Redox mechanisms in retinal cells

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Abstract

Oxidative stress is a key underlying component in the pathogenesis of retinal diseases, including glaucoma, diabetic retinopathy and age related macular degeneration (AMD). Intracellular and extracellular anti-oxidants counteract oxidative stress to prevent tissue damage and subsequent disease. The transcription factor Nrf2 is found in mammalian cells and is a major sensor of redox changes. In response to oxidative stress, it activates numerous intracellular anti-oxidant enzymes and cytoprotective proteins. Exogenous anti-oxidants also utilise this pathway to amplify the anti-oxidant response in cells. In retinal pigment epithelial cells and photoreceptor cells, the Nrf2 pathway has been involved in providing cytoprotection and delaying degeneration within the eye. Mueller cells play a supportive role within the retina, protecting the surrounding cells from oxidative stress. However, the effects of oxidative stress and the Nrf2 pathway have not previously been investigated in retinal Mueller cells. In this study, the effects of a range of pro-oxidants and the dietary anti-oxidant sulforaphane (SFN) on the Nrf2 pathway were investigated in the human Mueller MIO-M1 cell line. None of the pro-oxidants investigated, including hydrogen peroxide, high glucose concentrations, oxygen and glucose deprivation, lipopolysaccharide and TNF-α, activated Nrf2-mediated gene expression in MIO-M1 cells. Hydrogen peroxide however, induced the Nrf2-driven cellular anti-oxidant heme oxygenase-1 (HO-1) in the human ARPE-19 retinal epithelial cell line In contrast, SFN significantly increased HO-1 and NAD(P)H:quinine oxidoreductase 1 (NQO1), but not ferritin or Thioredoxin (Trx1) expression in MIO-M1 cells, which are Nrf2 target genes. SFN also increased Nrf2 protein expression but not Nrf2 *de novo* synthesis in MIO-M1

cells. SFN-induced Nrf2 and HO-1 expression was dose-dependently suppressed by Bisindolylmaleimide I (a protein kinase C inhibitor) and LY294002 (a phosphionositide 3-Kinase (PI3K) inhibitor). These results suggest that Mueller cells may be more resistant to oxidative stress than other retinal cell types and that SFN utilises the Nrf2 pathway to amplify the anti-oxidant response in these cells via PI3K and PKC regulation of Nrf2. In conclusion, the Nrf2 pathway may have a key role in amplifying neuroprotection in the diseased retina through SFN in the Mueller cells.

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AMD	Age related macular degeneration
ARE	Antioxidant regulatory element
ARPE-19	Human Retinal Pigment Epithelial Cell line
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BCP	1-bromo-3-chloropropane
bFGF	basic Fibroblast Growth Factor
BIS I	Bisindolylmaleimide I
BTB	Broad-Complex, Tramtrack and Bric à Brac
CAC	Carbonic anhydrase-C
CAPE	Caffeic acid phenethyl ester
cDNA	complementary DNA
CCL2	Chemokine (C-C motif) ligand 2
CNC Family	Cap and Collar family
CREB	cAMP-response-element binding protein
CTR	C-terminal region
Cul3-Rbx1-E3	Cullin3-Ring-box protien-1-ubiquitin ligase complex
Cys	Cysteine
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DGR	Double Glycine Repeat

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
dPBS	Dulbecco's Phosphate Buffered Saline
DR	Diabetic Retinopathy
ECACC	European Collection of Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinases
FAD(2H)	Favin adenine dinucleotide H_2
FCS	Foetal Calf Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCL	Glutamate-Cysteine Ligase
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter
GS	glutathione synthetase
GSH	Glutathione
GST	Glutathione S-Transferase
GR	Glutathione Reductase
HepG2	Liver Hepatocellular Cells
HIF-1-α	Hypoxia Inducible factor-1-α
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
HO-3	Heme oxygenase-3

H_2O_2	Hydrogen Peroxide
HORCs	Human Organotypic Retinal Cultures
IOP	Intraocular Pressure
IVR	Intervening Region
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
LY	LY 294002
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein Kinase
MCP-1	Monocyte Chemoattractant Protein 1
MIO-M1	Moorfields/Institute of Ophthalmology-Müller 1 cell line
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MMPs	Matrix Metalloproteinases
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger Ribonucleic acid
MTS	Cell Titer 96 AQ_{ueous} One Solution Reagent
Neh	Nrf2-ECH homology
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NTR	N-terminal region
NAD(P)H	Nicotinamide adenine dinucleotide
NMDA	N-methyl D-aspartate
NMDA-R-1	N-methyl D-aspartate Receptor 1
NQO1	NAD(P)H:quinine oxidoreductase 1

NQO2	NAD(P)H:quinine oxidoreductase 2
OGD	Oxygen and Glucose Deprivation
One way ANOVA	One Way Analysis of Variance
p38	p38 Mitogen-Activated Protein Kinases
PES	Phenazine Ethosulfate
PERK	PKR-like Endoplasmic Reticulum Kinase
Pen/Strep	Penicillin and Streptomycin
Pi	Phosphate
PKC	Protein Kinase C
PI3K	Phosphionositide 3-Kinase
POAG	Primary Open Angled Glaucoma
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RGC	Retinal Ganglion Cells
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RPE	Retinal Pigment Epithelial Cell
SEM	Standard Error of the Mean
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SFN	Sulforaphane
sMAF	small Musculoapotneurotic Fibrosarcoma Proteins
siRNA	small interfering Ribonucleic Acid

tBHQ	tert-Butylhydroquinone
TBST	Tris-buffered saline with Tween
THP-1	Human acute monocytic leukemia cell line
TLR4	Toll Like Receptor 4
Trypsin ETDA	Tryspin ethylenediaminetetraacetic acid
Trx1	Thioredoxin
TNFα	Tumour Necrosis Factor- alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor
UV	Ultra Violet

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Chapter 1

Introduction

1.1 Function and the Anatomy of the Eye

The eye is an extremely specialized organ; it intercepts light energy and transforms it into neuronal signals that the brain processes into visual images. Through the eyes, the brain is able to perceive the shapes and colours of the visual world. The human eye is an orbital shaped organ of approximately 2.5 cm in diameter consisting of different layers, as shown in Figure 1.1, which are divided into three sections or tunics: the fibrous coat, the uvea and the neuronal layer. The outermost tunic, the fibrous coat, consists of the cornea and sclera. The cornea is at the anterior of the eye and has a larger curvature compared to the rest of the eye. Its main functions include the refraction of light into the eye, transparency to allow light to enter the eye and the ability to protect the eye from damage and infection. The conjunctiva is a layer of translucent epithelial cells, which covers the cornea, and attaches the eye to the eyelid. The sclera covers the rest of the outer surface of the eye. This is a white fibrous coat made of collagen and elastin fibre, which maintains the shape of the eye and protects the fragile inner eye. The middle tunic is called the uvea or the uveal tract and consists of the choroid, ciliary body and the iris, which are continuous of each other. The majority of the uvea consists of the choroid, which is positioned between the sclera and the inner retinal layer. Its function includes the maintenance and nutrition of the outer layers of the retina and acts as a conduit for blood vessels going to other regions of the eye. The ciliary body divides the eye into the anterior and posterior regions (Forrester et al 2002).

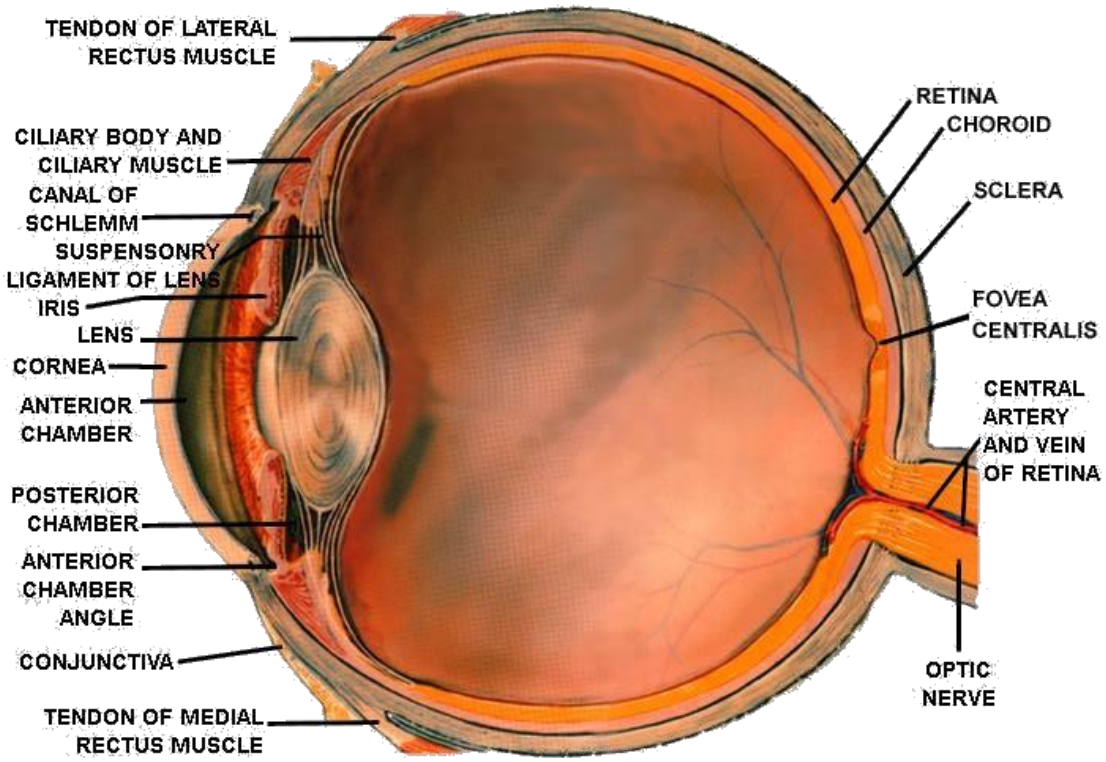

Figure 1.1 Anatomy of the Eye; A labelled cross section of a human eye http://user.it.uu.se/~jonasn/drug_delivery_eye.html accessed 2012

