REGULATION OF RETINAL ANGIOGENESIS BY A NOVEL LACTATE RECEPTOR, GPR81

by

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TABLE OF CONTENTS

LIST OF FIGURES

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Regulation of Retinal Angiogenesis by a Novel Lactate Receptor, GPR81

Background: Ischemic proliferative retinopathies as characterized by an exaggerated retinal neovascularization, are the major cause of visual impairment and blindness in children. Vascularization is essential for development and restoration of tissue integrity following an ischemic injury. Because vascular supply is coupled to tissue energy consumption, a role for metabolic intermediates such as lactate in angiogenesis is conceivable. Moreover, increase in lactic acid production has long been associated with angiogenesis in tumors. Given the recent identification of a novel G-protein coupled receptor for lactate (GPR81) and our detection of high levels of lactate in ischemic retina, we investigated the propensity of lactate to regulate retinal vessel growth via GPR81.

Hypothesis: We hypothesized that lactate may play an important role in the development of ischemia-induced retinal neovascularization by acting through specific cognate receptor GPR81.

Methods: Lactate levels were measured by a colorimetric assay in retinas of mice exposed to oxygen-induced retinopathy (OIR) model. GPR81 mRNA was analyzed by RT-PCR in the retina of mice exposed to OIR and in Retinal Ganglion Cells (RGC) and retinal endothelial cell cultures. GPR81 protein localization was evaluated by co-immunostaining with cell-specific markers in retinal cryosections. Pro-angiogenic response to lactate was measured *in vitro* by using endothelial cell tube formation assay and *in vivo* by analyzing the vascular density in retinal flatmounts of mice pups injected intravitreally with lactate.

Results: Lactate concentration increased 2.5 times in the retinas at P17 in animals exposed to OIR model compared to the control. GPR81 mRNA was detected in the mice retina and the cell cultures analyzed. The GPR81 protein was predominantly localized in the ganglion cells, endothelium and Muller Cells in the retina. Lactate showed a pro-angiogenic effect at 10 mM in the endothelial cell assay and a significant increase ($p<0.05$) in the retinal vascular density.

Conclusions: Our result suggest that the pro-angiogenic metabolite lactate produced during neovascularization phase in the OIR model acting via its GPR81 receptor expressed in the retina, may play an important role in the development of vasoproliferative retinopathies.

Régulation de l'angiogenèse rétinienne par un récepteur au lactate, GPR81

Contexte: Les rétinopathies ischémiques prolifératives caractérisées par une néovascularisation rétinienne exagérée sont les principales causes de déficience visuelle et de cécité chez les adultes et les enfants. Malgré le rôle important du VEGF dans cette néovascularisation, il existe des preuves que d'autres facteurs participent à ce processus. Notamment des facteurs qui sont sensibles à l'hypoxie-ischémie, les métabolites. Le succinate a été récemment montré comme exerçant un rôle important dans la régulation de l'angiogenèse rétinienne. En outre, l'augmentation de la production d'acide lactique a été associée à l'angiogenèse tumorale. Pour le moment, le rôle du lactate dans le développement des rétinopathies ischémiques n'est pas bien connu. Il a été récemment montré que le lactate exerce ces effets biologiques via un récepteur couplé à une protéine G, le récepteur GPR81.

Hypothèse: Nous avons émis l'hypothèse que le lactate peut jouer un rôle important dans le développement de la néovascularisation rétinienne induite par ischémie en agissant par le biais du récepteur GPR81.

Méthodes: Les taux de lactate ont été mesurés par un essai colorimétrique dans les rétines de rats soumises au modèle de rétinopathie induite par l'oxygène (RIO). L'ARNm de GPR81 a été analysé par RT-PCR dans les rétines de rats exposés à l'oxygène et dans des cultures de cellules gliales de Müller, de cellules ganglionnaires rétiniennes et de cellules endothéliales rétiniennes. La protéine

GPR81 a été localisée sur des cryosections de rétines par un co-immunomarquage avec des marqueurs cellulaires spécifiques. La réponse pro-angiogénique au lactate a été mesurée *in vitro* en utilisant un test cellulaire de migration de cellules endothéliales et *in vivo* par analyse de la densité vasculaire dans des montages à plats de rétines de ratons injectés avec du lactate par voie intravitréenne.

Résultats: La concentration de lactate est trois fois plus importante dans les rétines des animaux soumis au modèle RIO par rapport aux contrôles sous normoxie. L'ARNm de GPR81 est présent dans la rétine de rat et dans toutes les cellules analysées. La protéine GPR81 est principalement localisée dans les cellules ganglionnaires, endothéliales et de Müller. Le lactate a une concentration de 10 mM montre un effet pro-angiogénique dans le test de migration des cellules endothéliales et augmente significativement ($p < 0,05$) la densité vasculaire rétinienne dans les montages à plats.

Conclusions: Nos résultats suggèrent que le lactate est un métabolite pro-angiogénique. Le lactate, produit lors de la phase de la néovascularisation dans le modèle de RIO, joue un rôle important dans le développement des rétinopathies vaso-prolifératives, par l'intermédiaire de son récepteur GPR81.

Preface – Contribution of authors

This thesis is written in manuscript form as permitted by the McGill University. It is composed of one manuscript, as listed below, with contribution of each author.

This study was designed by Ankush Madaan, Dr. Carlos Rivera and Dr. Sylvain Chemtob. In this study, Ankush Madaan performed in *vitro* and *in vivo* experiments while intravitreal injections were performed by Dr. Carlos Rivera. The macro plugin in ImageJ to measure vascular density was developed by D. Hamel, a PhD student in Chemtob laboratory. Ankush Madaan collected most of the data. All the data analysis and manuscript writing was performed by Ankush Madaan with help from Dr. Sylvain Chemtob.

INTRODUCTION

The Eye

The eye can be divided into three sections: 1) Outermost sclera, which is continuous with the cornea; 2) Uvea, comprised of the choroid (a collection of blood vessels), the ciliary body (which holds the lens in place) and the iris; and 3) innermost layer consisting of the retina and its vasculature.

Retina

In the embryonic development of vertebrates, the retina and the optic nerve extend as outgrowths of the forebrain and are therefore considered to be a part of the central nervous system (Kolb 2003). The retina is a complex, layered structure with several layers of neurons that are interconnected by two layers of synaptic connections (Kolb 1994). Not only does the retina include light sensitive neurons, but also the neural circuitry that is involved in the initial processing of images that are transmitted to the brain (Kolb 2003). Many of the specific retinal cell types are common among different mammalian species (MacNeil and Masland 1998, Boycott and Wassle 1991) with similar retinal organization into three layers of neurons and two areas of neuropil (Kolb 1994).

The neurosensory retina is composed of three layers of nerve cell bodies; two synaptic (plexiform) layers and three types of glial support cells, which together process and transfer visual information to the optic never. The output neurons of the retina, the Ganglion Cells (GCs), lie innermost, whereas the rod and cone photo sensors are situated outermost. Therefore,

light is required to travel the thickness of the retina, a distance ranging from 200 μm in the far periphery to 500 μm centrally (Haq F *et al*. 2002) before activating the photosensors. Nevertheless, visual acuity is preserved by the fovea, a 'pit' devoid of blood vessels, composed entirely of cones, where the neuronal layers part allowing light to fall directly along the optical axis into this region.

Retinal Architecture

The retina is a thin tissue that lines the back of the eye between the choroid and vitreous, spanning a thickness of approximately half a millimeter (Kolb 2003). The vertebrate retina is made up of five neuronal cell types in addition to one glial cell type. The retinal cell bodies are found in three nuclear layers, while the synapses lie in the outer and inner plexiform layers (Dowling 1970). The retinal pigment epithelium (RPE) is bordered by the choroid on one side and by photoreceptor layer (PL) on the other. The photoreceptor layer includes both the outer segments (OS) and inner segments (IS) of the photoreceptors, while their nuclei lay in the outer nuclear layer (ONL). Following this is the first synaptic layer known as the outer plexiform layer (OPL), the inner nuclear layer (INL), the second synaptic layer known as the inner plexiform layer (IPL) and the ganglion cell layer (GCL). A schematic representation of the retina is shown in Figure 1.

Retinal Cell Types

Neurons

The rod and cone photoreceptors are composed of inner and outer segments, where the visual signal is initiated, as well as a cell body that resides in the outer nuclear layer and a synaptic terminal that reaches into the outer plexiform layer (OPL). Here the neurotransmission to bipolar neurons occurs vertically, whereas horizontal cells transmit information horizontally between photoreceptors. The bipolar cell bodies reside in the inner nuclear layer (INL), along with those of the horizontal and amacrine cells. Amacrine cells generally synapse with Ganglion Cells (GCs) in the inner plexiform layer (IPL), as bipolar cells, the dendrites of GCs, and/or other amacrine cells. At the GC layer, the ganglion axons group to form the optic nerve, which transmits the information onward to the lateral geniculate body of the brain and eventually the primary visual cortex.

Muller Cells

The bodies of Muller Cells, a type of retinal macroglia, reside in the inner nuclear layer (INL) while their processes span nearly the entire thickness of the retina. Their outer ends attach to the photoreceptor inner segments, creating the outer limiting membrane of the retina, and inwardly directed processes fuse their ‘endfeet’ to establish the inner limiting membrane. Muller cells also allow for appropriate neuronal activity by maintaining the ionic and neurotransmitter microenvironment (Newman and Reichenbach, 1996).

Microglia

Microglia, a type of glial cells, are the resident macrophages of central nervous system (CNS). They exhibit properties common to other tissue-specific macrophages as they phagocytose debris, as well as secrete numerous cytokines, free radicals and enzymes (Colton and Gilbert, 1987; Gottschall *et al*., 1995; Hu *et al*., 1996). The major dissimilarity between microglia and other tissue-specific macrophages is the ramified morphology characteristic of their resting state. Active (amoeboid) microglia are precursors to the resting (ramified) form which populate the parenchyma of the mature retina, residing throughout the inner layers until the INL/OPL interface (Diaz-Araya *et al*., 1995). However, in response to a variety of insults (e.g. infection, trauma, etc.) microglia will reassume their amoeboid shape and move to the site of injury. Based on the above, they are considered as local immune-competent cells. Interestingly, while all other glial cells derive from the neuroectodermal germ layer, it is now generally accepted that microglia are of hemangioblastic mesodermal origin (Streit WJ 2001).

Astrocytes

Astrocytes are a type of macroglia found only in the retinas of species with a retinal vasculature (i.e. present in humans, cats and rodents, but nearly absent in the virtually avascular retinas of the guinea pig and horse) (Stone and Drecher, 1987; Schnitzer, 1988). Likewise, they are neither present in the avascular fovea, nor in the peripheral developing retina prior to its vascularization (Stone and Drecher, 1987; Gariano *et al*., 1996b). Accordingly, astrocytes are key to proper vessel-related functions. The primary role of astrocytes in the maturing retina is to induce and guide retinal blood vessel formation. Astrocyte precursors migrate into the retina via the optic nerve (Ling and Stone, 1988; Wantanabe and Raff, 1988; Ling *et al*., 1989; Sarthy

et al., 1991), proliferate (Gariano *et al*., 1996a; Sanderocoe *et al*., 1999) and distribute ahead of the vascular front (Ling and Stone, 1988; Ling *et al*., 1989; Chan-Ling and Stone, 1991; Gariano *et al*., 1996b; Fruttinger, 2002). Once mature, astrocytes express the intermediate filament and common astrocyte marker, glial fibrillary acidic protein (GFAP) and serve as a physical template on which the endothelial cells (ECs) organize their vascular network (Jian *et al*., 1994; Dorrell *et al*., 2002).

After complete development, astrocytes maintain close contact with the blood vessels, including EC barrier properties (Janzer and Raff, 1987) and contributing, along with Muller Cells and microglia, to the perivascular glia limitans (Penfold *et al*., 1990; Hollander *et al*., 1991). In addition, astrocyte processes extend to neurons where they perform functions related to neurotransmitter and ion homeostasis (Walz, 1989; Danbolt, 1994).

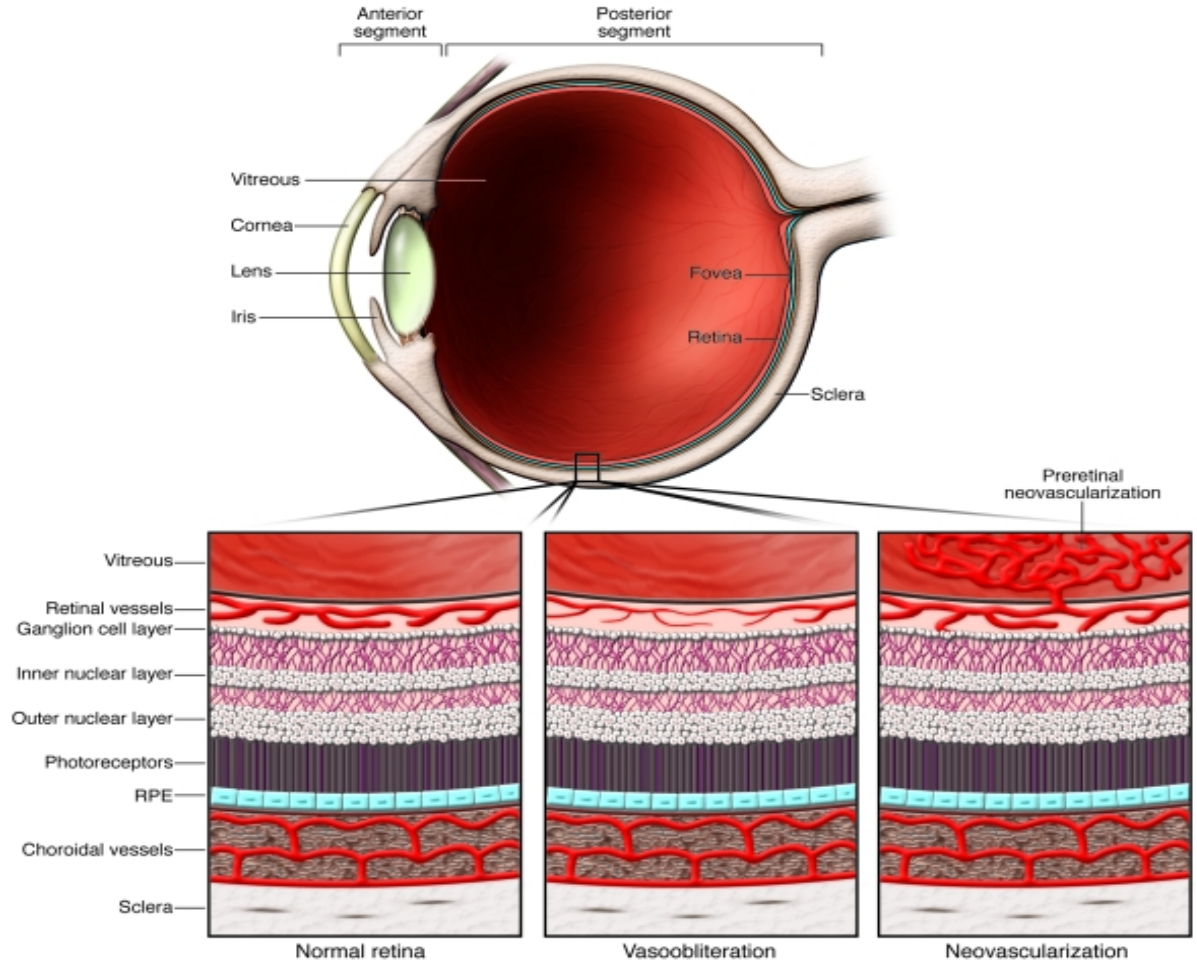
Endothelial Cells

The intima of every blood vessel is composed of a single layer of ECs, including the retinal capillaries, the smallest in the body with a diameter of 5 to 6μm (Alm, 1992). Unlike non-CNS vessels, those of the CNS are defined by their restrictive barrier properties. The blood-retinal barrier, like the blood brain barrier, serves to limit the exchange of substance between the blood and neural tissue (Cunha-Vaz *et al*., 1966;).

