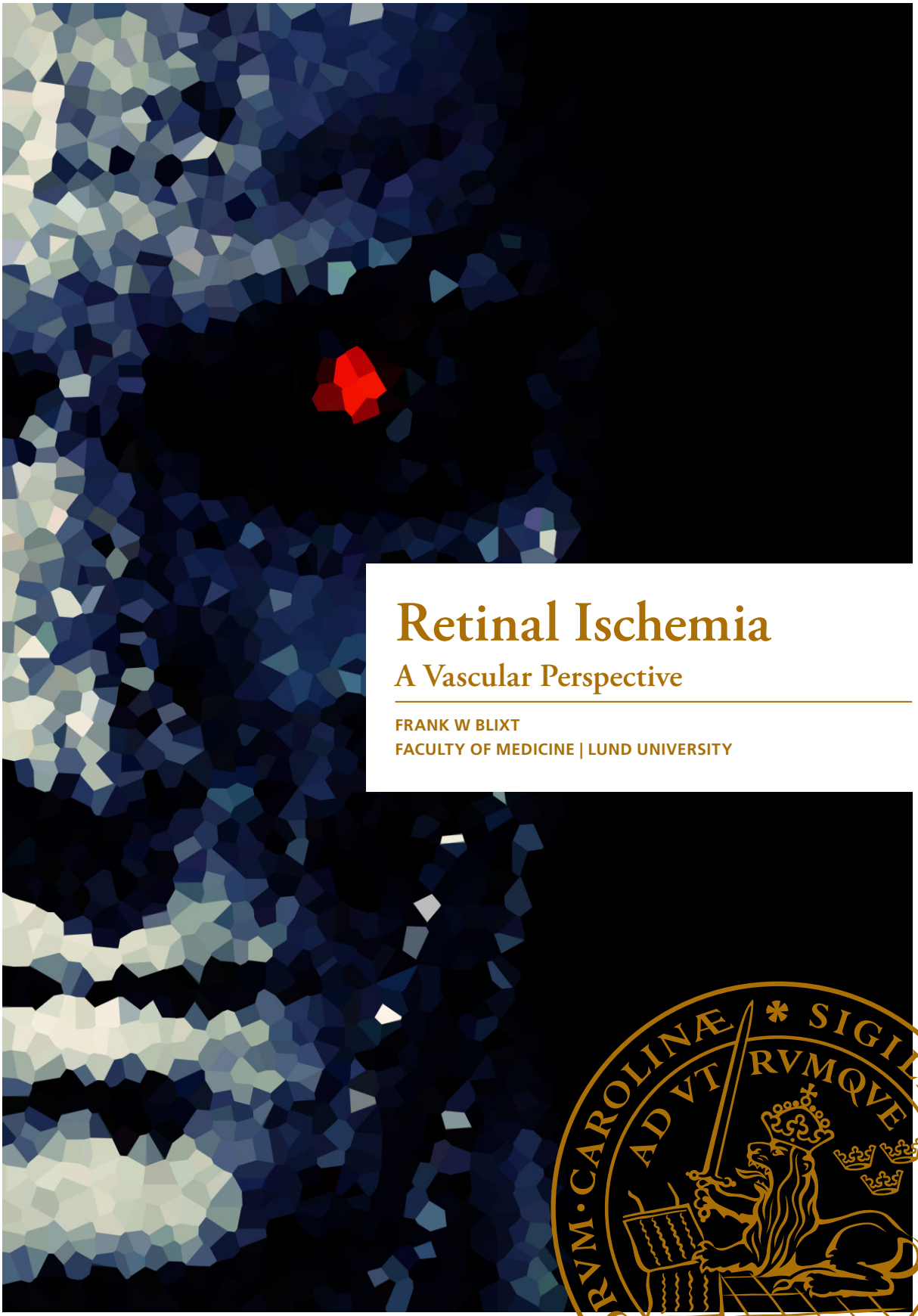
Retinal Ischemia - A Vascular Perspective

Blixt, Frank

2018

Document Version:
Publisher's PDF, also known as Version of record



Retinal Ischemia

A Vascular Perspective

FRANK W BLIXT
FACULTY OF MEDICINE | LUND UNIVERSITY

Retinal Ischemia

A Vascular Perspective

Frank W Blixt

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at 09:15 the 19th of April, 2018 at Segerfalksalen, Wallenberg Neuroscience Center, BMC, Lund, Sweden.

Faculty opponent
Professor Henrik Lund Andersen
Department of Clinical Medicine
Copenhagen University, Denmark

Organization LUND UNIVERSITY	Document name: Doctoral Thesis
Experimental Vascular Research Department of Clinical Sciences	Date of issue 19th of April, 2018
Author: Frank W Blixt	Sponsoring organization: Lund University

Title and subtitle: Retinal Ischemia – A Vascular Perspective

Abstract

This thesis aimed to highlight vasculature as a potential therapeutic avenue in treating retinal ischemia. With the recent advances in research on cerebral arteries following ischemia, the MAPK pathway, particularly the MEK/ERK1/2 activation, has been linked to key vascular changes. Following ischemia, vasoconstrictive receptors are significantly upregulated. Among these, endothelin-1 (ET-1) and 5-hydroxytryptamine (5-HT, serotonin), play key roles in cerebral arteries.

In the thesis, the role of ET-1 receptors was evaluated in the rat ophthalmic artery and pig retinal arteries in two ischemic models including global cerebral ischemia (GCI) and middle cerebral artery/ophthalmic artery occlusion (MCAO/OAO), as well as ischemia like conditions, through organ culture (OC). The MEK/ERK1/2 pathway was targeted in an effort to diminish the detrimental vasoconstriction. Finally, the overarching role of MEK/ERK1/2 and its link to possible protein changes was evaluated in the retina following ischemia.

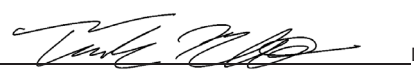
Main results:

1. GCI induced a significant increase of ET-1 mediated vasoconstriction in rat ophthalmic artery 48 hours after ischemia/reperfusion while 5-HT function seemed unaffected. ERG also exhibited a functional deficiency, confirming that the ischemic model caused retinal damage
2. 24 hour OC, mimicking ischemic conditions, allowed for the evaluation of two MEK1/2 inhibitors (U0126 and trametinib) on the rat ophthalmic artery. Both MEK1/2 inhibitors attenuated the OC induced vasoconstriction. ET-1 receptor ET_B was singled out as a key vasoconstriction mediator. ET_B, is expressed on the smooth muscle cells in contrast to on endothelial cells, mediated constriction rather than dilatation.
3. In vivo application of U0126 following MCAO/OAO showed that MEK1/2 inhibition can attenuate ET-1 mediated vasoconstriction successfully 48 hours after ischemia/reperfusion. ERG analysis also showed a diminished retinal function both in the ischemic and the contralateral eye of the operated animals suggesting a potential cross-talk between retinas.
4. Large scale proteomic analysis of the rat retina 48 hours after MCAO/OAO revealed that 143 out of 3023 identified proteins were altered in the ischemic eye compared to the contralateral control. These 143 proteins were sorted by function (metabolic processes, heat shock proteins, and protein synthesis) and by association to MEK/ERK1/2. Out of the MEK/ERK1/2 related proteins CD44, involved in the inflammatory response, and STAT3 linked to apoptosis of neuronal cells in the retina were found to be of future interest. The MEK/ERK1/2 pathway seems to be highly involved in the post-ischemic processes also in the retina and therefore there might be potentially positive secondary effects of the MEK1/2 inhibitors on the retina as well as the vessels, as treatment for retinal ischemia.

Key words: Retinal ischemia, MAPK, MEK/ERK1/2, endothelin-1,ET_A, ET_B, ophthalmic artery, retinal artery

Classification system and/or index terms (if any)

Supplementary bibliographical information Doctoral Dissertation Series 2018:38		Language: English
ISSN and key title 1652-8220		ISBN 978-91-7619-605-2
Recipient's notes	Number of pages 62	Price
	Security classification	

Signature  Date 2018-03-15

Retinal Ischemia

A Vascular Perspective

Frank W Blixt

LUND
UNIVERSITY

Cover designed by Hans Johnell

Faculty of Medicine
Department of Experimental Vascular Research

ISBN 978-91-7619-605-2
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2018:38

Printed in Sweden by Media-Tryck, Lund University
Lund 2018

MADE IN SWEDEN

To my Family

Table of Contents

List of Original Articles

1. **Blixt FW**, Johansson SE, Johnson L, Haanes KA, Warfvinge K, Edvinsson L. **(2016)** "*Enhanced Endothelin-1 Mediated Vasoconstriction of the Ophthalmic Artery May Exacerbate Retinal Damage after Transient Global Cerebral Ischemia in Rat*". PLoS One 11(6):e0157669

2. **Blixt FW**, Haanes KA, Ohlsson L, Christiansen AT, Warfvinge K, Edvinsson L. **(2017)** *"Increased Endothelin-1 mediated vasoconstriction after organ culture in rat and pig ocular arteries can be suppressed with MEK/ERK1/2 inhibitors"* Acta Ophthalmologica ahead of print doi: 10.1111/aos.13651

3. **Blixt FW**, Haanes K, Ohlsson, Dreisig K, Warfvinge K, Edvinsson L (2018) MEK/ERK1/2 sensitive vascular changes coincide with retinal functional deficit following transient ophthalmic artery occlusion (Manuscript)

4. **Blixt FW**, Cehofski LJ, Haanes KA, Warfvinge K, Honoré B, Edvinsson L, (2018) Proteomic changes following ophthalmic artery occlusion – with focus on MEK/ERK pathways (Manuscript)

Abbreviations

5-CT: 5-carboxamidotryptamine

5-HT: 5-hydroxytryptamine

5-HT_{1B}: 5-hydroxytryptamine receptor type 1B

ARVO: Association for Research in Vision and Ophthalmology

CRAO: central retinal artery occlusion

DMSO: Dimethyl sulfoxide

ERG: Electroretinography

ERK: extracellular signal-regulated kinases

ET-1: endothelin-1

ET_A: endothelin receptor type A

ET_B: endothelin receptor type B

GCI: Global Cerebral Ischemia

GFAP: Glial fibrillary acid protein

IPSP: Inhibitory post-synaptic potential

JNK: c-Jun N-terminal kinases

MAPK: Mitogen activated protein kinases

MCAO: Middle cerebral artery occlusion

MEK: Mitogen activated protein kinase/Extracellular signal-regulated kinase kinase

NMDA: N-methyl-D-aspartate receptor

OAO: Ophthalmic artery occlusion

OC: Organ culture

OP: Oscillatory potential

S6c: Sarafotoxin-6c

STAT3: Signal transducer and activator of transcription 3

STR: Scotopic threshold response

TNFα: Tumor necrosis factor alpha

VEGF: Vascular endothelial growth factor

Summary

This thesis aimed to highlight vasculature as a potential therapeutic avenue in treating retinal ischemia. With the recent advances in research on cerebral arteries following ischemia, the MAPK pathway, particularly the MEK/ERK1/2 activation, has been linked to key vascular changes. Following ischemia, vasoconstrictive receptors are significantly upregulated. Among these, endothelin-1 (ET-1) and 5-hydroxytryptamine (5-HT, serotonin), play key roles in cerebral arteries.

In the thesis, the role of ET-1 receptors was evaluated in the rat ophthalmic artery and pig retinal arteries in two ischemic models including global cerebral ischemia (GCI) and middle cerebral artery/ophthalmic artery occlusion (MCAO/OAO), as well as ischemia like conditions, through organ culture (OC). The MEK/ERK1/2 pathway was targeted in an effort to diminish the detrimental vasoconstriction. Finally, the overarching role of MEK/ERK1/2 and its link to possible protein changes was evaluated in the retina following ischemia.

Main results:

1. GCI induced a significant increase of ET-1 mediated vasoconstriction in rat ophthalmic artery 48 hours after ischemia/reperfusion while 5-HT function seemed unaffected. ERG also exhibited a functional deficiency, confirming that the ischemic model caused retinal damage

2. 24 hour OC, mimicking ischemic conditions, allowed for the evaluation of two MEK1/2 inhibitors (U0126 and trametinib) on the rat ophthalmic artery. Both MEK1/2 inhibitors attenuated the OC induced vasoconstriction. ET-1 receptor ET_B was singled out as a key vasoconstriction mediator. ET_B, is expressed on the smooth muscle cells in contrast to on endothelial cells, mediated constriction rather than dilatation.

3. In vivo application of U0126 following MCAO/OAO showed that MEK1/2 inhibition can attenuate ET-1 mediated vasoconstriction successfully 48 hours after

ischemia/reperfusion. ERG analysis also showed a diminished retinal function both in the ischemic and the contralateral eye of the operated animals suggesting a potential cross-talk between retinas.

4. Large scale proteomic analysis of the rat retina 48 hours after MCAO/OAO revealed that 143 out of 3023 identified proteins were altered in the ischemic eye compared to the contralateral control. These 143 proteins were sorted by function (metabolic processes, heat shock proteins, and protein synthesis) and by association to MEK/ERK1/2. Out of the MEK/ERK1/2 related proteins CD44, involved in the inflammatory response, and STAT3 linked to apoptosis of neuronal cells in the retina were found to be of future interest. The MEK/ERK1/2 pathway seems to be highly involved in the post-ischemic processes also in the retina and therefore there might be potentially positive secondary effects of the MEK1/2 inhibitors on the retina as well as the vessels, as treatment for retinal ischemia...

1. Background

1.1 Structure and Function of the Eye

The retina is the third and inner most layer of the eye in mammals. Its embryological origin as a part of the diencephalon means that it is, just as the brain, a part of the central nervous system (1). The retina is highly organized with strict layers separating different cell types based on function (Figure 1). From the photoreceptors in the outer most layer to the ganglion cells in the inner most, the retina has a marvelous capacity to detect photons of light and pass information onwards to the visual center of the brain. Light passes through the lens and is focused on the photoreceptors, causing an electrical stimulation via Inhibitory Post-Synaptic Potentials (IPSP) to be propagated inwards along the retinal structures towards the inner nuclear layer (2). The inner nuclear layer contains three categories of interneurons: bipolar, amacrine, and horizontal cells. These are not only responsible for the transmission of the signal from the photoreceptors to the ganglion cells, but also play a role in converging the signal from multiple photoreceptors and allowing for spatial and temporal patterns to be recognized. The electrophysiology of the retina can be divided into two parts: a hyperpolarization followed by a depolarization. As mentioned, photons cause an IPSP, hyperpolarizing the photoreceptors. This leads to the cessation of inhibitory neurotransmitter release at bipolar cells and thus a depolarization and activation of bipolar cells (3). As bipolar cells are activated they cause a release of neurotransmitters at the inner most ganglion cells that in turn cause a burst of impulses to be propagated along the nerve fiber layer and towards the optic nerve.

The distinct structure and electrophysiology of the retina allows for functional measurements and analysis which will be discussed later.

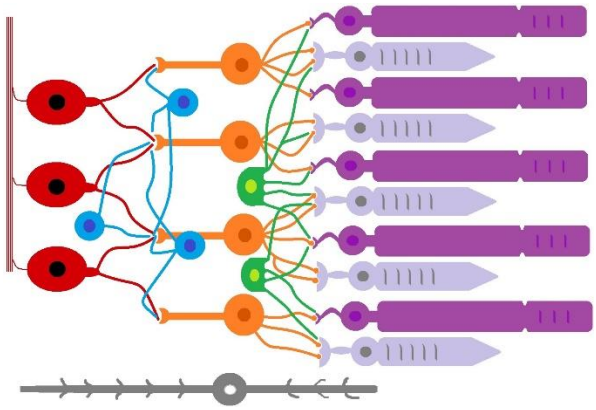
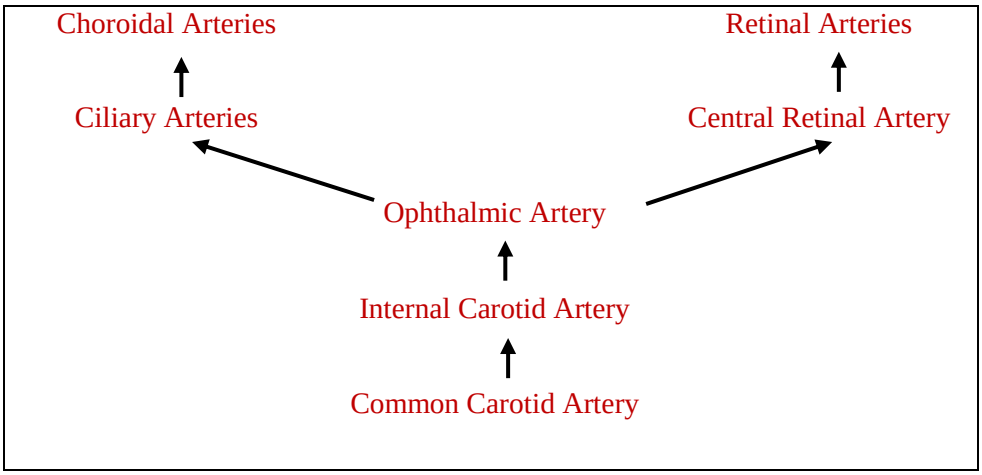


Figure 1. Neuroretinal structure from outer to inner: photoreceptors (purple), horizontal cells (green), bipolar cells (orange), amacrine cells (blue, including one displaced amacrine cell), ganglion cells (red), and a representative Müller cell (gray) spanning through the entire length of the retina.

The mammalian retina contains three types of glial cells: microglia, astrocytes, and Müller cells. Glial cells are supporting cells primarily involved in maintaining and regulating upkeep for neuronal cells. Spanning throughout the thickness of the retina are Müller cells, the chief glial cells of the retina (4). These cells are primarily involved in maintaining the homeostasis in the retina, monitoring pH levels, metabolism, retinal blood flow, ion concentrations, and neurotransmitter release/reuptake. This is accomplished as Müller cells provide an anatomical and functional link between the various cell types to the retinal vasculature, vitreous body, and the sub-retinal space (5). When retinal damage occurs, the Müller cells are first to respond in a process called gliosis. This process is commonly recognized by the increase of the structural Glial Fibrillary Acidic Protein (GFAP) and vimentin, highly associated with Müller cells (6, 7).

Most mammals, including rats and humans, have a dual blood supply to the retina. This is due to the high metabolic demand of the retina which cannot be met by inner retinal blood flow or choroid blood flow alone (2). The choroid layer is the middle layer of the eye consisting of a vascular bed which supplies the avascularized photoreceptors with blood/oxygen. The majority of retinal blood flow is provided

by the choroid, while retinal vasculature provides blood to the inner most layers. Both choroid and retinal vasculature stem from various vascular branches off the ophthalmic artery which in turn stems from the internal carotid artery. Choroid vessels stem primarily from posterior ciliary arteries that branch off the ophthalmic artery prior to reaching the eye. In contrast, retinal arteries stem from the central retinal artery, the final branch of the ophthalmic artery, which follows the optic nerve into the retina (8). Thus, to summarize the arterial chain of flow (Figure 2).



As mentioned before, the high metabolic rate and the organized structure of the retina are the driving forces behind the dual blood supply. Thick vasculature spanning the retina affects its function, which means that smaller capillaries or simple diffusion are needed to meet the metabolic requirements. Interestingly, nocturnal animals or those living in dark environments are quick to devolve their retinas and visual centre's in the brain in an effort to cut huge energy costs associated with vision (9).

1.2 Retinal Ischemia

Retinal ischemia is caused by the lack of blood flow to the retina. There are various clinical diagnoses that encompass retinal ischemia in their pathophysiology.

Some of these include retinal vein occlusion, retinal artery occlusion, diabetic retinopathy, ocular ischemic syndrome, and to some extent glaucoma. With this in mind, retinal ischemia is a leading cause of partial or complete blindness in the world (10-12).

Despite the close connection between the brain and the retina, the retina is significantly more resilient to ischemic damage than its cerebral counterpart. Evaluation of grown Rhesus monkey retinas show that a 97 minute ischemia elicits no observable retinal damage, while 240 minutes sets the limit for massive irreversible damage (13). The brain on the other hand cannot withstand more than 5 minutes of complete ischemia in general without devastating and permanent damage (14). The reason for this discrepancy is still under debate, but several factors such as the high amount of glucose readily available in the vitreous body, the no re-flow phenomenon, or presence of neuroglobin may all be responsible to some extent. The presence of Müller cells, which quickly become activated and initiate gliosis, is also attributed to the retina's ability to withstand damage. Müller cells are able to counteract the build-up of glutamate, ion imbalance, and other factors with impressive efficiency (5). Nevertheless, ischemia may have severe effects on the overall retinal function. Generally, the disruption of the retinal homeostasis caused by ischemia leads to several detrimental processes. Among the more well described and severe is excitotoxicity through excessive glutamate release.

Glutamate, the most prominent excitatory neurotransmitter acts through two main types of receptors: N-methyl-D-aspartate (NMDA) and non NMDA-receptors. The former is located on various retinal cell types including retinal ganglion cells (15) and has been pinpointed as the main target associated with retinal ganglion death following ischemia (16). Glutamate increases the flow of calcium into the cell, depolarizing it, and during conditions of high glutamate levels, the intracellular calcium concentration becomes toxic leading to cell death.

Inflammation is the natural response to harmful stimuli. Even though it has a protective role, some effects may be deleterious to specific tissue types under certain

conditions. In the retina, the inflammatory response causes elevated levels of Tumor Necrosis Factor Alpha (TNFα) which has been linked to triggering apoptotic processes causing cell death. However, it seems that this process is time dependent. TNFα neutralization immediately following ischemia has protective effects while similar treatment 48 hours later shows a significant worsening of retinal function (17).

1.3 Vascular Changes Induced by Ischemia

Vasculature is constantly regulated during normal physiological conditions to match the metabolic needs of the surrounding tissues. This process is upheld through the constant activity of ion channels, G protein-coupled receptors, transmembrane enzyme receptors and more. Within cerebrovascular research a handful of vasoactive receptors have been found to be upregulated following an ischemic insult. Among these, endothelin-1 (ET-1) has consistently been indicated in a wide range of ischemic animal models. ET-1 was first described as a potent vasoconstrictor in 1988 (18), and is one out of three isoforms of endothelin (ET-1/2/3) and is the best described and most relevant isoform to ischemic research. The function of ET-1 differs depending on which of its two G protein-coupled receptors it binds to. The ET_A receptor, found predominantly in vascular smooth muscle cells, has been described as a mediator of strong vasoconstriction both in vivo and in vitro (19). The second ET-1 receptor, ET_B, is more complex in regard to its vascular function. ET_B, primarily found in vascular endothelial cells, mediates a NO–facilitated vasodilation under physiological conditions (20). However, after ischemia, ET_B expression is increased in vascular smooth muscle cells, causing vasoconstriction (21-23). This dichotomy of ET_B function has become a focal point of vascular research within the scope of ischemia/reperfusion models. Furthermore,

the regulation of ET-1 receptor upregulation has been attributed to the cellular pathway named Mitogen-Activated Protein Kinase pathway (MAPK) (24, 25).

Another prominent vasoconstrictor that has been demonstrated to increase in abundance following ischemia is the 5-hydroxytryptamine (5-HT) (serotonin) receptor, specifically the 5-HT_{1B} subtype (26). As with ET-1 receptors, the increase of 5-HT_{1B} receptors span across several animal models (26-28) suggesting that there is an overarching increase of vasoconstrictive receptors following ischemia.