The posterior region of the ciliary body named the pars plana originates from the choroid at the transition region of the ora serrata. This is the flatter region of the ciliary body. The anterior region, known as the pars plicata, extends out into the interior of the eye in a triangular shape (Remmington 2005). This region contains the ciliary processes, which are responsible for the production of the aqueous humour. The aqueous humour is a gel-like viscous liquid. It maintains the eyes intraocular pressure and is contained within in the anterior and posterior chamber of the eye. The aqueous humour is also responsible for providing nutrients and for the removal of waste products of the lens and cornea

in the anterior chamber. In healthy eyes, the majority of aqueous humour is filtered through the trabecular meshwork and exits through the canal of Schlemn, which is drained by the collector channels and leads into veins at the conjunctiva, as demonstrated in Figure 1.2 (Remmington 2005, Grierson 2002).

Adjoining the ciliary body is the iris, which is a thin contracting disc that separates the posterior and anterior chambers. The iris regulates how much light is allowed into the eye by altering the size of the pupil. Directly behind the iris is the lens whose primary function is to focus the light on to the retina. This is held in place by the lens zonules, which are also connected to the ciliary body. Contraction of the ciliary muscle, which forms part of the ciliary body, allows the lens to focus. In the central region of the eye is the vitreous humour, which is attached to the lens, lens zonule, the ciliary body and the retina (Forrester et al 2002).

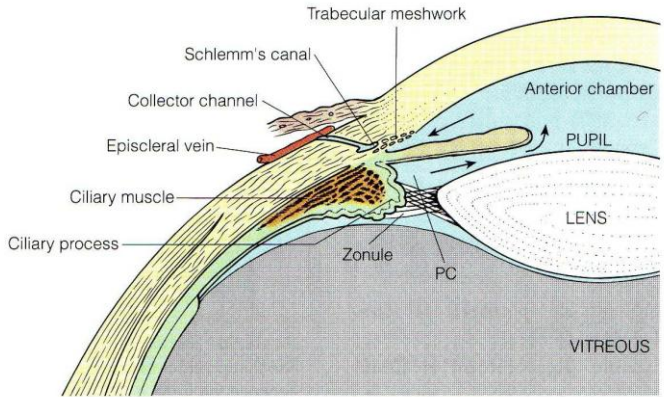

Figure 1.2. The anterior of the eye. The aqueous humour is produced by the ciliary body in the posterior chamber and flows into the anterior chamber. The majority of the aqueous humour exits through the canal of Schlemm which is drained by the collector channels which leads into the episcleral vein. (Forester 2002)

The inner most tunic, known as the fundus, includes the retina and its components. It is continuous with the optic nerve and situated between the choroid and the vitreous (Figure 1.1). Its function is to transform light signals into neural information that the brain can process into visual images via the optic nerve. The retina contains different regions within it, as shown in Figure 1.3. The central retina is a circular region with a diameter of approximately 5-6 mm. Cone photoreceptors and several layers of ganglion cells mainly populate this region (Forrester et al 2002). Within this section is the macular lutea, which has a slight yellow tint due to pigments in this area. There is a higher proportion of cones here than in the peripheral regions. The fovea is in the centre of the macula and is recognisable by having a concaved formed ridge, which consists entirely of cone photoreceptors, it is responsible for sharp central vision (Forester et al 2002).

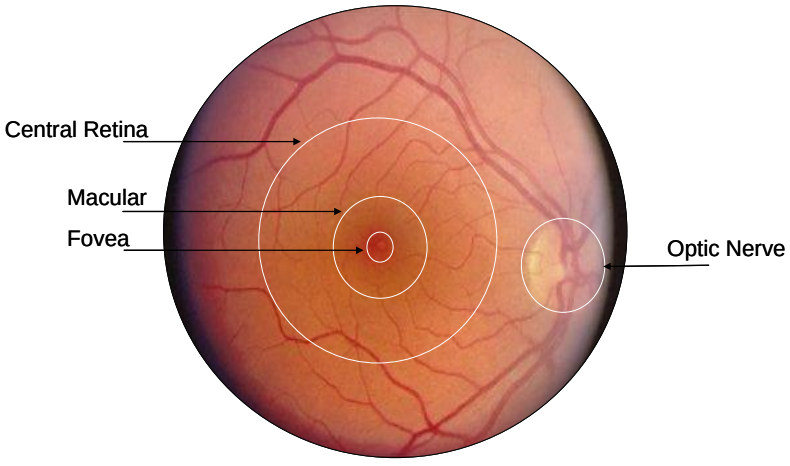

Figure 1.3. The different regions of the retina. The retina has different regions, which have different concentrations of rod and cone photoreceptors. The central retina consists mainly of rod photoreceptors and the macular and fovea has cone photoreceptors. The optic nerve is located towards the naval cavity. Adapted from www.daviddarling.info/images/fovea.jpg

The retina consists of several different transverse layers, which separate into the outer and inner layers of the retina, as shown in Figure 1.4 A. The outermost layer consists of retinal pigment cells, followed by the rods and cones, biopiolar cells, ganglion cells and the retinal neuronal fibre layer (Forrester et al 2002)

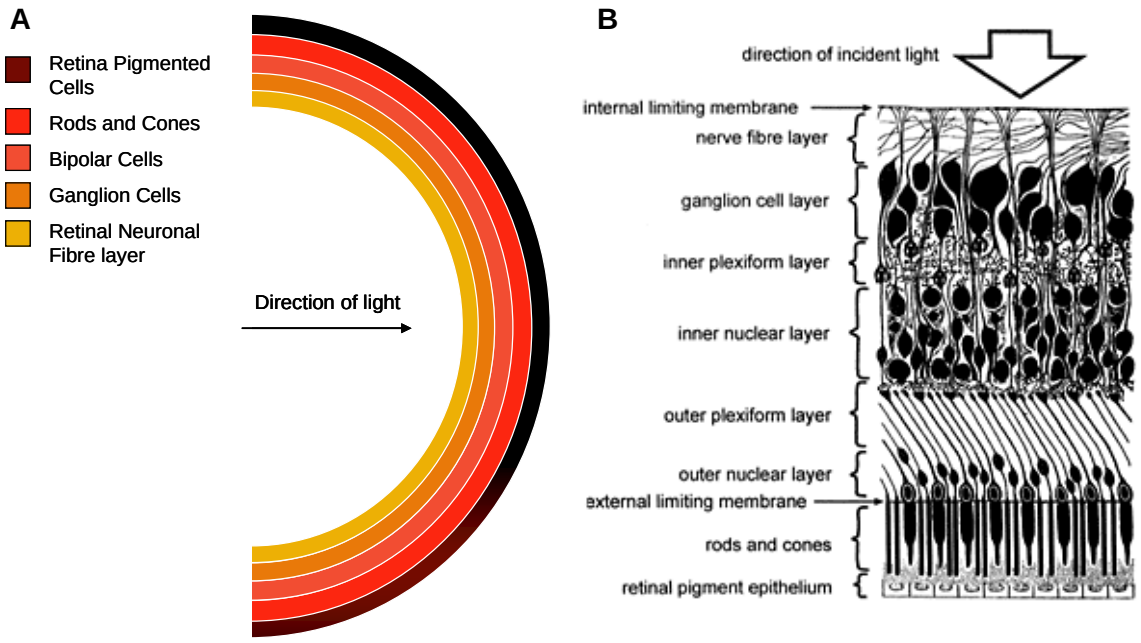

Figure 1.4. The layers of the retina. (A) The orientation of the layers of the retina. (B) A detailed view of the cells in the different layers of the retina (Modified from Brash 1951).

The retinal pigment epithelium (RPE) is not part of the neural retina layer, but plays an important role supporting visual function. It is a highly organised hexagonal monolayer of cells with a lower regeneration rate than other retinal cells that have a multitude of functions including maintaining the attachment of the retina. The RPE is a selective permeable barrier between the retina and the choroid (Forrester et al 2002). It aids the phagocytosis of rod photoreceptors and increases the resolution capacity of the eye by the absorption of light, which reduces the occurrence of light scatter. The RPE also plays a major role in the storage and transport of metabolites required by the eye (Forrester et al 2002, Dunn et al 1996).