Pericytes

Pericytes are contractile mural cells, located between glia limitans and endothelium, within the basement membrne of the retinal mcicrovasculature. Unlike the smooth muscle cells (SMCs) associated with larger vessels, pericytes envelop the endothelium without forming gap

junctions between one another, creating discontinuities that allow for contact between glial cells and ECs (Chakravarthy and Gardiner, 1999). Pericytes stabilize the vasculature via contact activation of latent transforming growth factor (TGF)-B (Antonelli-Orlidge *et al*., 1989; Sato and Rifkin, 1989; Benjamin *et al*., 1998). The extent of vessel coverage by pericytes has been positively correlated with neovascular protection during development and ROP.



(Sapieha *et al*. 2011)

Figure 1: Schematic depiction of the neural retina and the vascular beds that perfuse it. Retinal vessels (which form preretinal vascular tufts in ROP) are present adjacent to the retinal ganglion cell layer next to the vitreous body. As ROP progresses, there is an initial phase of vascular degeneration (vasoobliteration), followed by a secondary phase of compensatory (but pathologic) angiogenesis toward the vitreous of the retina (preretinal neovascularization). The choroidal vascular plexus, which supplies the outer retina and is affected in age-related macular degeneration, is present behind the photoreceptors at the back of the eye.

Retinopathy of Prematurity (ROP)

History

ROP is a potentially blinding retinal disorder resulting from the exposure of premature infants to postnatal hyperoxia and has been characterized by the occurrence of two notable epidemics over the past 60 years. ROP, referred to earlier on as retrolental fibroplasia (RLF), was first observed clinically in 1942 by Terry in both eyes of a number of children born prematurely by approximately 8 weeks and was characterized by a persistent fibroplastic sheath behind the crystalline lens (Terry 1942). Shortly thereafter, RLF was found to be associated with excessive oxygen use, and consequently, supplemental oxygen delivery to premature infants became very closely monitored (Patz 1952, Crosse 1952). The clinical modifications resulted in a sharp decrease in the incidence of the disease, where the prevalence of blindness due to RLF fell from 50% in 1950 to 4% in 1965 in the United States alone (Hatfield 1972). However, tight restrictions on oxygen use unfortunately led to an increase in morbidity and mortality due to respiratory distress and brain disease were found to accompany the cessation of this first RLF epidemic (McDonald 2001). Interestingly, despite increased control and monitoring of supplemental oxygen in preterm infants from the first wave of deaths resulting from RLF, a second epidemic nonetheless occurred in the late 1970s and 1980s. This time, however, the epidemic resulted from the increased survival of very low birth weight infants (75-999g), some of who were born at even less than 27 weeks of gestation (Phelps 1981). Despite the numerous attempts to regulate oxygen use, the number of infants with ROP has increased further, likely as a result of their increased ability to survive (Hussain 1999, Phelps

1981), which therefore suggests a complex association of both oxygen related and no oxygen related growth factors in generating the disease.

Vascular Development of the Human Retina

The development of the retinal vasculature normally begins within the fourth month of gestation (approximately 16 weeks) (Ashton 1970), starting at the optic disc in the posterior pole and gradually spreading peripherally as a wave of mesenchymal spindle cells towards the ora serrata. Endothelial cell proliferation and capillary formation follow the lead of these mesenchymal cells, and the new capillaries will subsequently form the mature retinal vessels. The nasal retina is completely vascularized by 36 weeks of gestation (Michaelson 1948). The last 12 weeks in a normally occurring pregnancy are therefore crucial for the final stages of the developing retinal vasculature and consequently the retina is almost completely vascularized in a full term born baby.

It has been suggested that retinal vascular growth proceeds from two distinct processes that occur at different times. First, an initial phase of vasculogenesis that is responsible for the formation of new vessels by the transformation of vascular precursor cells from the optic nerve head, which ultimately results in a primitive vascular network. This is then followed shortly after by a secondary phase of angiogenesis, where the superficial vascular plexus is expanded by the sprouting and branching of pre-existing vessels (Flynn 2006). Animal studies have suggested that the expression of vascular endothelial growth factor (VEGF) in microglia precedes the formation of superficial retinal vessels, while that expressed by Muller Cells (MS) located in the Inner Nuclear Layer (INL) closely precedes the formation of the deep layer of retinal vessels (Stone 1995). These microglia come together to interact with various neuronal

(Flynn 2006) as well as vasoformative elements. These include pericytes and smooth muscle cells, which have been implicated in vessel stabilization, limiting endothelial cell proliferation and preventing vessel regression (Hughes *et al*. 2004) in order to form a retinal vasculature that meets the needs of the metabolically active tissue.

Pathogenesis

Two distinct phases of ROP have been described: Initially, the premature infant is born prior to the time at which retinal vascular development is complete, which normally occurs by the end of a full term pregnancy. This results in an incomplete retinal vasculature which would have normally occurred in utero, a loss of some of the vessels that had already fully developed and a peripheral avascular zone which largely depends on gestational age (Chen 2007, Smith 2003, 2004). Regression of existing retinal vessels, also referred to as vaso-attenuation that is the hallmark of this first phase of ROP, is though to result from the combined effects of a relatively hyperoxic extrauterine environment along with the supplemental oxygen that is administered to premature infants. The relatively hypoxic environment in utero, where blood is approximately 70% saturated and where the PaO_2 is 30mm Hg, helps to drive retinal vascular development, while the relatively hyperoxic extrauterine environment, where blood is 100% saturated and the PaO_2 is 60-100 mm Hg, makes the vascularization process rather difficult (Chen 2007, Saugstad 2006). Eventually as the infant continues to mature, the non-vascularized retina becomes increasingly metabolically active, and the tissue hypoxia that ensues resulting from the absence of sufficient vasculature marks the end of this initial phase of ROP (Smith 2004).

The second phase of ROP is characterized by hyperoxia-induced retinal neovascularization (NV), where new vessels form at the interface between the vascularized retina and avascular zone. It was suggested early on by Michaelason (1948) that in the absence of sufficient oxygen levels, the increasingly metabolically active retina might release angiogenic growth factors. Later on, this hypothesis was proven true with two studies, which showed that hypoxia stimulated the production of VEGF, which is currently believed to be responsible for inducing Neovascularization (NV) (Saugstad 2006, Shweiki 1992, Ferrara 1992). Eventually, the disorganized growth pattern, which is characteristic of pathological NV, results in the development of a fibrous scar that extends from the retina throughout the vitreous and lens. Retraction of this scar that can lead to the separation of the retina from the RPE, ultimately leading to blindness (Chen 2007, Saugstad 2006). In addition to the incomplete retinal vascularization and exposure of the immature retinal vessels to an abnormally high oxygen environment, other factors have also been thought of being related to the incidence of ROP, incluing hypercapnia, acidosis, sepsis, blood transfusions, interventricular hemorrhage, mechanical ventilation and respiratory distress syndrome (Dani 2004, Hardy P 2000).

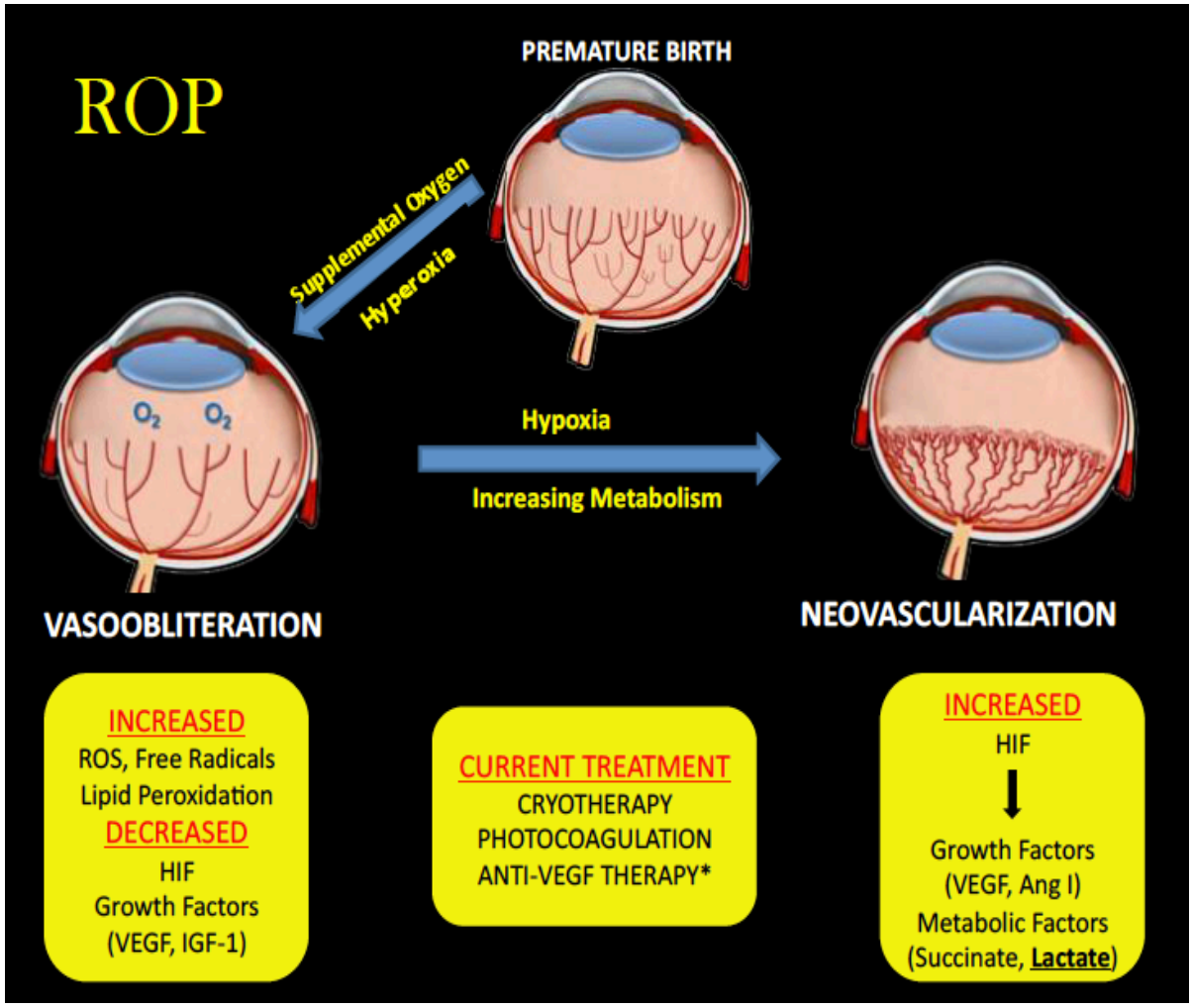


Figure 2: Summary illustration of the current concepts in ROP. When premature birth occurs, the retinal vasculature, which normally develops until birth, is immature. In addition, the premature infant is deficient in several maternally derived factors (such as ω-3 PUFAs and IGF-1, which are transferred during the third trimester) that are essential for healthy blood vessel development, thus further compromising the prognosis. Moreover, the premature patient is mechanically ventilated to overcome pulmonary insufficiencies, and as a consequence, the supplemental oxygen given during mechanical ventilation contributes to retinal vascular obliteration due to oxidant stress and suppression of oxygen-regulated proangiogenic factors such as VEGF and Epo. Following the initial phase of vascular dropout, a second phase of compensatory and destructive neovascularization results and is driven by hypoxia-induced angiogenic factors. Current therapeutic interventions rely on invasive procedures such as laser photocoagulation, whereby affected areas of the retina are cauterized. A number of future treatments, including anti-VEGF therapy and the use of antioxidants, are currently being evaluated.

Predisposing Factors

Birth Weight

Although the pathogenesis of ROP is multifactorial, very low birth weight (>1000 g and <1500g), extremely low birth weight (<1000g) of the baby as well as low gestational age have been consistently linked to the development of the disease. In fact, despite the careful monitoring of oxygen delivery since the link between its administration and ROP became apparent, as many as 30% of very low birth weight infants may progress to develop severe ROP and 8% have a high probability to become blind (Brown 1998). Others have shown that as many as 52% of premature infants that weight between 500-1250 grams at birth tend to develop the disease (Shohat 1983).

Oxygen

The exact role that supplemental oxygen plays in the pathogenesis of ROP has been an important area of research ever since a link between the two was discovered. Whether it is the length of time over which oxygen therapy is administered or the wide fluctuations in oxygen saturation levels that affect the development and progression of ROP, is an area of great debate and controversy. The significance of excessive oxygen delivery stems from the inability of the choroidal vasculature in the newborn to auto regulate in response to increasing oxygen tensions (Hardy 1996, Chemtob 1991), which is due to the combination of increased vasorelaxants, and insufficient vasoconstrictions in neonates (Hardy 1996).

Genetic Factors & Pigmentation

While excessive use of oxygen has been frequently associated with the incidence of ROP resulting in curtailed use in the clinic, it is not the only factor. In fact, many premature infants that are treated with supplemental oxygen never develop ROP, while other children that are nearly full term and receive little or no treatment will eventually develop the disease (Brown, 1998). The factors that determine which infants will progress to retinal detachment remain unknown. Studies have suggested that ethnicity is a significant factor, which influences the epidemiology of ROP (Saunders 1997, Schaffer 1993) where for example, a greater number of Caucasian infants have been shown to reach threshold ROP as compared to African American infants (Tadesse 2002, Saunders 1997). These studies have led to the conclusion that the African-American race offers more protection against the development of severe ROP and that fundus pigmentation may play an instrumental role for the same. Interestingly, however, other studies have suggested that an even greater incidence in the onset of threshold ROP exists in another darkly pigmented race, namely Alaskan natives, when compared to that in other racial groups (Lang 2005, Arnold 1994). Further elucidations of the role of pigmentation and/or genetics on the rate and severity of ROP are warranted, although it would seem that genetic, socioeconomic and possibly dietary factors might be involved (Saunder 1997, Arnold 1994).