1.4 MAPK Pathway

For cells to react and respond to stimuli, such as ischemia, they need a means of communication with each other. Cell signaling governs the basic behavior and coordination between individual and groups of cells. MAPK is a serine/threonine protein kinase family involved in several biological functions including cell proliferation, gene expression, differentiation, cell survival, and apoptosis. The MAPK family is comprised of three main proteins: Extracellular signal-Regulating Kinase (ERK)1/2, stress-activated protein kinase c-Jun (JNK), and p38. The ERK pathway described in Figure 3, has been linked to the upregulation of vasoconstrictive receptors in cerebral arteries in rat and humans. By inhibiting the ERK kinase (MEK), ERK1/2 is not phosphorylated and cannot reach its active state (pERK1/2), and thus vascular homeostasis is maintained. ERK has only been briefly examined in the retina within the context of ischemia with inconclusive results. The pathway has been linked to the activation and proliferation of glial cells as part of the retinas intrinsic and protective response to ischemia in chicken (29) suggesting a beneficial role following ischemia. The beneficial function of ERK1/2 is further supported where MEK1/2 inhibition increased retinal ganglion cell death (30). However, there are those who argue that ERK1/2 is involved in damaging apoptotic processes leading to retinal ganglion cells in rats where MEK1/2 inhibition

increased ganglion cell survival (31, 32). With that being said, ERK1/2 is demonstrably activated in the retina following ischemia in rat (32), pig (33, 34), and human (35), being involved in the processes following the onset of retinal ischemia. Therefore, my thesis will mainly revolve around ERK1/2 and specifically the MEK/ERK1/2 part of MAPK with focus on its vascular role.

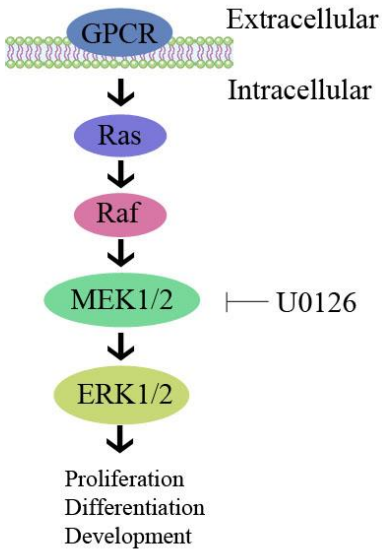


Figure 3: MEK/ERK1/2 signaling cascade.

1.5 Current Treatments

Current treatments of retinal ischemia are largely focused on prevention of neovascularization (formation of new vasculature) and vascular leakage. These processes may disturb the precise and important structural integrity of the retina and thus have a detrimental effect on retinal function. A key factor promoting the post-ischemic neovascularization and increased vascular permeability is Vascular Endothelial Growth Factor (VEGF). Therefore, intraocular anti-VGEF injections have become the norm in treating many ocular conditions including vascular occlusions. Although this treatment has in many ways revolutionized

ophthalmological care, improving vision significantly among 50% of patients with central retinal vein occlusion (36), this approach has not been free of complications. Among these are: ocular hemorrhage, persisting in approximately 10% of patients (higher incidence among patients on aspirin), intraocular inflammation and endophthalmitis among up to 5% of patients, and some systemic effects mainly among older patients have provided another subset of conditions in need of care (37). Furthermore, it is important to note that VEGF has been proven to have strong neuroprotective effects in the retina following ischemia (38) suggesting that the depletion of VEGF with anti-VEGF drugs may exacerbate retinal ganglion cell death and thus not be well suited for acute ischemic conditions.

In the case of Central Retinal Artery Occlusion (CRAO), caused by a thrombosis of the CRA, thrombolytic agents have instead been suggested as a form of treatment. However due to the high probability of hemorrhage side effects, few successful attempts have been made. It has however been recommended that treatment must be delivered within a specified time window for optimal efficiency. As within stroke treatment, and this has been stipulated to be within 6 hours of ischemic onset (39).

Therefore, with the pitfalls of the current therapies available it is warranted to seek alternative therapeutic options as either a supplementary or main therapeutic opportunity within the scope of retinal ischemia.

2. Aims

The central aim of this thesis was to translate knowledge from cerebral research into the ophthalmological field in an effort to bridge the gap between disciplines and in doing so, shed new light on the pathophysiology of retinal ischemia and offer a novel therapeutic suggestion.

Specific aims:

- We hypothesize that similar vascular changes are induced in ocular arteries as in cerebral vessels following ischemia, and aim to identify the major vasoconstrictors.
- Evaluate if MEK/ERK1/2 inhibitors are able to attenuate the increase of the above identified vasoconstrictive receptors in ocular arteries, as in cerebral arteries in vitro.
- Reduce the detrimental changes of vascular receptor expression with the goal of improved vascular and retinal function in vivo; and
- We hypothesize that MEK/ERK1/2 is involved in several processes outside the vasculature. Therefore, we aim to evaluate the MEK/ERK1/2 pathway role in the ischemic retina and the potential effects of MEK/ERK1/2 inhibitors.

3. Methods

3.1 Ethics

All procedures and animal treatment was performed in accordance with the guidelines of the ethics committee of Lund University, the guidelines of the Danish Animal Experimentation Inspectorate, and the statement of the Association for Research in Vision and Ophthalmology (ARVO) for The Use of Animals in Ophthalmic and Vision Research.

3.2 Global Cerebral Ischemia (Paper I)

Transient Global Cerebral Ischemia (GCI) was induced through a two-vessel carotid artery occlusion coupled with hypovolemia previously established by Smith et al (1984) (40). This method allows for an incredibly severe drop of blood flow to the brain and eye. Its extreme nature was utilized to try and cause concrete ischemic conditions for the ophthalmic artery with the goal of eliciting ischemia related changes in the vasculature. Rats were anesthetized using 3% isoflurane in a mix of 30% O_2 and 70% NO_2 and then intubated and artificially ventilated with 1.5-2% isoflurane in 30% O_2 and 70% NO_2 during the duration of the surgery. A catheter was inserted through the tail artery in order to measure mean arterial blood flow. Then, a neck incision was made and both common carotid arteries were isolated and loose ligatures were placed around them. Next, a polyurethane catheter filled with 300 IU/ml heparin was inserted into the external jugular vein into the right atrium. Rats were injected with 0.5 ml heparin and allowed to equilibrate for 15 min prior

to ischemia. Ischemia was achieved by withdrawing blood through the carotid vein until the mean arterial blood pressure stabilized at 40 mm Hg, followed by clamping of both common carotid arteries. These conditions were held for 15 minutes before the clamp was undone and blood was reinjected carefully to the rat, restoring normal arterial blood pressure and a dose of 0.5 ml of 0.6 M sodium bicarbonate was injected to counteract systemic acidosis. The incisions were closed and the rat was allowed to rest for 15-20 minutes before isoflurane was discontinued.

Blood values and body temperature were monitored throughout the surgery.

3.3 Ophthalmic Artery Occlusion via Occlusion of the Middle Cerebral Artery (Papers III & IV)

Ophthalmic Artery Occlusion (OAO) was performed by what is commonly known as the Middle Cerebral Artery Occlusion (MCAO) model. This model, first described by Koizumi in 1986 (41, 42), allows for ischemia reperfusion by the insertion of a silicone filament through the common carotid artery, into the internal carotid artery where it ultimately occludes the middle cerebral artery. Due to the proximity of the ophthalmic artery to the Middle Cerebral Artery (MCA), this model also occludes the ophthalmic artery (43). The model does not carry the same devastating brain damage as GCI, allowing for more flexibility in the duration of ischemia.

Rats were anesthetized using 3% isoflurane in a mix of 30% O_2 and 70% NO_2 and allowed to breathe autonomously on a constant supply of 2-3% isoflurane depending on the body weight. The neck and top of the head was shaved and disinfected with chlorhexidine prior to making an incision along the neck. The common, external, and internal carotid arteries were isolated with the former two being permanently ligated with sutures. A Laser-Doppler probe, attached to the skull of the rat, monitoring blood flow to the brain from the MCA was used to confirm

loss of blood flow to the brain. The silicone mono-filament was then inserted into the internal carotid artery through a small incision in the common carotid artery (Figure 4), until a clear drop in blood flow was observed by the Laser-Doppler reading. The filament was at that point secured, local anesthetics (Marcaine) were given, the neck and skull incision were closed, and the rat was allowed to wake up. A two-hour occlusion was allowed to transpire before the rat underwent a 6-point behavioral test (described in detail below) to confirm behavioral changes induced by the occlusion. Then the rat was re-anesthetized and the Laser-Doppler probe was attached in the same position as before. Next, the neck was reopened, the filament was retracted, and an increased Laser-Doppler reading confirmed reperfusion to the previously occluded area. Surgery was finalized by a new dose of Marcaine in both the neck and skull, and 0.9% NaCl in H_2O was given (1ml/100g body weight) to counteract any post-surgery dehydration. Blood flow readings were maintained for at least 10 minutes to confirm successful reperfusion. The animals were then allowed to recover with free access to water and food. Recovery ranged from 48 hours to 7 days depending on whether vascular changes or retinal function was to be evaluated respectively.

Rats destined for MEK/ERK1/2 inhibitor treatment were injected immediately after the completion of the surgery (0 hours), then at 6 hours, and 24 hours post reperfusion through intraperitoneal injections of U0126 30 mg/kg dissolved in dimethyl sulfoxide (DMSO).

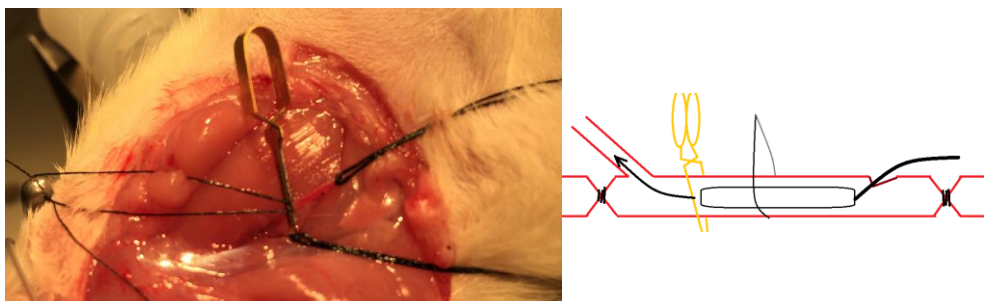


Figure 4: Image and schematic of filament insertion into the internal carotid artery

3.4 6 Point Neuroscore (Papers III & IV)

Sensory motor function was assessed pre-reperfusion and pre-sacrifice according to an established scoring system (44, 45). Scores were given according to the following criteria: 0 – no visible defects, 1 – contralateral forelimb flexion when held by tail, 2 – decreased grip of contralateral forelimb, 3 – spontaneous and free movement in all directions, however contralateral circling following tail pulling, 4 – spontaneous contralateral circling, and 5 – death.

3.5 Organ Culture (Paper II)

As in vivo methods require more time and higher costs, in vitro models become a helpful tool. This is even more accurate for assessing concepts and building a theoretical foundation prior to animal work. Organ Culture (OC) allows for arterial segments to experience ischemia-like conditions by removing the internal pressure of the arteries while maintaining nutrients, temperature, and pH at physiological levels (46), eliciting very similar vascular responses as seen in in vivo models. Therefore, dissected arterial segments of the rat ophthalmic and pig retinal arteries were incubated for 24 hours in Dulbecco's modified Eagles medium containing 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.25 µg/ml amphotericin at 37º C. Arterial segments destined for MEK/ERK1/2 inhibitors were incubated in medium containing either 10-6 M U0126 or 10-8 M trametinib (dissolved in cremophor + polyethylene glucose).

3.6 Myography (Papers I - III)

In order to assess the vascular properties of arteries both in brain and eye, myograph is the method of choice. Two 25 µm steel wires were inserted through

the lumen of 1 mm long ophthalmic artery segments and mounted in a Mulvany-Halpern myograph. One wire was attached to a force displacement transducer with a digital converter-unit, while the second wire was attached to a micrometer screw, dictating the distance between the two wires and in turn the initial vascular tone on the arterial segment (Figure 5). Arteries were normalized to 90% of their diameter under normal condition of 100 mm Hg, and tested for viable contractions by 65 mM K+ bicarbonate buffer, eliciting a strong vasoconstriction by smooth muscle contraction via membrane depolarization and influx of calcium (47). This constriction would later serve as each vessel's individual reference contraction.

Individual vascular receptors were then evaluated by increasing application of its agonists. 5-carboxamidotryptamine (5-CT, a 5-HT_1 serotonin analog) for the 5-HT_{1B} receptor, ET-1 for both ET_A and ET_B receptors, and sarafotoxin 6c (S6c) with high specificity for ETB receptors. To further evaluate which ET-1 receptors were active, specific ET_A and ET_B antagonists BQ123 and BQ788 were applied prior to the application of ET-1 or S6c.

The overall contraction of each arterial segment was presented as a percentage of its initial K^+ mediated contraction.

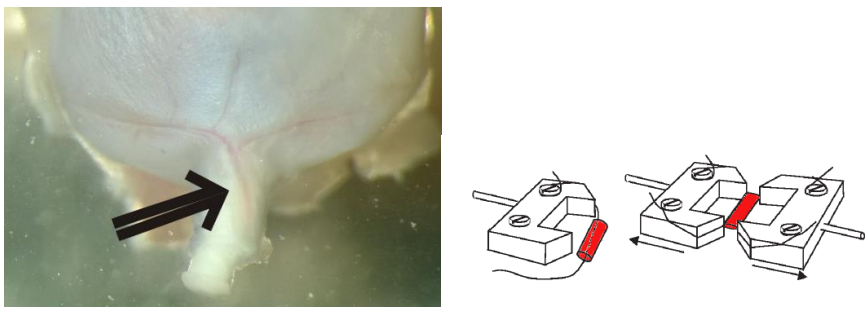


Figure 5: Ophthalmic artery seen running along the optic never (Image rights: KA Haanes 2014) and the myography set up.

3.7 Immunohistochemistry (Papers I - IV)

Immunohistochemistry was used to visualize specific targets in both the retina and arterial segments of the rat. In short, a 10 µm thick cryosectioned retina and ophthalmic artery were prepared with primary antibodies for GFAP, Vimentin, ET_A, and ET_B antigens. These were applied either separately (single staining) or in serial (double staining) fashion to the cryosections and incubated at 8ºC overnight. Secondary antibodies conjugated with a fluorophore, were applied to bind to the primary antibody and allow for it to be visualized through specific wavelength excitation in an epi-fluorescent microscope. Omission of the primary antibody was used as a negative control, allowing to separate nonspecific bindings of the secondary antibodies and to visualize auto fluorescent structures such as the elastic lamina.

All staining was performed a minimum of three times to ensure consistent results.

3.8 Electroretinography (Papers I & III)

Ultimately, the goal of all retinal ischemia research is to improve the retinal function (vision) of affected patients. Therefore, the functional analysis of the retina was evaluated by electroretinography (ERG) which measures both the effect of ischemia and any potential improvements caused by treatments. Dark adapted rats were anesthetized using ketamine (85 mg/kg) and xylazine (20 mg/kg) and placed on a heated pad inside the Ganzfield bowl equipped with both LED and xenon lamps. A series of eye drops including oxybuprocain (0.4%), tropicamide (1%), and phenylephrine (5%) were added to each eye for topical anesthesia and pupil dilation. Electrodes were placed on each cornea and in the mouth of the rat while a needle in the tail acted as ground and the retinas were stimulated with increasing strength of flashes with increasing inter-stimuli intervals. Finally, rats were monitored until they woke up from anesthesia and were then returned to their cages.

3.9 Proteomics Analysis (Paper IV)

Protein changes in retinas were analyzed through processing homogenized retinal sample prior to repeated centrifugations in order to separate proteins. This method is described in detail in paper IV. Eventually, the peptide mixture was separated by a mass spectrometer and the resulting proteins were compared to Uniprot databases for Rattus norvegicus (downloaded updated as off September 12th 2017) and Homo sapiens (as of September 22nd 2017).

4. Results and Discussion

4.1 Vascular changes in rat ophthalmic artery and pig retinal arteries (Paper I-III)

Our first and foremost task was to confirm that vascular changes occur in the ocular arteries following ischemia. In order to achieve this we opted for three different ischemic models that would either diminish the blood flow to the eye or simulate ischemia-like conditions in vitro. Myograph results were described in terms of E_{max} = the total relative contraction of the artery, EC_{50} = the agonist concentration at the half way point between baseline and E_{max}, and comparing values at specific agonist concentrations.

Paper I

With global cerebral ischemia we set out to evaluate two central vasoconstrictive ligands implicated in cerebral arteries following a 15 minute ischemic insult: ET-1 and 5-CT. We evaluated arteries at both 24 and 48 hours after reperfusion and observed only a significant increase in ET-1 mediated vasoconstriction at the later time point (Figure 6). Concentration-response curves of the ischemic arteries were significantly increased ($p<0.05$), with a weak leftwards shift compared to sham operated animals and with a significantly increased Emax ($p<0.01$). 5-CT mediated contraction did not differ between ischemic and sham operated arteries.

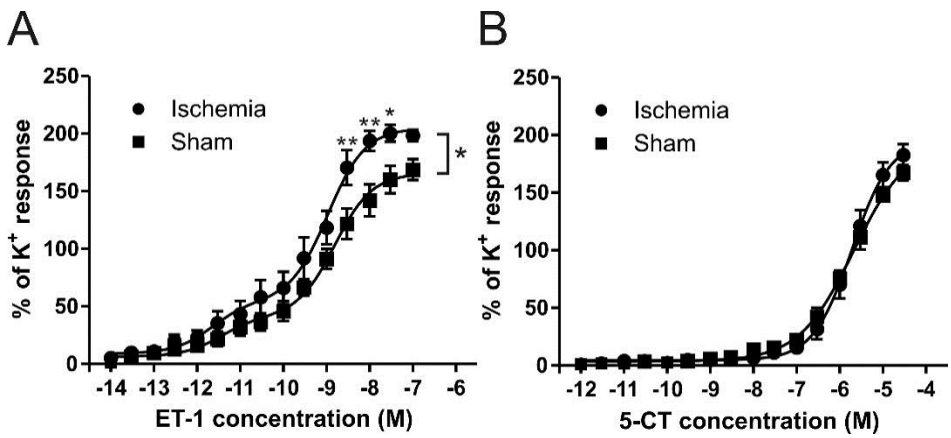


Figure 1: **Increased endothelin-1 induced constriction after 48 hours.** (A) Dose response curves for ET-1 on ischemic and sham ophthalmic artery 48 hours after ischemia are significantly different ($p<0.05$) with a left shift in the EC_{50}. Sham EC_{50} was 1.56nM (pEC_{50} 8.810±0.12M) and for ischemia it was 1.07nM (pEC_{50} 8.97±0.13M). (B) 5-CT response curves are identical with EC_{50} 1.91μM (pEC_{50} 5.72±0.08M) for ischemia and EC_{50} 2.09μM (pEC_{50} 5.68±0.11 M) for sham.

These initial results show a similar behavior of ET-1 mediated contraction 48 hours after ischemia as seen in cerebral arteries (26). The exact nature of ET-1 receptor expression was not evaluated to determine whether the observed contraction as due to an increase in ET_A receptors in vascular smooth muscles or the expression of ET_B receptors from the endothelial cells to the smooth muscle cells. Interestingly, the endothelium was intact prior to myography meaning that the potential vasodilatory effects of endothelial ET_B receptors could be present. Nevertheless, previous studies on receptor upregulation demonstrate negligible effects of endothelial ET_B (48). Cerebral and ophthalmic arteries do not seem to follow identical pathological patterns as the absence of 5-HT_1 mediated vasoconstriction in the ophthalmic artery. Whether this is due to methodological grounds or that the response of the ophthalmic artery varies from the cerebral arteries, is still unclear. These results however, indicated that ET-1 may be a key player in retinal ischemia leading to a secondary decreased blood flow after original reperfusion and could therefore be a valid target for further research.

Paper II

It had previously been established that the MEK/ERK1/2 pathway regulates the receptor changes in cerebral arteries. Therefore, our next goal was to evaluate if MEK/ERK1/2 inhibitors can prevent the vasoconstriction observed in the rat ophthalmic artery. Organ culture (OC) allowed for a practical way of testing dose concentrations of both U0126 and trametinib, MEK1/2 inhibitors, where the former having been prominently used in cerebral research. Furthermore, the specificity of ET_A and ET_B receptors were evaluated both by ET_B specific agonist S6c and the addition of BQ123 and BQ788, ET_A and ET_B antagonists respectively. Figure 7A shows the results obtained with a significant increase of ET-1 mediated vasoconstriction of OC arteries compared to fresh. The curves differ significantly ($p<0.001$) and there was a leftward shift of the biphasic curve with differences in pEC501 and pEC502 between the curves. We were also able to confirm that MEK1/2 inhibitors are able to offset the ischemia induced vascular changes $p<0.0001$ and $p<0.001$ for the U0126 and trametinib treated arteries respectively.

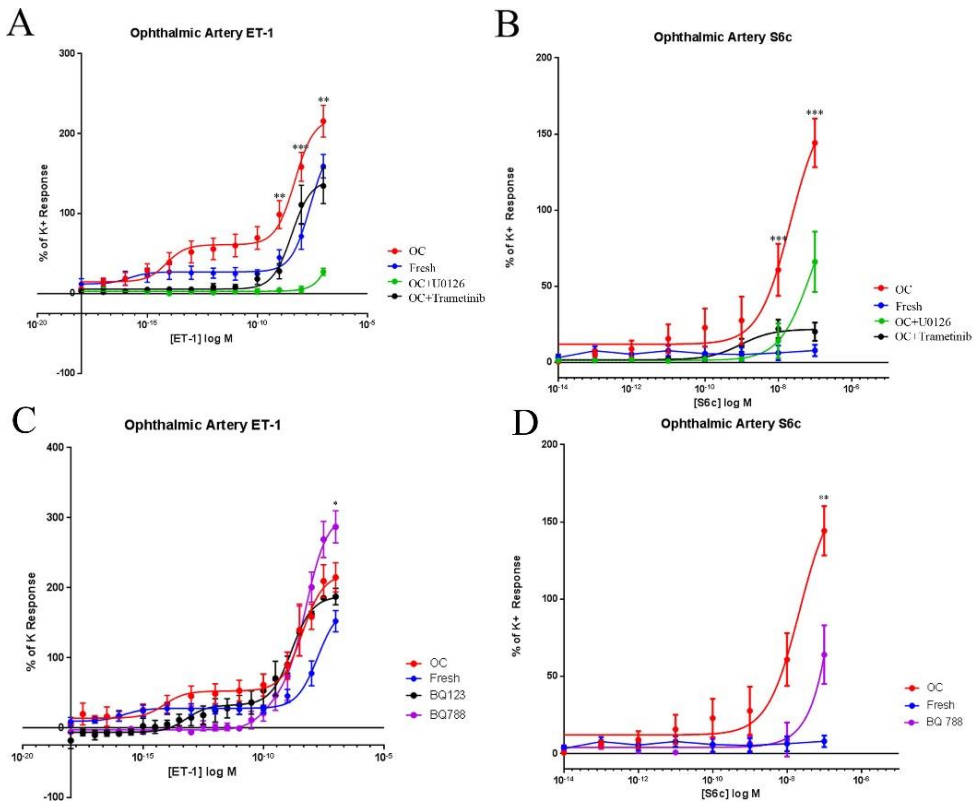


Figure 2: Rat ophthalmic artery after organ culture (A) Concentration–response curve showing an increased vasoconstriction of organ culture (OC, n = 14) rat ophthalmic artery in comparison with fresh (n = 13) and MEK/ERK1/2 inhibitors U0126 (n = 9) and trametinib (n = 10). Significant differences between fresh and OC at endothelin-1 (ET-1) concentrations 10-9M, 10-8M and 10-7M were also observed. (B) Concentration–response curve of ETB agonist sarafotoxin (S6c). Organ-treated arteries (n = 11) exhibited a significantly increased vasoconstriction compared to untreated fresh arteries (n = 9)with significance at concentrations 10-8M and 10-7M. Both U0126 (n = 6) and trametinib (n = 10) attenuated the increase. Full statistical review can be found in Table 2. (C) Concentration–response curve of ET-1 administration with ETA and ETB-specific antagonists BQ123 (n = 3) and BQ788 (n = 3), respectively. BQ123 having no significant effect on ET-1-mediated contraction while BQ788 abolishes the biphasic pattern and causes an overall increased contraction. (D) Sarafotoxin contraction is inhibited significantly by the use of ETB-specific antagonist BQ788.