The adjacent cell layer contains the photoreceptors, the rods and cones, which transduce photons of light, into neural signals by the process of phototransduction. Rods sense contrast, black and white, brightness and motions but without fine detail (Forrester et al 2002, Grierson 2002). Three different types of cones are involved in transforming colour images. They give high spatial resolution and sharpness. There are approximately 125 million rods and less than 7 million cones within each eye (Grierson 2002). The signal is then transferred to the bipolar cells, whose cell bodies reside within the inner nuclear layer. The primary function of the biopolar cells is to take information from the photoreceptors to the ganglion cells (Forester et al 2002).

Ganglion cells transmit the signal from the eye to the brain via the optic nerve. They form the innermost nucleated layer of the retina. The retinal ganglion cells (RGC) axon bundles are separated and are ensheathed by glial cells. These bundles form the optic nerve and synapse with the lateral

genicalate nucleus of the thalamus within the brain. Within the brain the ganglion cells are myelinated by oligodendrocytes. There are approximately 1.2 million ganglion cells per eye. Theoretically, this suggests that there are 100 rods per ganglion cell and 4-6 cones per ganglion cell (Forrester et al 2002). The number of layers of ganglion cell bodies varies within the retina. In the centre of the retina, there can be up to seven layers but on the peripheral retina, there can be only one layer. There are also several different subtypes of ganglion cells. In mammals there are 25 different types, with 18 found in human retinas (Forrester et al 2002). Ganglion cells are protected and supported by glial cells. There are two different types of glial cells; Mueller cells and astrocytes. Mueller cells provide structural support and are found throughout the retina with their nuclei located in the middle of the inner nuclear layer (Grierson 2002, Limb et al 2002). Astrocytes are associated with the small blood vessels and their regulation in the inner retina (Grierson 2002).

1.1.1 Mueller Cells

There are approximately 8 to 10 million Mueller cells within each human retina. They have many roles in the retina, for example, they provide structural support as they span all of the retinal layers (Bringmann et al 2006). In addition, they interact and provide parental support to other retinal cell types, for example, the feet of the cells are in direct contact with the retinal ganglion cells, and surround a column of neurons (Bringmann et al 2009a). They play a vital role in enabling neurons to maintain a healthy synaptic function, by providing them with trophic substances and removing their metabolic waste (Limb and

Jayaram 2010). Mueller cells also guide light to the photoreceptors and buffer mechanical deformations within the tissue. In addition, they play a vital role in regulating the volume, water and ion homeostasis of the extracellular space, and maintaining the inner blood retinal barrier (Bringmann et al 2009a).

Mueller cells remove excess toxic extracellular glutamate from the inner retinal tissue by the Glutamate Aspartate Transporter (GLAST). If this process malfunctions it can lead to the apoptosis of retinal ganglion and photoreceptor cells and this can result in retinal diseases including glaucoma and diabetic retinopathy (Harada et al 2007, Bringmann et al 2009b). Mueller cells regulate excess extracellular potassium within the retina through the potassium channel sub family known as Kir channels, especially via Kir4.1 and Kir2 (Bringmann et al 2003). These channels can decrease in function as a person ages and can be one of many factors leading to individuals becoming more susceptible to age-related retinal diseases (Bringmann et al 2003).

Mueller cells can be activated by a large and varied range of pathogenic stimuli, including, anoxia, hypoglycaemia and ischemia (Bringmann et al 2006). Upon activation, Mueller cells have a dual action that is yet to be fully understood, as they can be both neuroprotective and also contribute to neuronal degeneration; this activation is known as gliosis (Bringmann et al 2009a). This is a physiological mechanism designed to protect the retina from receiving more damage and promote tissue repair without tissue remodelling. Gliosis involves biochemical, physiological and morphological changes depending on the type and seriousness of the damage and/or insult (Bringmann et al 2006). A major and measurable sign of gliosis activation is the upregulation of glial fibrillary acidic protein (GFAP) and vimentin, which are intermediate

filaments and identifiable markers of Mueller cells. Mueller cells that under gliosis activation can proliferate, de-differentiate and increase in volume (Bringmann et al 2009a)

Mueller cells also aid in the modulation of inflammatory and immune responses. They release cytotoxic factors when the retina is subjected to Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), which recruits phagocytic cells of myeloid origin including microglial cells, monocytes and macrophages to the damaged area. This leads to the release of reactive oxygen species and cytokines, causing death to surrounding retinal cells (Nakazawa et al 2007). Upon activation Mueller cells also release the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) a hypoxia induced factor which stimulates the growth of new blood vessels (Tezel et al 2001, Wang et al 2010). These and other vascular factors play a role in several neurodegenerative eye diseases, including a form of age-related macular degeneration (AMD) and diabetic retinopathy (DR). Mueller cells may contribute to the development of abnormal vascular physiologies in diseased states (Wang et al 2010).

When the retina is in a diseased or damaged state Mueller cells also protect neuronal cells from cell death by secreting neurotrophic growth factors and antioxidants such as basic fibroblast growth factor (bFGF) and glutathione, respectively. This dual role of being protective and toxic is yet to be fully understood, but it may be related to the intensity of gliosis activation, with a higher activation of gliosis being more likely to result in neuronal degradation

compared to low levels of activation of gliosis being associated with cytoprotection (Bringmann et al 2006, Bringmann et al 2009a).

Mueller cells have also been reported to have stem cell like properties, which are dormant until the retina is subjected to injury, for example retinal detachment (Limb and Jayaram 2010). When this occurs, a population or sub-population of Mueller cells de-differentiate into retinal progenitor cells, which express neuronal and photoreceptor proteins. This is a relatively new area of research which is focusing on using this population for potential therapeutic regeneration of damaged retinas (Lawrence et al 2007). The majority of research conducted into Mueller cells thus far has been restricted to animal models and cultured Mueller cells; there have been no reports to date on human Mueller cells *in situ*.

1.2 Diseases of the Eye

Several different chronic diseases affect the eye; many, if left untreated will lead to a level of blindness. Most chronic eye disorders are more prevalent in the elderly and as the UK have an increasing aging population, more people will be affected. Treatments are limited and therefore an understanding of the pathologies of these diseases is required to identify new therapeutic targets.

1.2.1 Cataracts

The major cause of blindness worldwide is cataracts (Pascolini and Mariotti 2012). Cataracts can occur naturally as a part of the aging process, leaving the

lens with a yellow tinge or a cloudy mass, which can severely impair an individual's vision. In the UK, more than 50% of individuals over 65 have some form of cataract in one or both eyes (NHS Choices 2012). The lens becomes opaque, which blocks light from reaching the retina. Other types of cataracts can also occur, including; paediatric cataracts where an infant is born with the condition, diabetes related cataracts and UV damage related cataracts, where oxidative stress is a factor. In modern medicine, surgical removal of the cataract and the insertion of a new lens into the eye is a simple and quick procedure, which allows the patient's sight to be restored. However, this can still leave post operational complications including retinal detachments and posterior capsular bag tears (Varma et al 2011, Grierson 2002).

1.2.2 Age related macular degeneration

Age related macular degeneration (AMD) is a group of diseases which affect the macular region of the retina, resulting in the gradual loss of colour and central vision. The macula has a high concentration of cones, which are responsible for the colour and sharpness of images. As this disease progresses, the layers underneath the retina, including the RPE layer, are affected (Ambati and Fowler 2012). AMD can be early or late stage depending on the amount of damage that has occurred in the macular region. An individual's reading ability can be affected as the central vision is partially or completely lost. Objects that normally appear straight can be interpreted to be wavy by the patient. AMD is more prevalent in older people, with approximately 30% of the UK population aged over 75 having early symptoms of AMD and 7%

with more severe symptoms. In 2011, approximately 239,000 individuals in the UK suffered visual impairment caused by AMD (NHS Choices 2012). It is more common in women compared to men, possibly due to women generally having a longer lifespan than men (Evans et al 2004). Many factors have been associated with the aetiology of AMD, including oxidative stress, smoking, UV light, high blood cholesterol levels, high blood pressure, obesity and genetic makeup (Liang and Godley 2003, Bertram et al 2009, Cano et al 2010).

There are two main types of AMD, dry and wet AMD. Dry AMD affects eyes gradually, over many years, and accounts for 90% of all AMD cases and there is no current treatment available. In dry AMD there is atrophy of the RPE layer with cellular debris developing known as drusens. Wet macular degeneration can develop more rapidly and is more serious than dry AMD. Within two years of diagnosis, 70% of patients with wet AMD have lost a significant amount of their central vision. In wet AMD there is growth of abnormal blood vessels which leak blood and fluid into the retina, this is known as choroidal neovascularisation. Unlike dry AMD, there are several treatments available to treat wet AMD, which can slow down the rate of degeneration and in some cases restore some of the lost sight. However, the effectiveness of the treatments relies on them being given within the early stages of disease progression. VEGF is a cytokine released by Mueller and other cells, which stimulates the formation of new blood vessels. Recent therapies have targeted VEGF using antibodies (Witmer et al 2003). These include Macugen, Avastin and Lucentis and are aimed at reducing the vascular effects seen in AMD (NHS Choices 2012). Laser Surgery, also known as photodynamic therapy, is another treatment option for wet AMD. This involves injecting the patient intravenously

with a dye that collects in the newly forming abnormal blood vessels within the retina and then using a low power cold laser to seal off the highlighted blood vessels. Hot lasers have also been used, however, this is not preferred as it can cause damage to the surrounding healthy retinal tissue (Ambati et al 2012).