Current Treatments for ROP

An appropriate course of treatment is often chosen based on the location and stage of the disease. As mentioned before, stage 1 and 2 of ROP often only require observation of the infant, whereas more advanced cases of ROP, namely stage 3 through stage 5, require invasive surgeries on the eye such as laser treatment or cryo-therapy. The goal of the treatment is to destroy the areas of the retina that are devoid of vessels. This helps to shrink the newly forming abnormal vessels in the eye and prevents the formation of scarring that usually leads to increased traction on the retina, ultimately resulting in visual impairment, retinal distortion or retinal detachment. Laser treatment involves the use of a diode or argon laser that is delivered by transpupillary or transcleral routes, with a laser beam directed by a surface light on specific retinal areas or with a probe on the external scleral surface that is viewed as an indentation of the retina with an indirect ophthalmoscope (Fielder 2001). This type of therapy will "burn away" the periphery of the retina. On the other hand, cryo-therapy involves the use of instruments that generate freezing temperatures in order to touch spots on the surface of the retina that are affected. Both laser treatment and cryo-therapy slow down or reverse the abnormal growth of retinal blood vessels. While long-term effects are often debated and still remain unclear, these are still considered to be the most effective treatments for retinopathy of prematurity.

A promising strategy to counter ROP is the use of the anti-VEGF therapy, Bevacizumab, a VEGF-specific neutralizing antibody is currently used for treatment of several diseases including diabetic retinopathy and macular degeneration. Until recently, bevacizumab was used for ROP in the absence of randomized trials (Micieli *et al*. 2009); timing (stage/zone

of ROP), dose (0.4–12.5 mg intravitreal) and frequency of administration of bevacizumab, as well as cotreatment with photocoagulation, varied tremendously among reports. Nonetheless, there were generally favorable outcomes (controlled progressive neovascularization) in most reports. A prospective randomized double-blind trial of bevacizumab was recently reported and revealed that the recurrence rate of preretinal neovascularization was significantly greater in laser-treated patients than bevacizumab-treated ones exhibiting proliferative retinopathy in the central zone 1 (Mintz-Hittner *et al.* 2011). Moreover, revascularization of the peripheral retina occurred as expected in a normal subject, while conventional laser therapy led to destruction of the peripheral retina. Despite these promising beneficial effects of bevacizumab in the treatment of ROP, additional studies are needed to determine the optimum dosages, timing of administration, frequency and evaluation of possible collateral effects, especially for neurodevelopment.

It is important to note that while ROP is the ophthalmic condition most often associated with preterm birth, a number of other visual manifestations can result from the challenges that prematurity imposes on the developing ocular system (O'Connor 2007). These can include an increased incidence in refractive errors including myopia, astigmatism and anisometropia, as well as an increased incidence in strabismus. Aside from the effects of preterm birth on eye growth and movement, visual functions including visual acuity, contrast sensitivity, color vision and visual fields may also be compromised, thereby significantly implicating psychological and educational development (O'Connor 2007). The advancement of neonatal technologies has resulted in the increased survival of extremely premature infants and consequently the incidence of severe ROP has begun to emerge as a major cause of childhood blindness. While we now have a greater understanding of the postnatal risk factors for the

development of ROP, studies aimed at further elucidating the molecular pathogenesis of ROP are essential in order to identify new therapeutic modalities for neonatal care (Gilbert 2008).

Models of Retinopathy of Prematurity

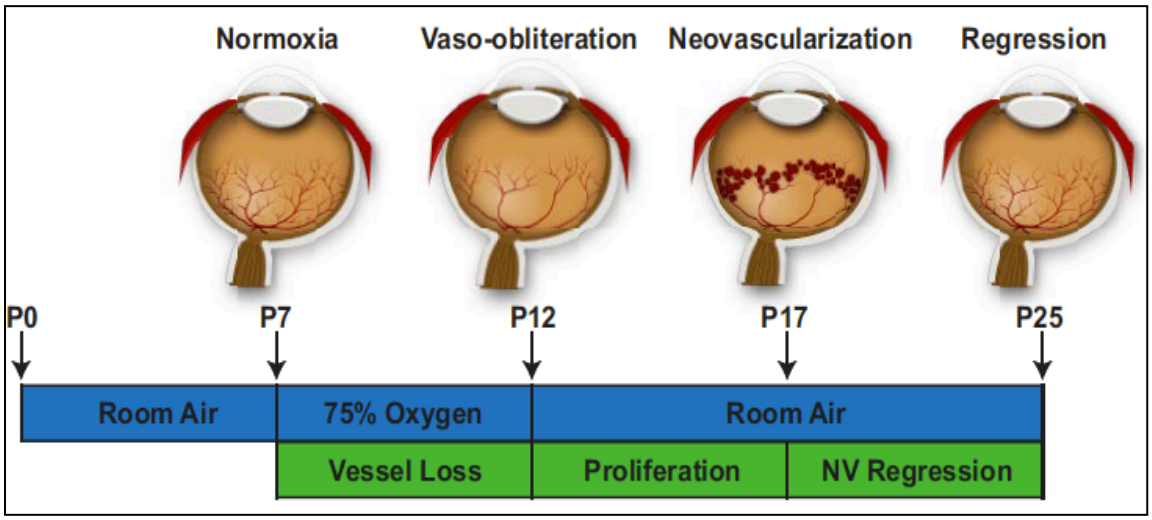
The characteristic features of ROP can be reproduced in several neonatal species, including rats, kittens, mice, puppies and piglets (Tasman 1970, Ricci 1990, Chang-Ling *et al* 1992, Penn *et al* 1993, Smith *et al* 1994, McLeod *et al* 1998). One of the major advantage of using these animal models, apart from the fact that their retinal vasculature forms and responds in a similar manner to that of humans, is that their retinal blood vessels are not fully developed at birth, thus enabling the study of an immature vessel network in an otherwise healthy subject at term.

The most prevalent and one of the best-characterized models of ROP are the highly reproducible rodent OIR (Oxygen Induced Retinopathy) protocols. At birth, the mice and rat have a retinal vasculature comparable to that of a 24 to 26 weeks old human fetus (Gyllensten and Hellstorm 1954, Ricci 1990). The superficial layer is completely formed by postnatal day (P) 11, followed by the deep plexus by P15 (Cairns 1959, Connolly *et al* 1988). The basic premise of this, and other OIR models, is that exposure to hyperoxia leads to vaso-attenuation, -constriction, and –obliteration of the developing retinal blood vessels such that upon return to room air, the inner retina is rendered ischemic as there is no longer sufficient O_2 to compensate for the blood vessels that have been lost. This hypoxia subsequently increases VEGF and neovascularization, as described above, depending on experimental design. Interestingly,

gradual withdrawal from hyperoxia does not reduce the severity of the retinopathy (Gyllensten and Hellstorm 1956, Patz 1957) but recovery in slightly elevated O_2 at a constant level, reduces the extent of aberrant neovascularization (Berkowitz and Zhang, 2000).

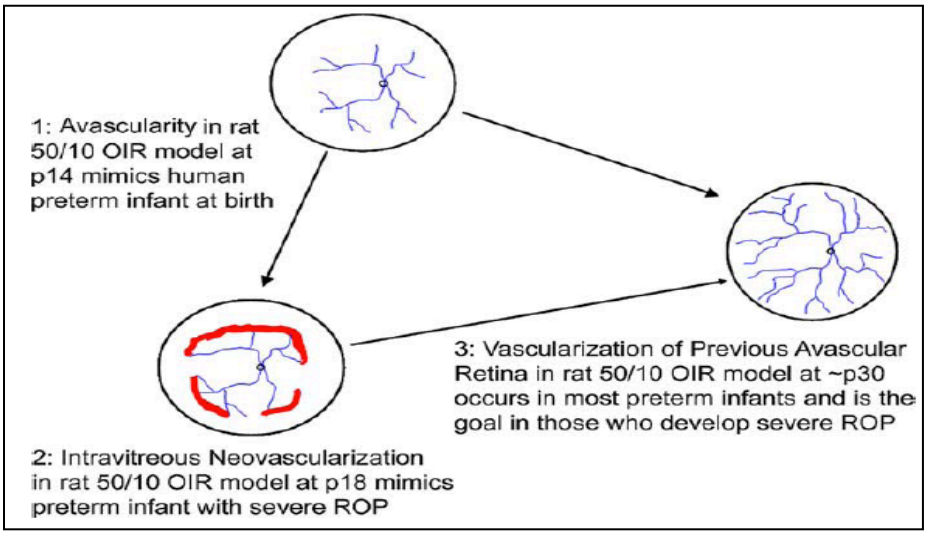
Two key differences exist between mice and rat OIR models. With regards to the rat protocol, raising the level of inspired O_2 does not produce a more severe ROP. Rather, alternating 24-hour exposure period between 50% and 10% O_2, as opposed to 80% and 40% O_2, for the first 14 days of life followed by room air recovery, leads to greater vaso-attenuation, as well as a higher incidence and severity of ROP (Penn *et al*. 1994). Moreover, this protocol also highlights the importance of relative hypoxia in the development of OIR, an element of ROP observed in the clinic (Reynaud 1994, Penn *et al*. 1994). Cunningham and colleagues mimicked clinical ROP intimately by translating PaO_2 fluctuations experiences by a preterm infant who developed severe ROP to the neonatal rat, and in doing so illustrated that clinically relevant levels of O_2 variability are capable of producing ROP (Cunningham *et al*. 2000). In mice, on the other hand, the most extensively used protocol derives from the extreme O_2-induced injury models based on ROP cases prior to restrictions on O_2 supplementation. With other models being proved inconsistent, the refined experimental design of Smith *et al.* (i.e. 75% O_2 from P7, for 5 days, followed by room air recovery until P17) (Smith *et al*. 1994) has become invaluable in studying the mechanisms of neovascularization (Madan and Penn 2003). However, mice subjected to this protocol present with a vascularized periphery and regions of central capillary dropout with neovascularization occurring at the junctions of the vascular and avascular retina, whereas rats typically exhibit peripheral vaso-attenuation and an ensuing neovascularization just posterior to the advancing edge of vessel growth (Madan and Penn 2003).

The similarities in addition to other advantages of the rodent model, including the large litter sizes (approximately 6 pups per dam in C57-BL mice and 15 pups per dam in rats depending on the strain), low costs associated with developing the model and knowledge that has already been acquired from the rodent with respect to various anit-angiogenic, anti-inflammatory and antioxidant therapeutics (Madan 2003) make it a very attractive model to study the pathogenesis of OIR and consequently better understand the sequence of ROP. The above-mentioned models were used for carrying out experiments that will be described in this thesis.



(Stahl et al. IOVS, June 2010, Vol. 51, No. 6)

Figure 3: The mouse model of OIR. Neonatal mice and their nursing mother are kept at room air from birth through postnatal day P7. From P7 to P12, the mice are exposed to 75% oxygen, which induces loss of immature retinal vessels and slows development of the normal retinal vasculature, leading to a central zone of vaso-obliteration (VO). After returning mice to room air at P12, the central avascular retina becomes hypoxic, triggering both normal vessel regrowth and a pathologic formation of extraretinal neovascularization (NV). Maximum severity of NV is reached at P17. Shortly thereafter, NV starts to regress and by P25 almost no VO or NV remains visible.



(M. Elizabeth Hartnett Doc *Ophthalmol* (2010) 120:25–39)

Figure 4: 50/10 OIR model in rats at different post-natal day ages (p) mimics what occurs in human preterm infants. (1) at birth, human preterm infants have incompletely vascularized retina; (2) approximately 6% of preterm infants born\1000 g in birth weight will develop severe ROP, at which time treatment with laser is recommended to reduce unwanted intravitreous neovascularization and consequences of retinal detachment; (3) most preterm infants will undergo vascularization of the avascular retina and 50% of infants with threshold severe ROP will also have regression of the intravitreous neovasculariza- tion with ongoing vascularization of the avascular retina. The goal of our lab is to promote vascularization of avascular retina and to reduce unwanted intravitreous neovascularization

G PROTEIN COUPLED RECEPTORS (GPCRs)

G protein-coupled receptors (GPCRs) are one of the largest and most commonly examined families of receptors (Jalink & Moolenaar 2010, Hill Stephen 2006, Launcer *et al*., 2001; Vender *et al*., 2001). They play a crucial role in an extensive number of physiological and pathophysiological processes. The human genome project has identified more than 800 different GPCR genes and yet the majority of GPCR drugs in current clinical practice (which represent more than 30% of all drugs) exert their actions on only approximately 30 of them (Wise *et al*, 2004). As a result, there are therefore enormous opportunities for further drug discovery in the field of GPCRs.

All GPCRs contain a central core constituted of 7 Transmembrane (TM) domains stretched of about 25 to 35 consecutive residues that show a relatively high degree of hydrophobicity and represent the α-helicies that span the plasma membrane; a change in conformation of this core domain is most likely responsible for the receptor activation. GPCR ligands are extremely diverse and include ions, light organic odorants, neurotransmitters, amines, peptides, proteins, and lipids. Despite years of efforts from both academic and industrial research to pair GPCRs to potential ligands, i.e. to deorphanize these receptors, more than 140 of the non-odorant GPCRs still remain orphan (Civelli, 2005). GPCRs are the targets of most modern drugs worldwide.

Although all GPCRs have similar structures, they demonstrate substantial diversity at the sequence level. On the basis of specific motif, GPCRs have been classified into three distinct families – A, B and C, each containing sub families that often have related ligands (Bjarnadóttir TK, 2006). Family A is by far the largest group, and includes the receptors for

light (rhodopsin) and adrenaline (adrenergic) receptors, and most other 7 TM receptor types, such as the olfactory sub group. Family B is limited to only ~ 25 members, including receptors for the gastrointestinal peptide hormone family (secretin, glucagon, vasoactive intestinal peptide, and growth hormone releasing hormone), corticotrophin releasing hormone, calcitonin and parathyroid hormone. All receptors in family B seem to couple mostly to activation of the effector adenyl cyclase through the G protein G_S. Family C is also relatively small, and is composed of the metabotropic glutamate receptor family, the $GABA_B$ receptor, and the calcium sensing receptor as well as some taste receptors. All members in family C have a very large extracellular amino terminus, which seems to be critical for ligand binding and activation. The development of chimeric G proteins (Conklin et al. 1993) has had a significant impact upon GPCR research and on orphan GPCR research in particular. The identification of specific GPCRs conveying signaling of the metabolic intermediates in retinal neovascularization offers new and potentially important targets to develop therapeutic interventions to optimize recovery. This was recently shown for the succinate receptor, GPR91 (Sapieha, Nat Med, 2008).