The ET_B specific agonist S6c also elicited an increased vasoconstriction in OC rat ophthalmic arteries (Figure 7B) suggesting that ET_B mediated vasoconstriction plays an important role in the overall ET-1 mediated vasoconstriction following ischemia. The OC artery exhibited a significantly higher contraction ($p<0.0001$), as fresh arteries showed no noticeable contraction, most likely due to ET_B receptors

not being present on smooth muscle cells under physiological conditions. Furthermore, both MEK1/2 inhibitors suppressed the ET_B contraction.

Pig retinal arteries were also evaluated under OC conditions (figure 8). Even more so than in rat, the arteries exhibited a significant ET-1 response after OC compared to fresh arteries with pEC_{50}^1 13.52±0.45 and 11.76±0.95 respectively, and pEC_{50}^2 9.00±0.22 and 8.69±0.35 respectively (Figure 8A). Furthermore, ET_B receptor mediated contraction was evaluated with and without the presence of U0126 (Figure 8B). Results showed a significant increase of vascular constriction in OC treated arteries compared to fresh pig retinal arteries. U0126 was also shown to successfully neutralize the increased vasoconstriction with pEC_{50} values for OC, fresh, and U0126 treated arteries being 8.31±0.18, 9.87±0.11, and 8.81±1.43 respectively.

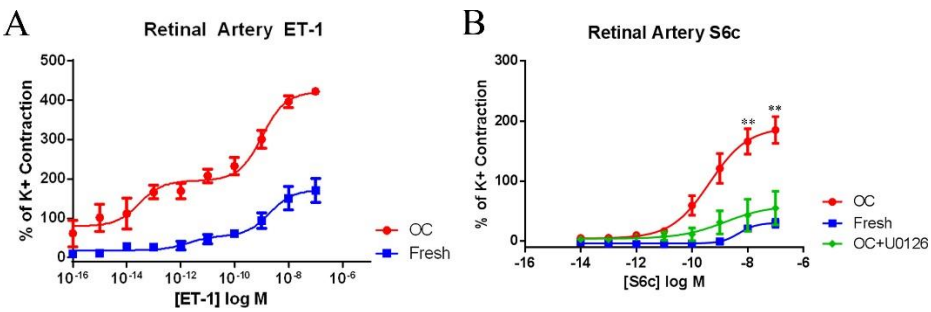


Figure 3: **Pig retinal arteries after organ culture** (A) Increased contractility of pig retinal arteries after organ culture (OC) (n = 4) compared to fresh (n = 4). (B) The increased contractility is, at least partly, due to increased ET_B contractility after sarafotoxin (S6C) stimulation. Significance is shown between OC (n = 4) and fresh (n = 4) at both 10^{-8}M and 10^{-7}M. U0126 (n = 4) also severely attenuates the observed contractility of ET_B.

In the second paper our hypothesis of vascular changes in the ophthalmic and retinal arteries following ischemia were elaborated on. Here the specific role of the individual ET-1 receptors were evaluated both with an ET_B specific agonist and $ET_{A/B}$ specific antagonists. The shift in ET_B function from endothelium-dependent NO vasodilation to constriction was confirmed in the ophthalmic artery for the very first time, coinciding with what has been described in cerebral arteries (26, 27, 49, 50). ET_B has also been shown to be involved in the clearance of ET-1 through the endothelial cells (51). The inhibition of ET_B by specific antagonist BQ788 might inhibit the clearance process further increasing the ischemia induced increase in

vasoconstriction resulting in the observed Figure 7C. However, the limited effect of BQ123 needs to be further analyzed before any major conclusion can be taken. As stipulated after the initial study following GCI, a potentially decreased blood flow to the surrounding tissue could exacerbate ischemic damage. Further validation of the role of ET-1 mediated vasoconstriction was provided by similar changes being observed in pig retinal arteries, suggesting a cross species phenomenon. These results lend further support to the relevance of ET-1 in the vascular pathophysiology of ischemia.

Furthermore, the underlying mechanism of the vascular changes was also determined for the first time in this study. Both U0126 and trametinib inhibited the overall increase of the ET-1 receptor function however with varying efficiency. The observed variation may be the result of trametinib being a highly potent and specific MEK1/2 inhibitor with little known effects on other kinases involved in the cellular communication (52) while U0126, on the other hand, acts as a strong inhibitor of MEK1/2 but also as a weak inhibitor of phosphokinase C, Raf, ERK1/2, and JNK (53). This difference between both inhibitors may be the reason for the discrepancy observed in the vascular function of the arterial segments. Nevertheless, the root mechanism of the observed increase of ET-1 mediated vasoconstriction seems to be found within the MEK/ERK1/2 signaling pathway, allowing us to identify a target for potential treatments.

Paper III

Finally, an in vivo assessment of MEK/ERK1/2 inhibition after transient ischemia was evaluated. The MCAO/OAO method allowed for a longer ischemic period than GCI, along with reperfusion and neurological assessment of the rat mid and post-surgery. U0126 at a dose of 30 mg/kg was used over trametinib in order to enable comparison with cerebral ischemic studies, at 0, 6, and 24 hours after ischemia. ET-1 contraction was evaluated and as in previous models ischemic arteries exhibited a more potent contraction than their control counterparts (Figure 9). Ischemic OA had

a leftwards shift with significant difference at ET-1 concentration of 10-7, but indistinguishable E_{max} values. Furthermore U0126 treatments inhibited the increased ET-1 contraction and had a significant different E_{max} than seen in ischemic arteries ($p<0.05$). ET_B mediated contraction showed a significant increase in ischemic OA compared to control while U0126 reduced the E_{max} with 41.6±9.2% compared to ischemic arteries ($p<0.05$) and 83.1±10.4% compared to control arteries ($p>0.05$) (Figure 10).

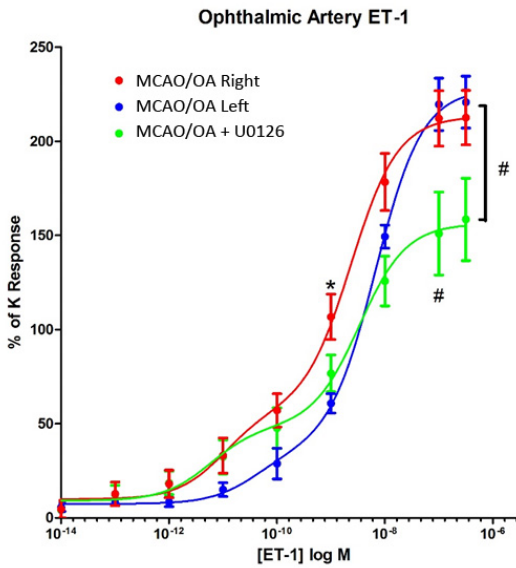


Figure 9: MCAO/OA ET-1 mediated vasoconstriction of MCAO/OA operated and U0126 treated rats.

As described in paper I and II, an ischemic event downstream from the ophthalmic artery elicits an increase in ET-1 mediated vasoconstriction. Comparatively to previous studies within cerebral ischemia it is interesting to note that the ophthalmic artery seems to have an ET_B mediated contraction even in control arteries (54, 55) which is not noted in myograph studies of the MCA (26, 50, 56). Whether this remains isolated to the ophthalmic artery or to all ocular arteries remains unknown. Furthermore, U0126 treatment seems to not only affect ET-1 facilitated vasoconstriction through ET_B receptors as seen in cerebral arteries,

but also affecting the ET_A facilitated constriction. However, the specifics around this occurrence also remain unknown.

These results, spanning over three different ischemic methods, all suggest that ET-1 receptors are highly involved in ocular arteries after ischemia 48 hours after reperfusion. Furthermore, the increase in ET_B mediated contraction suggests not only a presence of ET_B receptors in the arteries, but also a vascular smooth muscle location based on their function. Thus, the role of ET-1 receptors, particularly preventing their upregulation, may be a future consideration in acute treatment of ocular ischemic conditions with a vascular origin. Moreover, treatment of ischemic rats with U0126 will decrease the overall contraction response by ET-1 *in vivo*.

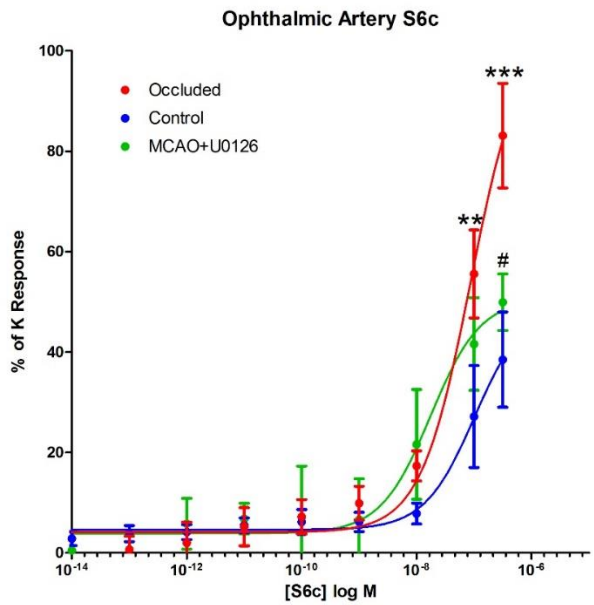


Figure 10: MCAO/OA S6c function of MCAO/OA operated and U0126 treated rats.

4.2 Immunohistochemical Analysis (Paper 1-IV)

Paper I, III, and IV

The retina was analyzed following both GCI and MCAO/OAO to confirm ischemic damage with the presence of gliosis markers glial fibrillary acid protein (GFAP) and Vimentin. A 15 minute GCI insult resulted in gliosis markers being noticeably increased at 72 hours after ischemia (Figure 11) while the 2 hour MACO/OAO period led to a retinal gliosis at 48 hours (Figure 12). GFAP has been established as a highly sensitive and non-specific indicator of retinal damage and the initiation of gliosis in multiple diseases pertaining to the retina (5). Vimentin, also an intermediated filament, is commonly co-expressed with GFAP in the retina. Both of which act as stabilizing proteins for Müller cell processes as well as being involved in signal transduction cascades in reactive gliosis (6). Their presence indicating that the structural or functional integrity of the retina has been compromised both after a 15 minute global cerebral ischemic, and a 2 hours MCAO/OAO insult.

Furthermore, the location of ET_A and ET_B were evaluated on fresh OA, after 24 hour OC, and after 24 hour OC + U0126/trametinib (Figure 13). As expected, ET_A was predominantly expressed in the smooth muscle cells of the artery. ET_B however was mostly found in endothelial cells of fresh arteries but following OC could only be seen in smooth muscle cells. Additionally, the presence of pERK1/2, the phosphorylated and active state of ERK1/2, was also stained for in the aforementioned conditions. Following 24 hour OC there was an increased presence of pERK1/2 in the smooth muscle cell layer of the ophthalmic artery which was completely abolished in arteries treated with either U0126 or trametinib. It is important to note that the activation of ERK1/2 is only present in the acute stage of ischemia in cerebral arteries in rat (57), human (58), and in the retina and retinal arteries (33, 59). Therefore the need to establish a therapeutic window where the inhibition of ERK1/2 provides the highest potential effect is crucial while it does

not interfere with other ERK1/2 mediated signaling which may have positive outcomes for patients.

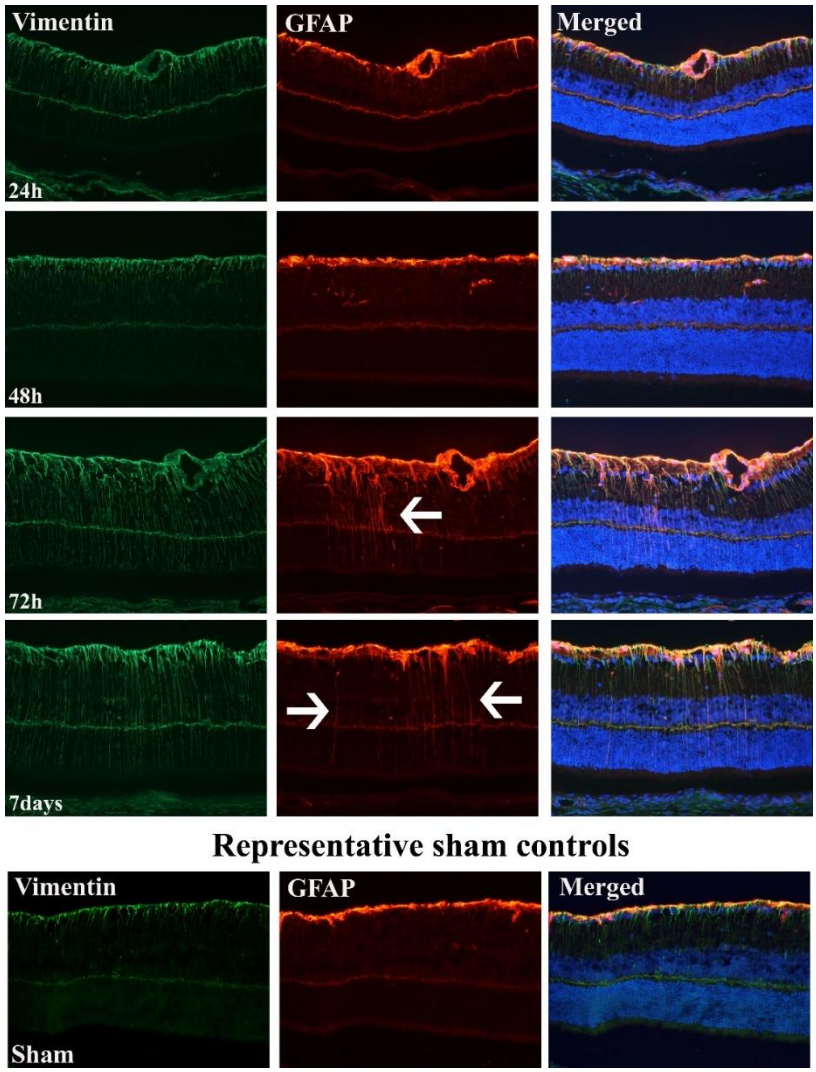


Figure 4: **GFAP and Vimentin expression increased at 72 hours and 7 days after GCI indicating active gliosis in the rat retinas.**

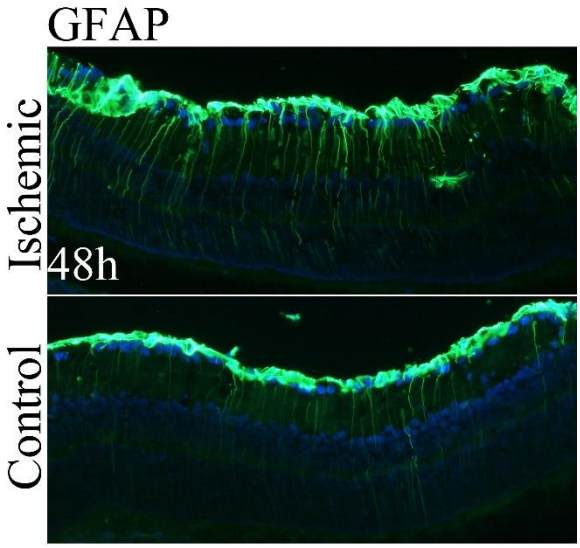


Figure 12: Gliosis in the retina following MCAO/OAO and TTC staining of brain to confirm a successful surgery.

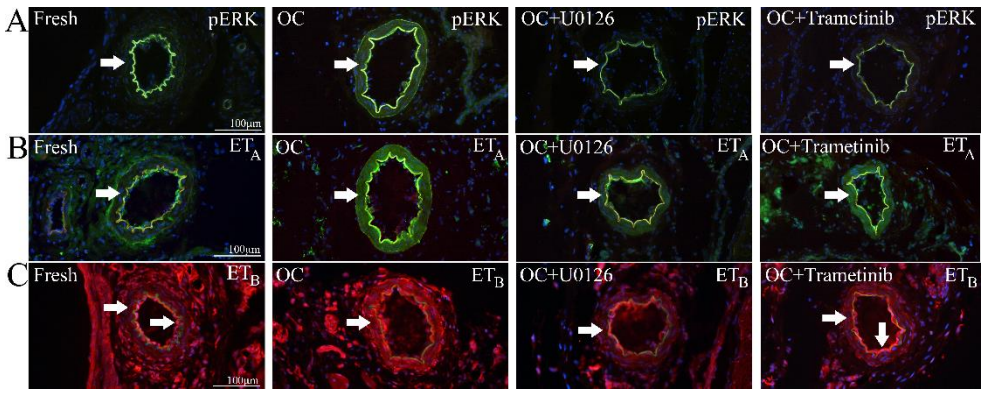


Figure 13: Immunohistochemistry of the rat ophthalmic artery following organ culture. A) ETA, B) ETB, and C) pERK1/2 antibodies in fresh controls, OC, OC + U0126, and OC + Trametinib cultures.

4.3 Electroretinography Analysis (Paper I and III)

The electrophysiology of the retina was evaluated, both after GCI and after MCAO/OAO. Primarily the photoreceptor activity and inner nuclear layer were evaluated by the a- and b-wave. Due to the severity of GCI, animals were only able to be put under anesthesia 3 days after the original operation to prevent increased mortality rates. This was due to the extent of the brain damage to the rat. Three days after surgery rats demonstrated a significant decrease in photoreceptor activity compared to sham operated animals at all but one luminance intensity (Figure 14). Interestingly this had recovered spontaneously at 7 days after ischemia suggesting that the extent of the retinal damage may not have been enough to cause permanent functional damage. The b-wave, highly dependent on the a-wave as bipolar cells process the signal provided by the photoreceptors, followed a similar pattern where all but three luminance intensities demonstrated a functional deficit in the ischemic retina compared to controls (Figure 14). As with the a-wave, a spontaneous recovery was observed at 7 days returning functional abilities of the retina to normal levels. We were also able to evaluate the oscillatory potentials of the b-wave which typically is a representation of proximal retinal cells (possibly amacrine cells). At day three, the amplitudes of the third and fourth wavelets (OP3 and OP4) of ischemic eyes were decreased by 30% and 40%, respectively compared to their control equivalents (Figure 14). This diminished functional reading was persistent up to 7 days after ischemia but had been reduced to 20% and 35% deficiency respectively ($p<0.05$).

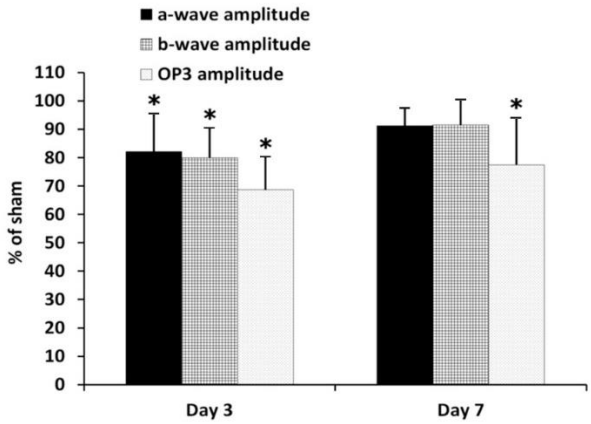
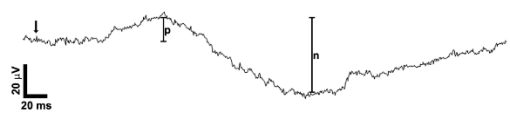


Figure 14: ERG recordings of a-, b-wave, and oscillatory potentials in rat retinas 3 and 7 days after enduring a 15 minute GCI.

Finally, the positive and negative scotopic threshold response (STR) was evaluated. The STR is generally attributed to ganglion cells, the bottleneck of visual stimuli processing. Again, a similar pattern was observed with a decrease in functional outcome at day three with a positive STR (pSTR) decreased to 50% ($p<0.05$) of those of sham operated animals (Figure 15). The negative STR (nSTR) amplitudes were also decreased by 30% ($p<0.05$) at day three. Both pSTR and nSTR had recovered at day three.

A



B

	3 days		7 days	
	Sham	Ischemic	Sham	Ischemic
pSTR	19.4±4.5	**9.5±6.6***	16.0±8.7	17.8±6.3
nSTR	47.6±7.3	**34.1±8.6***	45.4±5.8	35.7±11.7

Figure 15: pSTR and nSTR at 3 and 7 days following GCI.

The functional analysis of GCI operated animals presented clear retinal functional deficiencies. However, due to the higher mortality rate accompanied by a longer ischemic insult than the 15 minutes used, another ischemic method was preferred. Therefore, retinal ischemia via MCAO/OAO was utilized. As mentioned in the methods, MCAO/OAO allowed for a longer ischemic period without increasing the mortality rate compared to GCI and has significant support in the literature as a retinal ischemia model (60-62).

Paper III

ERG analysis of MCAO/OAO operated animals was performed at 1, 4, and 7 days post reperfusion (Figure 16). At day 1, functional photoreceptor activity was significantly diminished among ischemic animals at -1 to 2 log(cd*s*m2) ($p<0.05$ - $p<0.001$). This deficiency persisted to day 4 however, with a spontaneous recovery only giving a significant variance at luminance 1 to 2 log(cd*s*m2). The recovery persisted to 7 days where the functional difference between ischemic and sham operated animals was negligible.

It is noteworthy that bipolar cell activity showed no significant change between the ischemic and sham operated animals, however a trend could be observed at 1 and 4 days where ischemic eyes exhibited a lower amplitude than sham operated animals. By day 7, the trend had disappeared again suggesting a spontaneous ability to recover as seen with the photoreceptors.

The spontaneous functional recovery pattern of the retina was also observed in our previous research (63) and is not unique to our methods. It can suggest though that the ischemic insult was not enough to cause permanent functional deficit and that for future studies longer ischemia is needed to study the treatment potential of U0126 on retinal function.

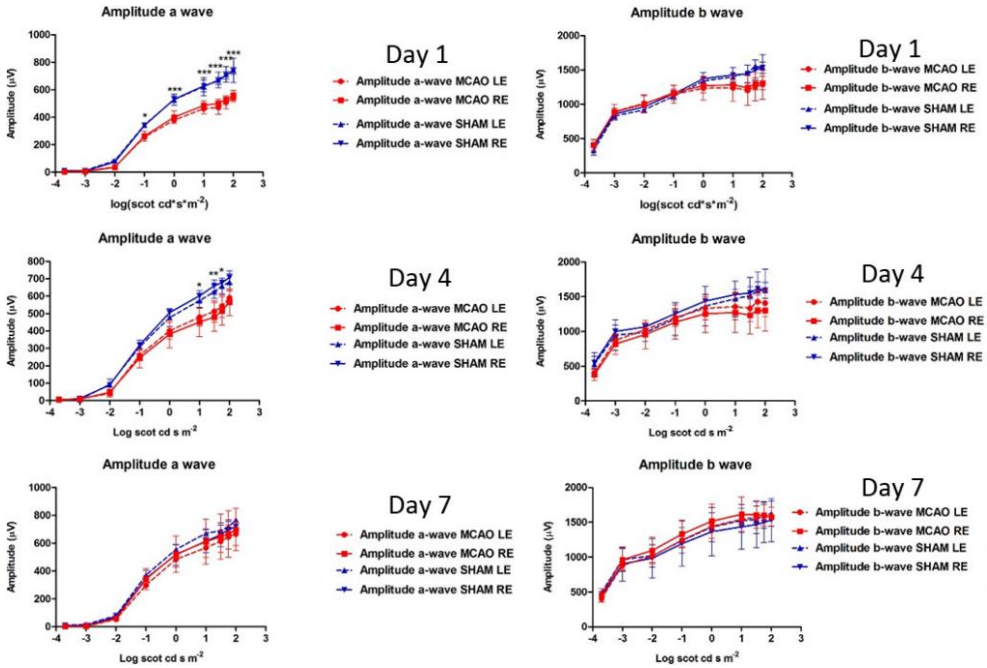


Figure 16: ERG recordings from MCAO/OAO operated animals at 1, 4, and 7 days after reperfusion.

