Signalling Through the PGC-1α Pathway Mediates an Inducible Stress Response in Retinal Astrocytes to Resist Oxidative and Metabolic Insults

by

Qi Jiang

A thesis submitted in conformity with the requirements for the degree of Master of Science
Laboratory Medicine and Pathobiology
University of Toronto

© Copyright by Qi Jiang 2015
Abstract

Glaucoma is a neurodegenerative disease characterized by the loss of retinal ganglion cells (RGCs) in the eye. Astrocytes are an adjacent cell type that provides metabolic support and antioxidant defense for the RGCs and the loss of these neurosupportive functions contributes to the pathogenesis of glaucoma. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a transcriptional co-activator that regulates the expression of metabolic and antioxidant genes through interactions with transcription factors. PGC-1α deficiency has recently been implicated in neurodegeneration, but its role in mediating astrocyte functions is not well understood. In this study, PGC-1α activity and its expression were induced in astrocytes following metabolic and oxidative stress. Under these conditions, pharmacological stimulation of PGC-1α improved antioxidant capacity, mitochondria biogenesis, and increase astrocyte viability through regulations of downstream gene expression. Thus, PGC-1α is a key mediator of astrocyte stress responses, which can be targeted to promote their neurosupportive functions.
Acknowledgments

I would like to express my sincere gratitude to Dr. Jeremy Sivak for being a caring advisor who dedicated a significant amount of his time in helping me throughout my project. I am thankful for his mentorship and expertise that continued to motivate and guide me towards my research goals. I would like to give special thanks to my committee chair Dr. Isabelle Aubert and committee member Dr. John Flanagan. I truly appreciate all your advice, support, and encouragement that allowed me to expand my learning and build confidence.

I would also like to acknowledge members in the Sivak and Flanagan labs for their help around the bench and constructive feedbacks during our meetings. Many thanks to Cindy, Darren, and Adrian for all the teaching and training without which I would not be able to complete my work efficiently. Thank you to Rachel, Nevena, Samih, Izzy, Tom, Ken, and Lee-Anne for being generous and supportive lab mates. It has been a wonderful experience working with all of you.

Finally, I would like to thank my family and friends. Thank you all for being my listeners. Your encouragement over the past two years has been invaluable.
## Table of Contents

Acknowledgments .................................................................................................................. iii  
List of Tables ........................................................................................................................... vii  
List of Abbreviations ............................................................................................................... viii  
List of Figures .......................................................................................................................... x  
List of Appendices ................................................................................................................... xi  

**Chapter 1 Introduction** ............................................................................................................. 1  
1 Introduction .............................................................................................................................. 2  
   1.1 Hypothesis ......................................................................................................................... 4  
   1.2 Specific Aims ...................................................................................................................... 4  

**Chapter 2 Literature Review** ................................................................................................... 5  
2 Literature Review .................................................................................................................... 6  
   2.1 Overview of Glaucoma ....................................................................................................... 6  
      2.1.1 Risk Factors for Glaucoma Development ................................................................... 7  
      2.1.2 Current Treatments for Glaucoma .......................................................................... 9  
   2.2 Mechanisms of RGC Degeneration .................................................................................. 11  
      2.2.1 RGC Degeneration Due to Metabolic Stress .............................................................. 11  
      2.2.2 Role of Oxidative Stress in RGC Degeneration ....................................................... 13  
   2.3 Astrocyte in Neuronal Support ......................................................................................... 14  
      2.3.1 Supportive Roles of Astrocytes for RGCs ................................................................. 17  
      2.3.2 Astrocyte in Glaucoma Pathogenesis and Progression ........................................... 17  
   2.4 PGC-1α: a Master Transcriptional Co-Regulator .............................................................. 19  
      2.4.1 PGC-1α in Metabolism ............................................................................................. 20  
      2.4.2 PGC-1α in Antioxidant Defense ............................................................................... 21  
      2.4.3 Regulation of PGC-1α ............................................................................................. 22  
      2.4.4 PGC-1α in Neurodegeneration ............................................................................... 25  
      2.4.5 Investigating PGC-1α in Retinal Degeneration ......................................................... 26  

**Chapter 3 Methods** .................................................................................................................. 29  
3 Methods ................................................................................................................................... 30  
   3.1 Cell Cultures ..................................................................................................................... 30  
      3.1.1 Primary Rat Retinal Astrocyte Culture ................................................................... 30
3.1.2 A7 Astroglial Cell Culture ................................................................. 30
3.2 Induction of Oxidative and Metabolic Stresses .................................... 31
3.3 Gene Expression Analysis Through qRT-PCR ..................................... 32
3.4 Western Blot ...................................................................................... 33
  3.4.1 Whole-Cell Lysate Collection and Nuclear Extraction .................... 33
  3.4.2 Protein Quantification .................................................................. 34
  3.4.3 SDS-PAGE and Protein Detection ............................................... 35
3.5 Pharmacological Treatments ............................................................... 35
3.6 PGC-1α siRNA Knockdown ............................................................... 36
3.7 Cell Viability Assay ........................................................................... 36
3.8 Measuring Intracellular ROS Level ................................................... 37
3.9 Determining Intracellular Level of GSH ............................................ 37
3.10 Determining Mitochondrial Content ............................................... 38
3.11 Statistical Analysis .......................................................................... 38

Chapter 4 Results .................................................................................. 39
4 Results ............................................................................................... 40
  4.1 Aim 1: Response of PGC-1α Pathway to Oxidative and Metabolic Stress in Astrocytes ........................................................................................................ 40
    4.1.1 Elevated Pathway Activity in Astrocytes Following Oxidative Stress ................................................................. 40
    4.1.2 Elevated Pathway Activity in Astrocytes Following Metabolic Stress ................................................................. 41
  4.2 Aim 2: PGC-1α Pathway Activity Regulates Astrocytic Resistance to Oxidative Stress ................................................................................................................. 44
    4.2.1 Pharmacological Stimulation of PGC-1α Induces Antioxidant Expression ............................................................................................................. 44
    4.2.2 Identification of GCLM as a PGC-1α Target in Astrocytes ......... 46
    4.2.3 Pharmacological Stimulation of PGC-1α Alter Cellular GSH Level ................................................................. 48
    4.2.4 PGC-1α Pathway Activation Influences Intracellular ROS Levels ................................................................. 49
    4.2.5 Stimulation of PGC-1α Pathway Improves Cell Viability Under Oxidative Stress ................................................................. 51
  4.3 Aim 3: The PGC-1α Pathway Activity Regulates Astrocytic Resistance to Metabolic Stress ................................................................................................................. 54
4.3.1 Pharmacological Stimulation of PGC-1α Regulators Influences Metabolic Gene Expression ................................................................. 54
4.3.2 Pharmacological Stimulation of PGC-1α Results in Increased Mitochondria Biogenesis ........................................................................ 56
4.3.3 Pharmacological Stimulation of PGC-1α Regulators Improved Cell Viability Under Metabolic Stress ........................................... 57

Chapter 5 Discussions .................................................................................................................. 59
5 Discussions ................................................................................................................................. 60
  5.1 The PGC-1α Pathway is Induced in Response to Oxidative and Metabolic Stress in Astrocytes ...................................................................................................................... 62
  5.2 PGC-1α Regulates Astrocytic Resistance to Oxidative Stress ........................................... 64
    5.2.1 PGC-1α Regulates Production of Key Antioxidants in Retinal Astrocytes ..................... 64
    5.2.2 PGC-1α is Crucial for ROS Buffering under Oxidative Insult ................................. 67
    5.2.3 Targeting PGC-1α Pathway Improves Astrocyte Viability under Oxidative Stress ........................................................................... 68
  5.3 PGC-1α Pathway Activity Regulates Astrocytic Resistance to Metabolic Stress ................. 69
    5.3.1 PGC-1α Signalling Induces Key Metabolic Genes and Mitochondria Biogenesis in Retinal Astrocytes ......................................................... 70
    5.3.2 Increased PGC-1α Signalling Improves Cell Viability under Metabolic Stress ......................... 72
  5.4 Conclusions .......................................................................................................................... 73
6 References .................................................................................................................................. 75
7 Appendices .................................................................................................................................. 92
List of Tables

1. List of primers used for quantitative PCR.
2. List of antibodies and dilutions for incubation.
List of Abbreviations

AICAR – 5-aminoimidazole-4-carboxamide ribonucleotide
AMPK – 5'-adenosine monophosphate-activated protein kinase
BDNF – brain-derived neurotrophic factor
CNTF – ciliary neurotrophic factor
CNS – central nervous system
CRE – cyclic AMP response element
ECM – extracellular matrix
GCL – glutamate cysteine ligase
GCLC – glutamate cysteine ligase catalytic subunit
GCLM – glutamate cysteine ligase modifier subunit
GDNF – glial cell line-derived neurotrophic factor
GFAP – glial fibrillary acidic protein
GLUT – glucose transporter
GSD – glucose and serum deprivation
GSH – glutathione
HIF-1α – hypoxia-inducible factor -1 alpha
IOP – intraocular pressure
LC – lamina cribrosa
MEF – myocytes enhancer factors
NAD - nicotinamide adenine dinucleotide
NR – nuclear receptors
NRF – nuclear respiratory factor
ONH – optic nerve head
PACG – primary angle-closure glaucoma
PGC-1α – peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PGC-1β – peroxisome proliferator-activated receptor gamma coactivator-1 beta
POAG – primary open angle glaucoma
PPARγ – peroxisome proliferated-activated receptor gamma
PQ – paraquat
PRC - PGC-1α related coactivator
RGCs – retinal ganglion cells
ROS – reactive oxygen species
SIRT1 – sirtuin 1
SOD – superoxide dismutase
TBP – TATA-binding protein
Tfam – mitochondria transcription factor A
TFs – transcription factors
TNF-α – tumor necrosis factor alpha
VEGF – vascular endothelial growth factor
List of Figures

1. Posterior structures of the eye susceptible to glaucomatous damage.
2. Neurosupportive functions of astrocyte.
3. The PGC-1α pathway and its downstream functions.
4. Retinal expression of PGC-1α target genes following excitotoxic damage induction.
5. Increased reactivity of retinal astrocytes in PGC-1α knockout mice.
7. Elevated PGC-1α pathway activity in astrocytes following metabolic stress.
8. PGC-1α-mediated regulation of antioxidant SOD enzymes.
9. PGC-1α-mediated regulation of GCL subunits in vitro and in vivo.
10. Pharmacological stimulation of PGC-1α regulators and PQ treatment elevated intracellular GSH levels.
11. PGC-1α-dependent buffering of intracellular ROS.
12. PGC-1α pathway stimulation improves astrocytic viability under oxidative stress.
13. PGC-1α-dependent upregulation of metabolic genes by pharmacological activation of PGC-1α regulators.
14. Pharmacological activation of PGC-1α regulators induced increased mitochondrial biogenesis.
15. Pharmacological activation of PGC-1α regulators affects astrocytic viability under glucose and serum deprivation (GSD).
16. Summary of the PGC-1α pathway and its role in retinal astrocytes as characterized in the present investigation.
List of Appendices

1. Confirmation of PGC-1α knockdown through mRNA and protein analysis.
2. Paraquat treatment dose-response cell viability curve.
3. Glucose and serum deprivation time-course viability determination.
Chapter 1
Introduction
1 Introduction

Glaucoma is the leading cause of irreversible blindness worldwide (Quigley, 2011). It is considered a neurodegenerative disease characterized by the progressive deterioration of retinal ganglion cells (RGCs) in the eye whose axons form the optic nerve that travels to and reaches targets in the brain. At the cellular level, metabolic and oxidative stresses are two major pathogenic insults associated with glaucoma development (Chrysostomou et al., 2010). With increasing age, vascular dysregulation and mitochondria dysfunction occur in the retina to compromise an efficient delivery of energy substrates, and disrupt the generation of ATP in RGCs (Delaney et al., 2006; Osborne et al., 2014). Since these cells are highly energetically demanding in their electrical transmission, they are particularly susceptible to metabolic insufficiencies (Chrysostomou et al., 2010; Yu et al., 2013). Likewise, oxidative stress is a prominent feature of age-related diseases including glaucoma. The accumulation of reactive oxygen species (ROS) in the RGCs is directly cytotoxic, leading to their apoptotic cell death (Tezel, 2006). Therapeutic strategies that can alleviate metabolic and oxidative injury may therefore prevent or slow RGC loss. Other than the deleterious mechanisms that occur intrinsically in RGCs, alterations in their surroundings, particularly glial cells, significantly contribute to their survival and loss. As such, studies characterizing the role of glial cells can reveal potential targets for increased neuroprotection (Tezel et al., 2009; Inman et al., 2011).

Astrocytes are a major glial cell type that is central to neuronal support in situations of metabolic needs and oxidative stress. Within the eye, they are present in the inner retina and surround the optic nerve where they maintain a homeostatic environment for RGCs (Büssow, 1980). Astrocytes are situated at the interface between neurons and vasculature where they can obtain, process, and release energy substrates for uptake and utilization by neurons (Pellerin et al., 2007; Fernandez-Fernandez et al., 2012). They are also equipped with a high antioxidant capacity and are able to provide neurons with precursors for their own defence (Sagara et al., 1993; Dringer et al.,
Accumulating evidence suggests that astrocytic dysfunction contributes to the pathogenesis of glaucoma (Morgan, 2000; Hernandez et al., 2008; Johnson and Morrison, 2009; Inman et al., 2011). Prolonged exposure to cellular stressors, such as oxidative insult, transform astrocytes from a quiescent to a reactive phenotype distinctly marked by increased staining for glial fibrillary acidic protein (GFAP). In gaining reactivity, astrocytes show altered behaviours that exacerbate RGC deterioration. They exhibit compromised metabolic capacity reflected by reduced ATP levels and a reduced expression of energy substrate transporters (Hernandez et al., 2002; Inman et al., 2011). In glaucomatous optic nerve, astrocytes also have a decreased antioxidant capacity to resist against oxidative stress (Malone and Hernandez, 2007). Given these deficiencies, molecular mediators need to be identified that can be targeted to maintain, restore, or augment astrocyte functions and improve RGC survival.

Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a transcriptional co-activator that has gained increasing attention in the field neurodegeneration. It is a master regulator of metabolism highly expressed in energy-demanding tissues such skeletal muscles and the brain (Esterbauer et al., 1999). More recently, PGC-1α is also recognized as a broad regulator of ROS-detoxifying enzymes (St-Pierre et al., 2006). In response to an increased metabolic demand and ROS accumulation, PGC-1α interacts with various transcription factors to elevate the expression of genes that lead to improved energy provision and antioxidant protection. Among the key targets of PGC-1α are genes involved in mitochondrial biogenesis (NRF-1 and NRF-2), angiogenesis (VEGF), and ROS detoxification (SOD-1 and SOD-2) (Liu and Lin, 2011).