GPR81 – A receptor for lactate

GPR81, among many other GPCRs, was considered as an orphan G Protein Coupled Receptor (GPCR) till Liu *et al*. (2009) found L-lactate to be the ligand for this receptor. GPR81 shares high sequence similarity to niacin receptors GPR109a and GPR109b (66% homology and 52% identity at the amino acid sequence level) (Lee *et al*. 2001) and is colocalized in humans on the same chromosome, 12q24.31, as GPR109A and GPR109B (Offermanns *et al.* 2006). GPR109A and GPR109B were recently identified as receptors for niacin, also known as

nicotinic acid (Offermanns *et al*. 2006, Gille *et al*. 2008). GPR81 expression has been found to be highest in adipose tissue among a number of human tissues tested (Wise *et al*. 2003) and is known to couple to the Gi signaling pathway (Ge, H. *et al*., 2008). Lactate activated both human and mouse GPR81 with an EC_{50} of 4.87 ± 0.64 mM when tested in the GTPγS binding assay. Lactate effectively inhibits lipolysis in adipocytes from humans, mice, and rats and adipocytes from GPR81-deficient mice lack responses to lactate, confirming that the antilipolytic effect of lactate is mediated by GPR81. The fact that GPR81 from mice, rats, pigs, cows, dogs, monkeys and humans all respond to L-lactate at the physiological concentration indicates that the function of the GPR81 is conserved among these species, and these results further offer strong support that L-lactate is indeed the endogenous physiological ligand for GPR81.

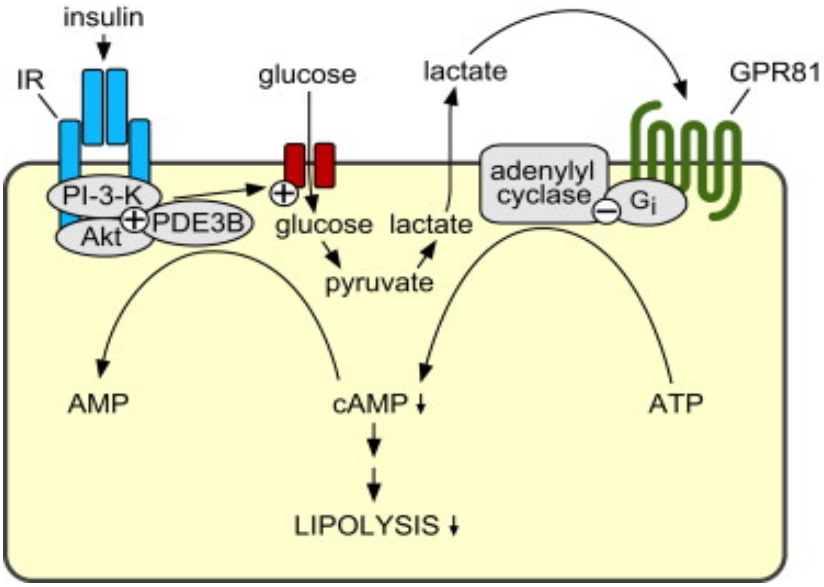


Figure 5: Model of the Mechanisms Underlying Insulin-Induced Inhibition of Adipocyte Lipolysis via PDE3B-Mediated cAMP Degradation and Lactate/GPR81-Dependent Inhibition of cAMP Formation PI-3-K, phosphatidylinositol-3-kinase; PDE3B, phosphodiesterase 3B. (Ahmed K *et al*. 2010)

History of Lactate

For much of the 20th century, lactate was largely considered as A) A dead-end waste product of glycolysis due to hypoxia, B) The primary cause of the O_2 debt following exercise, C) A major cause of muscle fatigue, and D) A key factor in acidosis-induced tissue damage. The period from the 1930s to approximately the early 1970s has been attributed to as the dead-end waste product era. During this period, lactate was largely considered to be a dead-end metabolite of glycolysis resulting from muscle hypoxia (Wasserman 1984). Numerous studies beginning with those of Pasteur (Keilin et al 1966) in the 18th century demonstrated that anoxia and hypoxia stimulate cellular lactic acid (HLa) production. Since the 1970s, a 'lactate revolution' has occurred. Lactate is now known to play various important biochemical processes. Increased lactate production and concentration as a result of anoxia or dysoxia, are often the exception rather than the rule. Furthermore, lactate has been found to be an important intermediate in the process of wound repair and regeneration while lactic acidosis is being re-evaluated as a factor in muscle fatigue (Trabold 2003).

Lactate is produced from glucose through glycolysis and the conversion of pyruvate by lactate dehydrogenase (LDH) (Meyerhof and Kiessling, 1935). It serves as a precursor for hepatic gluconeogenesis and may also be an energy substrate for aerobic oxidation via the citric acid cycle in various peripheral tissues (Brooks 2002; Kreisberg, 1980). It does not increase in concentration until the rate of lactate production exceeds the rate of lactate removal, which is governed by a number of factors, including monocarboxylate transporters (MCT's), concentration and isoform of LDH, and oxidative capacity of tissues (Brooks, 1999a).

The role of lactate in the delivery of oxidative and gluconeogenic substrates is described by the ‘‘lactate shuttle’’ concept (Brooks 2009, 2002, 1999b, 1985). The skeletal muscle is regarded as the major site of lactate production. While it forms and utilizes lactate continuously under resting conditions, lactate formation increases during exercise (Bergman *et al*. 1999; Margaria *et al*. 1933). Subsequently, consistent with the lactate shuttle hypothesis, results from studies on laboratory rodents, dogs and humans have established that lactate is a quantitatively important oxidizable substrate and gluconeogenic precursor. Furthermore, when lactate is released into the systemic circulation and taken up by distal tissues and organs, lactate also affects redox state in the cells, tissues and organs of removal (Brooks 2009). Although controversial only a few years ago, the concept of lactate shuttles within and between cells has been confirmed by others who have observed lactate exchange between diverse cells and tissues including astrocytes and neurons (Pellerin *et al*. 1998; Hashimoto *et al*. 2008). Recognition that there exist both intra- and extracellular effects of lactate production and removal has led to renaming of the original ‘lactate shuttle’ hypothesis (Brooks, 1985) to the ‘cell–cell lactate shuttle’ (Brooks, 1998).

The bulk of the evidence suggests that lactate is an important intermediary in numerous metabolic processes, a particularly mobile fuel for aerobic metabolism, and perhaps a mediator of redox state among various compartments both within, and between cells. Lactate can no longer be considered the usual suspect for metabolic ‘crimes’, but is instead a central player in cellular, regional and whole body metabolism. Overall, the cell-to-cell lactate shuttle has expanded far beyond its initial conception as an explanation for lactate metabolism during muscle contractions and exercise to now subsume all of the other shuttles as a grand description of the role(s) of lactate in numerous metabolic processes and possibly as a signaling molecule.

Recent findings have shown that facilitated transport of lactate across membranes is accomplished by a family of monocarboxylate transport proteins (MCTs), which are differentially expressed in cells and tissues (Halestrap *et al*. 2004, Brooks 2009).

Lactate as a signaling molecule - LACTORMONE

In strong contrast to the classical view terming lactate as the usual suspect of various metabolic crimes and taking into consideration its purported autocrine-, paracrine- and endocrine-like actions, lactate may be an important signaling molecule, hence lactormone. The discovery of lactate as the ligand of previously orphan GPCR, GPR81 suggested that this intermediate has un expected signaling functions beyond traditional roles of a metabolite; a surprising finding given the fact that the glycolytic and kreb cycle have been extensively studied for over sixty years. Lactate has been considered an alternative energy source to glucose (Dienel GA *et al*. 2001) and acutely is a vasorelaxant (Mori k *et al* 1998). Recently Liu *et al*. (2009) showed that lactate inhibits lipolysis in fat cells through activation of an orphan G-protein coupled receptor (GPR81) that acts as a lactate sensor, the response of which inhibition of lipolysis.

Role of GPR81 in Regulation of Retinal Angiogenesis and ROP

Ischemic retinopathies are a major cause of visual impairment in adults and childhood. Microvascular degeneration is commonly observed in ischemic retinopathies such as of diabetes and prematurity. This loss of retinal microvessels precedes a secondary phase wherein exaggerated preretinal neovascularization takes place and predisposes to retinal detachment. Of particular interest in context of this thesis is the GPCR, GPR81. As of now, data on GPR81

remains very limited with a very few research papers, but to our interest it was recently shown that the glycolytic pathway metabolite lactate was the ligand for this previously orphan receptor (Liu *et al*. 2009). The discovery of lactate as the ligand of previously orphan GPCR suggested that this intermediate has unexplored signaling functions beyond traditional roles of glycolytic pathway; a surprising finding given the fact that the TCA has been extensively studied for over sixty years.

Notwithstanding the important role of hypoxia-triggered vascular endothelial growth factor (VEGF) released in this post-ischemic neovascularization in retinopathy of prematurity (Mu D *et al* 2003, Hai J *et al* 2003, Arai Y *et al* 1998), there is abundant evidence that other factors also partake in this repair process (Conway *et al*. 2003, Harrigan *et al*. 2003, Wang *et al*. 2002) but need to be better elucidated. Of interest, factors that have been overlooked despite being significantly affected by hypoxic-ischemia are intermediates of carbohydrate metabolism like succinate, lactate and α-ketoglutarate (α-KG), which tend to accumulate during hypoxia-ischemia (Folbergrova J *et al*. 1986, Hoyer S *et al* 1974). For instance, the Krebs cycle metabolite succinate has recently been shown to exert important role in regulating retinal angiogenesis (Sapieha M 2008); specifically the release of numerous angiogenic factors by a G-protein-coupled receptor, GPR91 that is highly expressed in neurons. These Krebs cycle products have been postulated to link capillary function with tissue metabolic needs (Baysal BE 2006, King A *et al* 2006, Burns P 2003). However, hypoxia rapidly leads to increases in lactic acid prior to any changes in downstream Krebs cycle activity. This is consistent with the observation that the muscle type of lactate dehydrogenase (LDH 5), which is needed for high glycolytic rates, predominates in retina (Graymore, 1970). Interestingly, increases in lactic acid have been associated with angiogenesis in tumors (Brahimi *et al*. 2011, Chiche *et al*. 2009,

Heiden *et al*. 2009) and shown to cause vasorelaxation in retina (Brazitikos *et al*., 1993), suggesting a link between capillary function and tissue metabolic needs; yet the primary targets of lactate remained until this year unknown. Lactate was recently found to exert its biologic effects via an orphan receptor, GPR81 (Cai TQ 2008; Liu C 2009). GPR81 is mostly expressed in adipocytes (LiuC 2009). ***We herein hypothesize a novel concept whereby lactate, which acts on its cognate receptor GPR81, governs retinal angiogenesis; this effect is mediated in part by pro- and anti-angiogenic factors released from retinal ganglion cells;*** by complementing the effects of succinate on retinal ganglion cells, together Krebs cycle and glycolysis pathway mediators (notably succinate) and lactate, respectively, orchestrate angiogenesis. The identification of specific receptors conveying signaling of metabolic pathways in retinal neovascularization offers new and potentially important targets to develop therapeutic interventions to optimize recovery. Whether (and how) lactate via GPR81 regulates in retinal angiogenesis is the prinicipal focus of this thesis

MATERIAL AND METHODS

Animals

We used Sprague-Dawley rats and C57BL/6 mice from Charles River Laboratories in compliance with experimental procedures approved by the Animal Care Committee of the Centre Hospitalier Universitaire Ste-Justine (CIBPAR) and in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC).

Measuring L-Lactate in tissue extracts from normal and OIR mice

Lactate contents of retinas, from normal or oxygen exposed mice, was measured at different time points. Addition of cold 80% ethanol to the tissue (tissue/solvent ratio 1:8) followed by centrifugation at 10,000 g at 4 °C for 30 min allowed the extraction of the metabolites. The supernatants were collected and diluted with dH_2O at different dilutions (1:30 and 1:60). The lactate contents were then assayed using the lactate assay kit (Eton Bioscience, CA).

RGC-5 cells and conditioned media preparation

RGC-5 cells were terminally differentiated by exposing them to 1 mM staurosporine (Sigma) for 12 h, which effectively maintains RGC-5 cells in a post-mitotic, neuronal state. These were then stimulated with 0 (control), 5mM and 10 mM lactate for 12 h. The lactate-containing medium was removed and replaced with fresh DMEM (Invitrogen). The stimulated RGC-5 cells were then used to condition fresh medium for 16 h, at which point the medium was collected and filtered through a 20 um filter. This conditioned media was subsequently distributed to wells in both aortic explants and tube formation assays as described ahead.

Immunohistochemistry

Staining of different cell types and receptors in the retina was evaluated by staining cryosections (12 microns) from rat or mice eyes using epifluorescence microscope (Nikon Eclipse E800) and confocal microscope (Zeiss LMS5). The eyes were fixed in 4% paraformaldehyde for 1.5 hours, incubated with a 30% sucrose solution for 18 hours at 4 ^{o}C, embedded in OCT and sectioned (cryotom). The sections were subsequently washed with PBS and blocked with PBS 1% BSA and 1% Triton. The cryosections were labeled with DAPI (Molecular Probes). Cell localization of GPR81 in tissues was evaluated by co-immunostaining with polyclonal Ab 1:100 (AbNova Biosciences # PAB4669), of RGC with NeuN (1:100, Millipore # MAB377), of Muller Cells with CRAL BP (1:200, Thermo Scientific, Catalogue # ma1813), VEGF (1:500; Santa-Cruz Biotechnology). Other cell-specific markers used were: *lectin griffonia simplicifolia* 1:100 for endothelium, GFAP (Glial Fibrillary Acidic Protein) 1:250 for Astrocytes.

Microvascular sprouting from Aortic Explants

Microvascular Sprouting from Aortic Explants were prepared from young (1 month) C57/BL mice. 1 mm thick aortic rings were placed in 24-well tissue culture plates, covered with 40 μL growth factor – reduced Matrigel (BD Biosciences), and cultured for 4 days in EGM-2 medium (Clonetics). Explants were exposed to 5 and 10 mM lactate (pH controlled to 7.2) or in control DMEM from day 4 to 6 of culture. To establish the role of RGC expressing GPR81 in guiding angiogenesis, RGC were treated with lactate and conditioned media from these cells was applied to aortic explants. We took photomicrographs of individual explants and quantified

microvascular sprouting by measuring the area covered by outgrowth of the aortic ring with ImagePro Plus 4.5 (Media Cybernetics).