The ERG results for the MCAO/OAO operated animals exhibited an interesting pattern where the non-occluded eyes of the operated animals would also suffer from functional impairment, an observation described previously (60, 64). The severity of the occlusion has been attributed to the contralateral control eyes decreased function (60), suggesting that the rats within this study have been severely affected by the MCAO/OAO. The phenomenon of functional deficits on the non-occluded eye points towards a neurological cross talk between retinas that has been suggested in previous studies in frogs (65, 66), rats (67), and rabbits (68). A similar phenomenon has been described in the brain where areas outside the ischemic core and penumbra exhibit deficient function, coining the term diaschisis (69). Interestingly however, the retina seems to have a constant cross talk between each eye both in normal conditions where if one eye is exposed to higher frequency stimuli, the unstimulated retina will also produce a cross ERG response (70). The

details of the observed cross talk is still unknown, but their acknowledgement and importance may be of great value in future research.

4.4 Proteomic analysis (Paper IV)

Proteomic analysis was performed in order to gather a greater understanding of what effects MEK inhibition may have on the retina. In total, 3043 proteins were identified with 143 having significantly changed prevalence. Our results revealed that 21 of those proteins were significantly altered in the ischemic eyes compared to the control ($p < 0.05$) and had a link to the MEK/ERK1/2 pathway (Figure 17). These proteins included the CD44 antigen, signal transducer and activator of transcription 3 (STAT3), talin 1 (TLN1), RAS p21 protein activator (RASA1), microtubule-associated protein tau (MAPT), ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1), phosphoenolpyruvate carboxykinase 2 (PCK2), heat shock protein 90kDa alpha (HSP90AA1), fibronectin 1 (FN1), hemogrobin alpha 1 (HBA1), semaphoring 7A (SEMA7A), RhoGEF and pleckstrin domain protein 1 (FARP1), CCR4-NOT transcription complex subunit 6-like (CNOT6L), ankyrin 1 (ANK1), SMEK homolog 1 (SMEK1), ATPase H+ transporting lysosomal V0 subunit a1 (ATP6V0A1), protein phosphatase (PPM1H), dynamin 1 (DNM1), GTPase activating protein and VPS9 domains 1 (GAPVD1), COP9 constitutive photomorphogenic homolog subunit 7B (COPS7B), and GRIP1 associated protein 1 (GRIPAP1). Among these, a majority are involved in the movement of cell/subcellular components (Figure 17 blue), while four proteins were involved in the regulation of the MEK/ERK1/2 cascade (Figure 17 red)

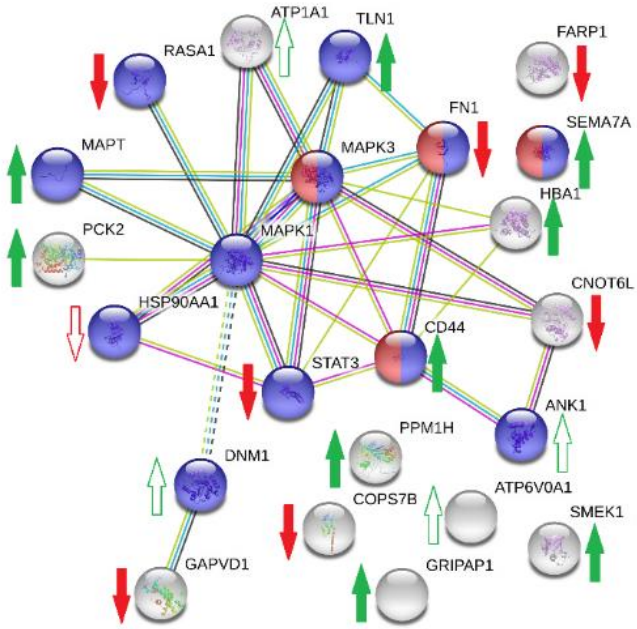


Figure 17. Proteins linked to the MEK/ERK1/2 pathway in the retina. Green arrows indicate an increase of proteins in the ischemic eye compared to the control (full arrow indicates >1.1 fold change), red arrows indicate a decrease of proteins in the ischemic eye compared to the control (full arrow indicates <0.9 fold change).

These proteins did not exhibit a clear up or down regulation pattern following ischemia, however a majority (15 out of 21) were severely changed (full arrow). The most severe change was that of CD44 (1.703-fold increase). CD44 function in the retina has been well described previously, activating the MEK/ERK1/2 pathway to trigger anti-apoptotic processes primarily in blood cells (71). However, the secondary inflammatory response which CD44 is an important component of, may have detrimental effects to the retina. CD44 deficiency has been linked to improved neurological function in mice following MCAO (72). CD44 and the MEK/ERK1/2 pathway has a demonstrably intricate relationship with MEK/ERK1/2 increasing CD44 RNA, facilitating CD44 translation (73).

STAT3 which had a 0.864 fold change in ischemic eyes is also very closely linked to MEK/ERK1/2. STAT3 is also a major signaling molecule for numerous neurotrophic factors and has been indicated in ischemia through subarachnoid

hemorrhage. Here, STAT3 levels were involved in the vasospasm of cerebral arteries following ischemia (74). Within retinal ischemia, STAT3 has been shown to be involved in the neovascularization processes triggered by hypoxia and play a role in proliferative retinopathy (75). However, other studies conclude that STAT3 may also play an important role in retinal ganglion cell survival (76). The exact role of STAT3 in retinal ischemia is thus far not fully understood, but its relevance seems nevertheless important.

Our proteomic results have shown the wide range of MEK/ERK1/2 involvement in the retina, spanning through CD44, STAT3, heat shock, metabolic, and protein synthesis regulated proteins. We can therefore validate our hypothesis that MEK/ERK1/2 involvement in the retinal ischemic response is clear, just as with ocular vasculature. Therefore, this pathway may play a very important role in future therapeutic options.

4.5 Concluding Remarks

This thesis aimed to bridge cerebral research pertaining to vascular function after ischemia with ophthalmological research and ocular arteries. Within the scope of the former, vascular changes have been highlighted as an important part of the pathophysiology of stroke (ischemia), thus presenting a novel approach to combat a multifaceted disease. In terms of retinal ischemia (and in particular thrombotic conditions), within ocular vasculature, we are in desperate need of new and impactful alternatives. Therefore, the results from the four studies included in this thesis may play an important role in doing just that: providing *a vascular perspective*.

Retinal ischemia is a tremendously complex condition in which there most likely will not be a ‘magic bullet’ treatment for. As described in the background, the pathophysiology suggests that a multitude of processes must be tackled either at the

same time or at certain time points to allow the eye to heal in the best possible way. Therefore multi-disciplinary efforts from neuroscience to ophthalmology as presented in this body of work are, in my opinion, crucial in the pursuit of a solution.

However, much work lays ahead in order to confirm the validity of the hypothesis presented. The need to evaluate these changes in larger animal models is imperative as the main limitation of rat retinal vascular changes is the scale of the retinal arteries. Animal models provide the vital and basic template for translational science but ultimately human arterial segments are needed. Therefore, the natural step moving forward lays in working towards applying our findings and knowledge to the clinical setting.

5. References

1. Lund RD, Bunt AH. Prenatal development of central optic pathways in albino rats. J Comp Neurol. 1976;165(2):247-64.
2. Adler FH, Hart WM. Adler's physiology of the eye : clinical application. 9th ed. St. Louis: Mosby Year Book; 1992. xiv, 888 p. p.
3. Shields CR, Tran MN, Wong RO, Lukasiewicz PD. Distinct ionotropic GABA receptors mediate presynaptic and postsynaptic inhibition in retinal bipolar cells. J Neurosci. 2000;20(7):2673-82.
4. Uga S, Smelser. Comparative study of the fine structure of retinal Muller cells in various vertebrates. Invest Ophthalmol. 1973;12(6):434-48.
5. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, et al. Muller cells in the healthy and diseased retina. Prog Retin Eye Res. 2006;25(4):397-424.
6. Bignami A, Dahl D. Radial Glia of Muller in the Rat Retina and Their Response to Injury - Immunofluorescence Study with Antibodies to the Glial Fibrillary Acidic (Gfa) Protein. Experimental Eye Research. 1979;28(1):63-9.
7. Lewis GP, Guerin CJ, Anderson DH, Matsumoto B, Fisher SK. Rapid changes in the expression of glial cell proteins caused by experimental retinal detachment. Am J Ophthalmol. 1994;118(3):368-76.
8. Michalinos A, Zogana S, Kotsiomitis E, Mazarakis A, Troupis T. Anatomy of the Ophthalmic Artery: A Review concerning Its Modern Surgical and Clinical Applications. Anat Res Int. 2015;2015:591961.
9. Country MW. Retinal metabolism: A comparative look at energetics in the retina. Brain Research. 2017;1672:50-7.
10. Laouri M, Chen E, Looman M, Gallagher M. The burden of disease of retinal vein occlusion: review of the literature. Eye (Lond). 2011;25(8):981-8.
11. Lee R, Wong TY, Sabanayagam C. Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss. Eye Vis (Lond). 2015;2:17.
12. Varma DD, Cugati S, Lee AW, Chen CS. A review of central retinal artery occlusion: clinical presentation and management. Eye (Lond). 2013;27(6):688-97.
13. Hayreh SS, Zimmerman MB, Kimura A, Sanon A. Central retinal artery occlusion. Retinal survival time. Experimental Eye Research. 2004;78(3):723-36.
14. Lee JM, Grabb MC, Zipfel GJ, Choi DW. Brain tissue responses to ischemia. Journal of Clinical Investigation. 2000;106(6):723-31.

15. Goebel DJ, Aurelia JL, Tai Q, Jojich L, Poosch MS. Immunocytochemical localization of the NMDA-R2A receptor subunit in the cat retina. Brain Res. 1998;808(2):141-54.
16. Joo CK, Choi JS, Ko HW, Park KY, Sohn S, Chun MH, et al. Necrosis and apoptosis after retinal ischemia: involvement of NMDA-mediated excitotoxicity and p53. Invest Ophthalmol Vis Sci. 1999;40(3):713-20.
17. Berger S, Savitz SI, Nijhawan S, Singh M, David J, Rosenbaum PS, et al. Deleterious role of TNF-alpha in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci. 2008;49(8):3605-10.
18. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332(6163):411-5.
19. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev. 1994;46(3):325-415.
20. Mazzuca MQ, Khalil RA. Vascular endothelin receptor type B: structure, function and dysregulation in vascular disease. Biochem Pharmacol. 2012;84(2):147-62.
21. Johnsson E, Maddahi A, Wackenfors A, Edvinsson L. Enhanced expression of contractile endothelin ETB receptors in rat coronary artery after organ culture. European Journal of Pharmacology. 2008;582(1-3):94-101.
22. Maddahi A, Edvinsson L. Enhanced expressions of microvascular smooth muscle receptors after focal cerebral ischemia occur via the MAPK MEK/ERK pathway. BMC Neurosci. 2008;9:85.
23. Eskesen K, Edvinsson L. Upregulation of endothelin ETB receptor-mediated vasoconstriction in rat coronary artery after organ culture. Eur J Pharmacol. 2006;539(3):192-4.
24. Maddahi A, Edvinsson L. Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway. J Neuroinflammation. 2010;7:14.
25. Henriksson M, Xu CB, Edvinsson L. Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries. Br J Pharmacol. 2004;142(7):1155-61.
26. Johansson S, Povlsen GK, Edvinsson L. Expressional changes in cerebrovascular receptors after experimental transient forebrain ischemia. Plos One. 2012;7(7):e41852.
27. Ahnstedt H, Saveland H, Nilsson O, Edvinsson L. Human cerebrovascular contractile receptors are upregulated via a B-Raf/MEK/ERK-sensitive signaling pathway. BMC Neurosci. 2011;12:5.
28. Edvinsson LI, Povlsen GK. Vascular plasticity in cerebrovascular disorders. J Cereb Blood Flow Metab. 2011;31(7):1554-71.
29. Fischer AJ, Scott MA, Tuten W. Mitogen-Activated Protein Kinase-Signaling Stimulates Muller Glia to Proliferate in Acutely Damaged Chicken Retina. Glia. 2009;57(2):166-81.

30. Akiyama H, Nakazawa T, Shimura M, Tomita H, Tamai M. Presence of mitogen-activated protein kinase in retinal Muller cells and its neuroprotective effect ischemia-reperfusion injury. Neuroreport. 2002;13(16):2103-7.
31. Gao S, Andreeva K, Cooper NGF. Ischemia-reperfusion injury of the retina is linked to necroptosis via the ERK1/2-RIP3 pathway. Molecular Vision. 2014;20:1374-87.
32. Roth S, Shaikh AR, Hennelly MM, Li Q, Bindokas V, Graham CE. Mitogen-activated protein kinases and retinal ischemia. Invest Ophth Vis Sci. 2003;44(12):5383-95.
33. Gesslein B, Hakansson G, Carpio R, Gustafsson L, Perez MT, Malmsjo M. Mitogen-activated protein kinases in the porcine retinal arteries and neuroretina following retinal ischemia-reperfusion. Mol Vis. 2010;16:392-407.
34. Wurm A, Iandiev I, Uhlmann S, Wiedemann P, Reichenbach A, Bringmann A, et al. Effects of ischemia-reperfusion on physiological properties of Muller glial cells in the porcine retina. Invest Ophthalmol Vis Sci. 2011;52(6):3360-7.
35. Abu-El-Asrar AM, Dralands L, Missotten L, Al-Jadaan IA, Geboes K. Expression of apoptosis markers in the retinas of human subjects with diabetes. Invest Ophthalmol Vis Sci. 2004;45(8):2760-6.
36. Yorston D. Anti-VEGF drugs in the prevention of blindness. Community Eye Health. 2014;27(87):44-6.
37. Falavarjani KG, Nguyen QD. Adverse events and complications associated with intravitreal injection of anti-VEGF agents: a review of literature. Eye (Lond). 2013;27(7):787-94.
38. Nishijima K, Ng YS, Zhong L, Bradley J, Schubert W, Jo N, et al. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. Am J Pathol. 2007;171(1):53-67.
39. Cugati S, Varma DD, Chen CS, Lee AW. Treatment options for central retinal artery occlusion. Curr Treat Options Neurol. 2013;15(1):63-77.
40. Smith ML, Bendek G, Dahlgren N, Rosen I, Wieloch T, Siesjo BK. Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. Acta Neurol Scand. 1984;69(6):385-401.
41. Koizumi J, Yoshida Y, Nakazawa T, Ooneda G. Experimental studies of ischemic brain edema. I. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn J Stroke. 1986;8:1-8.
42. Memezawa H, Minamisawa H, Smith ML, Siesjo BK. Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat. Exp Brain Res. 1992;89(1):67-78.
43. Block F, Grommes C, Kosinski C, Schmidt W, Schwarz M. Retinal ischemia induced by the intraluminal suture method in rats. Neurosci Lett. 1997;232(1):45-8.
44. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke. 1986;17(3):472-6.

45. Menzies SA, Hoff JT, Betz AL. Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery. 1992;31(1):100-6; discussion 6-7.
46. Adner, Geary, Edvinsson. Appearance of contractile endothelin-B receptors in rat mesenteric arterial segments following organ culture. Acta Physiologica Scandinavica. 1998;163(2):121-9.
47. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. Am J Physiol Cell Physiol. 2005;288(4):C769-83.
48. Skovsted GF, Pedersen AF, Larsen R, Sheykhzade M, Edvinsson L. Rapid functional upregulation of vasocontractile endothelin ETB receptors in rat coronary arteries. Life Sci. 2012;91(13-14):593-9.
49. Nilsson T, Cantera L, Adner M, Edvinsson L. Presence of contractile endothelin-A and dilatory endothelin-B receptors in human cerebral arteries. Neurosurgery. 1997;40(2):346-51; discussion 51-3.
50. Stenman E, Malmsjo M, Uddman E, Gido G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. Stroke. 2002;33(9):2311-6.
51. Brunner F, Doherty AM. Role of ET(B) receptors in local clearance of endothelin-1 in rat heart: studies with the antagonists PD 155080 and BQ-788. FEBS Lett. 1996;396(2-3):238-42.
52. Lugowska I, Kosela-Paterczyk H, Kozak K, Rutkowski P. Trametinib: a MEK inhibitor for management of metastatic melanoma. Onco Targets Ther. 2015;8:2251-9.
53. Duncia JV, Santella JB, 3rd, Higley CA, Pitts WJ, Wityak J, Frietze WE, et al. MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett. 1998;8(20):2839-44.
54. Haefliger IO, Flammer J, Luscher TF. Nitric oxide and endothelin-1 are important regulators of human ophthalmic artery. Invest Ophthalmol Vis Sci. 1992;33(7):2340-3.
55. Yao K, Tschudi M, Flammer J, Luscher TF. Endothelium-dependent regulation of vascular tone of the porcine ophthalmic artery. Invest Ophthalmol Vis Sci. 1991;32(6):1791-8.
56. Ahnstedt H, Mostajeran M, Blixt FW, Warfvinge K, Ansar S, Krause DN, et al. U0126 attenuates cerebral vasoconstriction and improves long-term neurologic outcome after stroke in female rats. J Cereb Blood Flow Metab. 2015;35(3):454-60.
57. Ansar S, Edvinsson L. Subtype activation and interaction of protein kinase C and mitogen-activated protein kinase controlling receptor expression in cerebral arteries and microvessels after subarachnoid hemorrhage. Stroke. 2008;39(1):185-90.
58. Ansar S, Eftekhari S, Waldsee R, Nilsson E, Nilsson O, Saveland H, et al. MAPK signaling pathway regulates cerebrovascular receptor expression in human cerebral arteries. BMC Neurosci. 2013;14:12.

59. Roth S, Li Q, Shaikh AR, Hennelly M, Graham CE, Bindokas V. Functional roles of mitogen-activated protein kinases in retinal ischemia. Invest Ophth Vis Sci. 2003;44:U80-U.
60. Allen RS, Sayeed I, Cale HA, Morrison KC, Boatright JH, Pardue MT, et al. Severity of middle cerebral artery occlusion determines retinal deficits in rats. Exp Neurol. 2014;254:206-15.
61. Ritzel RM, Pan SJ, Verma R, Wizeman J, Crapser J, Patel AR, et al. Early retinal inflammatory biomarkers in the middle cerebral artery occlusion model of ischemic stroke. Mol Vis. 2016;22:575-88.
62. Steele EC, Jr., Guo Q, Namura S. Filamentous middle cerebral artery occlusion causes ischemic damage to the retina in mice. Stroke. 2008;39(7):2099-104.
63. Blixt FW, Johansson SE, Johnson L, Haanes KA, Warfvinge K, Edvinsson L. Enhanced Endothelin-1 Mediated Vasoconstriction of the Ophthalmic Artery May Exacerbate Retinal Damage after Transient Global Cerebral Ischemia in Rat. Plos One. 2016;11(6):e0157669.
64. Lovasik JV, Kergoat H, Gagnon M. Experimentally Reduced Perfusion of One Eye Impairs Retinal Function in Both Eyes. Optometry Vision Sci. 2005;82(9):850-7.
65. Bohn RC, Stelzner DJ. Aberrant Retino-Retinal Pathway during Early Stages of Regeneration in Adult Rana-Pipiens. Brain Research. 1979;160(1):139-44.
66. Bohn RC, Stelzner DJ. The Aberrant Retino-Retinal Projection during Optic-Nerve Regeneration in the Frog .2. Anterograde Labeling with Horseradish-Peroxidase. J Comp Neurol. 1981;196(4):621-32.
67. Bunt SM, Lund RD, Land PW. Prenatal Development of the Optic Projection in Albino and Hooded Rats. Dev Brain Res. 1983;6(2):149-68.
68. Muller M, Hollander H. A Small Population of Retinal Ganglion-Cells Projecting to the Retina of the Other Eye - an Experimental-Study in the Rat and the Rabbit. Exp Brain Res. 1988;71(3):611-7.
69. Avellaneda-Chevrier VK, Wang X, Hooper ML, Chauhan BC. The retino-retinal projection: Tracing retinal ganglion cells projecting to the contralateral retina. Neuroscience Letters. 2015;591:105-9.
70. Tang XL, Tzekov R, Passaglia CL. Retinal cross talk in the mammalian visual system. Journal of Neurophysiology. 2016;115(6):3018-29.
71. Herishanu Y, Gibellini F, Njuguna N, Hazan-Halevy I, Farooqui M, Bern S, et al. Activation of CD44, a receptor for extracellular matrix components, protects chronic lymphocytic leukemia cells from spontaneous and drug induced apoptosis through MCL-1. Leukemia Lymphoma. 2011;52(9):1758-69.
72. Wang XK, Xu L, Wang H, Zhan YT, Pure E, Feuerstein GZ. CD44 deficiency in mice protects brain from cerebral ischemia injury. Journal of Neurochemistry. 2002;83(5):1172-9.
73. Robbins EW, Travanty EA, Yang K, Iczkowski KA. MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells. Bmc Cancer. 2008;8.

74. Samraj AK, Muller AH, Grell AS, Edvinsson L. Role of unphosphorylated transcription factor STAT3 in late cerebral ischemia after subarachnoid hemorrhage. J Cerebr Blood F Met. 2014;34(5):759-63.
75. Mechoulam H, Pierce EA. Expression and activation of STAT3 in ischemia-induced retinopathy. Invest Ophth Vis Sci. 2005;46(12):4409-16.
76. Zhang C, Li H, Liu MG, Kawasaki A, Fu XY, Barnstable CJ, et al. STAT3 activation protects retinal ganglion cell layer neurons in response to stress. Experimental Eye Research. 2008;86(6):991-7.

Svensk Sammanfattning

Flera olika ögonsjukdomar, t ex artärocklusion, ventrombos, diabetes retinopati, och till viss utsträckning vid glaukom, orsakas av nedsatt blodflöde till näthinnan (retina) - retinal ischemi. Det är en av de vanligaste orsakerna till delvis eller total synnedsättning och det finns få behandlingsterapier som kan hjälpa denna patientgrupp. Därför råder det stort behov av ett nytt perspektiv på att förstå problematiken kring retinal ischemi.

Inom forskningen kring stroke har man lyckats identifiera proteiner som påverkar kärlsammandragningen efter ischemi. Bland dessa är endotelin-1 (ET-1), som orsakar en kraftig kärlsammandragning även då blodflödet till hjärnan återställs. ET-1 agerar via två olika receptorer ($ET_{A/B}$) som man finner på insidan av kärl och i kärlväggen. Ökning av ET_A leder till en kraftig kärlsammandragning. Ökning av ET_B kan orsaka både vidgning och sammandragning av kärlen. I hjärnans kärl efter ischemi har man upptäckt en specifik signaleringsväg, som involverar aktivering av en typ av enzym som kallas Mitogen Activated Protein Kinase (MAPK). Aktiveringen av denna signaleringsväg leder till en ökad ET_B-relaterad sammandragning och ett efterföljande minskat blodflöde till det redan skadade området i hjärnan.

Arbetet i denna avhandling avser att undersöka om samma typ av kärlförändringar som uppstår i hjärnan efter stroke också återfinns i ögat efter retinal ischemi. Avhandlingen beskriver tre olika experimentella, retinala ischemiska modeller för ögat, där ET-1-styrd kärlsammandragning återfinns både i råtta- och grisnäthinna. Slutsatsen blir att den stroke-relaterade kärlsammandragningen, som man ser i hjärnan, också finns i näthinnan.

Genom att hämma kärlsammandragningen med två läkemedel, U0126 och trametinib som hämmar ett specifikt steg av MAPK signalvägen (MEK/ERK1/2), lyckades vi att motverka sammandragningen i både råtta- och grisnäthinna. Detta antyder att MEK/ERK1/2-hämmare kan ha roll som en behandlingsmetod för patienter med retinal ischemi.

Denna avhandling visar på ett nytt perspektiv att motverka skada efter retinal ischemi. Näthinnans kärl och dess förändringar vid retinal ischemi sätts i fokus. Genom att förhindra dessa förändringar, och därmed också förhindra kärlsammandragning, kan man motverka synförändringar hos patienter med en sjukdom som involverar retinal ischemi.