Reduced levels of PGC-1α have been associated with several neurodegenerative disorders and its upregulation leads to improved neuronal survival (Cui et al., 2006; St-Pierre et al., 2006; Qin et al., 2009; Liang et al., 2011). In the retina, high expression levels of PGC-1α is also evident in healthy states (Egger et al., 2012). Recently, PGC-1α has been implicated in the modulation of retinal angiogenesis (Saint-Geniez et al., 2013) and its knockdown was shown to increase apoptotic death of
photoreceptors in response to light-mediated damage (Egger et al., 2012). Our laboratory has also examined the retina of PGC-1α knockout mice and found compromised RGC homeostasis and their increased susceptibility to excitotoxic damage (Guo et al., 2014). In a genetic mouse model, DBA/2J, that is often used to study chronic glaucoma, the level of retinal PGC-1α showed a decline with age as RGC deteriorated (Guo et al., 2014). Importantly, in both PGC-1α knockout and DBA/2J mice, retinal astrocytes showed early and prominent reactivity demonstrated by increased GFAP staining.

Whether PGC-1α plays a role in astrocyte functions is largely uninvestigated. Considering that both astrocytes and PGC-1α function in energy provision and antioxidant defense, PGC-1α may be a key contributor to astrocyte neuroprotective activities with the potential to be targeted to improve RGC survival.

1.1 Hypothesis

In view of PGC-1α function and the role of astrocytes, I hypothesize that PGC-1α mediates astrocyte response to oxidative and metabolic stress in the retina, and can be targeted to increase resistance to such insults in the context of neurodegeneration.

1.2 Specific Aims

1. Characterize the activity of the PGC-1α pathway in response to oxidative and metabolic stresses in retinal astrocytes.

2. Determine how function of this pathway affects the resistance of astrocytes in the context of oxidative stress.

3. Determine how function of this pathway affects the resistance of astrocytes in the context of metabolic stress.
Chapter 2
Literature Review
2 Literature Review

2.1 Overview of Glaucoma

Glaucoma is the second most common cause of blindness after cataracts (Quigley, 1996). Currently, more than 60 million people worldwide are affected by the disease with a projected figure reaching 80 million by 2020 (Quigley and Broman, 2006; Quigley, 2011). Since vision loss from glaucoma development cannot be recovered, it has become the leading cause of irreversible blindness with more than 10% of those afflicted suffering from bilateral blindness (Pan and Varma, 2011; Quigley, 2011).

Vision loss in glaucoma is of a neurodegenerative nature characterized by the progressive deterioration of retinal ganglion cell neurons (RGCs). The RGCs are responsible for relaying electrochemical message from the eye to the primary visual processing centers in the brain. Their axons collect in the innermost nerve fiber layer of the retina and form the optic nerve that exits the eye (Figure 1). Where all RGC axons converge at the back of the eye is a specialized structural called the lamina cribrosa (LC), at which the sclera of the eye becomes perforated to allow the passage of the axons (Weinreb et al., 2014). Adjacent to the lamina cribrosa, the area formed by the bundled axons are termed the optic disc or optic nerve head (ONH), representing the beginning of the optic nerve. In addition to the RGC axons, astrocytes are a major neurosupportive glial cell type that surrounding the axonal bundles in the region of LC and ONH (Büssow, 1980). In the wall of the pressurized eye, the LC constitutes a weak point that renders the ONH particularly susceptible to insults from ocular fluid dynamics (Fechtner and Weinreb, 1994). As glaucoma develops and progresses, subtle structural changes accumulate in the ONH and nerve fiber layer, and up to 40% of the nerve fibers may be lost before changes in the visual field becomes detectable (Pan and Varma, 2011). As such, glaucoma remains asymptomatic until
late stages, and it is speculated that up to 50% of cases are undiagnosed (Quigley, 2011; Varma et al., 2011). Similar to other neurodegenerative diseases, glaucoma thus represents an epidemiological challenge as the number of cases rise for an aging population.

Figure 1. Posterior structures of the eye susceptible to glaucomatous damage. Retinal ganglion cell (RGC) axons collect in the innermost retinal layer, bundle together at the optic disc or optic nerve head (ONH), and exist the eye as the optic nerve through the lamina cribrosa (LC) (Weinreb et al., 2014).

2.1.2 Risk Factors for Glaucoma Development

The primary cause of glaucoma is unknown, with many contributing risk factors including age, ethnicity, family history, and increased intraocular pressure (IOP) (King et al., 2013). Epidemiological studies for the most common form, primary open angle glaucoma (POAG), have consistently found an increased prevalence with age shown by a steep rise in the
number of cases from 40 to 70 years (Burr et al., 2007). POAG is more commonly diagnosed in those of African American descent (Rudnicka et al., 2006), and a less common form, primary angle-closure glaucoma (PACG), is more highly prevalent in Asian populations (Quigley and Broman, 2006).

Familial aggregation for glaucoma has also been well established, where the first-degree relatives of POAG patients are three times more likely to develop the disease compared to non-relatives (Tielsch et al. 1994). As such, genetic studies have been carried out with the identification of numerous loci associated with glaucoma development (Takamoto and Araie, 2014). Genes that have been linked include myocilin (Stone et al., 1997), which influences intraocular pressure, and optineurin (Rezaie et al., 2002), which has a potential role in RGC apoptosis. Although these genes may be causative, they account for less than 10% of cases worldwide (Kwon et al., 2009).

Glaucoma has been most commonly associated with elevated IOP, which is a significant source of mechanical stress and strain on the posterior structures of the eye including lamina cribrosa and the ONH (Weinreb et al., 2014). Such pressure buildup is due to blocked or reduced drainage of the aqueous humor that fills the front of the eye. In addition to causing structural damages, increased IOP can further compromise vascular supply to the retina, leading to metabolic stress. In summary, glaucoma is a multifactorial disease with both genetic dispositions and environmental insults that ultimately undermine the integrity of the RGCs and their axons, resulting in the progressive loss of visual field.
2.1.3 Current Treatments for Glaucoma

At the present, there is no cure for glaucoma and the central goal in managing the disease is to prevent or slow further deterioration of visual functions. Currently, IOP is the only modifiable risk factor and glaucoma treatment is largely focused on methods of lowering IOP (Coleman, 1999; Lee and Higginbotham, 2005; King et al., 2013). This is achieved in a step-wise manner and would typically start with the topical application of pressure-lowering medications such as cholinergic agonists and prostaglandin analogues (Chae et al., 2013). If medications are ineffective, laser-based or surgical procedures follow to facilitate outflow from the eye and reduce IOP to target pressures (Morgan and Yu, 2012). Nevertheless, modulating IOP does not always slow vision loss in patients and studies have found 20-50% of surveyed glaucoma patients to exhibit IOP within normal ranges (Mudumbai, 2013).

Considering the neurodegenerative nature of the disease, increasing numbers of studies have started to explore neuroprotective strategies for a direct improvement in RGC survival (Chen et al., 2013). Neurotrophins are a family of proteins involved in neuronal support and have been shown to promote RGC integrity. In a number of animal studies, the application or upregulation of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) were able to improve RGC survival and visual functions (Koeberle and Ball, 2002; Lambiase et al., 2009; Pease et al., 2009). However, the effectiveness of these neurotrophins is limited by factors such as short half-life and demand for high dose (Chen et al., 2013).

Characterizing the mechanisms of RGC degeneration (further discussed below) has led to various studies that evaluated the effect of targeting pathogenic processes including apoptosis, oxidative stress, mitochondrial dysfunction, and excitotoxicity. In rescuing RGCs from cell
death, anti-apoptotic caspase inhibitors were tested and shown to offer protection (Chaudhary et al., 1999). Oxidative stress contributes to RGC injury, and treatment with antioxidants such as melatonin and α-lipoic acid was able to reduce RGC loss (Siu et al., 2004; Inman et al., 2011). Coenzyme Q10 in the mitochondria electron transport chain is an essential co-factor for energy production and its application protected against glaucomatous retinal damage (Cordeiro et al., 2007; Nucci et al., 2007). To counter neurotoxicity due to excess glutamate neurotransmitter, antagonists against glutamate receptor was also shown to reduce RGC apoptosis (Guo et al., 2006).

Neuroprotective strategies are a growing trend for glaucoma treatments, but the actual implementation of such methods is still limited at the present. Clinical trials that hope to assess neuroprotective agents face difficulties such as evaluating the preservation of RGCs following treatment (Chen et al., 2013). Since glaucoma progresses slowly, lengthy periods of assessments are also required (Chen et al., 2013). While these demands are addressed through technological improvements such as in imaging techniques, continued effort to characterize molecular events in glaucoma pathogenesis are crucial to reveal targetable mediators that can maximize RGC survival. Importantly, it is now recognized that in addition to pathogenic events that occur within the RGCs, their surrounding environment contribute to their injury (Hernandez, 2008; Chrysostomou et al., 2010; Almasieh et al., 2012; Yu et al., 2013). In particular, neighbouring neurosupportive glial cells exhibit altered behaviours that accelerate RGC damage (Hernandez, 2008; Garcia and Koke, 2008; Johnson and Morrison, 2009; Inman et al., 2011). Therefore, investigating RGC alone may not be able to prevent their loss, but examining deleterious alterations that also occur in neighbouring cells is crucial to identify additional factors that contribute to disease progression. As such, targeting neurosupportive cells surrounding RGCs is
emerging as a therapeutic strategy as it aims to provide a favourable immediate environment for neuronal survival. Current understandings of various events that contribute to RGC deterioration as well as how a glial cell type, the astrocytes, play a role in this process are discussed below.

2.2 Mechanisms of RGC Degeneration

At the molecular level, RGC death is influenced by multiple pathways that become aberrant in maintaining cellular homeostasis and their selective loss is attributed to some of the common pathogenic processes characteristic of neurodegenerative diseases (Sivak, 2013). Glutamate is a key neurotransmitter in the central nervous system including the retina. Similar to other neuronal types, RGCs are sensitive to elevated glutamate signaling, which triggers calcium fluxes into the cell and activates pro-apoptotic pathways (Casson, 2006). RGCs carry out transport of essential molecules including neurotrophin from axon terminals back to their cell bodies. Evidence suggests that this retrograde transport is blocked by elevated IOP and the resulting deprivation of neurotrophic factors contribute to RGC degeneration (Quigley et al., 2000). Protein misfolding and aggregation is a prominent feature of many neurodegenerative diseases and the characteristic amyloid-β peptide associated with Alzheimer’s disease has also been implicated in RGC apoptosis (Guo et al., 2007). Many of these pathogenic events are exacerbated by a metabolic deficiency where the lack of energy substrates compromise the cell’s ability to repair damages and restore homeostasis (Kong et al., 2009). In addition, ROS accumulation occurs in the retina with age that causes widespread damage to cellular macromolecules including proteins and DNA (Tezel, 2006). Both metabolic and oxidative insults are prominent aspects of RGC degeneration and further discussed in detail below.

2.2.1 RGC Degeneration Due to Metabolic Stress

Vascular insufficiencies lead to tissue damage due to compromised delivery of oxygen and nutrients. With age, there are changes in ocular vascular dynamics and reduced perfusion of the
retina and ONH has been reported (Groh et al., 1996; Boehm et al., 2005). In glaucoma patients of advancing age and often with the added biomechanical stress on blood vessels from elevated IOP, vascular etiology has thus been closely linked to its pathogenesis (Delaney et al., 2006). Numerous studies have found decreased blood flow to the ONH of glaucoma patients and the reduction of IOP improved circulation in some cases (Hafez et al., 2000, 2003). Since RGCs are some of the most energetically demanding cells in the body, they are particularly susceptible to unstable perfusion that lead to hypoxic and ischemic insults (Chrysostomou et al., 2010). In addition, the return of blood flow, or reperfusion, exacerbates RGC damage when the restoration of oxidative phosphorylation elicits a sudden elevation of reactive oxygen species (Flammer et al. 2002).

Contributing to metabolic stress on RGCs, mitochondria dysfunction also plays a key role in glaucomatous neurodegeneration. Significantly, the unmyelinated portion of RGC axons in the region of ONH and lamina cribrosa is highly concentrated in mitochondria, reflecting a high energetic demand in this area for electrical conduction into the brain (Bristow et al., 2002). With age, mitochondria undergo various changes that affect its function including increased structural disorganization, decreased efficiency of oxidative phosphorylation, and increased production of ROS (Lee and Wei, 2012). For the RGCs, their direct exposure to light exacerbates mitochondrial damage as light interacts with mitochondrial components to further generate ROS (Osborne et al., 2006, 2008). This increased susceptibility to mitochondrial dysfunction is also linked to reduced blood flow to the ONH where the RGCs are put in a state of low energy and damaging reactive oxygen intermediates cannot be removed efficiently (Osborne et al., 2006). In some glaucoma patients, it has been reported that there are increased levels of mitochondrial DNA mutations, many of which are potentially pathogenic (Abu-Amero et al., 2006). Furthermore, patients were found to exhibit deficiencies in mitochondrial respiration and ATP synthesis (Lee et al., 2012). Such bioenergetics failure is detrimental to RGC integrity as decreased ATP production reduces the amount of energy
available for cellular processes including repair mechanisms that are essential for cell survival (Kong et al., 2009).

2.2.2 The Role of Oxidative Stress in RGC Degeneration

Oxidative stress results when the production and exposure of ROS exceeds the level that can be efficiently handled by antioxidant mechanisms. ROS is generated as a by-product from the electron transport chain in normal mitochondrial respiration. Although ROS are involved in various signaling pathways under normal physiological conditions (Reczek and Chandel, 2014), a sustained high level causes severe damage to mitochondria and cellular macromolecules including proteins, lipids, and nucleic acids (Circu and Aw, 2010). To combat increased ROS levels, there are several intracellular antioxidant systems such as superoxide dismutases (SODs) that convert superoxide to hydrogen peroxide, catalase that catalyzes the decomposition of hydrogen peroxide to water, and glutathione peroxidase, which converts hydrogen peroxide to water using glutathione (Chaudière and Ferrari-Iliou, 1999).