1.2.3 Glaucoma

Glaucoma is a group of progressive neurodegenerative diseases of the optic nerve that result in apoptosis of the RGC. This leads to a reduction in the visual field and a specific pattern of irreversible damage to the optic nerve (Weinreb and Khaw 2004, Ritch 2007). The majority of individuals affected are older people. Opticians will actively test and monitor for signs of the disease in anyone over the age of 40. By 2020 it is predicted that 79.6 million people worldwide will be affected by a type of glaucoma. It is more common in individuals of Black-African or Black-Caribbean origins (Quigley and Broman 2006). It is often asymptomatic to the individual as peripheral vision is first affected and it is not until the central vision diminishes that the patient seeks professional help. Visual field tests and visual images of the retina often allow for a confirmation of the diagnosis by measuring the cup ratio of the retina. The cup enlarges due to the thinning of the neuroretinal rim and excavation of the optic nerve heads (Weinreb 2007). If the cup ratio is above 0.7 it is indicative of glaucoma. Several factors are thought to play a role in the aetiology of glaucoma, including raised intraocular pressure (IOP), oxidative stress, age, and genetics (Weinreb and Khaw 2004).

Primary Open Angled Glaucoma (POAG) is the most common type of glaucoma. POAG progresses slowly, often without noticeable symptoms, resulting in a delayed diagnosis. Over 58 million people worldwide are predicted to become affected by POAG by 2020 and approximately 480,000 individuals in England are currently affected (NHS Choices Quigley, 2006). In Caucasian Europeans 1 in 50 aged above 40 years old and 1 in 10 above 75 years are affected 2012 (Quigley and Broman 2006). POAG is more prevalent in individuals of African and Latin American origins. It also occurs more in women than in men, like AMD this may be due to women living for longer (Quigley and Broman 2006). In this type of the disease, the angle between the iris and cornea, which is important in aqueous humour drainage, is open as normal, meaning the IOP does not increase rapidly, as in acute closure glaucoma, but gradually. All current treatments of POAG are focused on reducing the IOP with a combination of medical and surgical approaches (Weinreb 2004).

There are a range of different eye drops, which work through different mechanisms that reduce IOP. Beta-blockers reduce IOP by reducing the rate of production of aqueous humour by the ciliary body. Prostaglandin analogues reduce IOP by increasing the outflow of the aqueous humour via the uveoscleral pathway. However, these drugs can often result in loss of pigmentation from the iris. Drugs that target adrenoreceptors, including dipovefrine and brimonidine reduce IOP by reducing the production rate of the aqueous humour as well as increasing its outflow. Carbonic anhydrase inhibitors like acetzolamide, brizolamide, and dorzolamide reduce IOP by reducing the aqueous humour production (Mincione et al 2008).

There are different surgical and laser treatments available including trabeculectomy. In this procedure an alternative route for the aqueous humour is created to increase its outflow. Often surgery has to be repeated due to the eye's ability to heal. Anti-wound healing drugs are used to prevent the trabeculectomy from healing. Even if the medication and surgery are successful in reducing the IOP, it can often increase later. Furthermore once the IOP has been brought into the normal range, glaucoma can continue to progress. This combined with the fact that glaucoma can occur with patients with normal IOP and the limited success rates of the current treatments available suggests that there is a need for research to be done into other potential mechanisms of glaucoma, resulting in better treatments for patients (Weinreb 2007; Vasudevan et al 2011).

In acute angle closure glaucoma the angle between the iris and cornea is narrowed, which induces a sudden increase in intraocular pressure (IOP). This causes the eye to become painful and red, resulting in damage to the RGCs. It is less common than POAG, affecting 1 in 1000 white individuals and 1 in 100 individuals from Asian origins in the UK (NHS Choices 2012). Immediate medical attention is required to prevent permanent loss of sight. Medication can be administered as a temporary solution until an iridectomy can be performed. This laser surgery results in a direct flow of aqueous humour being created between the posterior and anterior chambers (Grierson 2002).

Other forms of glaucoma include secondary glaucoma, a result of other eye conditions that can cause a rise in the IOP within the eye. This can lead to damage in the retina that ultimately results in glaucoma. Causes include a wide range of diseases including cataracts and uveitis. Congenital glaucoma occurs

when an infant has inherited the disease. Normal Pressure Glaucoma (open angled) is where the IOP is not elevated but cupping and loss of sight occurs (Harada et al 2007).

1.2.4 Diabetic Retinopathy

Diabetic retinopathy is a vascular disease of the retina which affects both eyes of an affected individual. Chronic high glucose concentrations and glycated proteins and oxidative stress cause abnormal changes in the microvessels of the retina, causing swelling and leaking eventually resulting in blindness (Crosby-Nwaobi et al 2012). There are four different stages in diabetic retinopathy, all of which can appear symptomless to the sufferer; these are mild, moderate, severe non-proliferative and severe proliferative retinopathy. In mild non-proliferative retinopathy, microaneurysms occur in the small blood vessels of the retina. The disease progresses when some of the blood vessels become blocked. In severe non-proliferative retinopathy several blood vessels are blocked, disrupting the blood flow to multiple areas of the retina. At this stage the retina tries to compensate by growing new blood vessels in the affected areas. This is a symptom of the final stage of the disease, proliferative retinopathy. The new blood vessels which grow along the surface of the retina and vitreous are always abnormal, weak and fragile. This leads to them leaking blood which causes the deterioration in vision and potentially blindness (Grierson 2002). If one of the blood vessels leaks in the area of the macular swelling, blurred vision can occur and result in macular

oedema. Whilst this can occur at any stage of this disease, it has a higher probability of occurring during the latter stages (Hatef et al 2011).

Individuals with either type 1 or type 2 diabetes are at risk of developing diabetic retinopathy, with the risk increasing the longer the individual suffers with from the disease. Gestational diabetes also increases the risk of developing this condition (Golbert and Campos 2008).

Regular eye examinations are required for individuals who have diabetes, as this can significantly reduce the risk of blindness due to the proper treatment and care being administered. No treatment is given for the first three stages of the disease. However, surgical procedures are used in macular oedema and proliferative retinopathy. The key to slowing down the progression of the first three stages of this disease is by managing the individuals blood pressure, blood glucose, and cholesterol levels (Forrester et al 2002). Poor management leads to a more rapid progression of the disease. Proliferative retinopathy is treated by scatter laser surgery, which helps to shrink the abnormal blood vessels by placing between 1000-2000 laser hits to the para macular and peripheral retina over two to three treatments. The risk of this treatment is a loss of peripheral, colour and night vision and this treatment is only effective if administered before multiple bleeds occur. If several bleeds have occurred, a vitrectomy may be performed to remove the excess blood from the eye (Grierson 2002). Macular oedema is also treated by laser surgery, with the laser treatment given in one session burning around the macular region, which reduces bleeds and excess fluid and blood within the retina. This procedure has a high success rate in reducing the loss of an individual's sight.

Drugs that may be used in treating macular oedema include Lucentis, Avastin, Aylea and steroid injections (Hatef et al 2001)

All of the ocular diseases described above occur more commonly in older people. One of the common factors in the pathogenesis of these diseases is oxidative stress. The eye is susceptible to oxidative stress, which is counterbalanced by anti-oxidants. During the aging process, it is thought that the ability to counteract oxidative stress diminishes. Without a defence against oxidative stress, the eye is more susceptible to neurodegenerative diseases which ultimately will lead to blindness. Understanding the role of oxidative stress within the eye and the mechanism that the eye has evolved to counteract oxidative stress is imperative to be able to enhance protection for eyes in a diseased state (Liang and Godley 2003; Jarrett and Boulton 2012).

1.4 Oxidative stress

Oxidative stress is caused by the production of reactive oxygen species (ROS). These are molecules that contain oxygen and are highly reactive. They can either be a free radical, where the species in question has one or more unpaired electrons, an oxidising reagent like hydrogen peroxide, or a species in a higher energy level like singlet oxygen (Bell et al 2011). They occur naturally within the body due to oxygen metabolism, cell respiration, cell signalling and other homeostatic processes. ROS can be produced in all cells, particularly in the mitochondria, leading to damaged DNA. High levels of oxidative stress can lead to the DNA, proteins and lipids of cells being damaged. However, there is a mechanism in place to counteract the effects of oxidative stress. Antioxidants are able to scavenge free radicals, decrease localised oxygen and through

different pathways inhibit oxidative stress. These antioxidants can are found in the diet and include vitamins C, E and flavonioids within the body such as anti-oxidant enzymes that are found in all cells (Eastwood 1999).