Differentiation Assay/ Tube Formation Assay

Differentiation assays essentially measure the formation of capillary-like tubules by ECs cultured on matrices consisting of fibrin, collagen or a basement membrane substance like Matrigel that enhance the attachment, migration and differentiation of ECs into tubules in a manner analogous to the in vitro situation. Rat Brain Endothelial Cells (RBEC) were plated in 24-well plates on growth factor– reduced Matrigel (BD Biosciences) and treated with either 5 mM, 10 mM lactate (pH controlled to 7.2) or incubated in conditioned media from RGC-5 cells as described before for aortic explants. Images were captured under phase contrast microscopy (Nikon Eclipse, TE300) and the number of tubes was quantified in non-overlapping fields with Image Pro Plus 4.5.

Intravitreal injections and developmental growth assessment

The vascular density of retinas was evaluated by making retinal flat mounts. Rats/mice pups at postnatal day 1 (P1) were anesthetized with isoflurane (2.5%), and 5 mM, 10 mM lactate (Sigma Chemicals) (final intravitreal concentration based on estimated eye volume) or control PBS was administered to their right eyes, (n=10/group) using a Hamilton syringe with glass capillaries of approximately 60 gauge (total volume injected ~1 µl). Pups were sacrificed at P4 and dissected retinas were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Retinal vasculature was revealed by incubating retinas with TRITC-labeled lectin (Griffonia Simplicifolia, Sigma Aldrich, Cat: L5264; 1/100) 18 hours at 4^{o}C. Flatmounts were

visualized by fluorescence microscopy (Nikon Eclipse E800). Retinal vascularization was quantified as the retinal surface covered at day 4 relative to total retinal surface using ImagePro Plus 4.5 (Media Cybernetics). Vascular density was determined using macros plug-in (developed by D. Hamel, Chemtob laboratory; article in preparation) in java-based image processing software, ImageJ.

OIR Model

The mice C57BL/6 were placed with an adoptive breast-feeding CD1 mother postnatal day 5 (P5) because they are less likely to cause cannibalism in hyperoxia as compared to the C57BL/6 mothers. These litters were placed in a hyperoxic chamber with 75 ± 2% oxygen from P7 to P12. As mothers are more vulnerable to the toxic effects of oxygen, they were exchanged between normoxic and hyperoxic cages everyday. The litters of mice were returned to normoxia or 21% oxygen five days later, on postnatal day 12 (P12). At postnatal day 17, when neovascularization is at its peak, the mice were sacrificed and enucleated to analyze their retina. The weight of mice was considered to exclude mice under developed eliminating the difference in weight gain as a variable factor (Stahl *et al*. 2010). Ischemic proliferative retinopathy in Sprague-Dawley rats was induced by exposing them to 24-h cycles of hyperoxia (50% O_2) and hypoxia (10% O_2) from post natal day 1 (P1) to P14 in chambers according to an established protocol (Beauchamp, 2001, 2002, 2004; Sennlaub, 2003; Kermorvant-Duchemin, 2005; Sapieha, 2008) and thereafter returned to room air (21% O_2)

Quantitative RT-PCR Analysis of mRNA Expression

mRNA was extracted from cells and tissues with Trizol (Invitrogen) followed by treatment with DNase I (QIAGEN) to remove DNA contaminants. Quantitative analysis of gene expression was done by converting RNA into cDNA by in house protocol using M-MLV reverse transcriptase (Invitrogen). Primers for rat, mice GPR81, VEGF, Ang1 were constructed using the Primer Bank and the software Primer NCBI Blast. Reverse transcription and amplification by polymerase chain reaction in real time (RT-PCR) performed with an ABI Prism 7700 Sequence Detection System and SYBR Green Master Mix kit (BioRad). The values of each sample were normalized with the expression levels of 18S.

Primers were synthesized by Alpha DNA (Montreal, Quebec, Canada); sequences of primers used were the following: GPR81 primer sequences used for amplifications were 5'-GGCTGAGAAAAGCGGTATGA-3' and 5'-TCGTTAACTCTCTCCGAGCTAG-3', 5'-AAAGCCAGCACATAGGAGAG-3' and 5'-AGGATTTAAACCGGGATTTC-3' for VEGF, 5'-AACGCTCTGCAAAGGGATGC-3' and 5'- TGAGTCAGAATGGCAGCGA -3' for Angiopoietin 1 (Ang I).

MTT Assay

In MTT assay, yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. Approximately 10^5 cells/well were plated into a 24 well culture dish (Sarstedt) with 500 μl of culture medium. Cells were starved

overnight. Subsequently cells were treated for 48 hrs with conditioned medium from lactate treated RGC. 50 μl MMT reagent (ATCC) was added per well and incubated for approximately 2 hrs. 200 μl detergent was added and incubated for 5 min. Absorbance was read at 550 nm with reference wavelength at 690 nm in a microtiter plate reader using Stat Fax-2100.

Generation of GPR81 Knock-out Mice

GPR81 deficient mice were generated by Lexicon (San Mateo, CA). The trans membrane domain 2 of mice GPR81 coding region (100 base pairs) is replaced by a 4-kb IRES-lacZ-neo cassette. Our laboratory is maintaining a colony of GPR81 and we are currently performing back cross to obtain a pure KO colony with C57/BL background which can be further used as OIR model for experiments.

Data analysis

Results on vascularity will be analyzed by 2-way ANOVA factoring for O_2 exposure or developmental age and treatment, and by comparison among means. Difference in GPR81 expression between O_2 exposure protocols, and difference for the same parameters and for vascular density or preretinal neovascularization between treatments in animals were analyzed with $\beta=0.2$ and $\alpha=0.05$, ~10 animals were required for each analysis factor, specifically vascularity, immunoblotting and immunochemistry. Statistical analyses. Data are presented as means ± s.e.m. We made comparisons between groups by one-way analysis of variance followed by post-hoc Bonferroni's test for comparison among means. $P < 0.05$ was considered statistically significant.

RESULTS

Lactate levels increase during oxygen-induced retinopathy

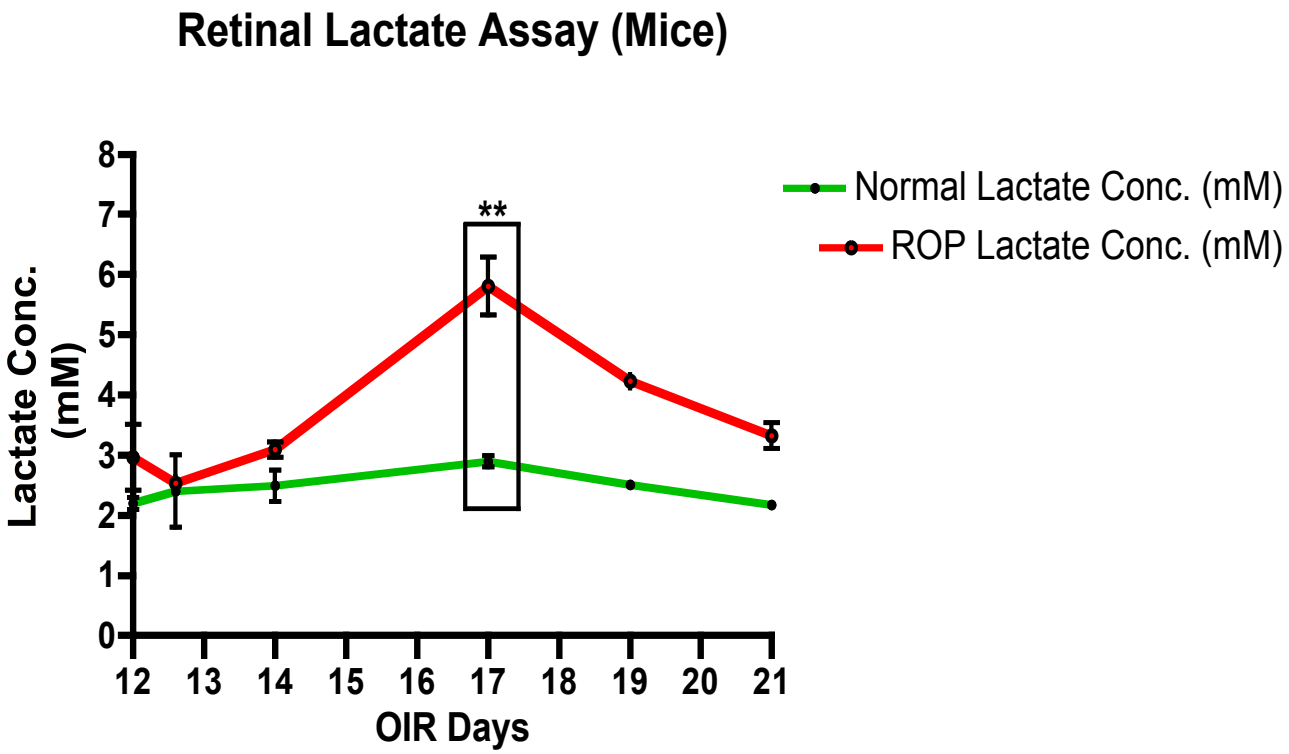


Figure 6: Retinal levels of lactic acid during OIR in mice subjected to oxygen induced retinopathy (OIR) (n=10) and age matched room-air normoxic controls (n=10), *** P< 0.001. The glycolysis pathway intermediate lactate was measured using lactate assay kit (Eton Biosciences, San Diego) in ischemic retinas of mice subjected to oxygen-induced retinopathy (OIR; exposure to 75% oxygen from postnatal day P7 to P12 (vasoobliterative phase) and resuming normoxic environment for 5 days afterwards (P12 TO P17) (ischemic vasoproliferative phase).

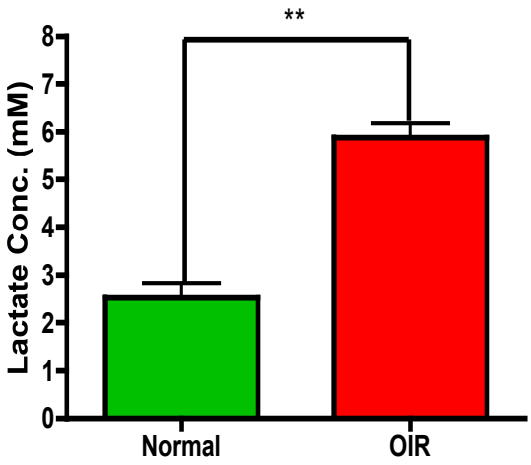


Figure 7: Lactate levels rise in the ischemic retina - Retinal levels of lactate at P17 in mice subjected to OIR (n = 10) and age-matched room-air normoxic controls (Control; n = 10). Values are shown relative to controls. **P < 0.001 compared to Control. Lactate abundance was 2.3 (app.) fold higher in ischemic retinas compared to normoxic controls (5.8 ± 1.68 mM versus 2.65 ± 0.56 mM in control mice).

As evident from Figure 6 & 7, lactate levels increase more than 2 fold in ischemic retina during the neovascularization (or ischemic vasoproliferative) phase of OIR. This data is consistent with previous reports of increased lactate concentrations in ischemic tissue. It confirms our hypothesis that levels of lactate increase many fold during the peak of neovascularization phase (P17) in OIR model as compared to age matched normoxic controls. This is consistent with the observation that the muscle type of lactate dehydrogenase (LDH 5), which is needed for high glycolytic rates, predominates in retina (Graymore, 1970).

Lactate increases viability of RBEC

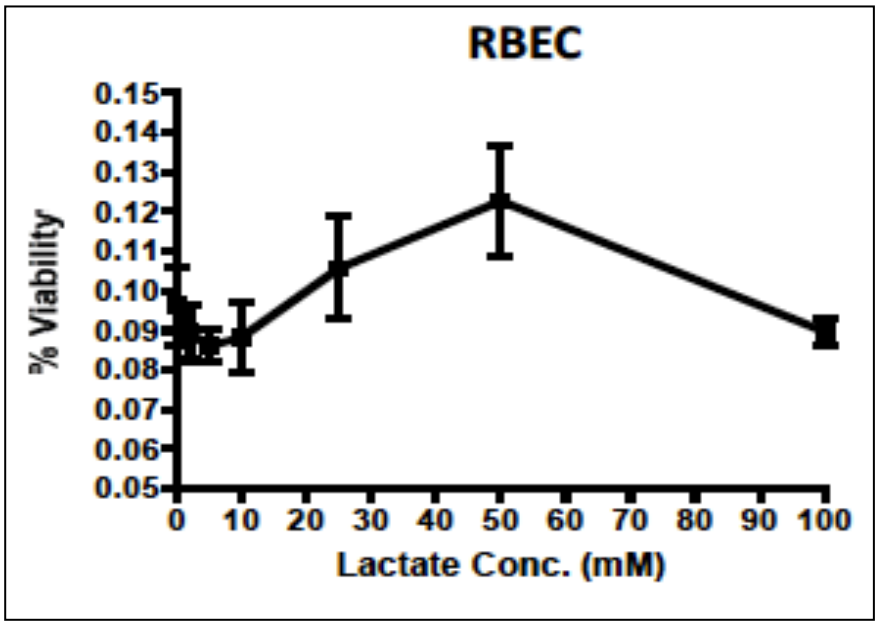
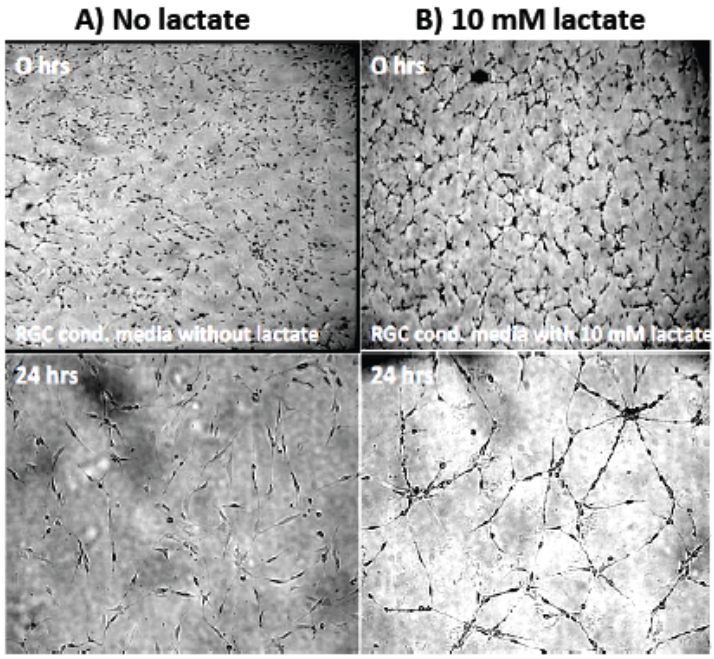


Figure 8: Viability of Rat Brain Endothelial Cells (RBEC) on treatment with different concentrations (0, 2, 5, 10, 25, 50, 100 mM) of lactate. The above graphs are representative of 3 different experiments repeated in triplicates per condition.