Several aspects of glaucoma pathogenesis are linked with development of oxidative stress. Studies have found that experimentally-induced elevation of IOP increased ROS levels in the retina and decreased retinal antioxidant capacity (Muller et al., 1997; Moreno et al., 2004, Ko et al., 2005). As discussed above, ischemia-reperfusion injury due to unstable retina perfusion and continuous light exposure can further elevate ROS levels in the retina. Clinical data also suggest the involvement of oxidative stress where in glaucoma patients, increased levels of ROS-modified protein products and reduced antioxidant capacity were found in the aqueous humour of eye as well as in serum (Chang et al., 2011; Erdumus et al., 2011). Thus, ROS accumulation is a common consequence of many pathogenic processes in glaucoma that expose RGCs to continuous oxidative insults.
Elevated ROS levels induce downstream signaling events such that RGCs become susceptible to apoptotic cell death. Using a proteomic approach, Tezel et al. (2005) found that ROS generation in glaucoma led to modification of many retinal proteins including the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, heat shock protein 72 involved in protein folding, and glutamine synthetase implicated in excitotoxicity. The authors proposed that such modifications block protein function and proteasome removal, and the accumulation of non-functional oxidation products reduce the ability of RGCs to cope with altered homeostasis, leading to neurodegeneration. In addition to being directly cytotoxic, ROS can also triggers apoptosis through pathways that are independent from caspase activation (Li and Osborne, 2008). Furthermore, oxidative damage to RGCs is exacerbated when ROS alters the function of neuroprotective glial cells, such that they release toxic agents such as TNF-α, facilitating neurodegeneration (Tezel and Wax, 2000). Once the cellular level of ROS overwhelms its antioxidant capacity, a positive feedback cycle occurs where ROS generation becomes self-propagating (Chrysostomou et al., 2013).

In summary, the pathogenesis of glaucoma involves the interplay of various cytotoxic insults such that the ability to repair damages caused by one factor is impaired by another, inevitably triggering degenerative loss. In addition to their own intracellular alterations, the RGCs receive various inputs from their surrounding environment that influence how they respond to stresses. In the following section, the role of neighbouring astrocytes as an important neurosupportive cell type for the RGCs and their contribution to glaucoma development will be discussed.

2.3 Astrocyte in Neuronal Support

Astrocytes are a highly abundant type of glial cells that are central to neuronal support (Figure 2). Under normal physiological condition, they perform various functions in the central nervous system (CNS) including modulating synaptic levels of neurotransmitters, regulating
extracellular pH and ion homeostasis, maintaining extracellular matrix, and secrete trophic and survival factors for neurons (Araque et al., 2001; Wordinger et al., 2002; Magistretti, 2006; Obara et al., 2008).

**Figure 2. Neurosupportive functions of astrocyte.** Astrocytes (blue) perform various supportive functions in the CNS to maintain neuronal (green) homeostasis including the release of neurotrophic factors (A), modulating synaptic transmission (B), shuttling of energy substrates from vasculature to neurons (C), and maintaining blood-brain (or blood-retinal) barrier (D) (Garden and La Spada, 2012).

Astrocyte supportive functions become especially critical in situations of metabolic need and cytotoxic stress. Their response to cellular stressors constitutes one of the earliest changes in the nervous system and they function through a gap junction-linked syncytium to help maintaining neuronal functions (Rouach et al., 2008). In the context of metabolic support, astrocytes are situated at the interface between neurons and vasculature where they obtain, process, and release energy substrates for uptake and utilization by neurons (Pellerin et al., 2007). Astrocytes are where glycogen
is predominantly stored, which acts as the main energy reserve in the CNS (Dringen et al., 1993; Swanson and Choi, 1993). When encountering low energy status, glycogen is broken down to glucose, which is then metabolized to pyruvate through glycolysis. Subsequently, pyruvate can be converted to lactate, which is then released from astrocytes and taken up by neurons for use as an energy substrate (Hamprecht and Dringen, 1993). In addition, in response to high levels of synaptic activity due to increased neuronal transmissions, calcium influx is triggered in astrocytes to signal for the release of vasodilators (Jakovcevic and Harder, 2007). As a result, astrocytic signalling allows for an increased local blood flow to match with higher neuronal activities to meet their greater energetic demands.

In the context of oxidative stress, astrocytes are well-equipped with a high antioxidant capacity that allows them to be more resistant to ROS-mediated insults compared to neurons (Fernandez-Fernandez et al., 2012). Under resting conditions, astrocytes express various antioxidant enzymes as well as antioxidant molecules such as vitamins C and E (Makar et al., 1993). A key system that controls cellular redox balance in the CNS is centered on the antioxidant glutathione (GSH). GSH is a cofactor required by the enzyme glutathione peroxidase to catalyze the reduction of hydrogen peroxide to water (Dringen, 2000). GSH synthesis is largely dependent on the activity of the rate-limiting enzyme glutamate cysteine ligase (GCL) composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM). Astrocytes have been found to exhibit a higher level of GSH and GCL activity in comparison with neurons and are able to provide neurons with GSH precursors for their own antioxidant defence (Makar et al., 1993; Sagara et al., 1993; Dringer et al., 1999; Hirrlinger et al., 2002). Thus, astrocytes play a critical role for neuronal redox balance where their absence, neurons exhibit reduced antioxidant capacity as shown by a quicker depletion of GSH levels (Sagara et al., 1993).
Despite their supportive functions, astrocytes have altered behaviours when they are subjected to prolonged cytotoxic stress. Their role in RGC neuroprotection and glaucoma pathogenesis will be discussed below.

2.3.1 Supportive Roles of Astrocytes for RGCs

Astrocytes are a major glial cell type supporting the RGCs and their neuroprotective functions are particularly important in the ONH where RGC axons are unmyelinated (Hernandez et al., 2008). Astrocyte cell bodies are arranged to form glial columns that surround optic nerve bundles to provide a structural support for RGC axons (Büssow, 1980). They are involved in the maintenance of the extracellular matrix (ECM) through the synthesis of macromolecules such as collagen (Hernandez et al., 1991, 2000). Astrocytic processes also line vasculatures that perfuse the retina where they contribute to the regulation of local blood flow to meet neuronal demands and maintain the blood-retinal barrier analogous to the blood-brain barrier in the CNS (Mackenzie and Cioffi, 2008; Inman et al., 2011). Recently, the supportive role of ONH astrocytes was further expanded when it was demonstrated that they are directly involved in RGC mitochondrial turnover (Davis et al., 2014). RGC axonal mitochondria were found to shed through membrane protrusions and evulsions that are subsequently phagocytosed and degraded by ONH astrocytes. Along with other general functions discussed above such as the maintenance of extracellular ions, astrocytes constitute an essential component in maintaining the homeostatic integrity of RGCs.

2.3.2 Astrocyte in Glaucoma Pathogenesis and Progression

Accumulating evidence suggests that aberrant astrocyte behaviour contribute to the pathogenesis of glaucoma (Hernandez et al., 2008). In response to stressed conditions and tissue injury, astrocytes transform from a quiescent phenotype to a reactive one distinctly marked by cellular hypertrophy and hyperplasia and an increased level for the intermediate filament proteins
glial fibrillary acidic protein (GFAP) and vimentin (Ridet et al., 1997). In gaining such reactivity, astrocytes in the optic nerve exhibit dichotomous behaviours where in attempts to combat stressors and restore homeostasis, they also adopt a number of deleterious behaviours that exacerbate RGC injury (Johnson and Morrison, 2009). They become prominently involved in the remodeling of the ECM by altering their expression of ECM components as well as upregulating the level of matrix metalloproteinases that degrade ECM proteins (Morrison et al., 1990; Hernandez et al., 1990, 2000; Agapova et al., 2001). Although such modifications were shown in some cases to promote axonal recovery (Ahmed et al., 2005; Johnson et al., 2007), they have largely been associated with harmful consequences including reducing the resilience and elasticity of the ONH and forming a glial scar that disallow axonal regrowth and exacerbates RGC injury (Ridet et al., 1997; Hernandez et al. 2000; Pena et al., 2001). In addition, reactive astrocytes are known to synthesize and secrete neurotoxic mediators such as nitric oxide and TNF-α that provoke RGC apoptosis (Morgan et al., 1999; Tezel et al., 2004).

Importantly, normal metabolic and antioxidant functions of astrocytes are also lost following prolonged exposure to stressed conditions. It has been demonstrated that elevated IOP leads to a loss of gap junction-mediated communication between astrocytes, blocking their syncytial and efficient regulation of neuronal homeostasis (Malone et al., 2007). Significantly, astrocytes in glaucomatous ONH have lower basal levels of antioxidant GSH compared to normal astrocytes and in glaucoma patients, a lower plasma level of GSH is also evident (Gherghel et al., 2005; Malone and Hernandez, 2007). This reduced capacity to protect against oxidative stress is exacerbated as reactive astrocytes are themselves a potent source of ROS generation (Tezel et al., 2006; Sheng et al., 2013). In turn, a vicious cycle is created as oxidative stress further impairs astrocyte functions including the recycling of glutamate, which is critical for preventing excitotoxicity (Muller et al., 1998).

The metabolic roles of astrocytes are also affected by disease development. In contrast to their normal ability to increase blood flow to meet neuronal demands, ONH astrocytes in glaucoma
patients exhibited decreased proangiogenic activities, reflecting an inability to counter ischemic insult to the RGCs (Rudzinski et al., 2008). Evidence also suggest that astrocytes subjected to increased IOP have reduced intracellular levels of ATP (Inman et al., 2011). In addition, the expression of glucose and monocarboxylate transporters was downregulated in ONH astrocytes from glaucoma patients (Hernandez et al., 2002). This would challenge the provision of energy substrates to RGCs as astrocytes themselves fail to receive fuel supply for regular metabolism.

In summary, considering the role of astrocyte in maintaining RGC homeostasis and that their normal capacities are lost with disease progression, identifying key regulators specifically in these cells can reveal mechanisms of neuroprotection that can then be upregulated to increase RGC survival. A candidate pathway that holds a high potential for modulating astrocyte function is the focus of the present study and will be discussed in the following section.

2.4 PGC-1α: a Master Transcriptional Co-Regulator

Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a member of a family of nuclear coregulatory proteins that are involved in the modulation of gene transcription (Lin et al., 2005). Rather than directly binding to DNA, these coregulators form large multiprotein complexes with nuclear receptors (NRs) and transcription factors (TFs) to regulate transcriptional activities. They do so by affecting DNA binding of NRs and TFs as well as contributing to chromatin modification, initiation of transcription, and mRNA processing (O’Malley and Kumar, 2009). The PGC-1 family of coactivator proteins, also including PGC-1β and PGC-1α related coactivator (PRC), is structurally characterized by an N-terminal transactivation domain that can interact with histone acetyltransferases, a central regulatory domain, and a C-terminal RNA binding domain that can facilitate the formation of transcription initiation machinery (Lin et al., 2005). Due to the lack of a DNA-binding motif, these domains mediate versatile binding with different protein factors to give rise to the transcriptional regulatory roles of the PGC-1 coactivators.
PGC-1α is the most well-characterized member in the family of PGC-1 coactivators. It was first cloned in mouse brown adipose tissue where it acted as a co-regulator of the NRs peroxisome proliferated-activated receptor gamma (PPARγ) and thyroid hormone receptor to drive adaptive thermogenesis (Puigserver et al., 1998). Subsequently, it was characterized in humans where it was found to be expressed in various tissues including heart, skeletal muscle, liver, and brain (Esterbauer et al., 1999). Since its discovery, its regulatory roles have continued to be expanded along with the identification of numerous binding partners influencing a wide range of physiological processes. Of particular interest in the present study, its role in metabolism and antioxidant defense are detailed below.

2.4.1 PGC-1α in Metabolism

One of the earliest established functions of PGC-1α is that it acts as a master regulator of mitochondrial biogenesis (Wu et al., 1999). As such, it is not surprising that it is abundantly expressed in tissues with high energetic demands including heart, skeletal muscle, and the brain (Wu et al., 1999; Esterbauer et al., 1999). The initiation of mitochondrial biogenesis is dependent on two TFs, nuclear respiratory factors 1 (NRF-1) and 2 (NRF-2), which are necessary for activating the expression of mitochondria transcription factor A (Tfam) involved in mitochondrial DNA replication (Scarpulla, 2008; Hock and Kralli, 2009). Not only does PGC-1α induce the expression of NRF-1 and 2, it is a critical coactivator that binds these TFs to enhance their transcriptional activities, leading to the expression of mitochondrial proteins and mitochondrial replication (Wu et al., 1999). Furthermore, PGC-1α is indispensable under normal physiological conditions for maintaining mitochondrial integrity and respiratory capacity such that when it is knocked down, multiple tissues including the heart and skeletal muscle exhibited diminished functions (Leone et al., 2005).

In addition to controlling mitochondria homeostasis across many tissue types, PGC-1α has organ-specific metabolic functions through interactions with distinct TFs. In the liver, PGC-1α is
involved in promoting gluconeogenesis, fatty acid metabolism, ketogenesis, and bile acid homeostasis by coactivating several hepatic TFs (Yoon et al., 2001; Puigserver et al., 2003; Zhang et al., 2004). In skeletal muscle, the expression of PGC-1α is elevated with exercise to meet increased energy demands (Baar et al., 2002). By increasing mitochondrial biogenesis, PGC-1α can drive the transformation of glycolytic muscles to oxidative muscles with a higher mitochondrial content and an increased fatigue resistance (Lin et al., 2002). Under PGC-1α upregulation, the expression of transporter GLUT4 is increased to facilitate the uptake of glucose into skeletal muscle cells (Michael et al., 2001). In addition to activating mitochondria biogenesis in the neonatal heart, PGC-1α is responsible for triggering a metabolic switch from glycolysis to oxidative phosphorylation in cardiac muscle cells (Lehman et al., 2000). More recently, PGC-1α has also emerged as an important regulator of angiogenesis by which new blood vessels are formed from pre-existing vessels (Arany et al., 2008). In response to nutrient and oxygen deprivation, it induced the expression of vascular endothelial growth factor (VEGF) to trigger angiogenesis in skeletal muscle cells independent of HIF-1α. Thus, PGC-1α can be considered a central molecular coordinator that responds to and regulates various aspects of metabolism to maintain cellular homeostasis.

2.4.2 PGC-1α in Antioxidant Defense

PGC-1α has also been recognized as a regulator of ROS-detoxifying enzymes in a number of cell types including neurons, endothelial cells and cardiac muscle cells (Valle et al., 2005; St-Pierre et al., 2006; Lu et al., 2010). Its expression was upregulated following oxidative stress and in turn, it activated the expression of a number of antioxidant enzymes including SOD-1, SOD-2, catalase, and glutathione peroxidase. On the contrary, deficiency of PGC-1α in cells increased their sensitivity to oxidative stress and in knockout animals, tissues such as the heart suffered from excessive oxidative damage as cells fail to upregulate antioxidant genes (St-Pierre et al., 2006). It was proposed that since mitochondria is a prominent source of endogenous ROS, at the same time that it promotes
mitochondria biogenesis, PGC-1α triggers an antioxidant gene expression program in order to maintain cellular redox balance. Considering the role of ROS in the development of several pathological conditions such as cardiomyopathy and neurodegeneration, targeting PGC-1α is an attractive strategy to induce cellular antioxidant defense. However, due to the difficulty of directly targeting transcriptional cofactors, studies have turned to the investigation of upstream proteins that are linked to PGC-1α in a signalling pathway. In the following section, regulation of PGC-1α will be discussed with particular focus on two upstream activators, 5′-adenosine monophosphate-activated protein kinase (AMPK) and Sirtuin 1 (SIRT1).