Several environmental factors can increase the levels of ROS in the eye, leading to oxidative stress and tissue damage. Since the eye is directly exposed to the environment, it is unsurprising that ROS cause damage in the eye and play a role in the pathogenesis of eye diseases (Sacca and Izzotti 2008, Varma et al 2011, Zhao et al 2011). In addition to ocular diseases, oxidative stress is associated with the pathogenesis of many other chronic age-related disorders including cardiovascular diseases, cancer, osteoporosis, arthritis and neurodegenerative diseases (Kim et al 2010).

The eye has one of the highest consumption rates of oxygen within the human body (Yu and Cringle 2005). The retina has a very high metabolic rate, the highest levels being in the RPE and the photoreceptors, which have a correlating high blood supply from the choroid. The inner retina has a slightly lower metabolic rate compared to the outer retina and is supplied by the retinal circulation (Forrester et al 2002). It is an organ where its function causes a high production of ROS, especially in the lens and in the fundus. This is due to the eye being exposed to UV and visual light exposure and because of the metabolism of polyunsaturated fatty acids contained within photoreceptors. When this is increased by environmental and external influences, it can lead to a state of oxidative stress and the resulting damage can be a contributing factor to eye diseases (Varma et al 2011).

1.3.1 Oxidative Stress stimuli and the eye.

Many different factors that play a role in the pathogenesis of eye diseases induce oxidative stress in the retina. These include increased intraocular pressure, glutamate, high glucose concentrations, advanced glycation end products, oxygen glucose deprivation (OGD)/ ischemic reperfusion, hydrogen peroxide, TNFα and lipopolysaccharide (LPS) (Flammer et al 2002, Gülgün 2008, Bordone et al 2012).

TNF-α is a pro-inflammatory cytokine that increases in neurons following ischemic attacks within the brain and therefore has been associated with neurodegenerative retinal diseases. TNF-α has been linked to the axonal degeneration and glial changes observed in the optic nerves of patients with AIDS (Scholz et al 2003). It also causes RGC death in glaucomatous retinas. TNF-α and TNF-α receptor 1 have found to be more prevalent in glaucomatous optic nerve heads in human donor eyes (Yan et al 2000). TNF-α induces apoptotic cell death through the TNF-α receptor -1 in a caspase mediated pathway. In primary co cultures with RGC and glial cells ischemia and increased pressure caused glial cells to release TNF-α, inducing RGC death, which was reduced by a TNF-α inhibitor (Fuchs et al 2005, Gülgün 2008, Tezel 2008).

LPS is an endotoxin from the outer membrane of gram negative bacteria. In the eye LPS plays a central role in the pathogenesis of uveitis (Kalariya et al 2012). In humans, LPS binds with LPS-binding protein, CD14 and the toll like receptor 4 (TLR4). LPS triggers a cascade of pro-inflammatory cytokines and other factors that result in an inflammatory response (Guha and Mackman

2001). LPS also induces ROS, resulting in oxidative stress in cells (Rushworth et al 2008).

Glutamate is the primary excitatory neurotransmitter in many retinal cell types including photoreceptors, biopolar and ganglion cells (Thoreson and Witkovsky 1999). However excessive levels of glutamate have been linked with neuronal and retinal ganglion cell death in vivo and in vitro. In 1957 Lucas et al first reported the toxic effect of high glutamate on the mammalian eye (Lucas 1957). Toxic levels of glutamate have been linked to several retinal diseases including glaucoma and diabetic retinopathy (Naskar et al. 2000). Young mice injected with glutamate demonstrated destruction of the inner retinal layers including the RGC. Injections of glutamate into adult albino rats also resulted in the degradation of ganglion and inner nuclear layers. An over stimulation of the glutamate receptor *N*-methyl-D-aspartate (NMDA) has been associated with causing increased intracellular calcium levels, leading to apoptosis. Non NMDA receptors have also been associated with glutamate neural degeneration within the retina. It is still unclear what leads to glutamate levels increasing to toxic levels in the retina; it may be an over load or a failure in the retinas glutamate reuptake and transporter (GLAST) mechanisms, which are still not fully understood. It is possible that that when RGC die they release intracellular concentrations of glutamate into the surrounding area, causing a cascade of glutamate toxicity. Drugs like memantine, an NMDA inhibitor, have been shown to inhibit the toxic effects of glutamate (Mansoor et al 2010).

Oxygen and glucose deprivation (OGD) with reperfusion is another stimulus that induces ROS and this has been carried out experimentally in vitro and in vivo (Tanaka et al 2007, Danilov et al 2009, Wang et al 2012). Mueller

cells may provide protection in OGD reperfusion episodes; these cells may be able to resist the effect of OGD insults more than other cell types in the retina, such as RGC (Bringmann et al 2009a). Glucose is required for the normal function of the retina and the majority of it is sourced from blood. The abnormal glucose levels caused by diabetes affect the eyes' ability to function. High blood glucose concentrations (hyperglycemia) are associated with the progression of the vascular component of diabetic retinopathy and cause neuronal degeneration within the retina. The biochemical changes that are induced by hyperglycemia result in higher levels of oxidative stress. In Mueller cells high glucose can stimulate the production of matrix metalloproteinases (MMPs), a family of proteins which can degrade the extra cellar matrix (Miyata et al 2012).

As mentioned above oxidative stress and the production of ROS are counteracted by anti-oxidants, which may be exogenous, (such as those derived from diet), or endogenous, which are produced by every cell type in the body. The transcription factor NF-E2 related factor 2 (Nrf2) is a major sensor of oxidative stress in cells and responds by activating cellular anti-oxidants to counteract cell damage. Both oxidative stimuli and extracellular anti-oxidants can activate this pathway to induce or amplify cytoprotective and anti-oxidative responses in cells. Nrf2 is thought to play a key role in protecting the eye and retinal cells from oxidative stress-associated damage (Yanagawa et al 2005).

1.4 The Nrf2 pathway

Nrf2 is a widely conserved protein across the animal kingdom with homologues found in nematodes, flies, fish and mammals. It is a 605 amino

acid protein that belongs to the Cap'N' Collar (CNC) family (Moi et al 1994). This CNC structure allows binding to small musculoapotneurotic fibrosarcoma proteins (sMAF) and to the antioxidant response element (ARE), an enhancer element in the regulatory regions of anti-oxidants and other cytoprotective proteins. Other mammalian family members include p45, NF-E2, Nrf1, Nrf3, Bach1 and Bach2 (Motohashi and Yamamoto 2004). Most of these enhance transcription when bound to the regulatory regions of target genes. However, Bach1 and Bach2 lack transcriptional activation domains and repress transcription (Oyake et al 1996)

Numerous pro-oxidative stimuli have been reported to activate the Nrf2 pathway, including heavy metals and toxins, pro-inflammatory stimuli, ischemia reperfusion, hypoxia, LPS and TNF-α (Tanaka et al 2007, White et al 2010, Sethy et al 2011). In addition, anti-oxidants utilize the pathway to amplify their anti-oxidant response within cells (Rushworth et al 2011).

Nrf2 regulates hundreds of genes that play a protective role in the cell (Lee and Johnson 2004). These include antioxidant enzymes such as catalase, superoxide dismutase, and heme oxygenase-1 (HO-1), metal-binding proteins including ferritin and metallothioneins, NADPH regenerating enzymes, Phase II detoxification enzymes including NAD(P)H:quinine oxidoreductase 1 (NQO1) and NAD(P)H:quinine oxidoreductase 2 (NQO2), glutathione-regulating enzymes Thioredoxin (Trx1) and Thioredoxin Reductase-1. Nrf2 also regulates itself, which may allow for more expression of Nrf2 once the pathway is activated (Johnson et al 2004, Tanito et al 2007).

The exact regulation of the Nrf2 pathway is not fully understood. Whilst the basic principles of the pathway are shown in Figure 1.6, certain aspects are still

under research. Nrf2 is inactive when bound to a homodimer of Keap1, a cytoplasmic inhibitor of Nrf2. Upon activation by oxidative stress or other stimuli, the Keap1/Nrf2 interaction is disrupted, resulting in Nrf2 moving into the nucleus and binding to a specific sequence on target genes. All of the genes regulated by this pathway have a 5'-flanking cis acting regulatory element sequence known as the antioxidant regulatory element (ARE) (Johnson et al 2004, Motohashi and Yamamoto 2004). The core ARE sequence has been identified as 5'TGACnnnGCA-3 and was first found in the rat glutathione S-transferase Yα subunit gene (Rushmore and Pickett 1990, Rushmore et al 1991) Other target genes include antioxidants, Phase II enzymes and other cytoprotective proteins that are then upregulated, which aids the protection of the cell. The Nrf2 pathway has been found to be upregulated and associated with several long-term chronic diseases, which include glaucoma (POAG) (Motohashi and Yamamoto 2004, Vargas et al 2008, Nguyen et al 2009).