Acknowledgements

My time as a PhD student has been one of some great challenges and several fantastic moments. More importantly I have learned to truly appreciate the people involved, either directly or indirectly, and wish therefore to thank you all!

First of all, my two supervisors have acted as a scientific dynamic duo worthy of endless praise. My main supervisor *Lars Edvinsson*, el presidente, has not only been an inspiration and constant motivator, but also a great supervisor. What has been lacking in administrative preparedness you have made up in being able to see the bigger picture of my research long before I did and pushed me forward with constant enthusiasm. You have allowed me to work independently and freely, allowing me to learn how to take responsibility for my work and therefore allowed me to grow into the role of a PhD student.

But a dynamic duo is only complete with a second key person: *Karin Warfvinge*, il professore. Karin, we have had a very special relationship due to your close connection to my family. This means that I probably have had a PhD experience unlike any other. When you weren't telling the rest of the lab about my various childhood business endeavors, you have been a blessing. You have provided me with the best possible working environment and have pushed me into thinking like a scientist. I am utterly grateful for all the conversations and times spent in the lab from when I was first introduced to the scientific world in Lund in 2011. Thank you!

Then I would like to thank my co-workers, especially *Kristian* who has become somewhat of a 3rd supervisor and a great friend. We have had tremendous times working long days, skiing in Poland, and, throughout my PhD time, having been a constant inspiration. You have an American spirit that I admire and I look forward to working with you in the future!

A huge part of my time has been spent in a fantastic work environment in Lund. Each one of you has over the years been forced to listen to my ramblings about movies, politics, and any of the odd topics we have talked about outside of science. I would especially like to thank *Saema* whom has shared office with me for a majority of my PhD time. I am very grateful for the experience and professionalism that you have shared with me, for the many discussion on academia, politics, and future life decisions. *Lena* for being the (only) one who truly appreciates a great joke or pun, no matter how dry and cheesy it may be, and for our dream team work

with the myographs. *Maryam* for having been my second office mate and PhD buddy going alongside me for a majority of my time here. Thank you for being a friend and keeping track of all the important dates and checkpoints during our time together! *Kajsa*, the only actually gluten intolerant person I know, for having gone from being a colleague to a friend and all the hours spent by the lunch table. *Mimmi* for putting the "fika" back in the "fika lab" with your baking skills. I also want to thank you for enduring my discussions and for the summers spent in the lab together. *Philip* for always making the lab a more exciting place to be in and the top notch banter. You have also become a very close friend! Thank you *André* for bringing the dankest of memes to the lab. *Diana* for coming all the way from California to prepare me for my half time and your helpful discussions. I also want to thank *Hilda* who has been a fantastic friend, co-worker, and training buddy as well as being very big part of my research career. I will see you in Houston soon! And *Saji* for our great times in the lab and constant Real vs Barca banter. And last but not least, thank you *Elisabeth* for being amazingly helpful and nice to me from the very first day at the lab.

Then I would like to thank all the other A13 people. *Miri* for having become a very good friend and your constant help and guidance during my PhD time, *Sarah* for letting me trade gallons of blood for movie tickets, *Jacob*, *Karsten*, *Ellinor*, *Andrea*, and *Kerstin* for providing an excellent atmosphere.

Thank you my friends at B11, *Oscar*, *Marina*, and *Belmin* for the times at ARVO, lunches, game nights, and for including me in your group and making me feel like I was a part of it. At A10 I want to especially thank *Michael* not only for our endless debates to get my mind of my experiments, but also for letting me be your friend, toastmaster at your defence, and for dishing out Pirlo-esque passes at football.

In Denmark, *Sara* for your help in the beginning of my project, for the countless surgeries and support as a fellow Swede in Denmark. *Leif* for being the ERG master and our multiple rides to and from Denmark. *Karin* for the great teamwork on our papers. *Stine*, *Ann-Sophie*, and *Hanne* for making Glostrup a welcoming place to a linguistically challenged Swede. *Anders* for the time spent at Panum and the help with our papers. Thank you *Lasse* for the incredible partnership that started at ARVO and lead to my fourth paper in this thesis. Your support and attitude has been invaluable.

I wish to also thank my many friends outside of the scientific community. In particular old and new friends at the gym, *Oskar*, *Daniel*, *Gustav*, and friends like *Josh*, *Ida*, *Tucker*, *Will*, *Ramin*, and everyone else who have been very supportive in more ways than I am able to list here. I appreciate having you all in my life.

I would also like to thank *Hans Johnell* for his great last minute help with the cover image, it's exactly what I wanted!

Finally but not least, I want to thank my family who has been incredibly supportive from day one. *My mom* for actively being curious about my research and providing me with a clinical insight, constantly encouraging me, and in many ways introducing me to BMC back in the early 2000's. *My dad* for his encouragement and constant nagging to keep pushing forward no matter what, to concentrate, and to always finish what I started. Your support has been absolutely vital for me and I am incredibly grateful to have you both. Also *Oscar*, who still is not convinced that I can read, for being the best brother one could ask for. I love you all.

Paper I

PLOS | ONE

RESEARCH ARTICLE

Enhanced Endothelin-1 Mediated Vasoconstriction of the Ophthalmic Artery May Exacerbate Retinal Damage after Transient Global Cerebral Ischemia in Rat

Frank W. Blixt[1]*, Sara Ellinor Johansson[2], Leif Johnson[3], Kristian Agmund Haanes[2], Karin Warfvinge[1,2], Lars Edvinsson[1,2]

1 Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, Lund, Sweden, **2** Department of Clinical Experimental Research, Glostrup Research Institute, Rigshospitalet, Glostrup, Denmark, **3** Department of Ophthalmology, Glostrup Research Institute, Rigshospitalet, Glostrup, Denmark

* frank.blixt@med.lu.se

Citation: Blixt FW, Johansson SE, Johnson L, Haanes KA, Warfvinge K, Edvinsson L (2016) Enhanced Endothelin-1 Mediated Vasoconstriction of the Ophthalmic Artery May Exacerbate Retinal Damage after Transient Global Cerebral Ischemia in Rat. PLoS ONE 11(6): e0157669. doi:10.1371/journal.pone.0157669

Editor: Alfred S Lewin, University of Florida, UNITED STATES

Received: April 26, 2016

Accepted: June 2, 2016

Published: June 20, 2016

Data Availability Statement: All relevant data are within the paper itself.

Funding: This work was supported by Hjärt-Lungfonden award number 20130271 (www.hjart-lungfonden.se) and Vetenskapsrådet (http://www.vr.se/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Cerebral vasculature is often the target of stroke studies. However, the vasculature supplying the eye might also be affected by ischemia. The aim of the present study was to investigate if the transient global cerebral ischemia (GCI) enhances vascular effect of endothelin-1 (ET-1) and 5-hydroxytryptamine/serotonin (5-HT) on the ophthalmic artery in rats, leading to delayed retinal damage. This was preformed using myography on the ophthalmic artery, coupled with immunohistochemistry and electroretinogram (ERG) to assess the ischemic consequences on the retina. Results showed a significant increase of ET-1 mediated vasoconstriction at 48 hours post ischemia. The retina did not exhibit any morphological changes throughout the study. However, we found an increase of GFAP and vimentin expression at 72 hours and 7 days after ischemia, indicating Müller cell mediated gliosis. ERG revealed significantly decreased function at 72 hours, but recovered almost completely after 7 days. In conclusion, we propose that the increased contractile response via ET-1 receptors in the ophthalmic artery after 48 hours may elicit negative retinal consequences due to a second ischemic period. This may exacerbate retinal damage after ischemia as illustrated by the decreased retinal function and Müller cell activation. The ophthalmic artery and ET-1 mediated vasoconstriction may be a valid and novel therapeutic target after longer periods of ischemic insults.

Introduction

Global cerebral ischemia (GCI) occurs when blood supply is significantly reduced to the entire brain, suddenly leaving the affected tissues severely deprived of oxygen and glucose. In clinical conditions, the most common source of global cerebral ischemia is cardiac arrest, a devastating

and common cause of death in the western world [1, 2]. Furthermore, ocular blood supply originates from the common and internal carotid arteries. Thus a carotid artery occlusion can lead to delayed ocular complication such as ocular ischemic syndrome as reviewed by Terelak-Borys and colleagues [3], thus global ischemia has an effect on the eye.

Recent data suggests that a key factor in ischemic pathophysiology is the regulation of vascular contraction. Endothelin-1 (ET-1) is a highly potent vasoconstrictor and its effect in cerebrovascular ischemic conditions is well described [4]. ET-1 acts on two main receptors, ET_A and ET_B. ET_A is largely found in smooth muscle cells of vasculature inducing strong vasoconstriction. ET_B function, however, is slightly more intricate. Under normal physiological conditions, ET_B is mainly found on the vascular endothelial cells, propagating NO release, which acts as a vasodilator agent [5]. Nonetheless, in certain conditions ET_B receptors are found to be upregulated and expressed on the vascular smooth muscle cells, causing strong vasoconstriction [6].

The brain and the eye share a common embryological origin, but very little has been done to investigate whether the ocular vasculature responds in a fashion similar to cerebral vasculature during ischemic conditions [6]. The ophthalmic artery, the key supplier of blood to the retina and eye, can potentially play an important role in ocular ischemic pathophysiology. Notably, active ET-1 receptors have been found on the ophthalmic arteries of humans [7] and rat [8]. Additionally, glial cell activation in the retina is a clear indicator whether homeostatic blood flow has been altered to the eye.

Müller cells are the chief glial cells of the retina and span from the inner limiting membrane to the outer limiting membrane. When activated after an ischemic insult they initiate gliosis, a process which attempts to re-establish homeostatic conditions in the retina [9]. The most efficient way of monitoring Müller cell activity is via glial fibrillary acidic protein (GFAP) [10] and vimentin [11] expression.

However, activation of Müller cells does not give any insight on whether vision has been compromised. To this end, electroretinography (ERG) can be used for the evaluation of functional changes to the retina. Using light stimuli, ERG results in a photoreceptor dependent a-wave, an ON-bipolar cell dependent b-wave, inner retinal dependent oscillatory potentials (OPs), and a (primarily) ganglion cell dependent scotopic threshold response (STR) [12]. By combining immunohistochemistry and ERG we can assess the severity of damage caused to the retina during global cerebral ischemia.

Retinal ischemia has been investigated before and one earlier study by Zhao and colleagues, did not observe any retinal changes 48 hours following 17 minutes of global ischemia. However, in our previous studies we have shown that using a 15 minute global cerebral ischemia model, an up-regulation of the 5-hydroxytryptamine/serotonin receptor, 5HT1B, and the ETB receptor occurs in the rat middle cerebral artery (MCA) and anterior cerebral artery (ACA) 48 hours after ischemia and reperfusion. The cerebral blood flow has been monitored in this method has been verified through MRI and laser Doppler [13, 14]. We therefore postulated that there might be a delayed ischemic damage caused by an upregulation of contractile receptors in the vasculature, and that significant retinal damage can be delayed. It follows that increased contractility to ET-1 leads to decreased blood flow as seen in the cerebral vasculature [7], where vascular receptor changes further decrease blood flow and exacerbates damage to the surrounding brain tissue after the original ischemic insult.

Therefore, we examined the contractility of the ophthalmic artery after ischemia and monitored ERG alterations occurring in parallel with activation of Müller cells at the earlier time points as well as at both 72 hours and 7 days after ischemia. We show that although the retina is resistant to ischemic damage, activation of Müller cells only occur in the retina after we observe an increased contractility in the ophthalmic artery. We therefore propose that changes

in the ophthalmic artery are an important factor to fully understand retinal ischemia, particularly for the delayed damage.

Material and Method

Animals

All animal experiments were approved under the license number 2012-15-2934-726 and were housed and treated under the strict national laws and guidelines issued by the Danish Animal Experimentation Incorporate. Rats were housed under 12 hour light dark cycle condition and fed standard rat chow with free access to water.

Global Cerebral Ischemia

Male Wistar rats weighing 260–380g were used for the experiment (Table 1). Prior to the operation the rats were fasted overnight with free access to water.

Reversible global cerebral ischemia was induced by a 15-minute two vessel carotid artery occlusion couple with simultaneous hypovolemia as previously described [14, 15]. In short, rats were anesthetized with 4% isoflurane (Abbott Laboratories) in N_2O/O_2 (70:30), then intubated and artificially ventilated with 1.5%-2% isoflurane in N_2O/O_2 (70:30). The tail artery and vein were used for blood pressure recording, regular blood sampling for gas analysis (Radiometer, Copenhagen, Denmark), and infusions, via inserted catheters. Both body and head temperature was monitored at all times during the surgery with a rectal and skull thermometer respectively, and the rat was kept on a heating pad set for 37°C for the duration of the operation. A heparinized soft poly urethane catheter was inserted via the external jugular vein towards the right atrium. Temporary ligatures were placed around both common carotid arteries. When the preparations were completed the rat received 0.5 ml heparin (100 IU/ml) and was equilibrated for 15–20 minutes.

After equilibration, reversible ischemia was induced by lowering the mean arterial blood pressure (MABP) to 40 mmHg by withdrawing blood through the jugular vein catheter, followed immediately by bilateral common carotid artery clamping. After 15 minutes, the clamps were released and the MABP was restored. Sodium bicarbonate (0.4 ml 0.6M) was injected additionally to counteract systemic acidosis. The rat was allowed to recover for 15–20 minutes after all catheters were removed and all incisions were closed before discontinuing isoflurane and extubation. Sham operated animals underwent the same procedure with the omission of bilateral common carotid clamping, lowering of MABP, and sodium bicarbonate injection. Rats were then kept alive for 24 hours, 48 hours, 72 hours, and 7 days.

Myography

Wistar rats were anesthetized using CO_2 and decapitated. The eyes were gently removed and the ophthalmic artery dissected out in Na-Krebs buffer containing: NaCl 119 mM, $NaHCO_3$ 15 mM, KCl 4.6 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.5 mM and glucose 5.5 mM. The ophthalmic artery segments (1–2 mm) were mounted on a pair of metal wires (25 μm) in an arterial myograph. One wire was attached to a micrometer screw which allows for fine adjustments of the distance between the wires, controlling the vascular tone. The second wire was connected to a force displacement transducer paired together with an analogue-digital converter (AD Instruments, Oxford, UK). All data was recorded on a computer with PowerLab unit software (AD Instruments).

Aerated bicarbonate buffer, composed of 95% O_2 and 5% CO_2, with a resulting pH of 7.4, was heated to 37°C and used to immerse the ophthalmic artery segments into the myograph.

Table 1. Total number or rats used (A) and group allocation (B).

A. Total Rats Operated			
	Total	*Ischemic*	*Sham*
No. of rats	36	16	15
B. Distribution of Rats in the Various Experimental Groups			
Myograph	12	6	6
Immunohistochemistry	24*	14	10
ERG	12	6	6

The total number of rats operated for this experiment.

*Rats used for immunohistochemistry included 4 ischemic and 4 sham operated rats from both the myograph and the ERG group.

doi:10.1371/journal.pone.0157669.t001

The segment was normalized to reach 90% of the internal circumferences that a fully relaxed vessel would have under a transmural pressure of 50 mmHg. A benchmark value of the contractile capacity was achieved by temporarily replacing part of the NaCl in the buffer solution with 60 mM K^+. Artery segments were omitted from the study if they failed to fulfill the inclusion criteria: a maximum contractile capacity of at least 0.5 mN.

All contractile responses are expressed as percentage of the average maximal contraction induced by the K^+ response. The concentration response curves were made in a cumulative manner for the ET-1 (PolyPeptide Group, Sweden) and 5-carboxamidotryptamine (5-CT, a 5-HT analog; Sigma Aldrich, Germany) agonists.

Tissue Preparation

Prior to dissection, eyes were marked at the inner angle with a diathermy burner (FIAB SpA, Italy). This allowed for consistent orientation when embedding and cryo-sectioning. The eyes were carefully dissected out and briefly put in 4% formaldehyde in phosphate buffer saline (PBS) to stabilize, allowing for the removal of the cornea and lens. Thereafter, the eyes were re-immersed in 4% formaldehyde in PBS for 3 hours. Next, the eyes were washed in rising concentration from 10% to 25% of sucrose in Sörensen's phosphate buffer (pH 7.2) for cryoprotection. Finally the eyes were embedded in a gelatin medium containing chicken egg albumin, and positioned according to the burned marking allowing for vertical cryostat sectioning, and stored at -20°C. Eyes were cut in vertical sections through the optic nerve for histology and immunohistochemistry.

Histochemistry

10 μm cryosections were stained with hematoxylin and eosin to evaluate the morphology of the dissected eyes prior to immunohistochemical evaluations. The slides were submerged in fresh hematoxylin for 1.5 minutes before rinsed in distilled water and washed in tap water for 5 minutes. After a brief submersion in fresh eosin for 1 minute, gradual dehydration was performed in rising concentrations of ethanol before the slides were immersed in xylene. Finally, they were mounted with Pertex mounting medium (Histolab, Gothenburg, Sweden).

Immunohistochemistry

Cryosections were washed in PBS with 0.25% Triton (PBS-T) for 15 minutes. Next, antibodies against GFAP (rabbit, 1:1500) and vimentin (mouse, 1:400) were applied (for details, see Table 2). The sections were then incubated overnight in incubation chambers at +8°C. The following day, the slides were washed 2x15 minutes in PBS-T followed by the application of

Table 2. Full list of antibodies used.

A. Primary Antibodies						
Name	*Host*	*Dilution*	*Source*	*Cat #*	*Antigen*	*Antibody Registry #*
GFAP	Mouse	1:400	Millipore, Billerica, MA, USA	IF03L	GFAP	AB_212974
GFAP	Rabbit	1:1500	Dako Cytomation, Denmark	Z0334	GFAP in cow spinal cord	AB_10013482
Vimentin	Mouse	1:400	Sigma Aldrich, St Louis, MO, USA	V6630	Vimentin in pig eye lens	V6630
B. Secondary Antibodies						
Name	*Host*	*Dilution*	*Source*	*Cat #*		
Alexa 594 anti-mouse	Goat	1:400	Invitrogen, Carlsbad, CA, USA	A-11032		
FITC anti-mouse	Donkey	1:200	Jacksson Immunoresearch, West Grove, PA, USA	735-095-151		
Alexa 594 anti-rabbit	Donkey	1:400	Jacksson Immunoresearch, West Grove, PA, USA	711-585-152		

The full list of primary (A) and secondary (B) antibodies used during immunohistochemistry, including dilution, host animal, and manufacturer.

doi:10.1371/journal.pone.0157669.t002

appropriate secondary antibodies. Slides were incubated for one hour at room temperature before washing 3x15 minutes with PBS-T and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame CA, USA).

For double staining, the staining was done sequentially. Considerations were made in selecting antibodies so that their host species would not conflict with each other. Each set of immunohistochemical staining was performed a minimum of three times, each coupled with a negative control where the primary antibody was omitted. A full list of antibodies used is found in Table 2.

Microscopy and Imaging

The slides were examined using an epifluorescence microscope (Nikon 80i, Tokyo, Japan) combined with a Nikon DS-2MV camera. Areas of interest were photographed with 10x, 20x, or 40x lenses. The images were then processed using Adobe Photoshop CS3 (v10.0 Adobe Systems, Mountain View, CA) and images taken with different wavelength filters were superimposed over each other to determine any potential co-localization.

Electroretinography

Rats were dark adapted overnight and then prepared for ERG recordings under dim red light. Each rat underwent three measurements, 24 hours before ischemia, 72 hours after, and 7 days after ischemia. The rats were anesthetized in accordance with previous studies [16] using ketamine (85mg/kg) and xylazine (20 mg/kg) to allow for comparisons with previously published ERG data. The rat was then placed on a heated platform to maintain body temperature at approximately 37°C. Next, one drop each of oxybuprocain (0.4%), tropicamide (1%) and phenylephrine (5%) were added to each eye for pupil dilation and topical anesthesia. A reference electrode was inserted into the mouth of the rat, gold ring electrodes were positioned on the corneas of the eyes and a subcutaneous needle in the tail served as a ground. ERGs were recorded using a Ganzfield bowl equipped with both LED and xenon lamps (model Q450 SCX, Roland consult, Siegburg, Germany) and digitized at 2.5 KHz over a 400 msec interval with Viking Select analysis software (Nicolet Biomedical Instruments, Madison, WI, USA). Scotopic

threshold responses (STR) were elicited with a stimulus of -5.8 log cd*s/m^2 and 20 responses were averaged, which was sufficient to elicit both a positive and negative STR. ERGs were then recorded for stimuli of -3.7, -3.0, -2.0, -1.0, 0.0, 1.0, 1.5, 1.75 and 2.0 log cd*s/m^2. The interstimulus interval for intensities of -1.0 log cd*s/m^2 and less was 5 seconds while for intensities above -1.0 log cd*s/m^2 it was increased incrementally up to 110 seconds. For the lower intensities, ten responses were averaged, while for the higher intensities the average was made from 2–7 responses.

The ERG trace raw data were exported into Microsoft Office Excel (2003). It has been shown that a delayed Gaussian function can be used to model the leading edge of the a-wave (P3) [17] as described by Hood and Birch [18]. A modification of this description has been used [19] to model P3 amplitude as a function of luminous energy (i, log cd*s/m^2) and time (t, seconds):

$$P3(i,t) = Rm_{P3}[1 - \exp(-i * S * (t - t_d)^2)],\ t > t_d$$

where Rm_{P3} is the saturated response amplitude, S (log m^{2}*cd^{-1}*s^{-3}) is sensitivity reflecting amplification of the phototransduction processes and t_d (seconds) is a delay in time due to the inherent lag of recording equipment as well as physiological processes involved in the photoreceptor response. In this study, this equation was used to model recorded responses to luminous energies of 1.5, 1.75 and 2.0 log cd*s/m^2. Minimization of the sum of square merit function was used to optimize the parameters Rm_{P3} and S with the solver module of Excel, while t_d was fixed at 3.6 ms.

The amplitude of the b-wave (V, μV) can also be modelled as a function of luminous energy (i, log cd*s/m^2) of the stimulus using the Naka-Rushton equation:

$$V(i) = V_{max} * \frac{i}{i + k}$$

where $Vmax$ (μV) is the saturated response amplitude and the semi-saturation constant k (log cd*s/m^2) is the luminous energy required for a half maximal response. Minimization of the sum of square merit function was used to optimize the parameters $Vmax$ and k with the solver module of Excel. Plotting b-wave amplitude versus energy resulted in a two branches, most likely due to cone contribution at higher energies, and so responses up to and including -2.0 log cd*s/m^2 and responses above this energy were modelled independently of one another [20, 21].

OPs were extracted from scotopic responses to a stimulus of 2.0 log cd*s/m2 with a band-pass filter of 75–300 Hz by using an add-in function for Microsoft Office Excel (www.web-reg.de/bp_addin.html#).

After ERG recording and while the rats were still anesthetized, fundus images were taken using the Micron IV (Phoenix Research Laboratories, Pleasanton, CA, USA) retinal imaging system and StreamPix software (NorPix, Inc., Montreal, QC, Canada).

Results

Global Cerebral Ischemia

The mortality rate of the global cerebral ischemia operation was 10% with 4 out of 40 rats dying prematurely. The MABP was measured before the ischemic insult and immediately after. Prior to ischemia the MABP was 107.5±18.4 mmHg, during the ischemia it dropped to an average of 39.3±1.2 mmHg, while after ischemia it was 121.5±18.3 mmHg. Thus the MABP returned to its normal range. Other parameters such as pH, pCO_2, pO_2, and temperature were

also monitored throughout the procedure and were all within the normal ranges (data not shown).