2.4.3 Regulation of PGC-1α

PGC-1α is regulated at many levels through transcription, nuclear translocation, and posttranslational modifications (Fernandez-Marcos and Auwerx, 2011). When it was first discovered in brown adipose tissue, its expression was found to be induced by cold exposure to promote adaptive thermogenesis (Puigserver et al., 1998). It is now recognized that PGC-1α expression is responsive to various physiological conditions including fasting and exercise that reflect cell’s energetic demands (Yoon et al., 2001; Baar et al., 2002). Under these situations, upstream regulators of PGC-1α are activated, which include transcription factors cAMP response element (CRE)-binding protein, p38 mitogen-activated protein kinase, and myocyte enhancer factors (MEF) (Herzig et al., 2001; Handschin et al., 2003; Akimoto et al., 2005). Moreover, PGC-1α expression can be regulated in an autoregulatory feed-forward loop to drive its own expression through expression of MEF (Handschin et al., 2003).

Since PGC-1α performs its regulatory roles in the nucleus, its translocation into the nucleus is necessary for its transcriptional activities. It has been demonstrated that exercise in both rodents and humans led to a shift of PGC-1α subcellular localization from the cytoplasm into the nucleus without changing its total protein content (Wright et al., 2007; Little et al., 2009). In a separate study,
exposure to oxidative stress was also found to increase nuclear levels of PGC-1α (Anderson et al., 2008). Thus, despite its predominant localization to the nucleus, the activity of PGC-1α is regulated at the level of nuclear translocation.

Following translation, PGC-1α can be modified in several ways among which phosphorylation and acetylation are the most influential to its transcriptional activity (Fernandez-Marcos et al., 2011). The serine/threonine kinase AMPK is one its key regulators that activates PGC-1α through phosphorylation at threonine-177 and serine-538 (Jager et al., 2007). As cell’s energy sensor, AMPK is responsive to increased AMP to ATP ratio as result of low energy status and activates catabolic processes to replenish ATP (Hardie, 2007). It is regulated by a number of upstream kinases including liver kinase B1 (LKB1) and its phosphorylation at threonine-172 leads to its activation (Hawley et al., 1996). By phosphorylating PGC-1α, AMPK augments its transcriptional activity, which is also partially accounted for by increased PGC-1α localization into the nucleus (Anderson et al., 2008). AMPK can also increase the expression of PGC-1α to promote mitochondrial biogenesis and function (Jorgensen et al. 2005).

A second well-characterized regulator of PGC-1α is the NAD⁺-dependent deacetylase SIRT1. It is most widely known as the mammalian homolog of yeast silent information regulator 2 (Sir2), which has been linked to prolonged lifespan in yeast (Tissenbaum and Guarente, 2001). Similar to AMPK, SIRT1 also senses perturbations to metabolic homeostasis through increased NAD⁺ levels or NAD⁺/NADH ratio (Houtkooper et al., 2010). The regulation of SIRT1 activity is however more complex involving posttranslational modifications and interaction with other proteins (Yang et al., 2007; Kim et al., 2008). In line with the role of PGC-1α, SIRT1 is also responsive to fasting, exercise, and oxidative stress (Yamamoto et al. 2013; Di Emidio et al., 2014; Lai et al., 2014). In turn, it deacetylates PGC-1α leading to its increased transcription activity (Rodgers et al., 2005). It has been suggested that the stability of PGC-1α in the nucleus is increased by SIRT1 deacetylation, allowing it to accumulate intranuclearly (Anderson et al., 2008; Chen et al., 2013).
The link between PGC-1α, AMPK, and SIRT1 has been established as a coordinated network to maintain cellular metabolic homeostasis (Figure 3). In contrast to PGC-1α, which is difficult to target, various pharmacologic agonists of AMPK and SIRT1 have been generated and tested for their potential to protect cells against metabolic and oxidative stress. These pharmacological agents such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which activates AMPK, and resveratrol, which stimulates SIRT1, have been able to improve many pathological conditions including cardiomyopathies and metabolic syndrome (Ahluwalia and Tarnawski, 2011; Bremer, 2014). Their mechanism of action have in turn been attributed to PGC-1α-mediated transcription (Frier et al., 2012; Chen et al., 2013; Suwa et al., 2014).

Figure 3. The PGC-1α pathway and its downstream functions. PGC-1α is regulated at many levels through posttranslational modifications and nuclear translocation. AMPK and SIRT1 are two of cell’s energy sensors that regulate PGC-1α through phosphorylation and deacetylation, respectively. PGC-1α activation leads to the upregulation of genes involved in mitochondrial biogenesis, antioxidant defense as well as angiogenesis.
2.4.4 PGC-1α in Neurodegeneration

PGC-1α has gained tremendous attention in the field of neurodegeneration through analyses of knockout mice. Animals carrying a deletion of the PGC-1α gene displayed prominent lesions in the subcortical striatum region (Lin et al., 2004), and exhibited increased susceptibility to oxidative stress in the brain (St-Pierre et al., 2006). In contrast, overexpression of PGC-1α was able to reduce neuronal death in culture following exposure to mitochondrial toxin (Weydt et al., 2006), and improve the motor performance and survival of a transgenic mouse model of amyotrophic lateral sclerosis (Liang et al., 2011; Zhao et al., 2011). Subjecting cortical neurons to glucose and oxygen deprivation induced PGC-1α expression while its overexpression led to reduced apoptosis (Luo et al., 2009). In terms of clinical data, reduced expression of PGC-1α has been reported in patients of several neurodegenerative diseases including Alzheimer’s, Huntington’s and Parkinson’s (Cui et al., 2006; Qin et al., 2009; Zheng et al., 2010). Furthermore, the age of onset and risk of developing Huntington’s and Parkinson’s disease have been associated with single nucleotide polymorphisms in PGC-1α (Weydt et al., 2009; Clark et al., 2011).

Considering the high energetic demands of the nervous system, it is not surprising that PGC-1α is involved in maintaining its integrity as a master regulator of metabolism. As such, investigations focusing on targeting PGC-1α are emerging in the field of neurodegeneration. At the present, no direct link between PGC-1α and glaucoma development has been made. However, considering the neurodegenerative nature of the disease and the similarity in the pathogenesis between neurological disorders, exploring the role of PGC-1α in the context of RGC degeneration holds high therapeutic potential. In support of this investigation, some identified roles of PGC-1α in retinal tissue are discussed below.
2.4.5 Investigating PGC-1α in Retinal Degeneration

There have been few studies as yet on PGC-1α signalling in the retina, but it has been implicated in the modulation of retinal angiogenesis through the transcriptional regulation of VEGF (Ueta et al. 2012; Saint-Geniez et al., 2013, Zhang et al., 2014). Its knockdown was also associated with increased apoptotic death of photoreceptors in response to light damage (Egger et al., 2012). Our laboratory has recently investigated the role of PGC-1α in the inner retina (Guo et al., 2014). When exposed to excitotoxic damage, the expression of PGC-1α, and several of its downstream genes including SOD-1, SOD-2, NRF-2, and VEGF, were significant induced (Figure 4). We also found that in PGC-1α knockout mice, there is an increased retinal sensitivity to excitotoxic damage marked by a higher level of apoptotic RGC death. When I analyzed for the retinal expression of Tfam involved in mitochondria biogenesis, I found its reduced level correlated with the loss of PGC-1α (Figure 5A). Furthermore, in a genetic mouse model, DBA/2J, which is often used as a model of chronic, IOP-induced glaucoma, the level of inner retinal PGC-1α expression was found to decrease with age as optic neuropathy developed. Strikingly, in both groups of mice, retinal astrocytes showed early and prominent reactivity demonstrated by increased GFAP staining (Figure 5B). These findings suggest that PGC-1α may play a major role in retinal response to stressed conditions, and increased signaling through this pathway may augment cellular protective mechanisms for improved resistance against metabolic challenges.
Figure 4. Retinal expression of PGC-1α target genes following excitotoxic damage induction. The expression of NRF-2, SOD-1, SOD2, VEGF, and HO-1 was rapidly increased following kainic acid injection that induced excitotoxicity. Data are presented as means±SEM. \( n = 6 \) mice,* \( p<0.05 \) versus baseline (Guo et al., 2014).

The significance of PGC-1α in neurodegeneration has continued to be consolidated. However, whether it contributes to the neurosupportive roles of astrocytes has largely been uninvestigated. Importantly, astrocytic functions in energy provision and antioxidant defense closely coincide with the functions of the genetic programs coordinated by PGC-1α signalling. A recent study has found that in multiple sclerotic lesions, astrocytes express a high level of PGC-1α (Nijland et al., 2014). This upregulation elevated the expression of glucose transporters for an increased uptake of energy substrates to meet metabolic demands. Taken together, PGC-1α may function as a key modulator of astrocyte neurosupportive activities and examining its role in this particular cell type holds a high potential towards identifying targetable mechanisms for neuroprotection.
Figure 5. Increased reactivity of retinal astrocytes in PGC-1α knockout mice. (A) Reduced expression of Tfam involved in mitochondria biogenesis in PGC-1α knockout mice under both PBS and kainic acid (KA)-treated excitotoxic condition. n=5 mice. (B) Increased staining for glial fibrillary acidic protein (GFAP) marks astrocyte reactivity in a dose-dependent manner with PGC-1α knockout. n≥7 mice. Data are presented as means±SEM. *p<0.05, **p<0.01 (Guo et al., 2014).
Chapter 3
Methods
3 Methods

3.1 Cell Cultures

3.1.1 Primary Rat Retinal Astrocyte Culture

Our laboratory has previously established the isolation and culturing of primary rat retinal astrocytes (Nahirnyj et al., 2013). Twenty-one day old Wistar rats were sacrificed by CO₂ asphyxiation according to a protocol approved by the University Health Network Animal Use and Care Committee. Eyes were dissected out and placed in ice-cold MEM-H17 media (Gibco, Ref#61100) supplemented with 5% fetal bovine serum (Multicell, Ref#080105), 5% horse serum (Invitrogen, Ref#16050-122) and antibiotics. Subsequently, retinas were removed from the eyes and digested in serum-free MEM-H17 supplemented with trypsin (Multicell, Ref#325-041-EL) and 5 µg/mL DNase I (Roche, Ref#10104159001) for 20 minutes. Digested retinas were washed with serum-free media, dissociated by trituration, and centrifuged at 1000 rpm for 8 minutes. Cells were resuspended in specialized astrocyte growth media (Lonza, Ref#CC-4123) and seeded into T-75 flasks (Corning, Ref#430641). On the following day, growth media was replaced with complete MEM-H17 to remove unattached debris and other cell types, and thereafter, media replacement was carried out three times per week. Upon reaching confluency, cells were shaken at 100 rpm for 5 hours after which media was replaced to remove microglial cells. One day after shaking, astrocyte-rich culture was detached and plated according to experimental needs.

3.1.2 A7 Astroglial Cell Culture

In vitro experiments were performed using an astroglial A7 cell line derived from the optic nerve of neonatal rat (Geller and Dubois-Dalcq, 1988). These cells were maintained in 100 mm polystyrene tissue culture dishes (Sarstedt, Ref#83.1802) at 37°C and 5% CO₂. They were
incubated with Dulbecco’s media H21 with antibiotics (Gibco, Ref#12800) that were supplemented with 10% fetal bovine serum (Multicell, Ref#095150). Media replacement took place two to three times a week and cells were passaged once a week or when reaching confluence. For detachment, cells were rinsed with TrypLE (Gibco, Ref#12604-013) after which complete media was added to inactivate trypsin in the solution. To pellet the cell suspension, centrifugation was carried out at 200 g for 5 minutes. Subsequently, the media was replaced to remove trypsin. Cell density was counted using a hemocytometer and plated according to experimental needs.

### 3.2 Induction of Oxidative and Metabolic Stresses

To induce oxidative stress, the redox cycling chemical paraquat (PQ) was applied (Sigma, Ref#75365-73-0), which elicits continuous ROS production through interaction with the mitochondria respiratory chain (Schmuck et al., 2002). A stock of 50 mM was reconstituted from powder form using sterilized Milli-Q® (MQ) water and further diluted with culture media for experimental use. For detection of early gene expression and protein level changes, cells were incubated with PQ at 300 µM for 6 hours. For longer term evaluation of intracellular ROS level and cell viability, cells were incubated with PQ at 100 µM for 18 hours. A lower concentration of PQ was used for the longer incubation to prevent cell death from the treatment.

For metabolic stress that models an ischemic condition, cells were incubated in glucose and serum deprived medium with no supplements added (Gibco, #11966-025). Optimization was carried out to determine the optimal duration for glucose and serum deprivation (GSD). For early evaluation of gene expression and protein level changes, cells were incubated in glucose and serum-deprived media for 6 hours. To determine cell viability, cultures were subjected to GSD for 48 hours.
3.3 Gene Expression Analysis through qRT-PCR

Total RNA was extracted from cultured cells using TRIzol® reagent (Life Technologies, Ref#15596018). To remove DNA contamination, resuspended RNA was digested using RQ1 RNase-free DNase (Promega, Ref#M6101). Subsequently, the RNA sample was reverse transcribed using Superscript III Reverse Transcriptase kit (Invitrogen, Ref#18080-093). Quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Ref#4367659) with 200 nM of primers (Table 1). The reaction mixture was loaded into the Mastercycler Eppendorf Realplex for running of 40 cycles followed by a melting curve analysis to confirm the purity of the products. Gene expression was determined using the comparative CT method with mRNA levels normalized to control, TATA-binding protein (TBP).

Table 1. List of primers used for quantitative PCR.