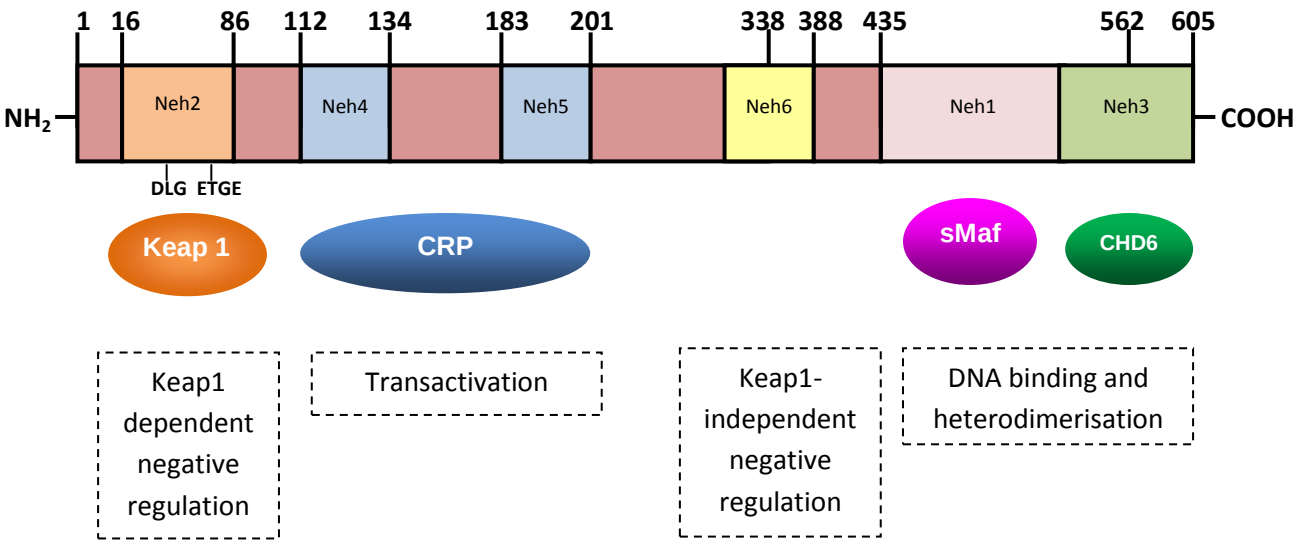

Figure 1.5 The Nrf2 domains and their interactions. There are 6 functional domains of Nrf2 depicted as Nrf2-ECH homology (Neh).

Nrf2 has six different functional domains known as Nrf2-ECH homology domains (Neh 1-6) Neh1 has a basic region-leucine zipper motif and a heterodimerisation domain that interacts with the small Mafs and binds to DNA as a hetreodimer. Neh2 interacts with Keap1 and is the negative regulatory domain (Itoh et al 1999). Currently there are several different theories on the exact interaction between these two proteins. However, Neh2 does contain two motifs where Keap1 heterodimers are known to interact. These are known as DLG and ETG (Tong et al 2006). Neh3 binds to the chromo-ATPase/helicase DNA binding protein family member CHD6, which is a transcriptional co-activator that promotes transcription of ARE-dependant genes (Nioi et al 2005). Neh4 and Neh5 together bind the transcription co-activator CBP. CBP is a cAMP-response-element binding protein (CREB) binding protein (Katoh et al 2001). The Neh6 domain controls the Keap1-dependent negative regulation of Nrf2 (McMahon 2004).

Nrf2 is tightly regulated in cells to enable the pathway to respond rapidly to oxidative stress. Levels of free Nrf2 are regulated by Keap1, which continually targets it for degradation by the 26S proteasome (Zhang 2006). Kelch-like ECH-associated protein 1 (Keap1) is an actin-binding cytoplasmic protein (Itoh et al 1999). Keap1 covers a cysteine rich sequence on Nrf2, which is then degraded by ubiquitination. Bound to Keap1 is the Cullin3-Ring-box protien-1-ubiquitin ligase complex (Cul3-Rbx1-E3) complex which helps target Nrf2 for ubiquitination degradation (Zhang et al 2004, Kaspar et al 2009). Keap1 was identified as the main negative regulatory protein for Nrf2 by in vivo data from keap1-deficient mice. Without it mice produced unregulated amounts of NQO1 and had major physiological problems and died a few days after birth

(Wakabayashi et al 2003). The domains that have been identified in Keap1 are the N-terminal region (NTR), the Broad-Complex, Tramtrack and Bric à Brac (BTB) and the Intervening Region (IVR), the double glycine repeat (DGR), which is also known as the Kelch domain and the C-terminal region (CTR) (Figure 1.6). The functions of these domains are still subject to much research. The BTB domain has been shown to form homodimers and heterodimers. The Kelch domain is named after the *Drosophila* egg-chamber regulatory protein Kelch and it interacts with the Neh2 domain of Nrf2 in the cytoplasm and is also attached to the actin cytoskeleton. The Kelch domain consists of 6 repeats of the Kelch motif which forms a 6 bladed β-propeller (Li et al 2004, Tong et al 2006, Baird and Dinkova-Kostova 2011).

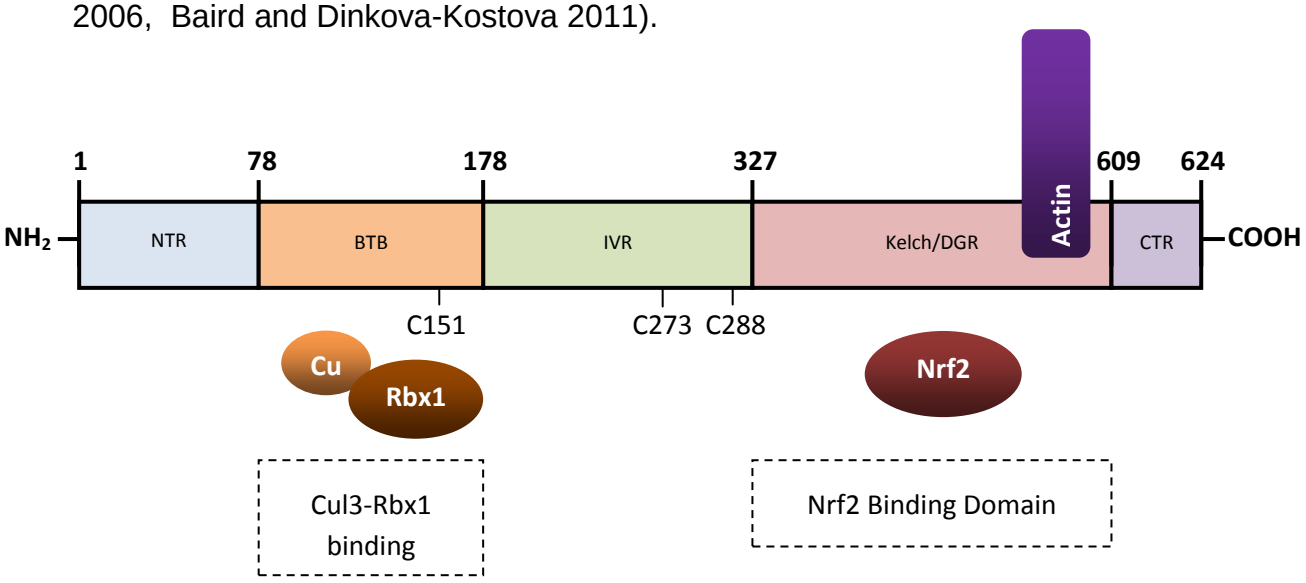

Figure 1.6 Keap1 domains and interactions. The domains are the N-terminal region (NTR), the Broad-Complex, Tramtrack and Bric à Brac (BTB) and the Intervening Region (IVR), the double glycine repeat (DGR), which is also known as the Kelch domain and the C-terminal region (CTR).

1.4.1 Nrf2 regulation

The exact mechanism by which Nrf2 is activated is still under investigation. There have are several pathways and intermediates associated with Nrf2 activation reported which include the ERK and p38 mitogen-activated protein kinases, a phosphoinositide 3-kinase (PI3K), and protein kinase C (PKC) (Keum et al 2006).

PKC is a large family multifunctional cyclic nucleotide independent protein kinases that phosphorylates serine and threonine residues in many target proteins. They have a key role in a broad range of cellular function through signalling transduction pathways including activation in cell proliferation, vasoconstriction, secretion and cell shape and volume. The PKC are able to have this broad range of functional effect through the different isoforms of PKC (Liu 2003). There are currently 11 different mammalian isoforms of PKC identified, divided into 3 different sub classes known as conventional, novel and atypical. The conventional PKCs consist of the isoforms α, βI, βII and γ and are activated by calcium and diacylglycerol (DAG) and phorbol esters. The novel PKC isoforms are ε, δ, η, and θ and are regulated by DAG and phorbol esters but not calcium. The atypical PKCs include ζ, λ and ι and are not activated by calcium, DAG or phorbol esters (Webb et al 2000).

PI3K is a kinase which is activated by a variety of cell surface receptors including tyrosine kinase receptors and is involved in intracellular signalling pathways. It is involved in a wide range of cellular processes including cell proliferation, cell migration, protein synthesis and metabolism. PI3K phosphorylates inositol phospholipids at the 3rd position of the inositol ring

Vanhaesebroeck et al 1997). There are three different sub classes of PI3K which are divided depending on the substrate specificity. Class I PI3K phosphorylates Phosphatidylinositol, Phosphatidylinositol 4,5-bisphosphate and Phosphatidylinositol -4-P and has a catalytic subunit of 11 to 120 kDa. Class II PI3K only phosphorylates Phosphatidylinositol and Phosphatidylinositol -4-P and Class III PI3K only react with Phosphatidylinositol (Vanhaesebroeck et al 1997).