Myograph

Firstly, we aimed to investigate if there was indeed an increase of contractile function in the ophthalmic artery after global cerebral ischemia, similar to what we have observed in the brain after 48 hours. The concentration-response curves for ET-1 were significantly different 48 hours after ischemia ($p<0.05$), with a weak leftward shift in the EC_{50} in the ischemic animals, with an EC_{50} of 1.07 nM (pEC_{50} 8.97±0.13 M) for the ischemic animals and an EC_{50} of 1.56 nM (pEC_{50} 8.81±0.12 M) for sham operated animals (Fig 1). There was a significant increase in E_{max} ($p<0.01$) which shows a significant increase of ET-1 mediated vasoconstriction (Fig 1). However, the concentration-response curves for 5-CT were identical between both ischemic and sham operated animals with EC_{50} values of 1.91μM (pEC_{50} 5.72±0.08 M) and 2.09 μM (pEC_{50} 5.68±0.11 M) respectively (Fig 1). This suggests no difference in 5-HT receptor regulation between the two groups 48 hours post ischemia.

Histology and Immunohistochemistry

To further investigate the retinal changes, we stained retinal cross sections using hematoxylin and eosin. We detected no noticeable differences in retinal thickness, cell density, or over-all morphology between the groups (data not shown).

GFAP and vimentin, which are markers of Müller cell activation, were clearly upregulated for the first time 72h after ischemia. This upregulation was maintained up to 7 days post ischemia with strong immunoreactivity spanning throughout the entire retina, characteristic of Müller cells [10]. The earlier time points, 24 hours and 48 hours post ischemia revealed only immunoreactivity along the nerve fiber layer which coincides with previous observations during normal physiological conditions [10]. Furthermore, the majority of GFAP and vimentin immunopositivity seem to co-localize, emphasizing that they are expressed in the same Müller cell projections spanning through the retina (Fig 2). GFAP positive staining in ganglion cell layer and nerve fiber layer is likely attributed to GFAP positive astrocytes. Two different GFAP antibodies were examined and they resulted in identical patterns of staining.

ERG

To investigate retinal function, ERG was performed and 3 days after surgery, both inner and outer retinal cell responses were compromised by ischemia.

Photoreceptor activity is reflected by the ERG a-wave. At 3 days after surgery, a-wave amplitudes of ischemic rats (n = 6) were approximately 20% lower than those of sham operated rats (n = 6) for luminances of -1.0 to 2.0 log cd^*s/m^2 ($p<0.05$; Fig 3A and 3B). Although dependent upon the a-wave, the magnitude of the b-wave reflects ON-bipolar cell activity and at this time point, b-wave amplitudes of ischemic rats (n = 6) were also approximately 20% lower than those of sham operated rats (n = 6) for luminances of -1.0 to 2.0 log cd^*s/m^2 ($p<0.05$; Fig 3A and 3C). Seven days after surgery, a statistically significant difference for either a- or b-wave amplitudes was not found ($p>0.05$; Fig 3). No differences in peak latencies were seen at either time point (not shown).

Mathematical modelling of responses across various luminances can be useful for further analysis of retinal function. Using a model similar to that formulated by Hood and Birch (1990) for photoreceptor responses to luminous energies of 1.5, 1.75 and 2.0 log cd^*s/m^2, the saturated response amplitude, Rm_{P3}, was found to be significantly reduced in ischemic animals 3 days after surgery, but not on day 7 (Fig 4A and 4C). No differences were found in sensitivity

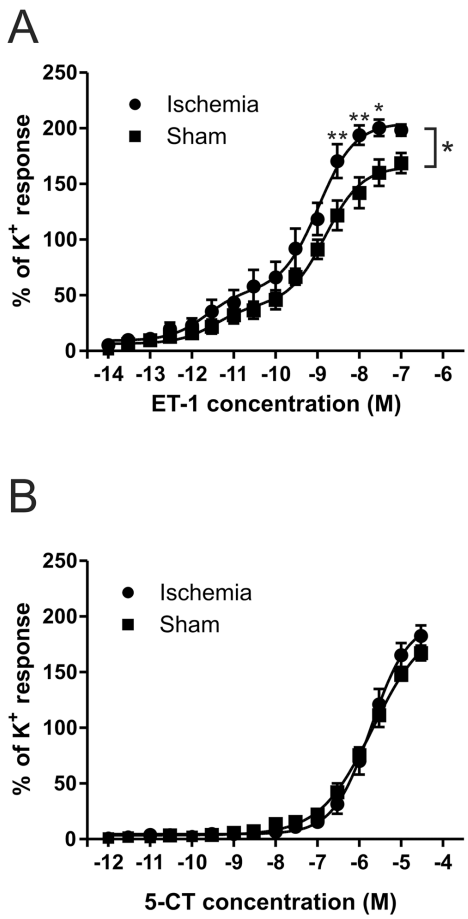


Fig 1. Increased endothelin-1 induced constriction after 48 hours. (**A**) Dose response curves for ET-1 on ischemic and sham ophthalmic artery 48 hours after ischemia are significantly different ($p<0.05$) with a left shift in the EC_{50}. Sham EC_{50} was 1.56nM (pEC_{50} 8.810±0.12M) and for ischemia it was 1.07nM (pEC_{50} 8.97 ±0.13M). (**B**) 5-CT response curves are identical with EC_{50} 1.91μM (pEC_{50} 5.72±0.08M) for ischemia and EC_{50} 2.09μM (pEC_{50} 5.68±0.11 M) for sham.

doi:10.1371/journal.pone.0157669.g001

(*S*) on either day (Fig 4C). The b-wave amplitudes were plotted against luminous energy and fitted using a Naka Rushton function (Fig 4B). The saturated response amplitude, *Vmax*, for the higher luminous energy branch (mixed rod and cone responses) was lower in ischemic animals as compared to sham operated rats on day 3, but not on day 7 and not for the lower branches (Fig 4C). No differences were found for the semisaturation constant, *k* (Fig 4C).

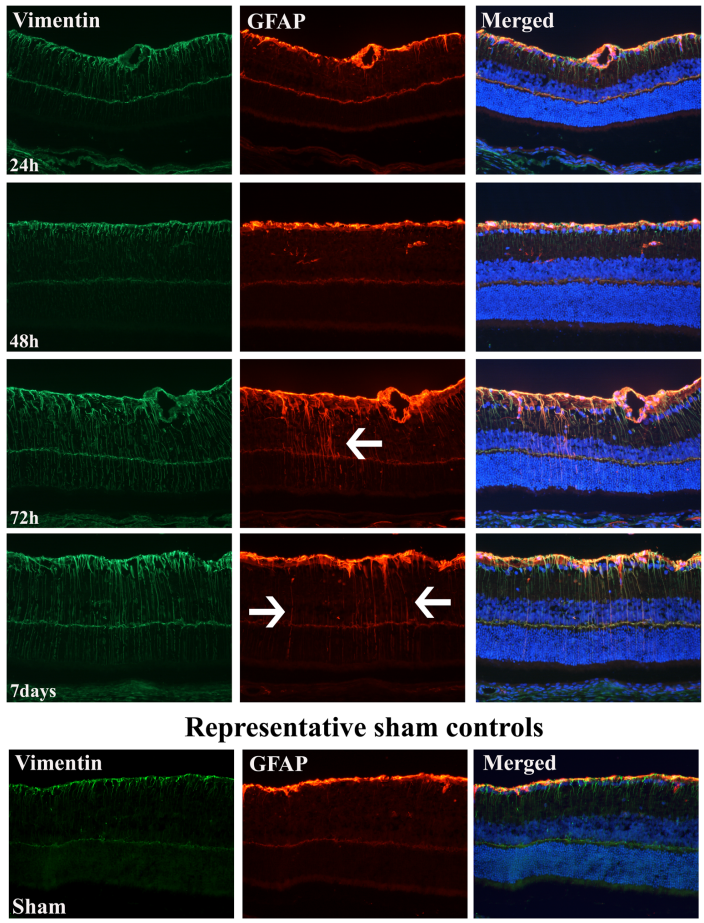
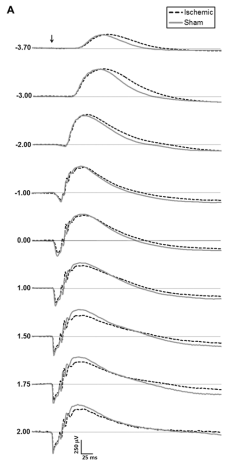


Fig 2. Increased GFAP and vimentin expression 72 hours and 7 days post ischemia. Increased GFAP and vimentin expression 72 hour and 7 days after transient global ischemia (1 day n = 4, 48h n = 8, 72h n = 4, 7 day n = 8). Arrows highlight GFAP positive staining spanning throughout the retina. Even though there was an increase in vasoconstriction on the ophthalmic artery at 48 hours, it took 72 hours for the retina to show signs of gliosis through Müller cell markers GFAP and vimentin upregulation. Müller cells were still active at 7 days after ischemia.

doi:10.1371/journal.pone.0157669.g002

Proximal retinal cell (possibly amacrine cell) activity is considered to give rise to the OPs on the rising face of the b-wave. These wavelets can be isolated by applying a band-pass filter and, when overlapped, differences between traces from the two experimental groups can be seen (Fig 5A). At day 3, the amplitudes of the third and fourth wavelets (OP3 and OP4) of ischemic rats were, respectively 30% and 40% lower than those of sham operated rats ($p<0.05$; Fig 5B).



B

a-wave amplitudes

Luminance:	3 days Sham	3 days Ischemic	7 days Sham	7 days Ischemic
-2.00	71±19	55±18	62±16	56±21
-1.00	376±20	**308±44***	373±37	345±33
0.00	625±20	**515±64***	608±71	564±28
1.00	718±34	**598±92***	727±92	649±76
1.50	797±47	**654±106***	795±94	725±48
1.75	821±36	**667±103***	799±101	753±62
2.00	865±59	**700±114***	864±114	792±73

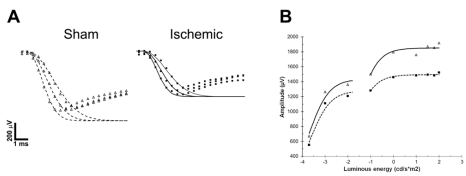
C

b-wave amplitudes

Luminance:	3 days Sham	3 days Ischemic	7 days Sham	7 days Ischemic
-3.70	670±175	552±67	624±104	601±78
-3.00	1263±154	1111±131	1197±169	1115±91
-2.00	1361±155	1208±186	1282±261	1177±80
-1.00	1500±136	**1281±189***	1453±223	1319±105
0.00	1726±131	**1462±214***	1685±223	1547±128
1.00	1760±130	**1483±234***	1760±238	1556±219
1.50	1831±116	**1487±252***	1791±296	1625±177
1.75	1855±136	**1483±193***	1778±287	1628±157
2.00	1918±184	**1521±278***	1826±267	1689±193

Fig 3. Representative Scotopic ERG response curves with average a and b-wave amplitudes. (**A**) Scotopic ERG responses representative of each experimental group to stimuli of increasing luminance on day 3. (**B**) Average a-wave amplitudes at day 3 and 7 after surgery. (**C**) Average b-wave amplitudes at day 3 and 7 after surgery. Arrow indicates time of stimulus; unit for luminance is log cd*s/m^2; unit for amplitudes is μV; * denotes p<0.05.

doi:10.1371/journal.pone.0157669.g003



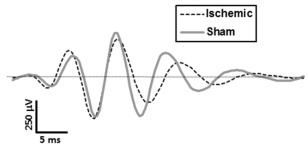
C

	3 days Sham	3 days Ischemic	7 days Sham	7 days Ischemic
a-wave				
RmP3	-946 ± 113	-725 ± 129*	-893 ± 113	-894 ± 55
S	5616 ± 1116	4984 ± 1078	5033 ± 813	5263 ± 1643
b-wave				
rods				
Vmax	1439 ± 162	1279 ± 188	1355 ± 256	1244 ± 85
k	-3,69 ± 0,20	-3,64 ± 0,06	-3,69 ± 0,07	-3,72 ± 0,08
rods + cones				
Vmax	1811 ± 136	1493 ± 226*	1779 ± 262	1638 ± 152
k	-1,70 ± 0,20	-1,83 ± 0,17	-1,65 ± 0,15	-1,32 ± 0,34

Fig 4. The falling phase of a-wave, Naka-Rushton modelling, and a representation plotted parameters of sham and ischemic rats. (**A**) The falling phase of a-wave responses to stimuli of 1.5, 1.75 and 2.0 log cd*s/m^2 were modelled for both sham (unfilled triangles) and ischemic animals (filled squares). (**B**) Average b-wave responses at day 3 for sham operated (unfilled triangles) and ischemic (filled squares) are plotted here against luminous intensity. These responses were modelled using a Naka-Rushton function (dotted and continuous lines, respectively). (**C**) Optimization of the parameters for these functions (A, B) were made and averaged for both experimental groups, shown here for both 3 and 7 days after operation. Unit for S is log m^2*cd^{-1}*s^{-3}; unit for k is log cd*s/m^2; unit for Rm_{P3} and *Vmax* is μV; * indicates p<0.05.

doi:10.1371/journal.pone.0157669.g004

A



B

	3 days		7 days	
	Sham	Ischemic	Sham	Ischemic
OP1	236±65	284±49	225±46	252±44
OP2	656±175	594±76	643±105	621±115
OP3	437±112	**300±50***	430±63	**333±70***
OP4	126±21	**78±15***	119±31	**79±19***

Fig 5. Oscillatory potential wavelets and average amplitudes or representative individuals from both sham and ischemic groups. (**A**) OP wavelets isolated from ERG traces (luminance: 2.0 log cd*s/m^2) from one animal which is representative of each experimental group on day 3. (**B**) Average OP amplitudes for each experimental group at 3 and 7 days after surgery. Unit for amplitudes is µV; * denotes p<0.05.

doi:10.1371/journal.pone.0157669.g005

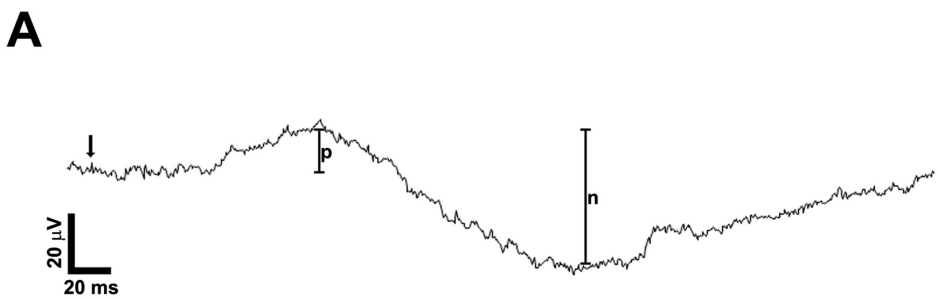
Seven days after surgery, OP3 and OP4 amplitudes were, respectively, 20% and 35% lower in ischemic rats ($p<0.05$; Fig 5B). No differences in wavelet latencies were seen at either time point (not shown).

The positive and negative scotopic threshold responses (STR; Fig 6A) are believed to mainly arise from the ganglion cells. In this study, the positive STR (pSTR) amplitudes of ischemic rats on day 3 were approximately 50% those of sham operated rats ($n = 6$; $p<0.05$; Fig 6B). The negative STR (nSTR) amplitudes of ischemic rats were also about 30% lower than those of sham operated rats on day 3 ($n = 6$; $p<0.05$; Fig 6B). On day 7, the amplitudes of the pSTR were similar in both groups (Fig 6B) and while the nSTR amplitudes of ischemic rats were still lower, this difference was not statistically significant (Fig 6B). No differences in peak latencies were seen at either time point (not shown).

Discussion

We hypothesized that there would be vascular changes occurring after the ischemic insult which could cause or contribute to delayed damage to the retina. Here we are shedding new light on the link between cerebral and ophthalmic vasculature within the context of global cerebral ischemia. We discovered, for the first time, that vasocontractility of the ophthalmic artery is significantly increased 48 hours after global ischemia, thus it is believed to reduce blood flow to the eye long after the original ischemic insult [22]. We observe retinal damage occurring at 72 hours after the ischemic insult. Therefore we propose that this is caused by changes in the retinal vasculature, suggesting that they are an important factor in fully understanding retinal ischemia.

Retinal ischemia has been investigated for decades with a variety of methods. However, compared to the brain the retina has an innate resistance to ischemic insult. Retinal ischemia can be induced for up to 120 minutes with both ocular pressure increase or with vascular occlusion [23, 24], leading to dramatic effects 48 hours after ischemia. Though generally, a minimum of 30 minute complete ischemia is needed to elicit lasting and reproducible damage in



B

	3 days		7 days	
	Sham	Ischemic	Sham	Ischemic
pSTR	19.4±4.5	**9.5±6.6***	16.0±8.7	17.8±6.3
nSTR	47.6±7.3	**34.1±8.6***	45.4±5.8	35.7±11.7

Fig 6. STR responses with average amplitudes of both sham and ischemic animals at 3 and 7 days post ischemia. (**A**) The amplitude of the pSTR was measured from baseline to the peak of the first positive potential (denoted by p) in response to a stimulus of -5.8 log cd*s/m^2. The amplitude of the nSTR was measured from this peak to the trough of the following negative potential (denoted by n). (**B**) Average pSTR and nSTR amplitudes for each experimental group at days 3 and 7 after surgery. Arrow indicates time of stimulus; unit for amplitudes is µV; * denotes $p<0.05$.

doi:10.1371/journal.pone.0157669.g006

the rat retina reviewed by Osborne and colleagues [25], and it has been shown that retinal ischemia is an ongoing process which is highly time dependent [26]. A recent study by Zhao and colleagues found a functional recovery after a 17 minute global cerebral ischemia [27]. However, our data on cerebral vasculature shows an upregulation of vascular contractile receptors which first occurs 48 hours after the ischemia [14]. We therefore propose that vascular changes can cause a second delayed ischemia and, crucially, even a shorter global ischemic insult of 15 minutes is sufficient to elicit delayed damage to the retina. Thus, judging from our results, we believe that the vasculature and the exuberated damage induced by receptor upregulation should be an important aspect in considering clinical targets for retinal ischemia.

Interestingly, our results show a similar increase of ET-1 mediated vasoconstriction occurring in the ophthalmic artery as observed in the cerebral arteries at 48 hours after ischemia [14]. The exact changes of ET-1 receptor expression in the ophthalmic artery was not evaluated in this study. However, in cerebral arteries ET_B expression in the smooth muscle cells of vasculature causes a significant portion of the observed increased vasoconstriction [6]. The exact expression of ET-1 receptors in the rat ophthalmic artery will be an aim of future studies. Still, the increased vasoconstriction which is determined after ischemia in this current study, could potentially lead to an increased ischemic damage to the retina. Another related aspect to be considered is the endothelium of the ophthalmic artery. Prior to the experimental procedure it

was shown to be intact (data not shown). Additionally, the endothelial cells do not have an influence on the contractile response when receptor upregulation is investigated [28]. Nonetheless, cerebral and ophthalmic arteries do not seem to follow identical pathological patterns, as cerebral vessels also exhibited a strong 5-HT_{1B} receptor mediated vasoconstriction [14]. This would suggest that even though 5-HT receptors are involved in cerebral vascular response to ischemia, they are not affected by global cerebral ischemia in the ophthalmic artery.

After acute ischemic stroke, the level of ET-1 in blood plasma is increased up to four times its normal level [29]. The vasoconstrictive property of ET-1 is so strong that it is used as a non-invasive method to induce local ischemia, by injecting ET-1 into the eye under the conjunctiva causing sudden and complete constriction of retinal vasculature [30]. Further, ET-1 has been found to be active within the eye during ischemic conditions such as elevated intraocular pressure models. Here, ET_B has been found to decrease retinal ganglion cell survival during glaucoma, suggesting that an ET-1 antagonist may be of therapeutic value [31]. Thus, with our results in mind, ET-1 may in addition decrease blood flow not only through the ophthalmic artery, but also in the retinal vasculature, as well as having a detrimental effect on the survival of retinal ganglion cells. However, ischemic damage has been of temporary character on the retina after vascular ischemia such as global or middle cerebral artery occlusion [24, 27]. However these studies did not continue past the timespan where we observed the vascular changes occur.

GFAP has been revealed to be an extremely sensitive and non-specific indication of retinal damage and the initiation of gliosis, as indicated by its upregulation in the retina in: glaucoma, ischemia- reperfusion, retinal detachment, diabetic retinopathy, inflammation, and proliferative retinopathies [9]. Thus, GFAP can be seen as a universal stress indicator in the retina. Vimentin, also an intermediate filament, is commonly co-expressed in the retina with GFAP. Both proteins act as stabilizers for Müller cell processes and are involved in the signal transduction cascades essential in reactive gliosis [10]. Within ischemia/reperfusion injuries glutamate toxicity and VEGF induced neovascularization are two key players that affect the function of the retina [9]. Glutamate has been shown to be accumulated in the neural processes disrupting the ionic balance [25, 32] and leading to excitotoxicity. Furthermore, once a shortage of blood flow is detected in the eye VEGF promotes the formation of new vasculature. However, this vasculature is not included in the retinal blood barrier and thus lead to leakage and faulty vascularization, leading to reduced vision [25]. Thus an upregulation of GFAP and vimentin suggests that structure and/or function of the retina has been compromised after 15 minutes of global cerebral ischemia.

Functional ERG results at 72 hours showed a significant reduction in the a-wave and b-wave of ischemic animals. The sensitivity parameters were not altered in either of these components (*S* and *k*, respectively), suggesting that transduction was unaffected. Retinal function, however, was affected as modelling the responses to the higher luminous energies showed that the saturated response amplitudes for both (Rm_{P3} and *Vmax*, respectively) were lower in ischemic rats. Oscillatory potentials of the animals were also significantly reduced. Finally, the scotopic threshold response (STR) primarily reflecting ganglion cell activity [33] was also decreased at 72 hours, indicating a complete reduction in retinal function. However, the reduced function of the retina was not permanent. Even though there were still signs of gliosis throughout the retina at 7 days, the a-wave, b-wave, and STR responses had recovered as significant differences between sham and ischemic animals were no longer detectable. The oscillatory potentials, however, did not recover completely by day 7.

A recent study by Zhao and colleagues [27] showed that the retina recovered almost completely following a 17 minute global ischemic insult and reperfusion at 48 hours. Here we demonstrate an increased contractile response in the ophthalmic artery 48 hours after global

cerebral ischemia, and thus a potential second round of reduced blood flow and ischemia. Interestingly, the increase in Müller cell activation was only observed after the increased contractility demonstrated in the ophthalmic artery. Therefore, the ophthalmic artery may be a valid and novel therapeutic target especially for longer ischemic episodes such as those following middle cerebral artery occlusions. Further studies are needed to evaluate the ophthalmic arteries vasoactive significance of the ophthalmic artery in other ischemic conditions.

Conclusion

In conclusion, this study shows that there is a similar response between the cerebral and the ophthalmic vasculature after a global ischemic insult. Myograph data shows an increase of ET-1 mediated vasoconstriction 48 hours after global ischemia. This increase of contractility may exacerbate retinal damage after ischemia, as seen by the increase of GFAP and vimentin along with decreased retinal function 72 hours post ischemia. No difference in GFAP or vimentin immunopositivity was observed 24 to 48 hours between sham operated and ischemic animals. These data suggest that decreased blood flow in the ophthalmic artery might play a role in ischemic damage, and that retinal ischemic changes should be monitored for longer than 48 hours to detect delayed damage that might follow the vascular upregulation.

Author Contributions

Conceived and designed the experiments: FWB LE KW. Performed the experiments: SEJ KAH FWB LJ. Analyzed the data: FWB LJ KAH SEJ. Contributed reagents/materials/analysis tools: LE. Wrote the paper: FWB KAH KW LJ SEJ LE.