<table>
<thead>
<tr>
<th>Target Protein (Gene)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBP (Tbp)</td>
<td>5’-ACAGGTGGCAGCATGAAGTG-3’</td>
<td>5’-GCAGGTTGATTTACGTGCAGA-3’</td>
</tr>
<tr>
<td>PGC-1α (Ppargc1a)</td>
<td>5’-ATGTGTGCGCTTCTTTGCTCT-3’</td>
<td>5’-ATCTACTGCTGGGGACCTT-3’</td>
</tr>
<tr>
<td>NRF-1 (Nrf1)</td>
<td>5’-AGCCCCATCTCGTACCAC-3’</td>
<td>5’-TCGTCCTGATGGTCATTTCA-3’</td>
</tr>
<tr>
<td>NRF-2 (Nrf2)</td>
<td>5’-GAGTGTGTAAGCCAGGC-3’</td>
<td>5’-TGTTGATATGCTTTGGTGC-3’</td>
</tr>
<tr>
<td>SOD-1 (Sod1)</td>
<td>5’-CCACTGCAGGACTCATTTT-3’</td>
<td>5’-CACCTTTTGCCCAAGTCATCT-3’</td>
</tr>
<tr>
<td>SOD-2 (Sod2)</td>
<td>5’-GGCCAAGGGAGATTTACAA-3’</td>
<td>5’-GCTTGATAGCCTCCAGCA-3’</td>
</tr>
<tr>
<td>HO-1 (Hmox1)</td>
<td>5’-TCTATCGTGCTCGATGAAC-3’</td>
<td>5’-AAGGCCGTTCTAGCCCTC-3’</td>
</tr>
<tr>
<td>VEGF (Vegfa)</td>
<td>5’-TGCTCTCTTGGGTGACTGG-3’</td>
<td>5’-TTCTCCGCTGTAACCAAGGC-3’</td>
</tr>
<tr>
<td>GCLC (Gclc)</td>
<td>5’-CTGGGGAGTGATTCTGCAT-3’</td>
<td>5’-AGATCTCCGTTGCGTGTC-3’</td>
</tr>
<tr>
<td>GCLM (Gclm)</td>
<td>5’-GAAATGAAATGGAGCTCCAAA-3’</td>
<td>5’-GACAGAGTCCAGCTGCAA-3’</td>
</tr>
</tbody>
</table>
3.4 Western Blot

3.4.1 Whole-Cell Lysate Collection and Nuclear Extraction

To collect whole-cell lysates, cultures were detached using TrypLE, washed with PBS, and lysed using ice-cold RIPA buffer (Cell Signaling, Ref#9806) supplemented with protease inhibitors (Roche, Ref#11836170001) and phosphatase inhibitor (Roche, Ref#04906845001). Following incubation for 10 minutes, lysates were centrifuged at 12,000 g for 10 minutes. The protein-containing supernatant was transferred into a separate tube and stored at -80°C until use.

Nuclear lysates were collected using the NE-PER Nuclear Protein Extraction kit (Thermo Scientific, Ref#78833). Briefly, following detachment with TrypLE and rinsing with PBS, cytoplasmic extraction reagent supplemented with protease and phosphatase inhibitor was added to the cell pellet. The mixture was vortexed for 15 seconds and incubated on ice for 10 minutes. Following incubation with a second cytoplasmic extraction reagent, the sample was centrifuged for 5 minutes at 16,000 g. The supernatant containing cytoplasmic extracts was transferred into a new tube and stored at -80°C until use. The remaining pellet containing the nucleus was lysed with supplied nuclear extraction reagent also supplemented with protease and phosphatase inhibitor. The lysate was incubated for 40 minutes on ice with 15-second vortex every 10 minutes. Lastly, the mixture was centrifuged for 10 minutes at 16,000 g. The supernatant containing nuclear proteins was transferred into a new tube and stored at -80°C until use.

3.4.2 Protein Quantification

All protein lysates including whole-cell, nuclear and cytoplasmic were quantified using the Bio-Rad DC Protein Assay kit based on the Lowry method of protein quantification (Bio-Rad, Ref#500-0116). Reagent containing an alkaline copper tartrate was added to samples to
produce cupper ions that subsequently reduce the Folin reagent in a second added solution (Lowry et al., 1951). The resultant colour change was analyzed using a spectrophotometer in which the absorbance of samples was measured at 605 nm. To determine protein concentrations, absorbance values were converted to concentrations based on a standard curve generated using serially diluted bovine serum albumin (Bio-Rad, Ref#500-0007).

3.4.3 SDS-PAGE and Protein Detection

Protein samples were diluted to equal concentrations using their respective lysis buffers and mixed with Laemmli Sample Buffer (Bio-Rad, Ref#161-0747) containing 10% β-mercaptoethanol (Sigma, Ref#M3148). Samples were then boiled for 5 minutes at 98°C and loaded onto 10% SDS-polyacrylamide gel. The gel was run in Tris-Glycine-SDS buffer (Bioshop, Ref#TGS222.1) at 60 V for 30 minutes followed by 100 V for 90 minutes. Resolved proteins were then transferred onto a PVDF membrane (Millipore, Ref#IPFL00010) at 25 V for 20 minutes using the Pierce Fast Semi-Dry Transfer System buffer (Thermo Scientific, Ref#PI-88217). Subsequently, membranes were blocked for 1 hour in 5% BSA (Fischer Scientific, Ref#9048-46-8) in Tris-buffered saline containing 0.1% Tween 20 (Fisher Scientific, Ref#9005-64-5). Primary antibody incubation was carried out overnight at 4°C with shaking (Table 2). Membranes were then washed three times for 10 minutes with TBS-Tween followed by incubation with secondary antibody (Table 2) for 1 hour with shaking at room temperature. After washing, membrane was scanned using the LI-COR Odyssey Infrared Quantitative Imaging System (LI-COR Biosciences). Lastly, protein densitometry was analyzed using ImageJ software.
Table 2. List of antibodies and dilutions for incubation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Supplier, Ref#</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1α</td>
<td>1:1000</td>
<td>Novus Biological, NBP1-04676</td>
</tr>
<tr>
<td>Phospho-AMPK</td>
<td>1:1000</td>
<td>Cell Signaling, 2531</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:10,000</td>
<td>Calbiochem, CB1001</td>
</tr>
<tr>
<td>Lamin B1</td>
<td>1:1000</td>
<td>Novus, NBP1-42594</td>
</tr>
<tr>
<td>Histone H3</td>
<td>1:1000</td>
<td>Abcam, ab24834</td>
</tr>
<tr>
<td>Donkey Anti-Rabbit IgG IRDye</td>
<td>1:10,000</td>
<td>Mandel Scientific, 926-68023</td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG IRDye</td>
<td>1:10,000</td>
<td>Mandel Scientific, 926-32212</td>
</tr>
</tbody>
</table>

3.5 Pharmacological Treatments

To stimulate the PGC-1α pathway, two pharmacological agents, AICAR (Calbiochem, Ref#123040) and SRT1720 (Selleckchem, Ref#S1129), were selected, which activate upstream regulators of PGC-1α, AMPK and SIRT1, respectively. AICAR was reconstituted from the powder form with sterilized MQ water to a concentration of 100 mM, which was subsequently diluted in culture media for treatments. SRT1720 was reconstituted from its powder form using sterile DMSO (Sigma, Ref#D8418) and further diluted with culture media for experimental use. DMSO at the same concentrations as SRT170 dilutions was used as controls. To determining the effects of these drugs against oxidative and metabolic stress, one or both compounds were added concomitantly with PQ or glucose and serum-free media incubation.

3.6 PGC-1α siRNA Knockdown

Small interfering RNAs (siRNAs) are short double-stranded RNA molecules that interfere with gene expression through complementarity and are used to study the effect of suppressing particular genes (Elbashir et al., 2001). siRNA targeting PGC-1α and non-targeting
(NT) siRNA were purchased from GE Dharmacon (GE Healthcare, Ref#D-099651-03) and reconstituted in siRNA buffer (Thermo Scientific, Ref#B-00200-UB-100). To transfect cultures, cells were plated on 12-well plates in antibiotics-free media the day before transfection. siRNAs were diluted to desired concentrations using serum and antibiotics-free media and mixed with DharmaFECT 1 Transfection Reagent (Dharmacon, Ref#T2001-02). Following incubation, the mixture was further diluted with antibiotics-free media and applied to plated cells for 24 hours after which transfection media was replaced with fresh media. To evaluate the knockdown, RNA was collected after 24 hours and analyzed using qRT-PCR. Proteins were collected after 48 hours and analyzed using western blot.

3.7 Cell Viability Assay

As a measure of cellular resistance to the stressed conditions, the colorimetric XTT viability assay kit (FroggaBio, Ref#20-300-1000) was used following treatments. This method is based on the activity of mitochondrial enzymes that inactivate following cell death (Scudiero et al., 1988). Thus, the generation of colorimetric dye is proportional to the number of viable cells in culture. The assay was carried out in 96-well plates in which cells were seeded. Following treatments, the XTT reagent was mixed with a second activation solution provided in the kit and added to each well. After an incubation period of 3 hours, absorbance was measured at 490 nm using a scanning multi-well spectrophotometer. Readings from blank wells with culture media only were subtracted from all other wells and the blanked absorbance was normalized to control wells for comparison between different treatments.

3.8 Measuring Intracellular ROS Level

Increased oxidative stress is reflected through elevated intracellular ROS levels. To determine whether treatments have an effect on the levels of ROS, cells were incubated with
2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), which upon oxidation, is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF). Immediately prior to use, H₂DCFDA (Invitrogen, Ref#D-399) was dissolved in DMSO, further diluted in serum-free media, and applied to cells for 15 to 30 minutes. Following incubation, cells were collected and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) at 530 nm.

### 3.9 Determining Intracellular Level of GSH

GSH is a key antioxidant in astrocytes and its level reflects the antioxidant capacity in these cells. Thus, the intracellular level of GSH was analyzed using the GSH-Glo™ Glutathione Assay (Promega, Ref# V6911) to determine the effect of different stress and pharmacological treatments. This assay is luminescence-based and depends on the conversion of a luciferin derivative to luciferin while GSH is consumed. Luminescence is then generated from luciferin under the enzymatic action of firefly luciferase and is proportional to cellular level of GSH. To carry out this assay, cells were plated in white, opaque polystyrene 96-well plates (Greiner, Ref#655083). Following treatments, culture media was replaced with GSH-Glo™ Reagent containing luciferin derivative and incubated for 30 minutes. Subsequently, luciferin detection reagent was added to generate luminescence for 15 minutes at which wells were analyzed using the luminometer function of Perkin Elmer Victor 3 Multilabel Counter. Readings from blank wells containing reagents only were subtracted from all wells. Luminescent values were normalized to control with no treatment for comparison.

### 3.10 Determining Mitochondrial Content

Mitochondria biogenesis is a well-established event downstream of PGC-1α activation. Thus, the effect of stressed conditions and pharmacological targeting of PGC-1α pathway was
determined through an analysis of intracellular mitochondria level. The MitoTracker® Red CMXRos fluorescent dye (Life Technologies, Ref# M-7512) stains mitochondria in living cells. It was reconstituted using DMSO and further diluted in serum-free media to a final concentration of 100 nM. Culture media was replaced with dye-containing media and incubated for 30 minutes. Subsequently, cells were collected and analyzed using the FACSCalibur flow cytometer at 585 nm.

### 3.11 Statistical Analysis

Statistics were analyzed using GraphPad Prism software, version 6.0. Data are presented as ± SEM values that were obtained from experiments performed from independently prepared astrocyte cultures. For experiments using the A7 cell line, at least 3 replicates were included in each biological repeat. For experiments using primary cultures, at least 3 biological replicates were performed. Statistical significance was determined using one-way ANOVA for multiple group comparisons and the Student’s t-test for comparison of gene expression and protein level with control. Statistical significance was defined as $P < 0.05$ indicated with one asterisk (*), and $P$ values of $<0.01$ and $<0.001$ were represented by two (**) and three (***), respectively.
Chapter 4
Results
4 Results

4.1 Aim 1: Response of PGC-1α Pathway to Oxidative and Metabolic Stress in Astrocytes

4.1.1 Elevated Pathway Activity in Astrocytes Following Oxidative Stress

To begin investigating the role of PGC-1α in astrocytes, I determined whether activities through this pathway are altered in response to oxidative stress. Increased nuclear concentrations of PGC-1α are one way through which its function is upregulated (Anderson et al., 2008; Chen et al., 2013). Thus, the level of nuclear PGC-1α was analyzed through western blot following nuclear extraction of cell lysates. In addition, upstream AMPK activation was determined with an antibody with specific activity against its activated and phosphorylated form. Using the A7 cell line as an astroglial cell model, oxidative stress was applied using PQ at 300 μM as previously established in our lab (Nahirnyj et al., 2013). Early responses were determined in these cells using 2 and 6-hour time points.

In the cytoplasmic fraction of A7 cell lysates, phospho-AMPK (pAMPK) was increased slightly after 2 hours of PQ treatment, and was significantly elevated at 6 hours by nearly three-fold (Figure 6A). Following a similar pattern, the level of nuclear PGC-1α also showed a significant increase of about 1.5-fold after 6 hours of oxidative stress (Figure 6B).

To confirm this finding, the 6-hour time point, which had the highest response, was chosen for repetition of oxidative stress-induced activity in primary retinal astrocyte cultures. Cytoplasmic and nuclear lysates were collected from primary astrocytes following PQ treatment, and both increased pAMPK activation (Figure 6C), and PGC-1α nuclear localization, were evident following 6 hours of ROS exposure (Figure 6D).
To provide evidence for the activation of PGC-1α transcriptional activity, a panel of genes known to be under its regulation was assessed for changes in mRNA expression in primary retinal astrocytes following PQ treatment. Furthermore, the response in expression of PGC-1α itself to oxidative insult was determined. A 6-hour time point was chosen for this analysis to capture early transcriptional changes that coincide with significant elevation of pathway protein signaling activity. Under PQ treatment, PGC-1α expression increased by three-fold along with a significant induction of its targets including metabolic genes, NRF-1 and VEGF, and antioxidant gene, SOD-1 (Figure 6E).

4.1.2 Elevated Pathway Activity in Astrocytes Following Metabolic Stress

A second key role of PGC-1α is its regulation of metabolism in response to energy deprivation. To test for this function in astrocytes, a metabolic stress model was established by incubating cell cultures with glucose and serum-deprived media. Pathway activity was similarly determined by the level of nuclear PGC-1α and cytoplasmic pAMPK. A7 cells under these conditions exhibited no change in pAMPK after 6 hours of GSD (Figure 7A). However, nuclear PGC-1α level was significantly increased (Figure 7B). These findings were confirmed in primary cultures similarly subjected to GSD for 6 hours (Figure 7C&7D).
Figure 6. Elevated PGC1-α pathway activity in astrocytes following oxidative stress. PQ-treated A7 culture exhibited increased cytoplasmic pAMPK (A) and nuclear PGC-1α (B) following 2 and 6 hours. Primary retinal astrocyte subjected to 6 hours of PQ treatment showed similar upregulations (C&D). n = 3. PQ induced increased expression of PGC-1α and some of its target genes (E). n = 4. *P<0.05, **P<0.01, and ***P<0.001.
Figure 7. Elevated PGC1-α pathway activity in astrocytes following metabolic stress. A7 culture incubated in glucose and serum-deprived medium (GSD) exhibited no change in cytoplasmic pAMPK (A) but an increased nuclear PGC-1α (B) following 6 hours. Primary retinal astrocyte subjected to 6 hours of GSD showed a similar result (C&D). n = 3. GSD induced increased expression of PGC-1α and some of its downstream genes (E). n = 4. *P<0.05, **P<0.01, and ***P<0.001.
Alterations in downstream gene expression patterns following nutrient deprivation in primary cultures were determined next. PGC-1α transcription under GSD was significantly elevated by more than 7-fold, higher than that induced by oxidative stress (Figure 7E). In addition, the expression of PGC-1α targets including NRF-1, VEGF, and SOD-1 were also significantly increased (Figure 7E).