In our laboratory, we have shown that PKC regulates Nrf2 or HO-1 expression by LPS, curcumin and epicatechin gallate (Rushworth et al 2006). Results suggest that LPS induction is mediated by a classical form of PKC, whereas upregulation by the dietary anti-oxidants may be mediated by PKCδ (Rushworth et al 2006, White et al 2010). How these different pathways interact to cause Nrf2 activation is unknown and is still under investigation. For example in response to the anti-oxidant tert-Butylhydroquinone (tBHQ) PKC has been reported to phosphorylate Nrf2 on Ser40, leading to dissociation from Keap1 and subsequent Nrf2 activation in HepG2 cells (Huang et al 2000, Huang et al 2002, Bloom and Jaiswal 2003). Nrf2 driven HO-1 expression has been upregulated in primary rat vascular smooth muscle cells by the plant extract oleanolic acid via the PI3K/Akt and ERK pathways and Nrf2 ability to decrease pigment-related molecules was regulated by PI3K (Huang et al 2000, Huang et al 2002, Rushworth et al 2006, Angeloni et al 2011, Feng et al 2011, Shin et al 2011).

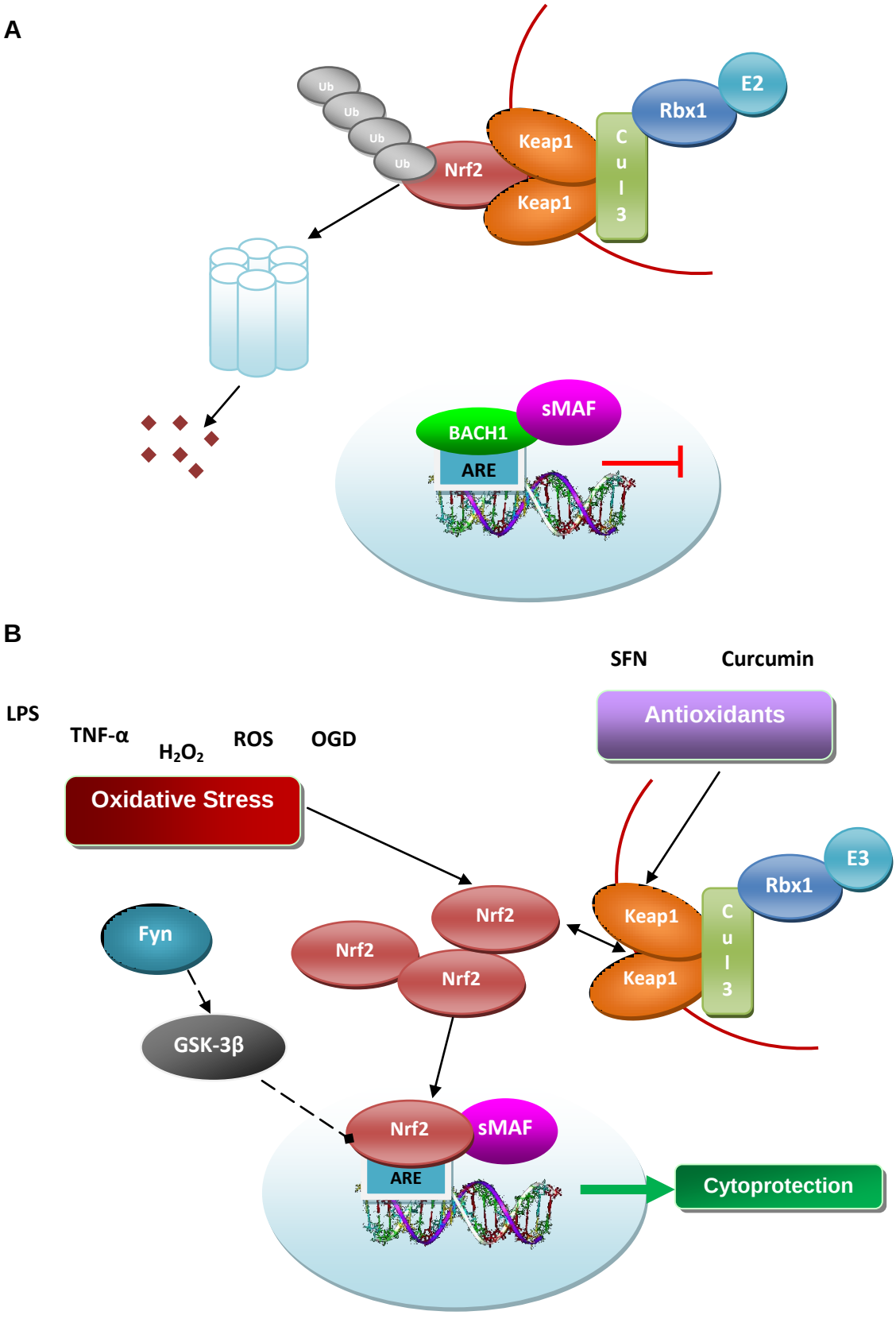

Figure 1.7 A schematic of the Nrf2 pathway. This depicts the current view of the Nrf2 pathway. (A) The unstimulated pathway. (B)T he stimulated pathway

Much evidence has accumulated about Nrf2 pathway that suggests it plays a major role in the anti-oxidant response in cells (Nguyen et al. 2009). In addition, Nrf2 knockout models have demonstrated its importance as an anti-inflammatory mediator, in a range of chronic inflammatory disease models (Thimmulappa et al 2006, Zhao et al 2011). There is still a lack of knowledge on the exact mechanisms of this pathway and the roles it could potentially play in several different diseases. Understanding the roles of Nrf2 driven genes is essential for maintaining a level of cytoprotection in healthy and diseased cells, tissues and organs. There are many Nrf2-ARE regulated genes with many different functions including efflux proteins like bile salt efflux pump and multidrug resistance-associated proteins, peroxiredoxins such as sulfiredoxin 1, xenobiotics including carboxylesterases and phase II enzymes and antioxidants like Trx1, HO-1, NQO1 and enzymes involved in regulating glutathione synthesis (Klaassen and Reisman 2010, Baird and Dinkova-Kostova 2011).

1.5 Genes targeted by Nrf2

1.5.1 HO-1

Heme oxygenase is the rate limiting enzyme within the heme catabolism pathway resulting in the formation of biliverdin, carbon dioxide and iron (Maines and Gibbs 2005). Biliverdin (which is metabolised to bilirubin by biliverdin reductase) functions primarily to scavenge peroxyl radicals (Wu et al 1991). CO at high amounts in the body causes death as it has a higher affinity to hemoglobin and myoglobin compared to oxygen. However, evidence suggests

that low levels of CO have cytoprotective effects within the cell related to anti - inflammatory and anti- apoptotic effects in ischemic reperfusion injuries (Berne et al 2012).

There have been three different heme oxygenase isoforms identified, the inducible heme oxygenase -1 (HO-1) which is also known as heat shock protein 32, heme oxygenase 2 (HO-2) which is constitutively expressed and heme oxygenase 3 (HO-3), which is still being characterised (McCoubrey et al 1997, Hayashi et al 2004). HO-1 is ubiquitous and found in detectable levels in many tissues (Jeong et al 2005). Induction and expression of HO-1 is associated with a strong role in cytoprotection, especially when cells are stressed by oxidative stress, inflammation, UV, ischemia, hypoxia, hyperoxia, hyperthermia, and radiation, cytokines, endotoxins, heat shock, heavy metals and other pathophysical responses involving oxidative stress (Keyse and Tyrrell 1989, Keyse and Tyrrell 1990, Terry et al 1998, Motterlini et al 2000, Castilho et al 2012). It has long been established that HO-1 has anti-oxidative and anti-inflammatory functions (Maines and Gibbs 2005). Individuals with HO-1 deficiencies have a higher susceptibility to oxidative stress and an increased pro-inflammatory state with high amounts of endothelial damage, which is thought to be caused by TNF-α and interleukin -1β (Terry et al 1998). Stimulating HO-1 expression has been suggested to give protection in OGD with reperfusion by providing a level of cytoprotection in a various animal and cell models (Cao et al 2006, Leonard et al 2006, Shah et al 2007, Sun et al 2010).