As shown above, the viability of endothelial cells increased on treatment with different concentrations of lactate (pH controlled to 7.2) up to 50 mM. This is consistent with previous findings, which have reported high concentrations of lactate (up to 25 mM) in conditions of hypoxia or stress (Ohkuwa et al. 2000, Kamijo, Y et al. 1987, Hughson, R. L 1984). Concentration as high as 100 mM lactate was tested to find out the level at which lactate becomes toxic to cells.

Conditioned media from RGC stimulated with lactate induces tube formation: Lactate is Pro angiogenic



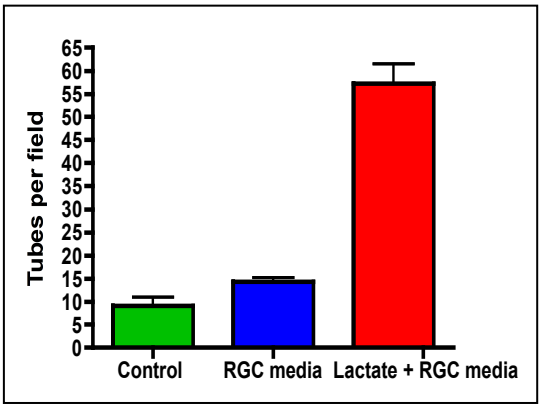
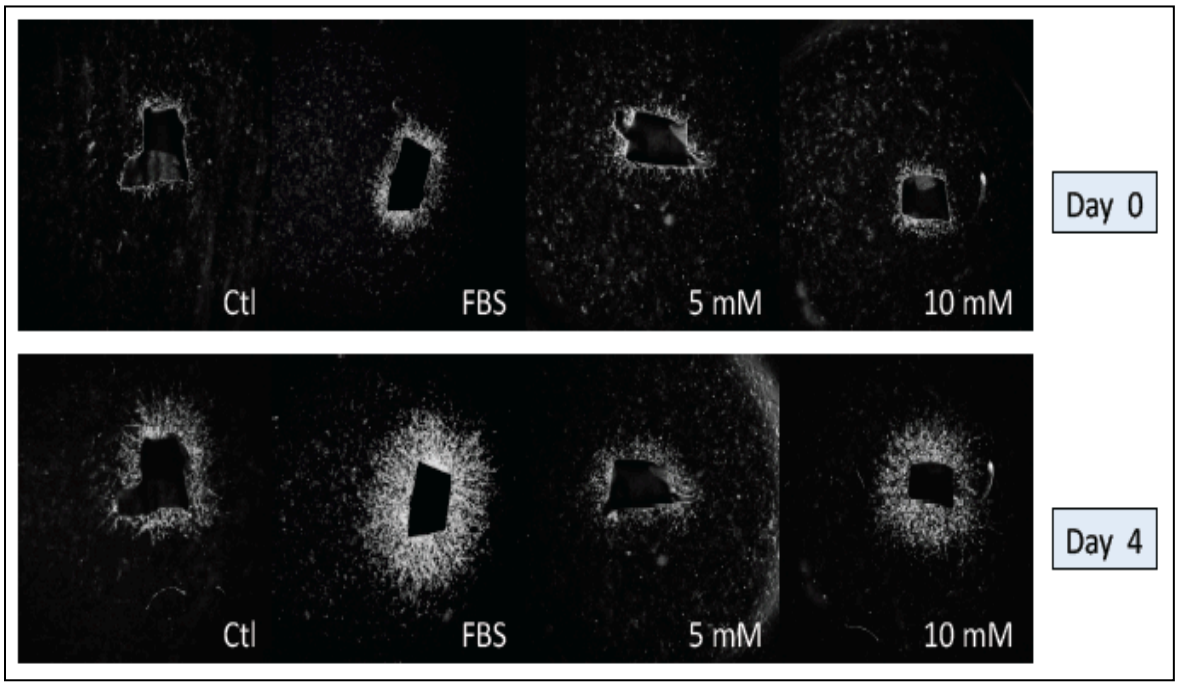


Figure 9: Representative micrographs showing RBEC tube formation (measured at 24 h after plating) mediated by control media (green), RGC-conditioned medium without lactate (blue) representing A wherein RBEC were treated from RGC media without lactate and medium conditioned by RGCs treated with 10 mM lactate (red) representing B. It indicates initial steps of cell alignment in control medium or medium conditioned by RGCs with and without lactate treatment. Scale bar, 100 mm. Quantification of the results is shown in the bar graph (n = 2 experiments) represented by control media (green), RGC-conditioned medium without lactate (blue) and medium conditioned by RGCs treated with 10 mM lactate (red). **P < 0.005 compared to control values.

The ability of lactate to vascular growth was tested in a three dimensional in vitro endothelial cell capillary tube formation with rat brain endothelial cells in Matri gel assay, also called as the tube formation assay. The above micrographs show the ability of conditioned media obtained from RGC treated with lactate to substantially promote the formation of tube like network after 24 hours of treamtment. This explains ours hypothesis that lactate receptor, GPR81 is believed to produce angiogenic mediators on stimulation with lactate. It is these mediators that are believed to produce the angiogenic effects on lactate treatment. The expression of these mediators was further investigated (Figure 17).

Lactate induces vascular sprouting in aortic explants



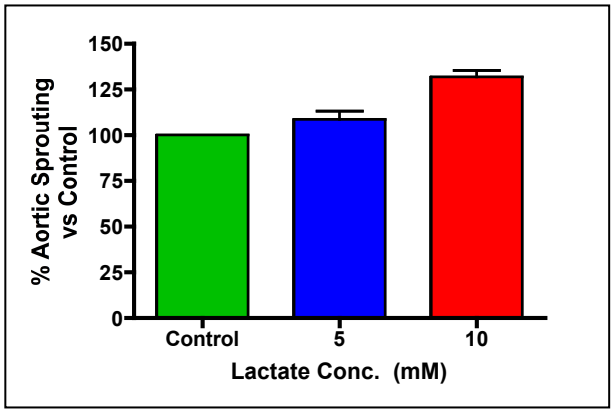


Figure 10: Representative microvascular sprouting from Matrigel embedded adult mice aortic explants treated with 5, 10 mM lactate or culture medium with or without FBS (Ctl). Scale bar, 1mm. The corresponding histogram values of results in 4b represent 4 separate experiments. *P < 0.05 for 10 mM lactate treatment compared to control (cult. med.) values

Conditioned media from RGC stimulated with lactate induces vascular sprouting in aortic explants

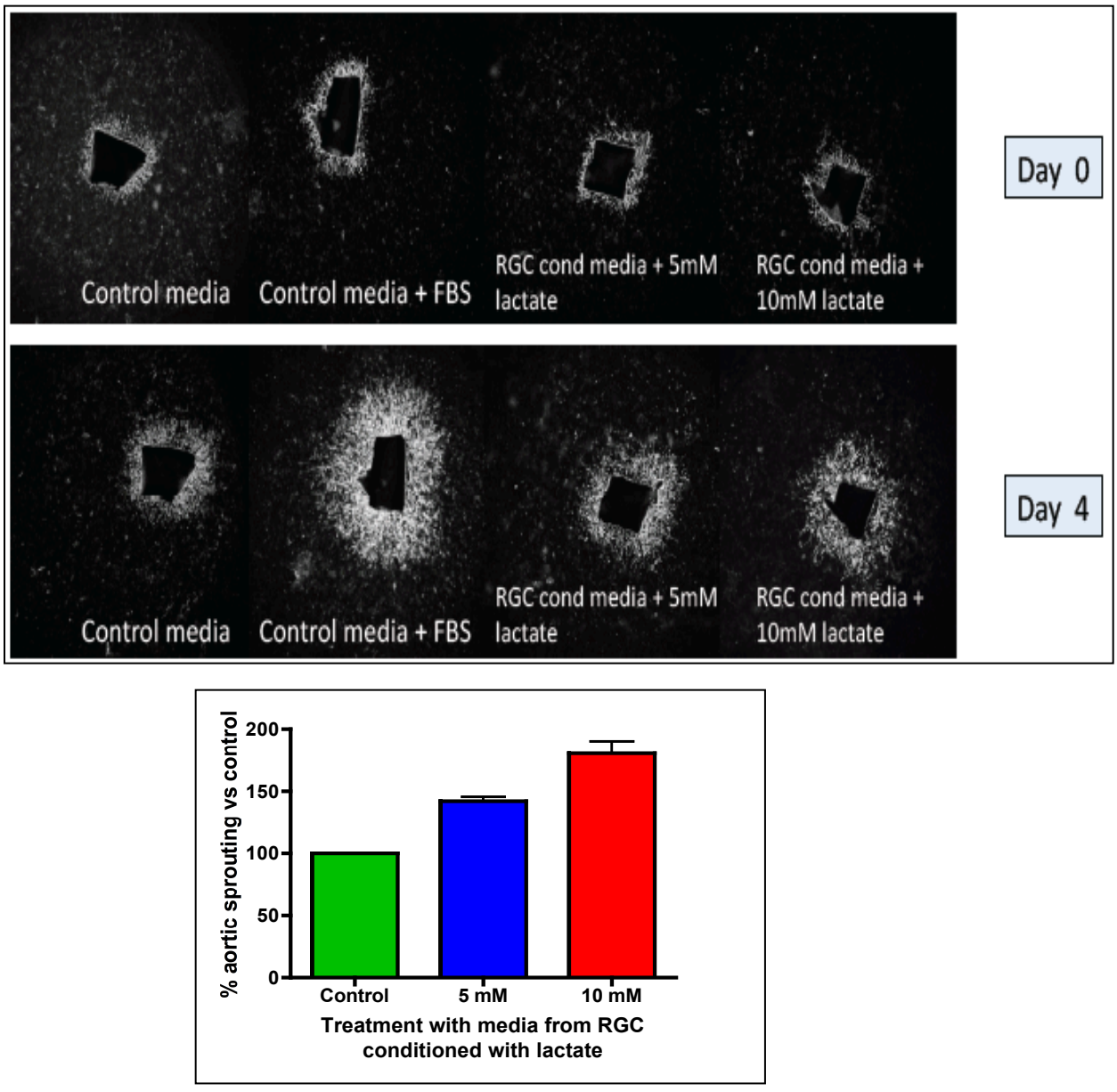


Figure 11: Representative microvascular sprouting from Matrigel-embedded aortic explants treated with lactate or culture medium conditioned by RGCs alone or RGCs treated with lactate (5, 10 mM). Scale bar = 1mm. (c) The corresponding histogram values of results in 5b represent 3 separate experiments. *P < 0.05 and ***P < 0.01 compared to control (RGC cult. media without lactate) values.

We postulated that GPR81 on RGCs could mediate vessel growth during an ischemic event and, hence, in response to lactate. We stimulated cultured RGCs (terminally differentiated RGC-5 cells) with lactate (5, 10 mM) and used these cells to condition fresh medium. The conditioned culture medium (containing factors secreted by lactate-stimulated RGCs) was collected, filtered and used to assay vascular sprouting from aortic ring explants. Medium conditioned by untreated RGCs induced low levels of sprouting when compared to non-conditioned control medium. Direct treatment of aortic rings with lactate resulted in minimal sprouting. However, conditioned medium obtained from RGCs treated with lactate for 6 h produced a two-fold increase, indicative of release of proangiogenic factors from lactate-treated RGCs.

GPR81 is expressed in Retina

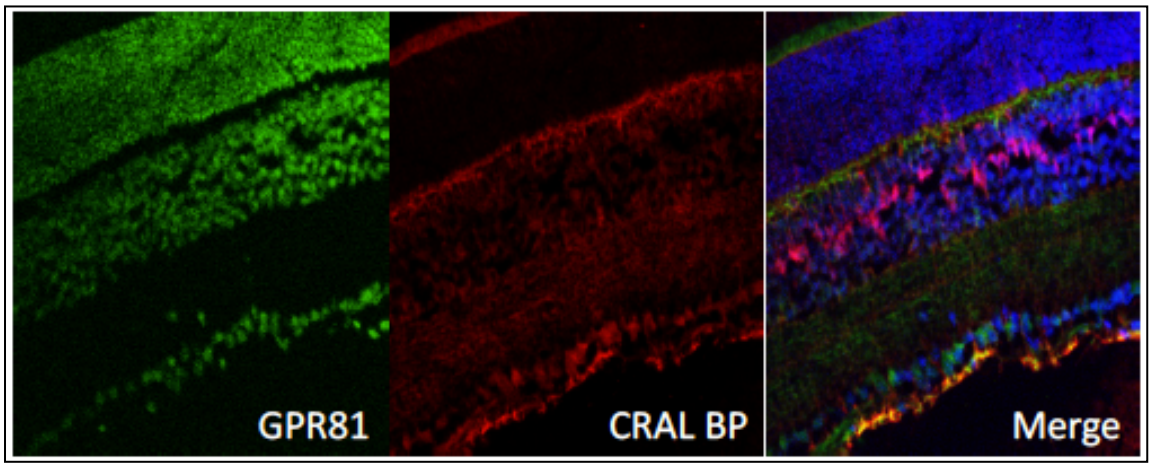


Figure 12: Immunohistochemical localization of GPR81 in retinal cryosections showing pronounced expression in the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). GPR81 expression in the retina was evaluated by staining cryosections (12 microns thick) of rat eyes using epifluorescence microscope (Nikon Eclipse E800) and confocal microscope (Zeiss LMS5). Cell localization of GPR81 in tissues was evaluated by co-immunostaining with cell-specific markers – GPR81, CRAL BP for Muller Cells, NeuN for RGC. Images are representative of two experiments. Immunohistochemistry showed that GPR81 was strongly expressed and predominantly localized in ganglion cell layer and Muller Cells. Hence the concerted effect of lactate on the RGC to instigate the release of angiogenic factors (discussed later) is regulated by GPR81.