References

1. Hossmann KA. Cerebral ischemia: models, methods and outcomes. Neuropharmacology. 2008; 55 (3):257–70. doi: 10.1016/j.neuropharm.2007.12.004 PMID: 18222496.
2. Chugh SS, Reinier K, Teodorescu C, Evanado A, Kehr E, Al Samara M, et al. Epidemiology of Sudden Cardiac Death: Clinical and Research Implications. Progress in Cardiovascular Diseases. 2008; 51 (3):213–28. doi: 10.1016/j.pcad.2008.06.003 WOS:000261605500004. PMID: 19026856
3. Terelak-Borys B, Skonieczna K, Grabska-Liberek I. Ocular ischemic syndrome—a systematic review. Med Sci Monit. 2012; 18(8):RA138–44. PMID: 22847215; PubMed Central PMCID: PMC3560693.
4. Edvinsson L. Cerebrovascular endothelin receptor upregulation in cerebral ischemia. Curr Vasc Pharmacol. 2009; 7(1):26–33. PMID: 19149637.
5. Schneider MP, Boesen EI, Pollock DM. Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. Annual Review of Pharmacology and Toxicology. 2007; 47:731–59. doi: 10.1146/annurev.pharmtox.47.120505.105134 WOS:000244461700024. PMID: 17002597
6. Edvinsson LI, Povlsen GK. Vascular plasticity in cerebrovascular disorders. J Cereb Blood Flow Metab. 2011; 31(7):1554–71. doi: 10.1038/jcbfm.2011.70 PMID: 21559027; PubMed Central PMCID: PMC3137480.
7. Haefliger IO, Flammer J, Luscher TF. Nitric oxide and endothelin-1 are important regulators of human ophthalmic artery. Invest Ophthalmol Vis Sci. 1992; 33(7):2340–3. PMID: 1607246.
8. Jarajapu YP, Grant MB, Knot HJ. Myogenic tone and reactivity of the rat ophthalmic artery. Invest Ophthalmol Vis Sci. 2004; 45(1):253–9. PMID: 14691181.
9. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, et al. Muller cells in the healthy and diseased retina. Prog Retin Eye Res. 2006; 25(4):397–424. doi: 10.1016/j.preteyeres.2006.05.003 PMID: 16839797.
10. Bignami A, Dahl D. Radial Glia of Muller in the Rat Retina and Their Response to Injury—Immunofluorescence Study with Antibodies to the Glial Fibrillary Acidic (Gfa) Protein. Experimental Eye Research. 1979; 28(1):63–9. WOS:A1979GH40500006.
11. Schnitzer J. Chapter 7 Astrocytes in mammalian retina. Progress in Retinal Research. 1988; 7(0):209–31. doi: 10.1016/0278-4327(88)90009-0

12. Weymouth AE, Vingrys AJ. Rodent electroretinography: methods for extraction and interpretation of rod and cone responses. Prog Retin Eye Res. 2008; 27(1):1–44. doi: 10.1016/j.preteyeres.2007.09.003 PMID: 18042420.

13. Johansson SE, Andersen XEDR, Hansen RH, Povlsen GK, Edvinsson L. Cerebrovascular endothelin-1 hyper-reactivity is associated with transient receptor potential canonical channels 1 and 6 activation and delayed cerebral hypoperfusion after forebrain ischaemia in rats. Acta Physiol. 2015; 214(3):376–89. doi: 10.1111/apha.12519 WOS:000356306300012.

14. Johansson S, Povlsen GK, Edvinsson L. Expressional changes in cerebrovascular receptors after experimental transient forebrain ischemia. Plos One. 2012; 7(7):e41852. doi: 10.1371/journal.pone.0041852 PMID: 22848635; PubMed Central PMCID: PMC3407123.

15. Smith ML, Bendek G, Dahlgren N, Rosen I, Wieloch T, Siesjo BK. Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. Acta Neurol Scand. 1984; 69 (6):385–401. PMID: 6464670.

16. Chaychi S, Polosa A, Lachapelle P. Differences in Retinal Structure and Function between Aging Male and Female Sprague-Dawley Rats are Strongly Influenced by the Estrus Cycle. Plos One. 2015; 10(8). ARTN e0136056 doi: 10.1371/journal.pone.0136056 WOS:000360299100058.

17. Lamb TD, Pugh EN Jr. A quantitative account of the activation steps involved in phototransduction in amphibian photoreceptors. J Physiol. 1992; 449:719–58. PMID: 1326052; PubMed Central PMCID: PMCPMC1176104.

18. Hood DC, Birch DG. A quantitative measure of the electrical activity of human rod photoreceptors using electroretinography. Vis Neurosci. 1990; 5(4):379–87. PMID: 2265151.

19. Bui BV, Vingrys AJ. Development of receptoral responses in pigmented and albino guinea-pigs (Cavia porcellus). Doc Ophthalmol. 1999; 99(2):151–70. PMID: 11097119.

20. Velten IM, Horn FK, Korth M, Velten K. The b-wave of the dark adapted flash electroretinogram in patients with advanced asymmetrical glaucoma and normal subjects. Br J Ophthalmol. 2001; 85 (4):403–9. PMID: 11264127; PubMed Central PMCID: PMCPMC1723928.

21. Charng J, Nguyen CT, Bui BV, Vingrys AJ. Age-related retinal function changes in albino and pigmented rats. Invest Ophthalmol Vis Sci. 2011; 52(12):8891–9. doi: 10.1167/iovs.11-7602 PMID: 22003116.

22. Macrae IM, Robinson MJ, Graham DI, Reid JL, McCulloch J. Endothelin-1-induced reductions in cerebral blood flow: dose dependency, time course, and neuropathological consequences. J Cereb Blood Flow Metab. 1993; 13(2):276–84. doi: 10.1038/jcbfm.1993.34 PMID: 8436619.

23. Buchi ER. Cell death in the rat retina after a pressure-induced ischaemia-reperfusion insult: an electron microscopic study. I. Ganglion cell layer and inner nuclear layer. Exp Eye Res. 1992; 55(4):605–13. PMID: 1483506.

24. Block F, Grommes C, Kosinski C, Schmidt W, Schwarz M. Retinal ischemia induced by the intraluminal suture method in rats. Neurosci Lett. 1997; 232(1):45–8. PMID: 9292888.

25. Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog Retin Eye Res. 2004; 23(1):91–147. doi: 10.1016/j.preteyeres.2003.12.001 PMID: 14766318.

26. Lafuente MP, Villegas-Perez MP, Selles-Navarro I, Mayor-Torroglosa S, Miralles de Imperial J, Vidal-Sanz M. Retinal ganglion cell death after acute retinal ischemia is an ongoing process whose severity and duration depends on the duration of the insult. Neuroscience. 2002; 109(1):157–68. PMID: 11784707.

27. Zhao Y, Yu B, Xiang YH, Han XJ, Xu Y, So KF, et al. Changes in retinal morphology, electroretinogram and visual behavior after transient global ischemia in adult rats. Plos One. 2013; 8(6):e65555. doi: 10.1371/journal.pone.0065555 PMID: 23776500; PubMed Central PMCID: PMC3679137.

28. Skovsted GF, Pedersen AF, Larsen R, Sheykhzade M, Edvinsson L. Rapid functional upregulation of vasocontractile endothelin ETB receptors in rat coronary arteries. Life Sci. 2012; 91(13–14):593–9. doi: 10.1016/j.lfs.2012.02.009 PMID: 22391414.

29. Ziv I, Fleminger G, Djaldetti R, Achiron A, Melamed E, Sokolovsky M. Increased plasma endothelin-1 in acute ischemic stroke. Stroke. 1992; 23(7):1014–6. PMID: 1615534.

30. Masuzawa K, Jesmin S, Maeda S, Kaji Y, Oshika T, Zaedi S, et al. A model of retinal ischemia-reperfusion injury in rats by subconjunctival injection of endothelin-1. Exp Biol Med (Maywood). 2006; 231 (6):1085–9. PMID: 16741054.

31. Minton AZ, Phatak NR, Stankowska DL, He S, Ma HY, Mueller BH, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma. Plos One. 2012; 7(8):e43199. doi: 10.1371/journal.pone.0043199 PMID: 22916224; PubMed Central PMCID: PMC3423444.

32. Napper GA, Pianta MJ, Kalloniatis M. Reduced glutamate uptake by retinal glial cells under ischemic/hypoxic conditions. Vis Neurosci. 1999; 16(1):149–58. PMID: 10022486.

33. Bui BV, Fortune B. Ganglion cell contributions to the rat full-field electroretinogram. J Physiol. 2004; 555(Pt 1):153–73. doi: 10.1113/jphysiol.2003.052738 PMID: 14578484; PubMed Central PMCID: PMC1664823.

Paper II

Acta Ophthalmologica

Increased endothelin-1-mediated vasoconstriction after organ culture in rat and pig ocular arteries can be suppressed with MEK/ERK1/2 inhibitors

Frank W. Blixt,[1] Agmund Kristian Haanes,[2] Lena Ohlsson,[1] Anders Tolstrup Christiansen,[3] Karin Warfvinge[2] and Lars Edvinsson[1,2]

[1]Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, Lund, Sweden
[2]Department of Clinical Experimental Research, Glostrup Research Institute, Rigshospitalet, Glostrup, Denmark
[3]Department of Ophthalmology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

ABSTRACT.

Purpose: **Even though retinal vascular changes following ischaemia have been poorly understood, the upregulation of vasoconstrictive endothelin-1 (ET-1) receptors (ET_A/ET_B) following global cerebral ischaemia has been described. The aim of this study was to investigate whether or not the MEK/ERK1/2 pathway is involved in the observed upregulation and whether specific MEK/ERK1/2 inhibitors U0126 and trametinib can prevent it.**

Methods: **The aim was also to localize ET_A and ET_B receptors using immunohistochemistry in both fresh rat ophthalmic arteries and after 24-hr organ culture and study the receptors functionally using myography. Pig retinal arteries also underwent 24-hr organ culture to validate similar responses across species and the retinal vasculature.**

Results: **Results showed that following organ culture there is a significant increase in ET-1-mediated vasoconstriction, in particular via the ET_B receptor. Furthermore, immunohistochemistry revealed a clear increase in pERK in the smooth muscle cells of rat ophthalmic artery. U0126 and trametinib were successful in attenuating the functional vasoconstriction in both rat and pig, as well as restoring immunofluorescence of pERK to fresh levels and counteracting ET_B expression in the smooth muscle cells of the rat ophthalmic artery.**

Conclusion: **This is the first study to show that the MEK/ERK1/2 pathway in responsible for the increase in functional vasoconstriction via ET-1 receptor in rat ophthalmic and pig retinal arteries. Furthermore, this study is the first to suggest a way of inhibiting and preventing such an increase. With these results, we suggest a novel approach in retinal ischaemia therapy.**

Key words: endothelin-1 – ischaemia – MEK/ERK1/2 – ophthalmic artery – organ culture – retinal artery – vasoconstriction

Acta Ophthalmol.

doi: 10.1111/aos.13651

Introduction

Retinal ischaemia is caused by a lack of sufficient blood supply to the tissue. The main artery supplying the retina with blood is the ophthalmic artery, which branches further to become retinal arteries. Retinal ischaemia research has been mainly focused retinal cells and neuroprotection (Osborne et al. 2004), thereby neglecting the importance of the vasculature and role of the ophthalmic artery and its branches. Recently, it was discovered that changes in the cerebral vasculature play a significant role in cerebral ischaemic injury in several experimental models such as global cerebral ischaemia (Johansson et al. 2012), middle cerebral artery occlusion (Ahnstedt et al. 2015) and subarachnoid haemorrhage (Hansen-Schwartz et al. 2003; Ansar et al. 2007). In these studies, endothelin-1 (ET-1) was consistently involved in delayed cerebral vasoconstriction after ischaemia that leads to a prolonged decrease in blood flow, exacerbating neuronal damage and worsening the overall neurological outcomes.

Endothelin-1 (ET-1) is a powerful vasoconstrictor that was first described in 1988 (Yanagisawa et al. 1988). Its vascular function is mediated through two main receptors: ET_A and ET_B. Activation of vascular smooth muscle ET_A causes strong vasoconstriction while endothelial ET_B causes nitric oxide-mediated vasodilation. The presence of both has been shown in fresh rat ophthalmic artery previously (Jarajapu et al. 2004). Interestingly, ET_B also appears in the smooth muscle cells of cerebral arteries after organ culture *in vitro* (Adner et al. 1996) and experimental ischaemia *in vivo* (Edvinsson & Povlsen 2011), facilitating vasoconstriction and being tied to detrimental neurological and behavioural outcomes. Furthermore, ET-1 levels have been shown to increase significantly in several ocular diseases as demonstrated

in blood plasma levels after retinal vein occlusions in humans (Iannaccone et al. 1998) and glaucoma (Emre et al. 2005), adding further relevance to why ET-1-mediated vasoconstriction can have an impact on patients.

In the brain, upregulation of vascular smooth muscle ET_B receptors has been linked to the intracellular mitogen-activated protein kinase (MAPK) signalling pathway (Edvinsson & Povlsen 2011). The MAPK family consists of three kinases: extracellular signal-regulated kinase (ERK1/2), p38 and protein kinase c-Jun N-terminal kinase (JNK). U0126, an ERK1/2 specific inhibitor, does indeed reduce the upregulation of vasoconstrictive ET-1 receptors after cerebral ischaemia in rat (Maddahi & Edvinsson 2010; Ahnstedt et al. 2015; Povlsen & Edvinsson 2015). This has also been demonstrated using the organ culture model, where inhibition of Raf, the first signalling molecule in the MEK/ERK1/2 pathway in human cerebral arteries, prevents receptor upregulation (Ahnstedt et al. 2011). Thus, ET-1-mediated vasoconstriction has been shown to be both important in the pathophysiology of cerebral ischaemia and being an experimentally valid therapeutic target. ERK1/2 levels have been shown to be significantly increased in porcine retinal arteries and the neuroretina after ischaemia induced through increased intraocular pressure (Gesslein et al. 2010). This suggests that ischaemia in the pig retina may induce similar vascular changes in retinal arteries.

To test this, we have used our established model for ischaemic changes, where we incubate isolated arteries in organ culture for 24 hr. We examined the rat ophthalmic artery and pig retinal arteries for changes in endothelin receptors, and tested whether or not these changes could be attenuated by the MEK/ERK1/2 inhibitors U0126 and trametinib. This study shows that ET-1-mediated vasoconstriction after organ culture is significantly increased in both rat ophthalmic artery and pig retinal arteries and that the upregulation of contractile receptors can be prevented by treatment with MEK/ERK1/2 inhibitors. We propose that the vascular changes in the ophthalmic artery and retinal arteries are important contributors to retinal ischaemia, and understanding of these factors may lead to a novel treatment approach.

Materials and Methods

Animal ethics

The study was performed under the rules and regulations of the Regional Ethical Review Board in Lund, Sweden (M17-15).

Tissue preparation

Thirty male Sprague Dawley rats (250–350 g) were used for this study. Rat ophthalmic arteries were obtained by dissecting out the eye carefully and severing the optic nerve as distally from the eye as possible. The dissected eye was put in Na-Krebs buffer (containing: NaCl 119 mM, $NaHCO_3$ 15 mM, KCl 4.6 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.5 mM and glucose 5.5 mM), and the dura sheath containing the ophthalmic artery, flanked by both posterior ciliary arteries, was cut free. The artery was measured, and if it was over 2 mm, it was divided into two equally long segments. For the myograph experiments, the ophthalmic arteries had a 25-μm wire inserted carefully into the lumen to facilitate the removal of surrounding tissue and isolate the artery. The artery was either immediately placed in the myograph (fresh) or in organ culture medium overnight. U0126 (10^{-6} M) or trametinib (10^{-8} M) was added to the organ as appropriate.

Retinal arteries were isolated from pig eyes that were harvested less than 30 min after death by electric shock (16 eyes, 8 pigs ~50 kg) and placed in cold Na-Krebs buffer. Eyes were bisected by a frontal section behind ora serrata, the lens and vitreous were removed, and an approximately 2-mm segment of the superior artery was taken within 5 mm from the optic disc.

Arteries used for immunohistochemistry were dissected and immediately fixed in 4% paraformaldehyde for 2 hr before cryoprotection in sucrose Sörensen's buffer with increasing sucrose concentrations of 10% and 25%. Finally, the arteries were embedded in an egg albumin-based protein medium and frozen for cryosectioning.

Organ culture

Ophthalmic and retinal artery segments were incubated in 2-ml wells with Dulbecco's modified Eagle's medium (DMEM, Gibco, Denmark) in combination with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) for 24 hr at 37°C. This method for studying upregulation of contractile receptors has been described previously (Adner et al. 1998). The arteries were cultured with vehicle, 10^{-6} M U0126 [dissolved in dimethyl sulfoxide (DMSO)] or 10^{-8} M trametinib (GSK, dissolved in cremophor + polyethylene glycol).

Myography

Rat ophthalmic artery segments (1–2 mm) and pig retinal arteries (2 mm) were mounted in an arterial myograph. Only one segment of retinal artery was harvested from each pig eye. Two wires (25 μm) were inserted through the arterial lumen. One wire was attached to a micrometre screw which allowed for fine adjustments of the distance between the wires. The second wire was attached to a force displacement transducer, paired together with an analog–digital converter (AD Instruments, Oxford, UK). The data were recorded using PowerLab unit software (AD Instruments, Oxford, UK). The arteries were submerged in tissue baths at 37°C containing a Na-Krebs bicarbonate buffer. All solutions were aerated with gas composed of 95% O_2 and 5% CO_2.

Both the rat ophthalmic and pig retinal arteries were given a pretension of 1.0 mN. The optimum pretension for the rat artery was established previously (Blixt et al. 2016), and the value for the pig was determined experimentally. The arteries were devoid of endothelial function, tested by the precontraction with 10^{-5} M $PGF_{2\alpha}$ followed by 10^{-5} M carbachol. In addition, we tested the dilation in fresh arteries with 30 mM K+ contraction followed by 10^{-7} M sarafotoxin (S6c) and 10^{-7} M Substance P. However, no relaxation responses were observed in fresh vessels, suggesting no functional endothelium.

A reference contraction value for each segment using 60 mM K^+ (part of the NaCl was replaced with KCl) and calculating contraction from baseline for the rat arteries and from a Ca^{2+}-free baseline for the pig arteries, due to their high myogenic tone. These values were considered as 100% contraction, and the data from each segment were expressed as a percentage of the value

determined from that segment. The arteries were allowed to stabilize before the application of the ET_B-specific agonist S6c or ET-1 to generate concentration–response curves. Furthermore, both ET_A- and ET_B-specific agonists BQ123 and BQ788, respectively, were used to evaluate specific receptor-mediated contraction. For all substances, a cumulative concentration–response curve was obtained.

Immunohistochemistry

Ten micro meter cryosections were washed in PBS containing 2.5% Triton (PBS-T). Next, the primary antibodies for pERK, ET_A and ET_B were added (Table 1). The sections were incubated in moisturized chambers at +8°C overnight. The following day, the cryosections were washed in PBS-T twice for 15 min before appropriate secondary antibodies (Table 1) were applied for 1 hr at room temperature in a dark room and then washed three times for 15 min. Finally, the slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame CA, USA). The described procedure was performed in triplicate to ensure reproducibility. Further, negative controls were included by omitting the primary antibody. The full list of antibodies used can be found in Table 1.

Microscopy and imaging

The slides were examined using an epifluorescence microscope (Nikon 80i, Tokyo, Japan) combined with a Nikon DS-2MV camera. Areas of interest were photographed with 10x, 20× or 40× lenses. The images were then processed using Adobe Photoshop CS3 (v10.0 Adobe 3 Systems, Mountain View, CA, USA), and images taken with different wavelength filters were superimposed to determine any potential colocalization.

Statistical analysis and chemicals

Two-way ANOVA test with Bonferroni post hoc test was applied to analyse the concentration–response curves to determine significance among the individual data points in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Endothelin-1 (ET-1) (cat# 155-001-PC05) was acquired from Enzo, San Diego, CA, USA, and S6c (cat# 167-002-PC05) from Enzo. Substance P (cat# 1156) from endothelin receptor antagonists BQ123 (cat# 1188) and BQ788 (cat# 1500) were acquired from Tocris, Bristol, UK. Eagle's medium used in this study was purchased from Gibco, Invitrogen, Carlsbad, CA, USA.

MEK/ERK1/2 inhibitor U0126 (cat# U-6770) was acquired from LC Laboratories, Woburn, MA, USA, while trametinib (GSK) (cat# S2673) was supplied by Selleck Chemicals, Houston, TX, USA. Both Cremophor EL (cat# 238470-1SET) and PEG (cat# 8074850050) were from Merck Millipore, West Point, PA, USA, while DMSO (cat# D2438) was from Sigma-Aldrich.

Results

Rat ophthalmic artery

We have previously used the organ culture model to study upregulation of contractile receptors in the brain and coronary vasculature. However, the vasculature of the eye is also susceptible to ischaemia, and we wanted to investigate whether these arteries responded in a similar manner.

Rat ophthalmic arteries subjected to 24 hr of organ culture exhibited a significant increase in vasoconstriction induced by ET-1 (Fig. 1A). The concentration–response curve from cultured arteries was statistically significant from that of fresh arteries ($p < 0.001$). There was a leftward shift of the biphasic curve with differences in pEC_{50}^1 14.13 ± 0.53 compared to pEC_{50}^1 15.82 ± 1.2 for fresh and pEC_{50}^2 8.30 ± 0.20 for cultured versus 7.60 ± 0.18 for fresh. The curves showed significant differences at ET-1 concentrations 10^{-9} M ($p < 0.01$), 10^{-8} M ($p < 0.001$), and 10^{-7} M ($p < 0.01$). For the ET_B-specific agonist, S6c, a similar difference between cultured and fresh arteries was observed with the curves being statistically significant ($p < 0.0001$) (Fig. 1B). In fresh arteries, there was little to no response to S6c, while after organ culture, the arteries contracted to S6c with a pEC_{50} of 7.67 ± 0.21. At S6c concentrations of 10^{-8} M ($p < 0.0001$) and 10^{-7} ($p < 0.0001$), there was a significant difference between cultured and fresh ophthalmic arteries (Table 2).

Specific ET-1 receptor antagonists were added to highlight the individual receptor function following 24-hr organ culture. ET_A antagonist BQ123 had little overall effect on the rat ophthalmic artery with a pEC_{50}^1 of 13.2 ± 0.38 and pEC_{50}^2 8.83 ± 0.11 (Fig. 1C). However, the administration of ET_B-specific antagonist BQ788 abolished the biphasic concentration–response curve and only exhibited one contraction phase with a pEC50 8.23 ± 0.16, resembling the pEC_{50}^2 contraction of the ET-1 concentration–response curve (Fig. 1C). However, E_{max} was significantly increased ($p < 0.05$). Sarafotoxin (S6c) concentration–response curves in relation to BQ788 show a significant effect ($p < 0.01$) by eliminating the S6c-mediated contraction (Fig. 1D).

The observed increase in ET-1-mediated vasoconstriction after culture could be attenuated by U0126 or trametinib treatment (Fig. 1A). pEC_{50}^1 values were significantly altered by trametinib and U0126 EC_{50}^1 14.13 ± 0.53 versus 12.76 ± 3.60 after trametinib. For U0126-treated arteries, the EC_{50}^1 could not be accurately

Table 1. A detailed list of all the antibodies used along with working dilutions, manufacturer and article number for the immunohistochemical analysis.