Therefore, in response to both oxidative and metabolic insults, the activity of PGC-1α and upstream AMPK were upregulated along with the expression of PGC-1α associated genes involved in mitochondria biogenesis (NRF-1), angiogenesis (VEGF) and antioxidant response (SOD-1).

4.2 Aim 2: PGC-1α Pathway Activity Regulates Astrocytic Resistance to Oxidative Stress

4.2.1 Pharmacological Stimulation of PGC-1α Induces Antioxidant Expression

With the establishment that PGC-1α pathway activity and expression in astrocytes is induced by oxidative stress, I next evaluated whether stimulating this pathway affects astrocytic antioxidant capacity. This was carried out pharmacologically using two selected small molecule compounds targeting upstream regulators of PGC-1α, AMPK and SIRT1, AICAR and SRT1720. AICAR is an allosteric activator of AMPK that promotes its phosphorylation (Corton et al., 1995). SRT1720 was identified as a specific agonist of SIRT1 with high potency (Milne et al., 2007). As both compounds converge downstream on PGC-1α activation from different regulators, common PGC-1α-mediated effects can be identified and consolidated.

Considering the involvement of PGC-1α in antioxidant regulation, treatment with AICAR and SRT1720 were tested for effects on the expression of antioxidant enzymes in
Figure 8. PGC-1α-mediated regulation of antioxidant SOD enzymes. Pharmacological targeting of PGC-1α regulators, AMPK and SIRT1, by AICAR (A) and SRT1720 (B), respectively, significantly increased SOD-1 expression while SOD-2 mRNA levels were unaltered. \( n = 4 \). PGC-1α knockdown blocked both AICAR (C) and SRT1720 (D)-induced upregulation of SOD-1. SOD-2 expression was again unaffected. \( n = 3 \). *\( P<0.05 \) and **\( P<0.01 \).
primary retinal astrocytes. Similar to stress-induced analyses, the 6-hour time point was chosen to evaluate early responses. Following 6 hours of AICAR treatment, the expression of SOD-1 was significantly upregulated while SOD-2 was unaffected (Figure 8A). A similar profile was produced when cells were treated with SRT1720 (Figure 8B). Conversely, to determine the dependence of antioxidant gene expression on PGC-1α, siRNA targeting PGC-1α was applied to cultures prior to AICAR or SRT1720 treatment. PGC-1α knockdown was confirmed through mRNA and protein analysis (Appendix 1A, B). The induction of SOD-1 by both AICAR (Figure 8C) and SRT1720 (Figure 8D) was blocked by PGC-1α knockdown in comparison with cultures treated with non-targeting siRNA (NT). In contrast, the regulation of SOD-2, which was unaffected by AICAR and SRT1720 treatment, also did not change in response to PGC-1α knockdown (Figure 8C, D).

4.2.2 Identification of GCLM as a PGC-1α Target in Astrocytes

The glutathione (GSH) antioxidant system is central to astrocytic defense against oxidative stress. The synthesis of GSH is dependent on the rate-limiting enzyme, glutamate cysteine ligase (GCL), consisting of a catalytic subunit, GCLC, and modifier subunit, GCLM. Considering the oxidative stress induced activity of PGC-1α in astrocytes, and the prominence of GSH in these cells (Makar et al., 1993; Sagara et al., 1993; Dringer et al., 1999; Hirringer et al., 2002), I determined whether PGC-1α is also involved in the regulation of GCL. Under AICAR and SRT1720 treatment, the expression of GCLC did not change while GCLM exhibited a significant upregulation (Figure 9A, B). When PGC-1α was knocked down, the induction of GCLM by AICAR and SRT1720 was blocked while the expression of GCLC remains unaffected (Figure 9C, D).
Figure 9. PGC-1α-mediated regulation of GCL subunits in vitro and in vivo. Pharmacological targeting of PGC-1α by AICAR (A) and SRT1720 (B) significantly increased GCLM expression while GCLC mRNA levels were unaltered. n = 4. PGC-1α knockdown blocked both AICAR (C) and SRT1720 (D)-induced upregulation of GCLM. n = 3. Under PBS and KA treatments, PGC-1α transgenic mice did not differ in GCLC expression (E). In a dose-dependent manner, expression of GCLM was blocked under KA treatment in PGC-1α knockout mice. n = 4-6. *P<0.05, **P<0.01, and ***P<0.001.
In order to confirm that expression of GCLM is under transcriptional control of PGC-1α *in vivo*, I took advantage of retinal tissues from PGC-1α wild-type, heterozygous and knockout mice previously obtained in the lab for analyses of gene expression alterations (Guo et al., 2014). These mice had been subjected to kainic acid (KA)-induced excitotoxic damage to the retina, where the expression of PGC-1α and its targets were induced in wild-type, but blocked in knockout animals. The mRNA level of GCL subunits was analyzed in these retinal extracts from the various genotypes. Similar to the *in vitro* results, the expression of GCLC was not affected by PGC-1α knockdown (Figure 9E). Under unstressed condition, the mRNA level of GLCM subunit also did not alter in knockout mice. However, when the retina was subjected to excitotoxic damage, the expression of GCLM was significantly lower in PGC-1α knockout animals (Figure 9F). In addition, the level of GCLM expression in heterozygotes fell in between that of wild-type and knockout mice, suggesting a direct correlation to PGC-1α gene dosage.

### 4.2.3 Pharmacological Stimulation of PGC-1α Alter Cellular GSH Level

Since the GCL enzyme controls the rate-limiting step of GSH synthesis, its level determines the amount of intracellular GSH available for antioxidant defense. With the finding that PGC-1α is involved in the regulation of GCLM subunit, the ability of this pathway to increase cellular GSH was evaluated. Primary cultures were treated with AICAR or SRT1720 after which intracellular GSH was determined using the GSH-Glo™ luminescence assay. Under 6 hours of AICAR treatment, the level of GSH was significantly increased while SRT1720 induced a trend towards GSH elevation (Figure 10). In addition, the effect of PQ-mediated ROS generation on this system was studied. PQ treatment also led to a significant GSH induction consistent with an increased astrocytic antioxidant response. Under PQ treatment, the presence of
AICAR further elevated GSH levels while SRT1720 had minimal effects (Figure 10). Therefore, AMPK activation induced a stronger GSH response under oxidative stress conditions.

### 4.2.4 PGC-1α Pathway Activation Influences Intracellular ROS Levels

Considering the involvement of PGC-1α in the regulation of antioxidant enzymes and GSH antioxidant capacity, I next investigated whether increased pathway activity ultimately leads to a reduction in intracellular ROS. To elicit ROS production, primary cultures were incubated overnight with PQ for 18 hours. A dose-response optimization was first performed using a cell viability assay to select a concentration of PQ causing minimal cell death (Appendix 2). Intracellular ROS levels after 18 hours of PQ treatment were evaluated by flow cytometry for DCF fluorescence.

Primary retinal astrocytes treated with PQ exhibited a 50% increase in intracellular ROS in comparison with controls (Figure 11A). However, in the presence of AICAR, ROS levels
Figure 10. PGC-1α-dependent buffering of intracellular ROS. PQ-mediated ROS generation was significant reduced by AICAR but not SRT1720 treatment (A). PGC-1α knockdown did not affect intracellular ROS under unstressed conditions (B), but exacerbated PQ-mediated elevation in ROS and blocked the ROS-lowering effect of AICAR (C). Reduced ROS-buffering capacity is evident through compromised induction of antioxidant genes, SOD1 and GCLM, under PGC-1α knockdown (D). n = 3. *P<0.05, **P<0.01, and ***P<0.001.
were significantly reduced nearly to baseline levels. In contrast, SRT1720 treatment did not affect PQ-mediated generation of intracellular ROS.

Next, PGC-1α-dependence of ROS buffering capacity was assessed by combining its knockdown with subsequent PQ and pharmacological treatments. Under unstressed condition without PQ, PGC-1α knockdown did not affect intracellular ROS levels (Figure 11B). However, with PQ treatment, knocking down PGC-1α caused a further increase in ROS generation (Figure 11C). Furthermore, PGC-1α deficiency significantly blocked the ROS-lowering effect of AICAR treatment. While unable to buffer ROS accumulation, under SRT1720 treatment, PGC-1α knockdown also led to a slight increase in ROS level.

PQ-mediated oxidative stress was found to induce PGC-1α-regulated antioxidants. In turn, the upregulation of these enzymes is crucial for ROS-buffering capacity. With the finding that PGC-1α knockdown exacerbated increases in ROS level, whether this was due to blocked expression of antioxidant enzymes was determined. Under PQ treatment, mRNA levels of SOD-1 and GCLM showed a significant increase of nearly two and three-fold, respectively (Figure 11D). PGC-1α-dependence of these upregulations were then confirmed where its knockdown blocked the induction of both enzymes following PQ treatment (Figure 11D).

4.2.5 Stimulation of PGC-1α Pathway Improves Cell Viability Under Oxidative Stress

Having characterized the antioxidant roles of the PGC-1α pathway in astrocytes, the effect of pharmacological treatments on astrocytic survival under oxidative stress was investigated next. A colorimetric cell viability assay was applied following various treatments. To induce oxidative stress-mediated cell death, a higher concentration of PQ (300 μM) was
selected from the optimization curve (Appendix 2) for 18 hours of treatment that caused more than 50% reduction of cell viability. To determine whether activating the PGC-1α pathway offers protection, AICAR and SRT1720 were applied in three different doses concomitantly with PQ after which any improvement in cell viability was assessed.

To investigate PGC-1α-dependence of the upregulated expressions, it was knocked down with siRNAs prior to AICAR and SRT1720 treatments. The induction of NRF-1 and NRF-2 by AICAR was blocked by PGC-1α knockdown (Figure 13C). VEGF also showed a significant decrease in expression levels in response to PGC-1α deficiency. A similar result was obtained under SRT1720 treatment where the upregulation of the three targets were significantly reduced due to PGC-1α knockdown (Figure 13D). Therefore, in the absence of PGC-1α, a blocked metabolic response in astrocytes is evident.

Under AICAR treatment, it was found that the intermediate dose of 1 µM significantly improved the reduction in cell viability caused by PQ (Figure 12A). However, at the highest concentration administered, its protective effect was abolished. A similar dose-dependent curve was found following SRT1720 treatment, where an intermediate concentration increased cell viability significantly (Figure 12B). The protection was abrogated at the highest applied dosage.

Considering that AICAR and SRT1720 act through different upstream PGC-1α regulators, I assessed whether combining their treatment leads to added protection. For each compound the optimal dosage that conferred improved cell viability was selected for the combined treatment; 1 µM AICAR, and 3 µM SRT1720. With the application of both compounds, cell viability was further improved compared to either alone (Figure 12C). This provides support that the increased stimulation of PGC-1α activators leads to an additive protection that significantly augments cell survival in the context of oxidative stress.
Figure 12. PGC-1α pathway stimulation improves astrocytic viability under oxidative stress. AICAR (A) and SRT1720 (B) treatments significantly increased cell viability at an intermediate applied dosage. At the highest administered concentration, their protective effects were however abolished. Combined treatment with optimal doses of AICAR and SRT1720 further improved cell viability compared to either alone (C). n =3-4. *P<0.05, **P<0.01, and ***P<0.001.

Taken together, these results demonstrate that PGC-1α pathway activation led to the upregulation of antioxidants SOD-1 and GCLM which reduced intracellular ROS and improved astrocyte viability under oxidative stress.
4.3 Aim 3: The PGC-1α Pathway Activity Regulates Astrocytic Resistance to Metabolic Stress

4.3.1 Pharmacological Stimulation of PGC-1α Regulators Influences Metabolic Gene Expression

A second aspect of PGC-1α function is its contribution to the maintenance of metabolic homeostasis. As reported earlier in the present study, PGC-1α activity and expression in astrocyte cultures were responsive to nutrient deprivation. Thus, to characterized PGC-1α metabolic functions in astrocytes, pharmacological stimulation of its upstream activators was again utilized in to investigate downstream effects.

Alterations in metabolic gene expression were first determined in response to pathway activation. AICAR and SRT1720 were applied to primary astrocyte cultures for 6 hours. Subsequently, mRNA levels were analyzed for PGC-1α targets NRF-1 and NRF-2, regulating mitochondrial biogenesis, and VEGF, promoting vascular permeability and angiogenesis. Under AICAR treatment, the upregulation of all three targets were evident, each increased by more than two-fold (Figure 13A). Similarly, SRT1720 caused a significant induction of these genes by about three-fold (Figure 13B). Thus, the activation of upstream PGC-1α regulators is able to promote the expression of its metabolic targets.
Figure 13. PGC-1α-dependent upregulation of metabolic genes by pharmacological activation of PCC-1α regulators. AICAR (A) and SRT1720 (B) treatments led to a significant induction of NRF-1, NRF-2 and VEGF in primary retinal astrocytes. \( n = 4 \). PGC-1α knockdown significantly reduced the upregulation of these genes (C&D). \( n = 3 \). *\( P<0.05 \) and **\( P<0.01 \).
4.3.2 Pharmacological Stimulation of PGC-1α Results in Increased Mitochondria Biogenesis

With the finding that PGC-1α mediates the increased expression of NRF-1 and 2, involved in regulating mitochondria biogenesis, this parameter was examined next. As a measure of mitochondria biogenesis, intracellular levels of the organelle were quantified through fluorescent staining using the MitoTracker® Red CMXRos fluorescent dye.

Under unstressed conditions, primary retinal astrocytes were treated with AICAR or SRT1720 for 18 hours after which mitochondria staining was carried out for analysis by flow cytometry. AICAR treatment did not affect mitochondrial levels, while SRT1720 led to a significant increase of more than 50%, providing evidence for increased mitochondrial biogenesis (Figure 14).

**Figure 14. Pharmacological activation of PGC-1α regulators induced increased mitochondrial biogenesis.** AICAR treatment alone did not affect mitochondria level while SRT1720 induced a 50% increase. Under glucose and serum deprivation (GSD), both AICAR and SRT1720 treatments led to a significant increase in mitochondria level. *n = 3. ***P<0.001.*
Next, primary cultures were subjected to 18 hours of glucose and serum deprivation to determine alterations in the level of mitochondria under metabolic stress. AICAR and SRT1720 were also applied under this condition to assess whether they affect mitochondrial homeostasis in a low energetic condition. Following 18 hours of nutrient deprivation, baseline mitochondrial levels in primary astrocytes were unaltered. However, under this condition, both AICAR and SRT1720 treatment led to a significant increase of mitochondria level by 50% (Figure 14). This suggests that while mitochondria biogenesis can be triggered by SRT1720 alone, its induction by AICAR only occurs in the presence of metabolic stress along with PGC-1α upregulation.