GPR81 expression increases in OIR

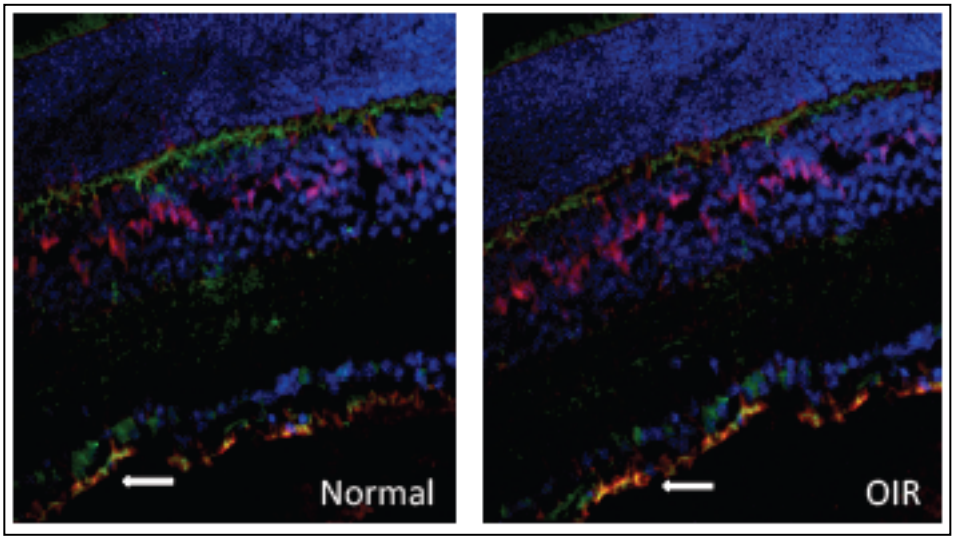


Figure 13: Immunohistochemical localization of GPR81 in retinal cryosections. GPR81 expression in the retina was evaluated by staining cryosections (12 microns thick) of retina from age-matched normal and OIR rat eyes using epifluorescence microscope (Nikon Eclipse E800) and confocal microscope (Zeiss LMS5). Cell localization of GPR81 in tissues was evaluated by co-immunostaining with cell-specific markers – GPR81, CRAL BP for Muller Cells, NeuN for RGC. The expression of GPR81 seems to be up regulated in the above immunostaining. This is further confirmed by our qPCR analysis of mRNA expression of GPR81 in RGC stimulated with different concentrations of lactate (discussed ahead). The above results suggest that GPR81 may be up regulated in conditions of ischemia and OIR followed after an increase in concentration of its ligand, lactate.

GPR81 expression increases in Endothelial Cells

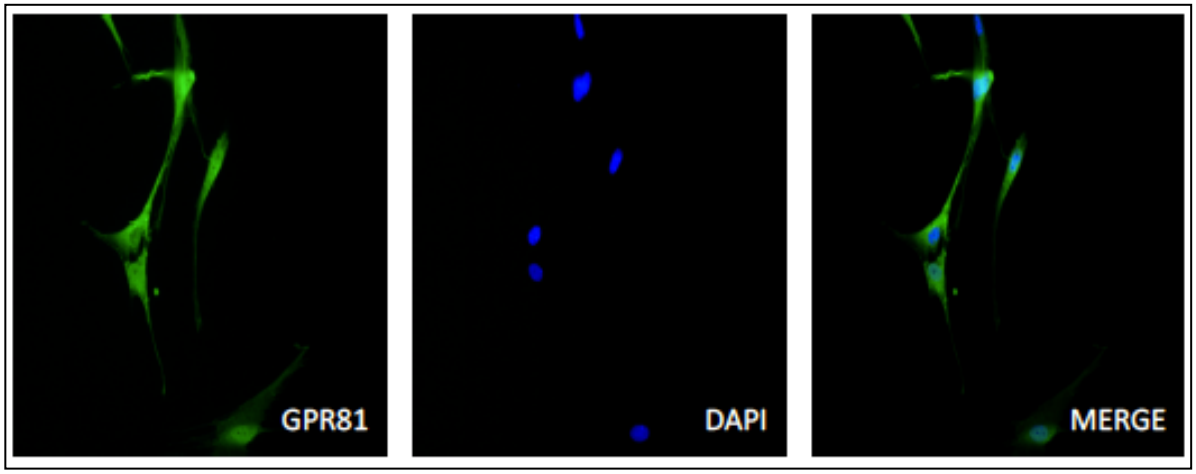


Figure 14: Immunohistochemical localization of GPR81 in endothelial cells (RBEC). Immunohistochemical localization of GPR81 expression in the endothelial cells was evaluated by staining them using epifluorescence microscope (Nikon Eclipse E800) and confocal microscope (Zeiss LMS5). Cell localization of GPR81 was evaluated by co-immunostaining with cell-specific markers – GPR81 (1:100; polyclonal AbNova Catalogue # PAB4669). This shows that GPR81 is expressed in endothelial cells and these cells can be stimulated with lactate. Western blot analysis showed some expression of GPR81 in endothelial cells (shown below in **figure 15**).

GPR81 expression in retinal cell lines

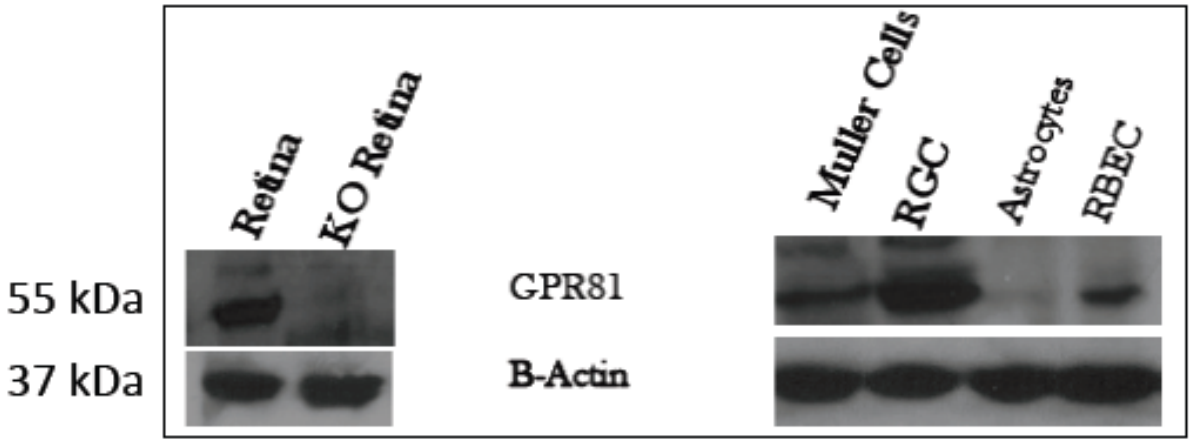
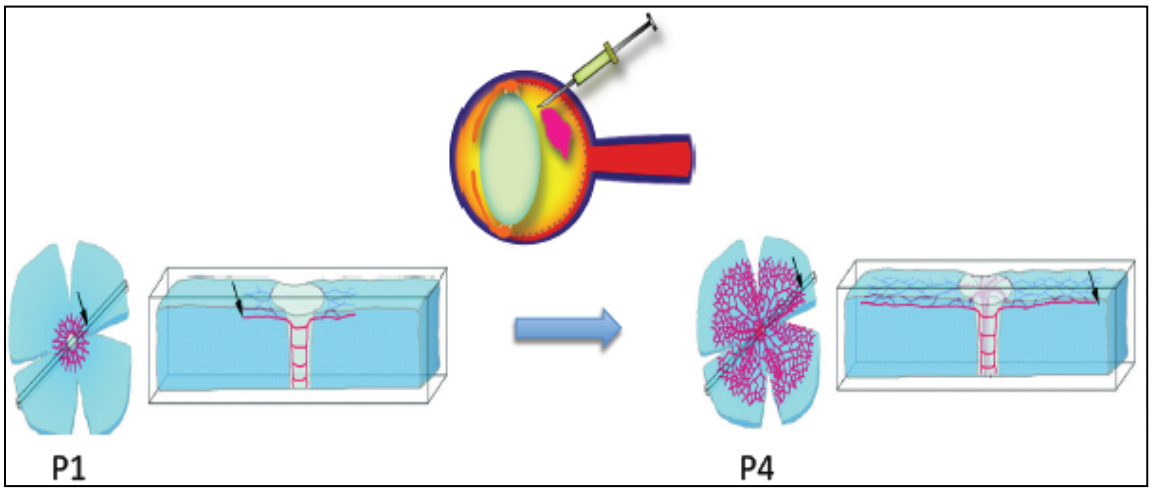
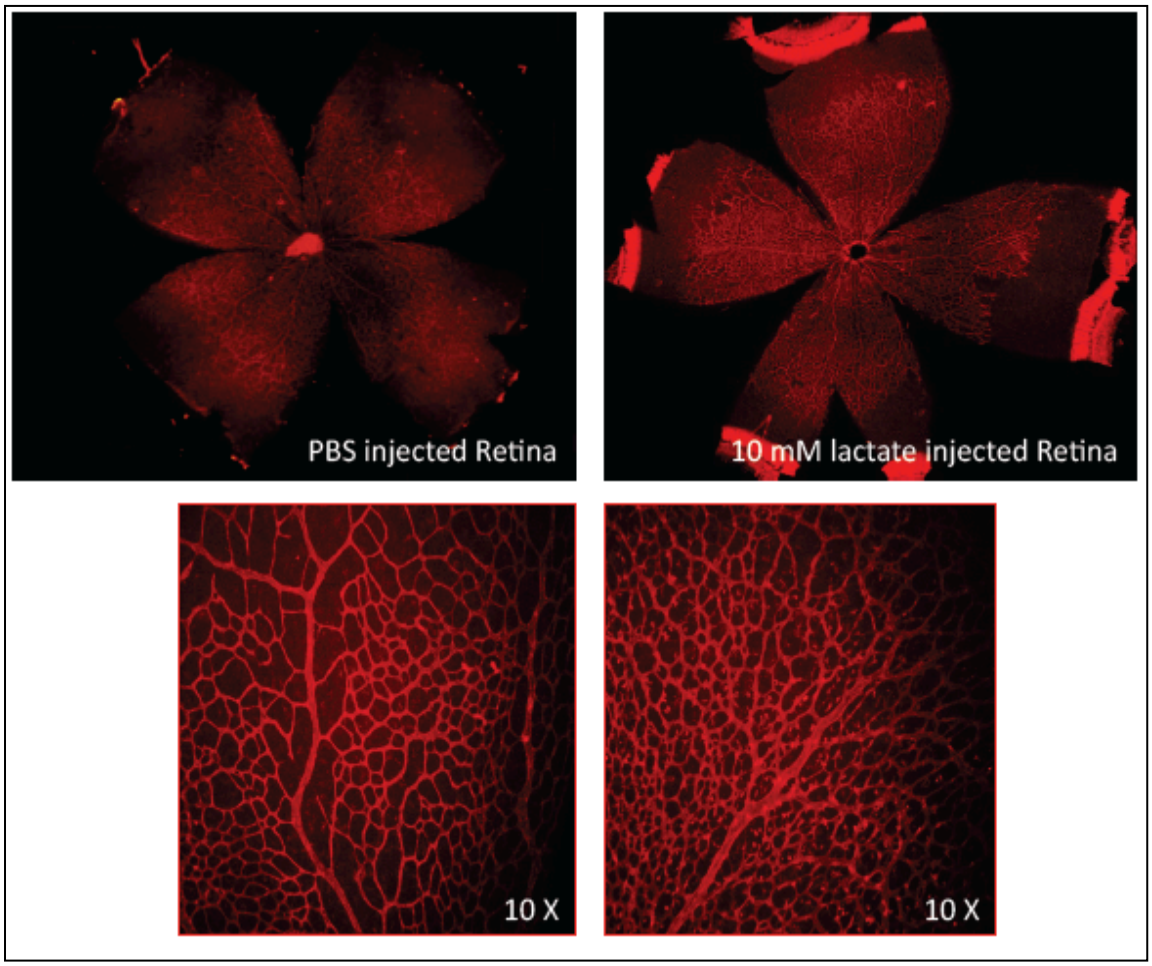


Figure 15: GPR81 protein expression was analyzed by Western Blotting using GPR81 antibody (1:75) (AbNova, Catalog # PAB4669) on cells like Muller Cells, RGC, Astrocytes and RBEC. Specificity of GPR81 antibody was confirmed by testing on normal and GPR81 KO retina. β-Actin was used as internal control.

From figure 15 it is evident that GPR81 is highly expressed in RGCs while it is also expressed in Muller Cells and to some extent in endothelial cells. GPR81 is not expressed in astrocytes at all. This supports our findings mentioned above in immunostaining experiments (Figure 13, 14) where GPR81 was expressed in RGC, Muller Cells and RBEC. The specificity of GPR81 antibody used in this research project was confirmed by testing on retina from normal and GPR81 KO mice. GPR81 expression in endothelial cells is comparatively less as compared to that in Muller Cells and RGC. This may explain the significant result obtained in microvascular sprouting of aortic explants on stimulation with 10 mM lactate but not 5 mM (figure 10).

Lactate has an angiogenic effect *in vivo*





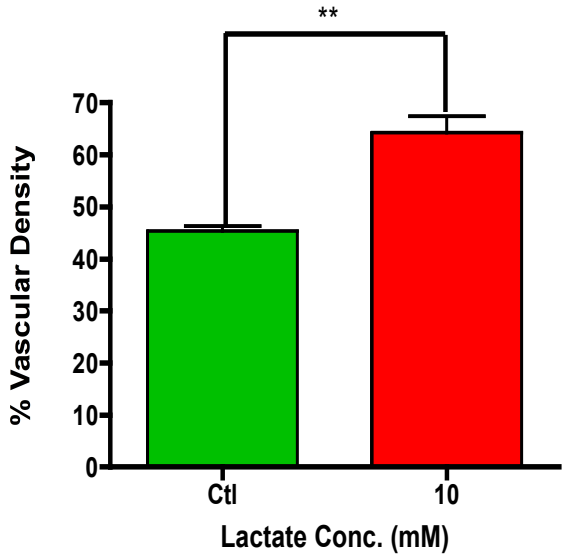


Figure 16: Model of intra vitreal injections for lactate. Rats/mice pups at postnatal day 1 (P1) were anesthetized with isoflurane (2.5%), and injected with 10 mM lactate (Sigma Chemicals) (10 mM final intraocular) or control PBS was administered to their right eyes, (n=10/group) using a Hamilton syringe with glass capillaries of approximately 60 gauge (total volume injected ~1 µl). Pups were sacrificed at P4. Retinal vasculature was revealed by incubating retinas with TRITC-labeled lectin (*Griffonia Simplicifolia*, Sigma Aldrich, Cat: L5264; 1/100) for 18 hours at 4°C. Flatmounts were visualized by fluorescence microscopy (Nikon Eclipse E800). Retinal vascularization was quantified as the retinal surface covered at day 4 relative to total retinal surface using ImagePro Plus 4.5 (Media Cybernetics). Vascular density was determined using macros plug-in (developed by D. Hamel, Chemtob laboratory; article in preparation) in java-based image processing software, ImageJ. Images are representative of 9–12 separate rats per treatment group. Scale bar, 1 mm. Quantification of the vascular areas from experiments in bar graph represents changes in developmental retinal vascularization represents changes in lactate-induced retinal vascularization. ***$P < 0.01$ compared to corresponding control values. It shows that lactate induces increased retinal angiogenesis *in vivo*. This result confirms the above shown results *in vitro* where in lactate was able to stimulate production of angiogenic mediators that induce retinal angiogenesis.