Primary antibodies				
Antibody	Host	Dilution	Manufacturer	Art. Nr
pERK	Mouse	1:200	Abcam, Cambridge, UK	ab50011
ET_A	Rabbit	1:100	Santa Cruz Biotechnology, Santa Cruz CA, USA	Sc33535
ET_B	Sheep	1:100	Enzo Life Sciences, Farmingdale, NY, USA	ALX-210-506A

Secondary antibodies			
Antibody	Against	Dilution	Manufacturer
FITC	Mouse	1:100	Jackson Immunoresearch, West Grove, PA, USA
FITC	Rabbit	1:200	Cayman Chemical, Ann Arbor, MI, USA
Texas Red	Sheep	1:50	Jackson Immunoresearch, West Grove, PA, USA

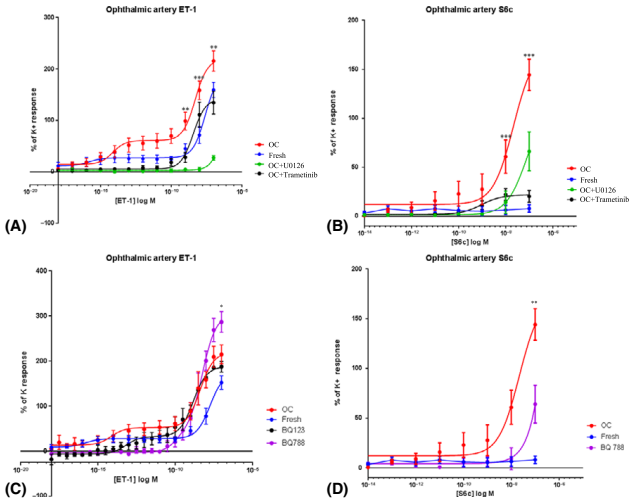


Fig. 1. (A) Concentration–response curve showing an increased vasoconstriction of organ culture (OC, $n = 14$) rat ophthalmic artery in comparison with fresh ($n = 13$) and MEK/ERK1/2 inhibitors U0126 ($n = 9$) and trametinib ($n = 10$). Significant differences between fresh and OC at endothelin-1 (ET-1) concentrations 10^{-9} M, 10^{-8} M and 10^{-7} M were also observed. (B) Concentration–response curve of ET_B agonist sarafotoxin (S6c). Organ-treated arteries ($n = 11$) exhibited a significantly increased vasoconstriction compared to untreated fresh arteries ($n = 9$) with significance at concentrations 10^{-8}M and 10^{-7} M. Both U0126 ($n = 6$) and trametinib ($n = 10$) attenuated the increase. Full statistical review can be found in Table 2. (C) Concentration–response curve of ET-1 administration with ET_A and ET_B-specific antagonists BQ123 ($n = 3$) and BQ788 ($n = 3$), respectively. BQ123 having no significant effect on ET-1-mediated contraction while BQ788 abolishes the biphasic pattern and causes an overall increased contraction. (D) Sarafotoxin contraction is inhibited significantly by the use of ET_B-specific antagonist BQ788.

Table 2. Statistical summary of significance between the complete dose–response curves.

Rat ophthalmic artery				
Endothelin-1				
	Fresh	OC	U0126	GSK
Fresh		***	***	NS
OC			***	***
Sarafotoxin				
	Fresh	OC	U0126	GSK
Fresh		***	NS	NS
OC			*	**
Porcine retinal arteries				
Endothelin-1				
			OC	
Fresh			***	
Sarafotoxin				
	Fresh	OC	U0126	
Fresh		***	NS	
OC			***	

Analysis is performed by the ANOVA test with Bonferroni post hoc test to compare the complete dose–response curves. NS represents $p > 0.05$, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

determined. The concentration–response curves for cultured arteries with and without U0126 and trametinib differed significantly $p < 0.0001$ and $p < 0.001$, respectively. Interestingly, U0126 seemed to almost completely suppress ET-1-mediated vasoconstriction, the curve being significantly lower than fresh ($p < 0.0001$), whereas trametinib-treated ophthalmic arteries did not differ statistically from fresh ($p > 0.05$). Similarly, S6c-mediated vasoconstriction also was significantly decreased when treated with U0126 ($p < 0.05$) and trametinib ($p < 0.001$) (Fig. 1B). Again, trametinib-treated ophthalmic artery resembled fresh arteries ($p = 0.60$).

Control experiments were performed including treatment of arteries with vehicle controls (without the active inhibitor) and fresh arteries with U0126. Both of these controls exhibited no difference compared to fresh untreated arteries (data not shown).

Immunohistochemistry on the rat ophthalmic artery

To further evaluate whether the organ culture caused ERK activation and ET-1 receptor upregulation in the rat ophthalmic artery, we performed immunohistochemistry. The phosphorylated and active form of ERK, pERK, was indeed found in the vascular wall of the rat ophthalmic artery after organ culture (Fig. 2A). Immunolabelling was located throughout the smooth muscle cell layer of the artery with no presence detected in the endothelial cells. In contrast, fresh arteries or arteries incubated in the presence of U0126 or trametinib did not show any pERK immunolabelling, suggesting that the activation of ERK1/2 was caused by the organ culture, and this response could be inhibited by the two inhibitors.

We also investigated whether the distribution and expression of ET-1 receptors was altered after organ culture. ET_A presence was clear in the smooth muscle cells following organ culture as compared to in fresh arteries (Fig. 2B). When arteries were kept in organ culture in the presence of either U0126 or trametinib, the immunolabelling remained at levels seen in fresh arteries. The ET_B distribution following organ culture followed a similar pattern (Fig. 2C). ET_B receptors

normally found in the endothelial cells of fresh arteries were absent in organ-cultured arteries; in the latter case, ET_B was mainly found in the smooth muscle layer. This is in concurrence with the myograph data presented above, and illustrate that the functional change in the nature of the ET_B response, dilation versus contraction, is dependent on the where the receptor is expressed, endothelium versus smooth muscle. Arteries incubated with U0126 lacked both endothelial and smooth muscle ET_B expression. However, for arteries cultured in the presence of trametinib, the arteries maintained endothelial ET_B positivity, similarly to what is seen in the fresh arteries.

Pig retinal arteries

Since retinal arteries play a key role in retinal ischaemia, we proceeded to investigate whether a similar mechanism was present in pig retinal arteries, as retinal arteries from the rat are too small to be studied in the myograph. In the pig arteries, we saw a clear increase in the contractility to ET-1 following organ culture. This was most likely due to as upregulation of ET_B receptors, which have an EC_{50} value that is much lower than for ET_A. This was observed as a leftward shift in the concentration–response curve and as an increase in contractility at the lower ET-1 concentrations (Fig. 3A). The pEC_{50}[1] for fresh retinal arteries was 11.76 ± 0.95 and 13.52 ± 0.45 for cultured arteries, and the pEC_{50}[2] 8.69 ± 0.35 and 9.00 ± 0.22, respectively. The increase in contraction was confirmed to be due to the increase in functional ET_B receptors as can be seen in Fig. 3B, where the specific ET_B agonist S6c only gives a strong contraction in the organ-cultured arteries. pEC_{50} values for fresh, organ-cultured, and U0126-treated arteries were 8.31 ± 0.18, 9.87 ± 0.11, and 8.81 ± 1.43, respectively. As a proof of concept and based on our previous studies (Gesslein et al. 2010), we applied the MEK/ERK1/2 inhibitor U0126. Here, we show that it also significantly inhibited the functional increase in ET_B receptors in pig retinal arteries, confirming that functional increase in ET_B not only occurs in both the ophthalmic and retinal vasculature but also is similar between the two species.

Discussion

In this study, we used an organ culture model that incudes vascular injury responses that mimic what is observed following tissue ischaemia (Henriksson et al. 2003). In both rat ophthalmic artery and porcine retinal artery, 24 hr of organ culture caused a significant functional increase in ET-1-mediated vasoconstriction. Furthermore, this increase in ET-1-mediated contractility can be diminished by the application of the MEK/ERK1/2 inhibitor U0126 in both rat ophthalmic artery and pig retinal artery, and in addition, trametinib in rat ophthalmic artery. What is more, the ET-1 receptors have been immunohistochemically investigated in the rat ophthalmic artery, further confirming the expressional changes following organ culture. Thus, we propose that the vasoactive changes of the ophthalmic artery and retinal arteries may play a significant role in the pathophysiology of retinal ischaemia, and thereby opening the door to a novel treatment target.

Because of the embryological origin of the retinal structures in the diencephalon, the eye and the brain are closely linked together. Therefore, it is not unreasonable that cerebral and retinal vasculature might share similar vascular properties. The rat ophthalmic artery is also the key artery supplying blood to the entire retina with a diameter of approximately 150 μm, making the ophthalmic artery a feasible vessel for myograph studies. For further insight on the retinal vasculature, the pig retinal arteries were chosen. These two arteries thus generate a more complete picture of the retinal vascular bed and ocular circulation. The retinal vasculature remains relevant to ischaemia both for stroke research and other ischaemic conditions such as glaucoma (Venkataraman et al. 2010), central

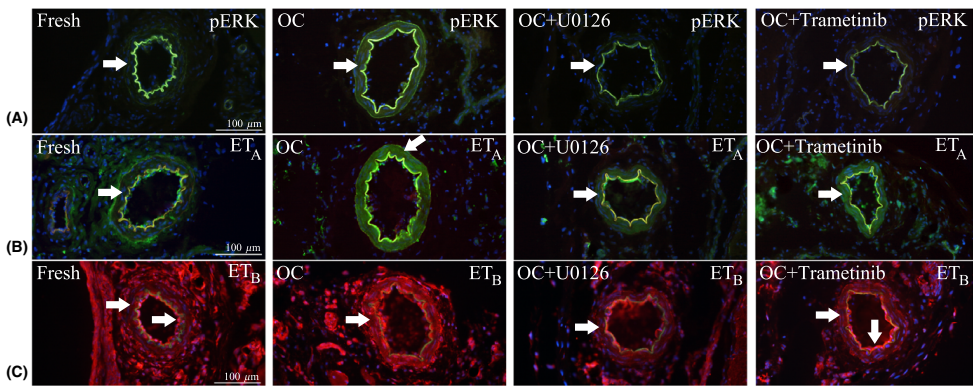


Fig. 2. (A) Immunolabelling of pERK in the rat ophthalmic artery. Organ culture-treated arteries show a clear expression in the smooth muscle cells of the artery. Fresh, U0126- and trametinib-treated arteries exhibit no immunopositivity. (B) ET_A distribution in the ophthalmic artery. Organ culture (OC) reveals clear presence of ET_A receptors in the vascular smooth muscle cells. Slight expression is observed in both treated arteries; however, fresh seems not to show any positive immunolabelling. (C) ET_B, present in endothelial cells of fresh arteries, are exclusively expressed in the smooth muscle cells after OC. MEK/ERK1/2 inhibitor treatment seems to prevent smooth muscle cell expression of ET_B.

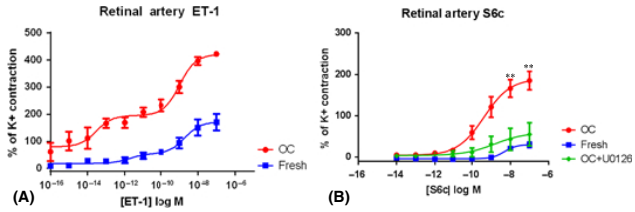


Fig. 3. (A) Increased contractility of pig retinal arteries after organ culture (OC) ($n = 4$) compared to fresh ($n = 4$). (B) The increased contractility is, at least partly, due to increased ET_B contractility after sarafotoxin (S6C) stimulation. Significance is shown between OC ($n = 4$) and fresh ($n = 4$) at both 10^{-8} M and 10^{-7} M. U0126 ($n = 4$) also severely attenuates the observed contractility of ET_B. Full statistical review can be found in Table 2. ET-1 = endothelin-1.

retinal artery occlusion (Varma et al. 2013) and ocular ischaemic syndrome (Mendrinos et al. 2010), hence leading to a broad range of possible application for these findings.

The vascular changes in cerebral ischaemia, exemplified by the upregulation of ET-1 receptors, are mediated via the MEK/ERK1/2 pathways (Lennmyr et al. 2002; Edvinsson & Povlsen 2011). In pig, pERK levels in the retina have been shown to spike immediately after increased intraocular pressure induced ischaemia (Gesslein et al. 2010). Therefore, we focused on pERK in this study. We opted for a visual confirmation of pERK manifestation in rat ophthalmic artery as a proof of concept that the pathway is indeed active and potentially involved in the regulation of vasoconstrictive receptors in rat ophthalmic artery. Phosphorylation of ERK and inhibition of the ERK1/2 pathway by U0126 have previously been demonstrated in rat cerebral vasculature after cerebral ischaemia (Maddahi & Edvinsson 2010; Ahnstedt et al. 2015; Povlsen & Edvinsson 2015). This further supports the hypothesis of the involvement of the activity of MEK/ERK1/2 pathways and potential pharmacological intervention to stop downstream regulation of vasoconstriction receptors within the ophthalmic artery and retinal artery.

The vasoconstrictive response in the rat ophthalmic artery has been evaluated previously with ET-1 being described as a potent vasoconstrictor with a biphasic dose–response curve (Jarajapu et al. 2004). In the present study, the expression of ET-1 receptors was evaluated both through immunohistochemistry and more importantly, functionally, utilizing the myograph. ET_A and ET_B were clearly increased in the smooth muscle cell layer of the ophthalmic artery in rat, much like in cerebral arteries after ischaemia (Stenman et al. 2002; Hansen-Schwartz et al. 2003). Interestingly, ET_B expression which is usually found in the vascular endothelial cells seems to be completely absent after organ culture, described here for the first time for the rat ophthalmic artery. We also show a significant increase in vasoconstriction mediated by ET-1 receptors and specifically an increase in ET_B-mediated vasoconstriction after organ culture. This was confirmed via the addition of specific ET_B antagonist BQ788 (Fig. 1C, D). Interestingly, the subsequent E_{max} (ET_A mediated) was enhanced in relation to organ culture-treated arteries without antagonist. The shift in ET_B function from mediating endothelium-dependent NO vasodilation to causing severe constriction has been described functionally in cerebral vasculature (Nilsson et al. 1997; Stenman et al. 2002; Ahnstedt et al. 2011; Johansson et al. 2012), and here for the first time shown to be functionally relevant in rat ophthalmic artery. ET_B receptors have been involved in clearance of ET-1, usually localized in the endothelial cells (Brunner & Doherty 1996). Expressed on smooth muscle cells, this clearance will lead to contraction upon binding of the ligand to the receptor. Applying BQ788 might inhibit the scavenging process at lower ET-1 concentrations, hence the lack of initial contraction. However, blocking the scavenger receptor leads to a higher E_{max} mediated through the ET_A receptor. Nevertheless, the minimal effect of ET_A-specific antagonist BQ123 on EC_{50}[2] needs further studies. The increased vasoconstriction will probably reduce the blood flow to the surrounding tissue, exacerbating the ischaemic effects as suggested previously (Blixt et al. 2016). As E_{max} is increased in the presence of BQ788, targeting the upregulation itself, rather than the ET_B receptor, seems more clinically relevant.

Our results support that ET-1-mediated vasoconstriction may be involved in the pathophysiology of retinal ischaemia, as proposed previously (Blixt et al. 2016). The present study revealed that the upregulation of the ET-1-mediated vasoconstriction is regulated by the MEK/ERK1/2 pathway as well as occurring in both rat and porcine arteries.

Both U0126 and trametinib are known to inhibit the MEK/ERK1/2 pathway; however, they have different specificities. Trametinib is a highly potent and specific MEK1/2 inhibitor with little effect on other kinases involved in the cellular communication (Lugowska et al. 2015). U0126, however, not only inhibits MEK1/2 strongly, but also elicits a weak inhibition on phosphokinase C, Raf, ERK and JNK (Duncia et al. 1998), suggesting that the discrepancy in our myograph results may be a result of weaker secondary inhibitions following U0126 but not trametinib. U0126 does not directly modulate contractility on fresh arteries. However, higher concentrations of U0126 have been shown to activate other kinases, for example, AMPK which could further complicate the interpretation of the results (Dokladda et al. 2005). The role of the MEK/ERK1/2 pathway in retinal ischaemia has been described as a double-edged sword, regulating vasoconstriction as well as promoting Müller cell activation and cell survival (Bringmann et al. 2006). Therefore, an optimized time window of the MEK/ERK1/2 inhibition must be carefully studied in *in vivo*, as well as constant monitoring of retinal damage and recovery. Within cerebral ischaemia, it has been suggested that administration of U0126 immediately after ischaemia and up to 24 hr later yields better outcomes both behaviourally and morphologically in rats (Maddahi & Edvinsson 2010; Ahnstedt et al. 2015).

The use of two species, both rat and pig, in this study provides a stronger foundation for future studies. Interspecies variations may alter the results of experiments and experimental

outcomes due to among others anatomical differences between species. Here, we show that MEK/ERK1/2 regulates an increase in ET-1 receptor function, which we believe is valid across several species.

The combined data from functional myography, coupled with immunohistochemistry, show that organ culture induces a functional increase in ET-1 receptors *in vitro*, in both rat ophthalmic artery and pig retinal arteries, similar to what has been observed for ischaemia *in vivo*. The increased vasoconstriction is primarily mediated through the ET_B receptor. Furthermore, this functional increase is mediated via the MEK/ERK1/2 pathway, shown using the ERK1/2 inhibitors U0126 and trametinib. These results strongly suggest that the rat ophthalmic artery and pig retinal arteries share similar characteristics with cerebral arteries following ischaemia. Furthermore, the MEK/ERK1/2 inhibitors U0126 and trametinib may be used as therapeutic agents for ocular vascular ischaemia.

References

Adner M, Cantera L, Ehlert F, Nilsson L & Edvinsson L (1996): Plasticity of contractile endothelin-B receptors in human arteries after organ culture. Br J Pharmacol **119**: 1159–1166.

Adner M, Geary GG & Edvinsson L (1998): Appearance of contractile endothelin-B receptors in rat mesenteric arterial segments following organ culture. Acta Phyiol Scand **163**: 121–129.

Ahnstedt H, Saveland H, Nilsson O & Edvinsson L (2011): Human cerebrovascular contractile receptors are upregulated via a B-Raf/MEK/ERK-sensitive signaling pathway. BMC Neurosci **12**: 5.

Ahnstedt H, Mostajeran M, Blixt FW, Warfvinge K, Ansar S, Krause DN & Edvinsson L (2015): U0126 attenuates cerebral vasoconstriction and improves long-term neurologic outcome after stroke in female rats. J Cereb Blood Flow Metab **35**: 454–460.

Ansar S, Vikman P, Nielsen M & Edvinsson L (2007): Cerebrovascular ETB, 5-HT1B, and AT1 receptor upregulation correlates with reduction in regional CBF after subarachnoid hemorrhage. Am J Physiol Heart Circ Physiol **293**: H3750–H3758.

Blixt FW, Johansson SE, Johnson L, Haanes KA, Warfvinge K & Edvinsson L (2016): Enhanced Endothelin-1 Mediated Vasoconstriction of the Ophthalmic Artery May Exacerbate Retinal Damage after Transient Global Cerebral Ischemia in Rat. PLoS ONE **11**: e0157669.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN & Reichenbach A (2006): Muller cells in the healthy and diseased retina. Prog Retin Eye Res **25**: 397–424.

Brunner F & Doherty AM (1996): Role of ET (B) receptors in local clearance of endothelin-1 in rat heart: studies with the antagonists PD 155080 and BQ-788. FEBS Lett **396**: 238–242.

Dokladda K, Green KA, Pan DA & Hardie DG (2005): PD98059 and U0126 activate AMP-activated protein kinase by increasing the cellular AMP:ATP ratio and not via inhibition of the MAP kinase pathway. FEBS Lett **579**: 236–240.

Duncia JV, Santella JB 3rd, Higley CA et al. (1998): MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett **8**: 2839–2844.

Edvinsson LI & Povlsen GK (2011): Vascular plasticity in cerebrovascular disorders. J Cereb Blood Flow Metab **31**: 1554–1571.

Emre M, Orgul S, Haufschild T, Shaw SG & Flammer J (2005): Increased plasma endothelin-1 levels in patients with progressive open angle glaucoma. Br J Ophthalmol **89**: 60–63.

Gesslein B, Hakansson G, Carpio R, Gustafsson L, Perez MT & Malmsjo M (2010): Mitogen-activated protein kinases in the porcine retinal arteries and neuroretina following retinal ischemia-reperfusion. Mol Vis **16**: 392–407.

Hansen-Schwartz J, Lovland Hoel N, Nilsson E, Tfelt-Hansen P & Edvinsson L (2003): Endothelium-dependent relaxant responses to selective 5-HT(1B/1D) receptor agonists in the isolated middle cerebral artery of the rat. J Vasc Res **40**: 561–566.

Henriksson M, Stenman E & Edvinsson L (2003): Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. Stroke **34**: 1479–1483.

Iannaccone A, Letizia C, Pazzaglia S, Vingolo EM, Clemente G & Pannarale MR (1998): Plasma endothelin-1 concentrations in patients with retinal vein occlusions. Br J Ophthalmol **82**: 498–503.

Jarajapu YP, Grant MB & Knot HJ (2004): Myogenic tone and reactivity of the rat ophthalmic artery. Invest Ophthalmol Vis Sci **45**: 253–259.

Johansson S, Povlsen GK & Edvinsson L (2012): Expressional changes in cerebrovascular receptors after experimental transient forebrain ischemia. PLoS ONE **7**: e41852.

Lennmyr F, Karlsson S, Gerwins P, Ata KA & Terent A (2002): Activation of mitogen-activated protein kinases in experimental cerebral ischemia. Acta Neurol Scand **106**: 333–340.

Lugowska I, Kosela-Paterczyk H, Kozak K & Rutkowski P (2015): Trametinib: a MEK inhibitor for management of metastatic melanoma. Onco Targets Ther **8**: 2251–2259.

Maddahi A & Edvinsson L (2010): Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway. J Neuroinflammation **7**: 14.

Mendrinos E, Machinis TG & Pournaras CJ (2010): Ocular ischemic syndrome. Surv Ophthalmol **55**: 2–34.

Nilsson T, Cantera L, Adner M & Edvinsson L (1997): Presence of contractile endothelin-A and dilatory endothelin-B receptors in human cerebral arteries. Neurosurgery **40**: 346–351; discussion 351-343.

Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M & Melena J (2004): Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog Retin Eye Res **23**: 91–147.

Povlsen GK & Edvinsson L (2015): MEK1/2 inhibitor U0126 but not endothelin receptor antagonist clazosentan reduces upregulation of cerebrovascular contractile receptors and delayed cerebral ischemia, and improves outcome after subarachnoid hemorrhage in rats. J Cereb Blood Flow Metab **35**: 329–337.

Stenman E, Malmsjo M, Uddman E, Gido G, Wieloch T & Edvinsson L (2002): Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. Stroke **33**: 2311–2316.

Varma DD, Cugati S, Lee AW & Chen CS (2013): A review of central retinal artery occlusion: clinical presentation and management. Eye (Lond) **27**: 688–697.

Venkataraman A, Kubicki M, Westin CF & Golland P (2010): Robust feature selection in resting-state fMRI connectivity based on population studies. Conf Comput Vis Pattern Recognit Workshops, **6**: 3–70.

Yanagisawa M, Kurihara H, Kimura S et al. (1988): A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature **332**: 411–415.

Received on October 12th, 2016.
Accepted on October 23rd, 2017.

Correspondence:
Frank W. Blixt, PhD
Department of Clinical Sciences,
Division of Experimental Vascular Research,
Lund University
Sölvegatan 17
BMC A13
SE-221 84
Lund
Sweden
Tel: +46 46 222 08 25
Email: frank.blixt@med.lu.se

Retinal ischemia is a condition that takes many forms, from vein or arterial occlusions, to diabetic retinopathy, to ocular ischemic syndrome and to some extent glaucoma. Its wide spectrum of manifestations mean that it is a common cause of reduced vision complete blindness in the world.

Many efforts have been made to tackle the disastrous effects of retinal ischemia yet few have been successful. Anti-VGEF treatment having become the first step towards reducing the impact caused by some of these conditions. With this thesis, new vascular perspective is proposed in the battle against retinal ischemia, drawing inspiration from research done on cerebral arteries after stroke, a condition sharing many pathophysiological processes. The results that lay within this thesis show that there is a significant increase of vasoconstrictive receptors present following ischemia that prolong and exacerbate ischemic damage by preventing normal blood flow to the retina, even after blood flow has been restored. We have also identified the cellular pathway responsible for this upregulation, providing a tangible therapeutic target for future studies.

We believe that our research may provide new insight into the treatment of retinal ischemia and to provide a new avenue in which to tackle one of the main causes of blindness in the world.

Printed by Media-Tryck, Lund 2018 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Lund University
Department of Experimental Vascular Research

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2018:38
ISBN 978-91-7619-605-2
ISSN 1652-8220