4.3.3 Pharmacological Stimulation of PGC-1α Regulators Improved Cell Viability Under Metabolic Stress

Pharmacological stimulation of PGC-1α activators promoted an increase in intracellular mitochondrial levels. Under metabolic stress, this can potentially improve the efficiency by which energy is supplied through the higher number of mitochondria present. In turn, this can result in a higher cellular resistance to low energy conditions. To characterize whether activation of the PGC-1α pathway provides protection under metabolic stress, astrocyte cultures were evaluated using the cell viability assay following incubation in GSD media. Subsequently, treatment with AICAR and SRT1720 were tested for their ability to improve viability.

Initially, an optimization experiment was carried out to determine the length of time for glucose and serum deprivation that led to a significant reduction of cell viability by more than 50% (Appendix 3). The 48-hour incubation time was selected for subsequent analyses. Three doses of AICAR and SRT1720 were applied to astrocyte cultures similar to oxidative stress experiments. Consistent with previous oxidative stress results, treatment with AICAR at an intermediate dose significantly increased cell viability under glucose and serum deprivation.
(Figure 15A), but at the highest concentration the protective effect of AICAR was abrogated. However, none of the applied doses of SRT1720 were able to improve cell viability under glucose and serum deprivation (Figure 15B). At the highest dosage, cell viability appeared to be even lower than baseline.

In summary, under a metabolic deficiency, AMPK and SIRT1 activation upstream of PGC-1α led to increased mitochondria biogenesis through PGC-1α mediated expression of NRFs. While this translated into improved astrocyte viability with AMPK activation, stimulating SIRT1 did not achieve such protection under nutrient deprivation.
Figure 15. Pharmacological activation of PGC-1α regulators affects astrocytic viability under glucose and serum deprivation (GSD). AICAR treatment at an intermediate dose led to a significant increase in cell viability under GSD (A). This protective effect was abolished at the highest dosage. \( n = 4 \). SRT1720 treatment did not affect cell viability under GSD at all doses applied (B). \( n = 3 \). \(* P < 0.05\).
Chapter 5
Discussion
5 Discussion

Metabolic and oxidative stresses are key contributors to the degenerative loss of RGCs in glaucoma pathogenesis (Chrysostomou et al., 2013). Considering the high metabolic demands of these neurons, and their direct exposure to light, they are particularly vulnerable to a lack of energy, and accumulation of ROS-mediated oxidative damage (Izzotti et al., 2006; Tezel, 2006; Chrysostomou et al., 2013; Yu et al., 2013; Osborne et al., 2014). In particular, the axons of RGCs converge at the ONH, a structurally weak area that is highly vulnerable to biomechanical strain and energetic deprivation (Fechtner and Weinreb, 1994; Bristow et al., 2002). Furthermore, this region lacks myelination, which indicates an absence of efficient saltatory conduction of nerve impulse and necessitates a greater energy supply for electrochemical transmission (Yu et al., 2013). With age, vascular abnormalities and ROS accumulation develop such that the RGCs are put under immense metabolic deficiencies and oxidative insult. When they are unable to counteract such stresses and repair damages, these cells inevitably deteriorate through apoptosis (Almasieh et al., 2012).

Adjacent to the RGCs, astrocytes are crucial for the maintenance of a homeostatic environment that promotes RGC survival (Hernandez et al., 2000, 2008; Garcia and Koke, 2008). They provide support under both metabolic and oxidative stress through the provision of energy substrates and antioxidant precursors, respectively (Dringer et al., 1999; Hirrlinger et al., 2002; Fernandez-Fernandez et al., 2012). Astrocytes are early responders to homeostatic alterations, but tissue injury and age can also elicit altered behaviours that become deleterious (Johnson and Morrison, 2009; Inman et al., 2011). In particular, astrocytes lose their normal metabolic and oxidative capacity and are thus unable to provide protection under these stressed conditions (Johnson et al., 2000; Hernandez et al., 2000, 2008; Malone and Hernandez, 2007;
Johnson and Morrison, 2009). With the recognition that astrocytic dysfunction contributes to RGC loss, targeting the neurosupportive functions of these cells is emerging with increasing efforts aimed at characterizing the molecular mediators and mechanisms underlying astrocyte behaviour in response to pathogenic insults.

In the present studies, the central goal was to identify a mediator of astrocyte metabolic and antioxidant functions that can be targeted to maintain their neuroprotective roles and ultimately improve RGC survival. Based on a recent study carried out in our laboratory, the transcriptional co-activator PGC-1α is crucial for protecting the inner retina under metabolic and oxidative injury (Guo et al., 2014). In the absence of PGC-1α, RGCs exhibited dramatically increased susceptibility to excitotoxic damage to the retina and pathological astrocyte activation. A number of other studies have also characterized PGC-1α as an essential regulator of metabolic and antioxidant genes in the context of neurodegeneration (Puigserver et al., 1998; Wu et al., 1999; Lin et al., 2005; St-Pierre et al., 2006). Considering that these functions closely match with that of astrocytes, I hypothesized that PGC-1α mediates astrocytic stress responses, and can be targeted to improve their resistance against metabolic deficiencies and oxidative insults. In my work, the activity of PGC-1α, its upstream activator AMPK, and downstream target gene expression were determined under conditions of oxidative and metabolic stress. Having established their responsiveness to these conditions, the downstream effector functions of the PGC-1α pathway were further characterized in detail. This was carried out pharmacologically using compounds that activate upstream regulators of PGC-1α, AMPK and SIRT1. Through analyses of gene expression, ROS level, and mitochondrial biogenesis, I found that stimulating this pathway significantly increased astrocytic antioxidant and metabolic capacities. Ultimately, these responses translated into higher astrocyte viability under oxidative and metabolic stress, demonstrating an improved cellular resistance to these challenges.
5.1 The PGC-1α Pathway is Induced in Response to Oxidative and Metabolic Stress in Astrocytes

The role of PGC-1α as a broad transcriptional regulator has been closely studied in context of skeletal muscles, heart, liver, and more recently the CNS. Its activity and expression are responsive to various conditions including exposure to oxidative stressors (St-Pierre et al., 2006), hypoxia (Zhu et al., 2010), fasting (Yoon et al., 2001), and exercise (Baar et al., 2002; Norrobom et al. 2004). While the expression of PGC-1α is enriched in energetically demanding tissues like the heart, it is sometimes expressed at low levels but becomes strongly inducible in other organs such as the liver (Matiello et al., 2010). PGC-1α is involved in hepatic gluconeogenesis and its expression is minimal in the liver under fed conditions to prevent unnecessary glucose output (Yoon et al., 2001). However, in response to fasting, the expression of PGC-1α is significantly elevated to replenish systemic glucose through gluconeogenesis (Yoon et al., 2001; Rhee et al., 2003). In the context of skeletal muscle, both PGC-1α activity and expression can be induced by exercise to increase cellular mitochondrial content to match energetic demands (Goto et al., 2000; Terada et al., 2002; Pilegaard et al., 2003; Akimoto et al., 2005; Wright et al., 2007). Furthermore, oxidative stress elevates PGC-1α expression in neuronal cells, which increases cellular antioxidant enzymes (St-Pierre et al., 2006). These findings highlight the importance of PGC-1α as an inducible factor for the maintenance of metabolic and redox homeostasis.

The role of PGC-1α has not been previously characterized in detail in astrocytes. In the present studies, PGC-1α level was initially determined from whole-cell lysates of astrocyte cultures. Possibly due to due to its low concentration, it was initially difficult to detect through western blot. However, following exposure to both metabolic and oxidative stress, PGC-1α activity and expression were both significantly elevated. In support of these observations, a
recent study reported that the expression of PGC-1α in brain astrocytes is normally low in healthy white matter (Nijland et al. 2014). However, in multiple sclerotic lesions characterized by an increased metabolic demand, reactive astrocytes exhibited significant upregulation of PGC-1α (Nijland et al., 2014). This induction also led to increased expression of glucose and monocarboxylate transporters, which suggested an effort to increase the uptake of energy substrates. In accordance with this metabolic response, I found similar upregulation of PGC-1α-dependent genes involved in mitochondria biogenesis (NRF-1), angiogenesis (VEGF), and antioxidant defense (SOD-1), under conditions of nutrient deprivation and oxidative stress. NRF-1-mediated mitochondria biogenesis would increase the efficiency of energy provision as well as replenish damaged mitochondria due to ROS damage to prevent apoptosis (Wu et al., 1999; Piantadosi et al., 2008). The upregulation of VEGF would induce vascular modulations to restore blood supply to deprived retina (Kubota and Suda, 2009). The antioxidant enzyme SOD-1 would facilitate the buffering of ROS and minimize damages caused by oxidative stress (St-Pierre et al., 2006; Geng et al., 2011). Together, the induction of these genes would allow astrocytes to counteract metabolic deprivation and redox imbalances. These results provide evidence that PGC-1α is an inducible factor in astrocytes that responds to homeostatic alterations in cellular environment. Similar to its hepatic regulation, the level of astrocytic PGC-1α is normally kept low under unstressed conditions to prevent unnecessary gene induction. When encountering metabolic needs and oxidative insults, PGC-1α expression and activity are promoted to counteract those stresses through the regulation of metabolic and antioxidant genes.

In addition to PGC-1α, its upstream regulator AMPK was also responsive to oxidative stress in cultured retinal astrocytes. Upregulation had been similarly reported in primary astrocyte cultures from mouse cerebellum where hydrogen peroxide induced the activation of AMPK (Kuang et al., 2012). Unexpectedly, when the retinal cultures were subjected to nutrient
deprivation, there was no change in AMPK activity. This result was surprising as AMPK is expected to respond to a lack of energy substrates. However, a recent study exposed rat cortical astrocyte cultures to oxygen and glucose deprivation over a time-course and found that AMPK activation peaked at 2 hours, and returned to baseline at 6 hours (Gabryel et al., 2014). Thus, the time point of 6 hours chosen in my study could have missed a rapid regulation of AMPK. This also suggests that the latency to AMPK activation is different in retinal astrocytes in response to different types of cellular insults. Nevertheless, increased PGC-1α activity and downstream gene expression were still evident, which could have resulted from earlier AMPK activation. In summary, retinal astrocytes challenged with metabolic deficiency and redox imbalance converge on increased signalling through PGC-1α, leading to the upregulation of a protective gene expression program.

5.2 PGC-1α Regulates Astrocytic Resistance to Oxidative Stress

Previous studies in neural and cardiac cells demonstrated that increased PGC-1α protein confers protection against oxidative stress while its knockdown reduced the expression of antioxidant enzymes (St-Pierre et al., 2006; Lu et al., 2010; Geng et al., 2011). Based on its upregulation in astrocytes in response to oxidative stress, PGC-1α is therefore potentially targetable to increase their antioxidant capacity.

5.2.1 PGC-1α Regulates Production of Key Antioxidants in Retinal Astrocytes

Through a pharmacological approach, PGC-1α signalling was stimulated using activators of its upstream regulators, AMPK and SIRT1, leading to elevated expression of the antioxidant enzyme SOD-1. The AMPK agonist AICAR had previously been shown to induce SOD-1 expression in lymphocytes (Fehér et al., 1988). Signalling through AMPK/PGC-1α has also been
associated with SOD-1 upregulation in renal mesangial cells (Hong et al., 2014). Similarly, activation of SIRT1 by an agonist led to increased SOD-1 level in renal cells (Huang et al., 2014). In the present study, SOD-1 expression was induced in retinal astrocytes by AMPK and SIRT1 activators, but in each case was subsequently blocked by PGC-1α knockdown. This reflects a PGC-1α-dependent antioxidant mechanism through AMPK and SIRT1 activation, and consolidates that these upstream regulators converge on PGC-1α transcriptional activities to trigger protection against oxidative stress.

The regulation of SOD-2 by PGC-1α has also been established in several cell types such as endothelial and myocardial cells (Valle et al., 2005; Lu et al., 2010). Unexpectedly, SOD-2 did not respond to AICAR or SRT1720 treatments, and was not affected by PGC-1α knockdown. This could be due to differential regulation of this SOD variant in astrocytes. A recent study found that treating ONH astrocytes with an antioxidant coenzyme Q10 reduced the expression of SOD-2 (Noh et al., 2013). With coenzyme Q10 able to buffer ROS, the authors speculated that SOD-2 was downregulated as it was no longer necessary to counteract oxidative stress. The current study suggests that SOD-1 is the main astrocyte enzyme responsive to oxidative stimuli through PGC-1α activity, while SOD-2 levels are constitutive.

GSH is a key astrocyte antioxidant that is crucial for neuronal protection against oxidative stress through provision of GSH precursors (Dringer et al., 1999). GSH-synthesizing enzyme, GCL, catalyzes the rate-limiting step of GSH synthesis and its regulation has not been previously linked to PGC-1α. In vitro, both AMPK and SIRT1 activation upregulated the modifier subunit of the enzyme, GCLM, while PGC-1α knockdown blocked this upregulation. This was supported in vivo as PGC-1α knockout mice exhibited a deficiency in GCLM expression. Therefore, in retinal astrocytes, GCLM represents a novel PGC-1α target that can be
induced by both AMPK and SIRT1 activation. In comparison, the catalytic subunit of GCL, GCLC, was not affected by pharmacologic treatment, or PGC-1α knockdown. This differential regulation of GCL subunits has previously been reported in endothelial cells, where GCLM expression alters in absence of changes in GCLC (Lu et al., 2014). GCLM is the rate-limiting subunit in most tissues and its increased concentration is able to drive GSH synthesis (Chen et al., 2005). In accordance with this finding, GCLM upregulation correlated with elevations in GSH activity in retinal astrocytes following treatments with AMPK and SIRT1 agonists. Therefore, increased signaling through the PGC-1α pathway can confer improved astrocyte antioxidant capacity driven by PGC-1α-mediated induction of GCLM and subsequent increase in GSH. Maintaining GSH is particularly important for astrocytic neuroprotection under oxidative insult. Co-culturing neurons with GSH-deficient astrocytes increased their susceptibility to oxidative stress (Gegg et al., 2005). Conversely, elevated GSH in astrocytes correlated with increased GSH export to neurons (Scheiber and Dringen, 2011). Based on my findings, the induction of GCLM and GSH through PGC-1α in astrocytes can increase GSH provision to potentially improve antioxidant defense of neighboring neurons. To test this, future studies may establish neuron-astrocyte co-cultures to examine if activating PGC-1α in astrocytes confers increased neuronal protection under oxidative stress.