Lactate treatment induces an increase in mRNA expression of GPR81 and angiogenic factors like VEGF and Ang I

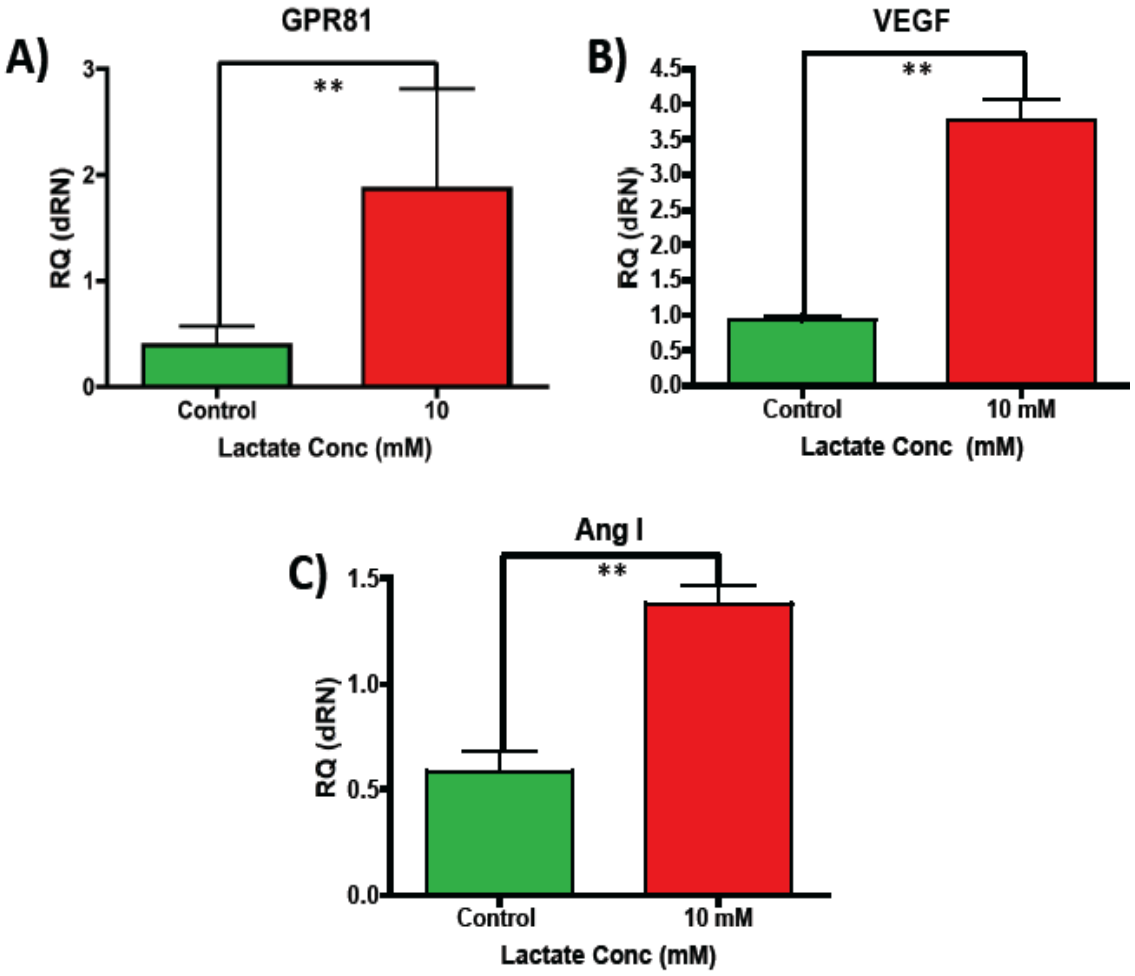


Figure 17: Analysis by qRT-PCR was performed on RGC treated with lactate (5 and 10 mM). This demonstrated an increased expression of certain angiogenic factors like VEGF and Angiopoietin I. The values were normalized with the expression of 18S and are expressed as relative amount of control.

The above result confirms that GPR81 expression is increased on stimulation with lactate. These results confirm the increase in GPR81 expression as seen in immunohistochemical studies on retinal cryosections, as shown in figure 13. The mRNA expression of angiogenic

mediators like VEGF and Ang I was also analyzed on lactate stimulation of RGC. Expression of VEGF and angiopoietin I increased on stimulation with lactate, as shown in figure 17. This result is consistent with our hypothesis that activation of GPR81 is believed to produce angiogenic mediators like VEGF and Angiopoietin. It is these mediators that are believed to produce the angiogenic effects on lactate treatment as shown above in vitro and in vivo experiments.

Lactate has no angiogenic effect *in vivo* in GPR81 KO

(Pilot experiment)

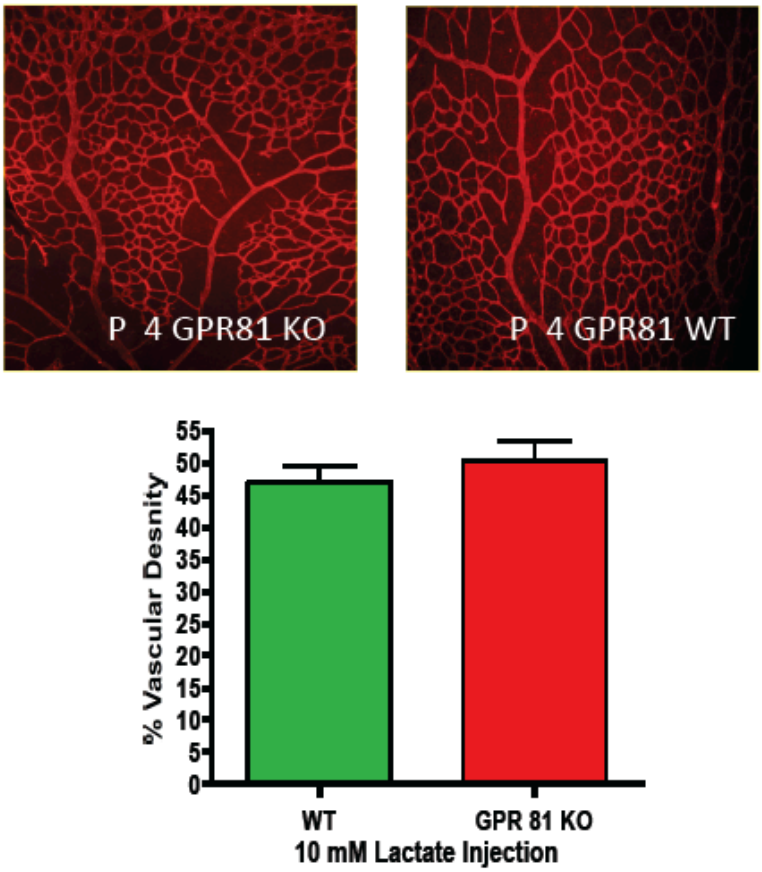


Figure 18: GPR81 KO mice (mixed background) at postnatal day 1 (P1) were anesthetized with isoflurane (2.5%), and injected with 10 mM lactate (Sigma Chemicals) (10 mM final intraocular) or control PBS was administered to their right eyes, (n=10/group) using a Hamilton syringe with glass capillaries of approximately 60 gauge (total volume injected ~1 µl). Pups were sacrificed at P4. Retinal vasculature was revealed by incubating retinas with TRITC-labeled lectin (*Griffonia Simplicifolia*, Sigma Aldrich, Cat: L5264; 1/100) for 18 hours at 4°C. Flatmounts were visualized by fluorescence microscopy (Nikon Eclipse E800). Retinal vascularization was quantified as the retinal surface covered at day 4 relative to total retinal surface using ImagePro Plus 4.5 (Media Cybernetics). Vascular density was determined using macros plug-in (developed by D. Hamel, Chemtob laboratory; article in preparation) in java-based image processing software, ImageJ. Images are representative of 1 pilot experiment with n=3 mice per treatment group. Scale bar, 1 mm. Quantification of the vascular areas from experiments in bar graph represents changes in developmental retinal vascularization represents changes in lactate-induced retinal vascularization.

SUMMARY AND CONCLUSIONS

For a long time lactate has been considered as the usual suspect for all the metabolic crimes in the body. The discovery of GPR81 as a receptor for lactate has opened new avenues to explore its role as a potential therapeutic target to alleviate pathological conditions like ROP. It has raised the possibility of physiological properties of lactate beyond its traditional role as a glycolytic pathway metabolite. Our results have been consistent with previous studies showing the potential of lactate in inducing angiogenesis.

Our results successfully show that lactate concentration increases as much as three times (6 – 7 mM) in the OIR retina as compared to age related normal mice. Conditioned media collected from RGC stimulated with lactate has been shown to induce tube formation. Furthermore, GPR81 is expressed in normal and OIR retina, especially in RGC, Muller Cells and Endothelial Cells. *Ex vivo* treatment of aortic explants with conditioned media from RGC treated with lactate was able to induce microvascular sprouting. This further confirms that RGC on stimulation with lactate release pro-angiogenic mediators like VEGF and Ang I. Intra-vitreal injections of 10 mM lactate in P1 rats and mice show a significant increase in retinal vascular density, further elucidating the possible role of GPR81 in stimulating retinal angiogenesis.

Presently, therapeutic options for ROP are limited primarily to antagonists of VEGF. GPR81, as a regulator of various angiogenic factors including VEGF and angiopoietins, provides an alternative target to govern revascularization after ischemia. More broadly, our findings uncover lactate GPR81 signaling as a new paradigm for the ability of metabolites to reinstate tissue energy balance by acting as a signaling molecule and not just a dead end metabolic by product.

DISCUSSION

In this study, we put forward two new concepts pertaining to the involvement of lactate in retinal angiogenesis. First, we show that lactate is a biologically active molecule that, via its cognate receptor GPR81, bridges the gap between a hypoxic retina and the ensuing vascularization processes. Secondly, we show that retinal ganglion neurons are the principal respondents to lactate and consequently identify these cells as key sensors of hypoxic stress, capable of reinstating vascular supply to an ischemic retina by governing the release of a number of major angiogenic factors. Hypoxia is a well-established modulator of retinal vascularization (Gariano *et al*. 2005). Although hypoxia-triggered events have classically been considered to depend on pathways involving HIF and VEGF (Gariano *et al*. 2005, Arjama 2006), it is noteworthy that hypoxia can itself provoke a constellation of metabolic changes. We therefore wished to explore whether these perturbations in metabolite levels aid in reinstating an energetic equilibrium to hypoxic tissue.

Although the angiogenic effects of certain metabolites have already been shown *in vitro* and *in vivo* (Folbergrova, *et al*. 1974, Hoyer *et al*. 1986, Lee *et al*. 2001, Neuman *et al*. 1958), their role in eliciting neovascularization after ischemia has generally been overshadowed by the focus on VEGF as the central regulator of hypoxia-induced vessel growth (Shweiki *et al*. 1992). Lactate abundance increases considerably in hypoxic tissue, as shown in our results in OIR model. Moreover, as the pro-angiogenic effect of lactate signaling through GPR81 is seen in mice, we suggest that GPR81 may be an attractive target for treating diseases such as diabetic retinopathy that is characterized by an aberrant neovascularization in the mature retina. Retinovascular changes have not been reported in the GPR81 knockout mice; however, these

mice (from Lexicon Pharmaceuticals, San Diego, USA) will be available in our laboratory for future experiments. Taken together all the results described above regarding the involvement of GPR81 in retinal angiogenesis, we suggested that RGCs might directly sculpt their vascular environment—a mechanism that under normal conditions would assure adequate local control of oxygen and nutrient supply. This inference is consistent with early studies that suggested that the pattern of vascularization of the inner retina is dictated by the distribution and density of neural cells within the ganglion cell layer, where capillary density is greater in areas of high cell numbers (Francois *et al*. 1974, Johnson *et al*. 1968, Michaelson *et al*. 1954), a probable consequence of the elevated metabolic needs of these cells. In line with a direct role for RGCs in influencing their vascular environment via GPR81, lactate concentrations increased in the medium of cultured RGCs exposed to a hypoxic stress (consistent with changes in retinal lactate abundance detected *in vivo* during hypoxia-ischemia), suggesting an autocrine activation of GPR81. Our data show that the interplay between lactate and GPR81 could be a major part of the mechanism by which neurons sense hypoxic stress and the ensuing energy deficit and thus signal for vessel growth.

Pilot studies have shown the expression of GPR81 in glia, particularly Muller Cells. Evidence exists to show that Muller Cells from mammalian retina release lactate for uptake and metabolism by retinal photoreceptor cells (Poitry-Yamate *et al*., 1995). Many previous studies have demonstrated specific transport of lactate into neurons and glia (Dringen *et al*., 1993; Nedergaard and Goldman, 1993; Tildon *et al*., 1993), where glia appears to be responsible for the release of lactate into the extracellular space under hypoxic or hypoglycemic conditions (Walz and Mukerji, 1988). This two-way communication between Muller Cells and retinal neurons indicates that Muller Cells may also play an active role in retinal function

(Newman and Reichenbach. 1998). The conditioned media from these cells will be further used to investigate the possibility of involvement of these cells in releasing angiogenic (or anti angiogeneic) factors on stimulation with lactate. This can further elucidate the possibility of an interplay between lactate and more than one cells in retina, in release of angiogenic factors that trigger revascularization. In turn, this can provide more therapeutic targets for alleviation of pathological conditions.

The above results confirm how lactate has an angiogenic role, both *in vitro* and *in vivo*. But to answer the question if this angiogenic response on stimulation with lactate was actually mediated via GPR81, pilot data from our ongoing experiments on GPR81 KO mice reveals that the intra vitreal lactate injections in KO mice fail to increase the vascular density (n=3, analysis in progress) as compared to wild type animals. This pilot data confirms our hypothesis that the angiogenic effects of lactate are in turn mediated by stimulation of GPR81. Additionally, importance of GPR81 in normal retinal vasculature development is being currently being investigated by collecting retina at different time points of retinal vasculature development (between day 1 and day 15) and further analyzing the difference of vascular density as compared to age matched wild type mice. Pilot results (n=3) so far have shown reduced vascular density in retina of GPR81 KO mice as compared to wild type. Additional experiments we will perform in future will confirm the above-mentioned results.

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