechanisme van resveratrol kon niet volledig worden opgehelderd, maar bleek wel endotheel-onafhankelijk te zijn en K^+ kanalen, koolstofdioxide en de myosine fosfatase signalisatieweg leken deels betrokken te zijn.

Samenvattend, de RRF lijkt enkel uit één of meerdere wateroplosbare moleculen kleiner dan 0.1-0.5 kDa te bestaan, die dezelfde activiteit op retinale als op niet-retinale bloedvaten vertonen. Vasculaire cellen, astrocyten en oligodendrocyten stellen de RRF sowieso niet vrij, en Müller cellen en microgliacellen vermoedelijk ook niet. De vrijstelling van de RRF, die niet kon worden veroorzaakt door veratridine, is deels Ca^{2+}-afhankelijk en deels Ca^{2+}-onafhankelijk. Daarnaast hebben serotonine, glutamaat, L-cysteïne, inhibitie van de cytochroom P450 signalisatieweg en inhibitie van de cyclooxygenase signalisatieweg geen invloed op de RRF-geïnduceerde relaxatie. Flufenaminezuur en carbenoxolon versterken de RRF- of retina-geïnduceerde relaxatie wel, maar het achterliggende mechanisme is nog onduidelijk. Ten slotte bleek de RRF resistent te zijn tegen oxidatieve stress en heeft het antioxidant resveratrol een sterk relaxerend effect op retinale arteries van runderen. Door het onderzoek in deze thesis konden enkele eigenschappen van de RRF worden bepaald, toch is verder onderzoek noodzakelijk om de identiteit en het werkingsmechanisme van de RRF verder te ontrafelen. Hierdoor kan de rol van de RRF in de regeling van de retinale doorbloeding duidelijk worden en de kennis van de RRF eventueel worden toegepast in de behandeling van ziektes met een verminderde retinale doorbloeding.

Curriculum vitae

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- *7th European Congress of Pharmacology (EPHAR2016)*, Istanbul, Turkey (2016)
- *Knowledge for Growth*, Ghent, Belgium (2016)
- *Belgian Society of Physiology and Pharmacology: autumn meeting*, Brussels, Belgium (2015)