In summary, the regulation of SOD-1 and GCLM suggests that PGC-1α is a broad modulator of oxidative stress response in astrocytes where it can upregulate an antioxidant program through distinct mechanisms. Targeting such a central regulator is ideal since it can maximize the number of molecular mediators recruited to counteract oxidative stressors.
5.2.2 PGC-1α is Crucial for ROS Buffering under Oxidative Insult

St-Pierre et al. (2006) found that in PGC-1α deficient fibroblasts, ROS level is higher under steady-state resting conditions. However, in the present study, when PGC-1α was knocked down in primary retinal astrocytes, ROS level was unaffected under unstressed conditions. It is possible that in astrocytes, PGC-1α is not necessary for regulating the basal level of ROS, which is important for normal physiological signaling (Reczek and Chandel, 2014). Consistent with this finding, a separate study has also found that in myocardial tissue, knocking down PGC-1α did not alter oxidative stress markers under normal conditions (Lu et al., 2010). It would follow that without stressors, knocking PGC-1α down would not affect intracellular conditions dramatically. The significance of PGC-1α-deficiency was revealed when cultures were exposed to oxidative insult, where the elevation in ROS level was exacerbated in comparison with wild-type cultures. This finding highlights the role of PGC-1α as an induced factor that becomes crucial with exposure to cellular stressors.

Based on the regulation of antioxidant enzymes, activating PGC-1α through AMPK and SIRT1 was hypothesized to reduce ROS levels. A previous study reported that AMPK activation decreased ROS level in endothelial cells (Xie et al., 2008). Consistent with this finding, I found that AMPK agonism reduced ROS elevation in primary retinal astrocytes. PGC-1α-dependence of this ROS buffering capacity was then confirmed as knocking down PGC-1α blocked the reduction in ROS. This result suggests that the antioxidant effect associated with AMPK activation is largely dependent on PGC-1α-mediated transcriptional regulation. Surprisingly, activating PGC-1α through SIRT1 did not affect ROS levels despite its induction of antioxidant enzymes. This is contradicted by a previous finding where the SRT1720 agonist reduced ROS production through activating SIRT1 in endothelial cells (Gano et al., 2014). However, in a
separate study that applied SRT1720 to a cancer cell line, ROS level was significantly increased through an unspecified mechanism (Chauhan et al., 2011). It is possible that depending on the cell type and metabolic status, SRT1720 has an off-target effect that elevates intracellular ROS. If this occurs, PGC-1α-mediated upregulation of antioxidant enzymes would be balanced by inadvertent ROS production through another pathway, resulting in the absence of overall antioxidant effect. Nevertheless, PGC-1α-dependent ROS buffering was evident as in its absence, elevation in ROS levels was exacerbated. Therefore, in retinal astrocytes, the PGC-1α axis is crucial in counteracting oxidative insult and promoting its activity can confer improved control of intracellular ROS.

5.2.3 Targeting PGC-1α Pathway Improves Astrocyte Viability under Oxidative Stress

Under AMPK and SIRT1 activation of PGC-1α signaling, increased antioxidant capacity translated into elevated astrocyte viability under oxidative stress. Moreover, combined AMPK and SIRT1 activation further increased viability compared to either alone. This additive effect is consistent with a previous characterization that the distinct AMPK and SIRT1 modifications of PGC-1α together augment its transcriptional activity (Cantó et al., 2009). This improved ability to resist damage can generate prolonged neuroprotection where astrocytes are better able to maintain their supportive functions and minimize damages that trigger cell death. Future investigations can explore the long-term effect of activating PGC-1α in vivo, using, for example, the DBA/2J mouse model of chronic glaucomatous injury. Astrocyte and RGC markers can be evaluated to determine if cell survival can be improved through PGC-1α upregulation.

It should be noted that at the highest level of AMPK and SIRT1 agonists used, the protective effects were abolished. As discussed above, SIRT1 activator SRT1720 may induce ROS production through an unknown mechanism such that high concentrations neutralize any
protective effects by the stimulation of an off-target process. The bell-shaped cell viability curve found in this study for AICAR has also been reported elsewhere (Łabuzek et al., 2010). AICAR-induced AMPK activation can be pro- or anti-apoptotic through the up- or down-regulation of ROS, respectively (Dyck and Lopaschuk, 2006; Kim et al., 2007; Sauer et al., 2011; Lee et al., 2012). For both SIRT1 and AMPK agonists, the concentration used, length of treatment, cell type, cellular energy status, and other coexistent environmental stimuli, are implicated in the resulting effect (Kim et al., 2007; Sauer et al., 2011; Lee et al., 2012). Therefore, in considering their therapeutic potential, the deleterious mechanisms associated with off-targets or overstimulation also need to be investigated. In the context of the PGC-1α pathway, the present study provided support for an antioxidant function downstream of AMPK and SIRT1 activation that protected retinal astrocytes under oxidative stress.

In summary, the PGC-1α pathway involving AMPK and SIRT1 is a targetable mediator of astrocyte antioxidant defense that can be stimulated to increase their antioxidant capacity and improve cell survival.

5.3 PGC-1α Pathway Activity Regulates Astrocytic Resistance to Metabolic Stress

The involvement of PGC-1α in maintaining metabolic homeostasis has been well-characterized across various tissue types. When it was first identified, PGC-1α was described to upregulate nuclear respiratory factors (NRFs) to stimulate mitochondria biogenesis (Wu et al., 1999). Its ability to match energy supply to demand through transcriptional regulation was further expanded to involve the reconstitution of vascular supply in response to skeletal muscle ischemia (Arany et al., 2008; Fraisl et al., 2008). To characterize the metabolic perspective of PGC-1α in retinal astrocytes, downstream genes were analyzed following pathway activation.
5.3.1 PGC-1α Signalling Induces Key Metabolic Genes and Mitochondria Biogenesis in Retinal Astrocytes

Several studies in cell types such as neurons and myocytes have reported that AMPK activation promoted mitochondria biogenesis associated with PGC-1α and NRF upregulations (Kukidome et al., 2006; Fu et al., 2008; Yu and Yang, 2010). Similarly, SIRT1 stimulation has been found to induce this process in muscle and renal cells (Seo et al., 2014; Li et al., 2013; Funk et al., 2010; Khader et al., 2014). In the present study, the NRF transcription factors were upregulated in a PGC-1α-dependent manner in response to AMPK and SIRT1 activation. When subjected to nutrient deprivation, pathway activation resulted in increased mitochondria levels, reflecting their biogenesis associated with PGC-1α-mediated NRF induction. These results suggest that in a low energy status such as ischemic insult, promoting PGC-1α transcriptional activity can induce mitochondria biogenesis to increase the efficiency of energy provision and meet energetic demands. Maintaining this metabolic capacity would allow the preservation of astrocyte function in providing energy substrates to neurons (Hamprecht and Dringen, 1993). In support of this hypothesis, PGC-1α has recently been implicated in the regulation of glucose transporters in astrocytes (Nijland et al., 2014). Future studies may thus investigate if targeting PGC-1α improves the export of metabolic substrates from astrocytes to neurons through coculturing experiments. Furthermore, considering that mitochondrial damage contributes to RGCs degeneration and astrocyte dysfunction (Osborne et al., 2006; Osborne et al., 2008; Lee et al., 2012; Noh et al., 2013), triggering biogenesis through increased PGC-1α may reduce release of reactive byproducts and apoptotic mediators (Osborne et al., 2014). As a result, healthy astrocyte functions can be maintained to slow or prevent RGC loss.

Importantly, vascular regulation by retinal astrocytes through PGC-1α was also evident from this study. AMPK and SIRT1 activation led to a PGC-1α-dependent upregulation of the
angiogenic gene VEGF. The induction of retinal angiogenesis by PGC-1α has previously been established (Ueta et al. 2012; Saint-Geniez et al., 2013). However, the source of VEGF release has not been linked to astrocytes. My findings suggest that retinal astrocytes may be an importance contributor to vasculature regulations through PGC-1α transcriptional control of VEGF. Within the eye, the ability to increase vascular supply to meet energy demands is crucial as retinal vasculature is limited by a requirement for optic transparency (Yu et al., 2013). In glaucoma pathogenesis, decreased blood flow to the retina and reduced proangiogenic activities of ONH astrocytes are evident (Hafez et al., 2000; Hafez et al., 2003; Rudzinski et al., 2008). Therefore, targeting PGC-1α can potentially restore these deficiencies through VEGF upregulation. However, it is important to note that VEGF has also been associated with pathological angiogenesis and increased vascular permeability in the context of neovascular glaucoma and diabetic retinopathy (Zhang et al., 2009; Arden and Sivaprasad, 2012; SooHoo et al., 2013). Dysregulation of VEGF signalling leads to vascular leakage and proinflammatory tissue response that exacerbates retinal damage (Witmer et al., 2003). In contrast, the benefit of upregulating VEGF is attributed to its role as a neurotrophic factor that prevents neuronal apoptosis and promotes their survival (Zachary, 2005). Thus, the implications of PGC-1α-mediated VEGF induction in astrocytes need to be further characterized in vitro and in vivo. Future studies may examine how retinal astrocytes exhibiting increased PGC-1α activity influence the behaviour of vascular endothelial cells. Furthermore, how targeting the pathway remodels retinal vasculature and influences RGC survival in vivo should be investigated. Previous studies have described the proangiogenic effects of AICAR-mediated AMPK activation (Ouchi et al., 2005; Zwetsloot et al., 2008; Ahluwalia and Tarnawski, 2011). Thus, as part of in vivo investigations, AMPK agonists can be tested for its effect on ischemic retinal damages in mouse models of glaucomatous degeneration.
In summary, my findings suggest that retinal astrocytes exhibit several PGC-1α-dependent mechanisms of protection against energy deficiency. Through mitochondrial biogenesis and vascular regulation, astrocytes can maintain their metabolic capacity for neuroprotection under ischemic insult.

5.3.2 Increased PGC-1α Signalling Improves Cell Viability under Metabolic Stress

Similar to my findings under oxidative stress, stimulating PGC-1α signalling through AMPK activation led to increased astrocyte viability under nutrient deprivation. This increased tolerance of metabolic deficiency as a result of AMPK agonism has previously been reported, including characterization of its dependence on PGC-1α transcriptional activity (Spasić et al., 2009; Ting et al., 2010; Xu and Si, 2010; Dasgupta et al., 2012). In contrast, however, SIRT1 activation did not confer any protection. As discussed above, off-target and cellular context-dependent effects may have masked the beneficial outcomes mediated through PGC-1α, especially at higher concentrations of the SIRT1 agonist administered. It should be noted that although in several cell types SIRT1 maintains homeostasis in response to nutrient deficiency (Gerhart-Hines et al., 2007; Kanfi et al., 2008; Ramadori et al., 2008), its role in astrocyte has not been previously characterized. A recent study demonstrated that SIRT1 activation reduced pathological astrocyte reactivity in the context of Alzheimer’s disease (Scuderi et al., 2014). It is likely that SIRT1 agonism can maintain astrocyte integrity, but the underlying mechanism needs to be clarified. Based my finding that SIRT1 activation did not protect astrocytes under metabolic stress, it is also important to investigate any deleterious mechanisms associated with SIRT1 stimulation and how they can be blocked such that the protective effects through the PGC-1α axis can be maximized.
In the context of the current study, AMPK and SIRT1 activation has been highly useful in investigating a PGC-1α-dependent mechanism of retinal astrocyte stress response. With the finding that they improve astrocytic resistance to oxidative and/or metabolic stresses, pharmacological agents can be further applied and investigated in vivo where targeting of the PGC-1α pathway has not been previously sought for protection against RGC neurodegeneration. In addition to a pharmacological approach, PGC-1α upregulation can also be evaluated in vivo using viral-mediated methods to determine whether it can rescue RGC loss (Koerber et al., 2009). In the DBA/2J mouse model of chronic glaucomatous degeneration, our laboratory found a decreasing expression of retinal PGC-1α with age (Guo et al., 2014). It can be speculated that neurodegeneration in these mice is partly due to deficiency of PGC-1α-mediated response. With these considerations, targeting the PGC-1α axis holds high therapeutic potential in restoring astrocyte neuroprotection and improve RGC survival.

5.4 Conclusions

This project aimed to characterize the role of PGC-1α, its regulators and associated downstream genes in retinal astrocytes in response to oxidative and metabolic insults. These stresses occur in glaucomatous degeneration and compromise the neurosupportive functions of retinal astrocytes. Thus, identifying astrocytic pathways that are cytoprotective holds therapeutic potential for increasing neuronal protection. Although previously not investigated in astrocytes, this study revealed that the PGC-1α signaling axis mediates both an antioxidant and metabolic response. Pharmacological stimulation of AMPK and SIRT1 improved astrocyte resistance to both oxidative and metabolic stresses in a PGC-1α dependent manner through increased antioxidant capacity and mitochondria biogenesis, respectively. Future investigations can expand the characterization of these mechanisms and design therapeutic strategies to induce this pathway
in vivo. Based on several cytoprotective functions, targeting this pathway may hold therapeutic promise in preventing RGC neurodegeneration in glaucoma. Targeting PGC-1α signalling in astrocytes may not only rescue neurons, but also extends to increasing the ability of neighbouring glia to counteract insults. A schematic representation of these conclusions is summarized in Figure 16.

**Figure 16. Summary of the PGC-1α pathway and its role in retinal astrocytes.** Under oxidative and metabolic insults or with pharmacological treatments, the PGC-1α pathway is activated to upregulate metabolic and antioxidant genes. This leads to increased astrocytic resistance against stresses through the buffering of ROS and mitochondria biogenesis.


Gegg, M.E., Clark, J.B. & Heales, S.J. Co-culture of neurones with glutathione deficient astrocytes leads to increased neuronal susceptibility to nitric oxide and increased glutamate-cysteine ligase activity. *Brain Res.* **1036**, 1-6 (2005).


Li, G.Y. & Osborne, N.N. Oxidative-induced apoptosis to an immortalized ganglion cell line is caspase independent but involves the activation of poly(ADP-ribose)polymerase and apoptosis-inducing factor. Brain Res. 10, 35-43 (2008).


Appendices

Appendix 1. Confirmation of PGC-1α knockdown through mRNA and protein analysis. (A) Expression level of PGC-1α following 24 hours of transfection with non-targeting (NT) and PGC-1α siRNA. n = 3. (B) Protein level of PGC-1α 48 hours following siRNA transfection.

Appendix 2. Paraquat (PQ) treatment dose-response cell viability curve. PQ at 300 uM was selected for viability experiments following pharmacological treatments. PQ at 100 uM was selected for determination of intracellular ROS level. n = 6.

Appendix 3. Glucose and serum deprivation (GSD) time-course viability determination. 48-hour incubation period was selected for viability assay following pharmacological treatments. n = 6.