Studies conducted on HO-1 knockout mice have shown they have multiple organ malfunctions including in the lung, heart and liver also the blood

system is impaired. Inflammation and oxidative stress levels in the mice are also highly raised (Yet et al 1999, Chen-Reotling et al 2005, Tanaka et al 2007)

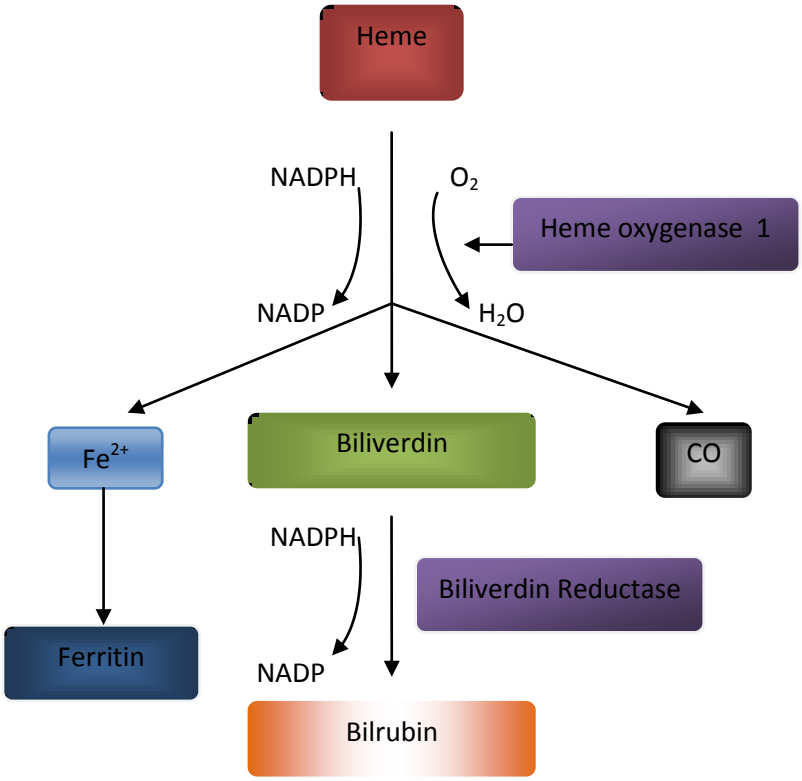

Figure 1.8 The Heme pathway showing the role of heme oxygenase 1.

Induction/activation of HO-1 has been associated with a protective role in a large range of different oxidative stress- related disease states. These include hypertension, atherosclersosis, diabetes, lung injury, Alzheimer's disease, renal injury, endotoxic shock, transplantation and ischemia reperfusion injury (Tanaka et al 2007). In the retina induction of HO-1 has been shown to be protective in the retina. In rats with DR symptoms were given hemin to induce HO-1 expression which gave cytoprotection to RGC. HO-1 has also been shown to give cytoprotection to the retina when induced by cobalt against ischemic injury

et al Sun 2010). In ARPE-19 cells curcumin has also been shown to upregulate HO-1 and provide cytoprotection against oxidative stress (Woo et al 2012).

1.5.2 Ferritin

Ferritin is an iron sequestering protein that is regulated by Nrf2. Free iron can participate in a reaction known as the Fenton reaction (He et al 2007). This reaction can cause tissue and membrane damage and can produce one of the most damaging types of ROS, a hydroxyl radical. During the metabolism of heme, free iron is released. Ferritin works with an ATPase pump to remove iron from the cell. The amount of ferritin within a cell is proportionate to the amount of free iron within the cell and this is regulated by a post transcriptional mechanism (Hahn et al 2004, He et al 2007). The iron regulatory protein binds to the iron responsive element, which inhibits the translation of ferritin mRNA. Ferritin expression has been reported in several regions of the eye, including the inner nuclear layer, the choroid, RGCs and RPE (Hahn et al 2004). There are several cell types within the retina that require iron including RPE and photoreceptors.

1.5.3 NQO1

The Phase II detoxification enzyme NQO1 is another target of Nrf2, which displays anti-oxidant activities (Baird and Dinkova-Kostova 2011). NQO1 is a FAD-dependent flavoprotein which can reduce a range of quinines, nitro-aromatics and azo dyes. It was discovered by Lars Ernster and was first

identified as DT-diaphorase (Conover and Ernster 1960). One of the main functions of NQO1 is to be an enzyme in a catalytic reaction in a two electron reduction of a variety of quinines to the respective hydroquinones by having NADPH or NADH as the hydride donor (Dinkova-Kostova and Talalay 2010). NQO1 reduces the opportunity for ROS intermediates to be produced as a result of redox cycling and for the depletion of intracellular sulfhydryl pools. Other NQO1 functions include its ability to scavenge superoxides and the stabilisation of microtubules. NQO1 can also stabilise and stop the degradation of tumour suppressing proteins such as p53 (Dinkova-Kostova and Talalay 2010, Park et al 2011). NQO1 has been reported to be expressed in RPE cells. Pro-oxidative chemicals from cigarette smoke and the exogenous anti-oxidants vitamin E and eriodictyol (a citrus flavoniod) have been shown to induce NQO1 expression in RPE cell cultures (Cano et al 2010).

1.5.4 Thioredoxin 1

The redox enzyme thioredoxin 1 (Trx1) is also regulated by Nrf2. Trx1 is a 13 kDa ubiquitous protein, which is found in organisms from yeast to mammals (Zhong et al 2000). In the presence of ROS, its two redox-active cysteine residues (-Cys-Gly-Pro-Cys-) become oxidised (Holmgren 1985, Zhong et al 2000). Once Trx1 has been oxidised it can be reduced back to its active form via Thioredoxin reductase, another Nrf2-regulated enzyme, with the aid of NADPH. Other functions of Trx1 include the protection of neuronal cells from ischemia reperfusion, reduction of protein disulfides, and protection from hypoxia during birth (Bertini et al 1999). It has also been reported to aid the

regulation of specific transcription factors and play a role in DNA synthesis. Trx1 expression has been detected in photoreceptor cells and RPE cells in the retina, where it protects these cells from light damage (Tanito et al 2005).

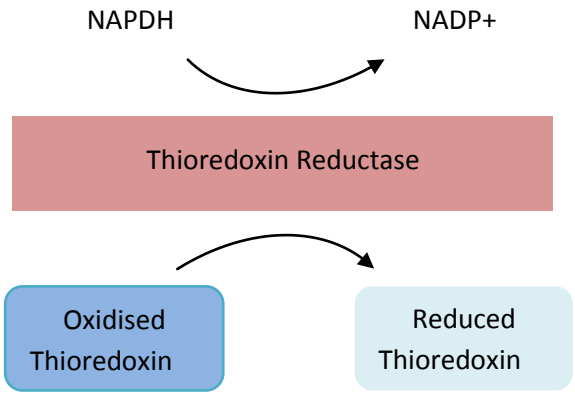

Figure 1.9 Reaction of Thioredoxin, Thioredoxin reductase with NAPDH returns Thioredoxin to its reduced state so it can provide protective effects within the cell.

1.5.5 Glutathione

Glutathione (GSH) is the major cellular anti-oxidant found in cells as it is found in millimolar concentrations. Several enzymes regulate glutathione synthesis and metabolism and many of these are regulated by Nrf2 (Lu 2009). They include glutathione S-transferase (GST), glutathione synthase (GS) glutathione reductase (GR) and glutathione cysteine ligase (GCL). GCL is the rate-limiting enzyme in glutathione biosynthesis. GSH is a substrate in reduction and conjugation reactions in cells, and these reactions are catalysed by GST. Glutathione is maintained in a reduced state in cells by the action of GR and

presence of NADPH, which allows the cells to maintain a reduced and healthy state (Wu and Juurlink 2001, Lu 2009). Glutathione has been reported to be present and protective in the retina. Low glutathione S-transferase (GST) expression has been reported in retinas from patients with glaucoma (Tezel 2006).

1.6 Nrf2 pathway activation by exogenous anti-oxidants

As discussed, the Nrf2 pathway can be activated by several stresses and pro-oxidative stimuli. It can also be activated by several natural products that possess anti-oxidant properties that utilities the pathway to amplify their antioxidant response in cells and tissues. For example in THP-1 cells *α*-lipoic acid from tomatoes, curcumin from turmeric and resveratrol from grapes been shown to induce HO-1 mRNA expression in THP-1 cells (Ogborne et al 2004, Rushworth et al 2006, Wang et al 2011). Curcumin also increased Nrf2 driven expression of GCL, in immortalized human bronchial epithelial (HBE1) cells 90 (Dickinson et al 2003). Resveratrol also has induced NQO1 expression in Hepa1c1c7 cells and HO-1 expression in rat pheochromocytoma (PC12) (Chen et al 2005, Park et al 2011). Caffeic acid phenethyl ester (CAPE) is derived from bee's honey and in rat renal epithelial cells induced Nrf2 driven HO-1 expression (Balogun et al 2003). Another well characterised compound from broccoli is sulforaphane

1.6.1 Sulforaphane (SFN)

Isothiocyanates are organosulphur compounds, which are characterised by a sulphur containing N=C=S functional group and are found in various cruciferous vegetables. They include allyl isothiocyanates found in cabbage, mustard and horseradish, phenethyl isothiocyanate from watercress and garden cress and SFN from broccoli, cauliflower, watercress and kale. SFN exists in the vegetables in the precursor form of a thioglucoside, which in broccoli is known as glucoraphanin. Glucoraphanin, like all glucosinolates is not bioactive until hydrolysed into sulforaphane, an indole-3 carbinol, a reaction that is catalysed by myrosinase (Zhang et al 1992, Danilov et al 2009, Keum 2011). This enzyme reaction occurs when the vegetable is disrupted either by harvesting, processing or chewing.

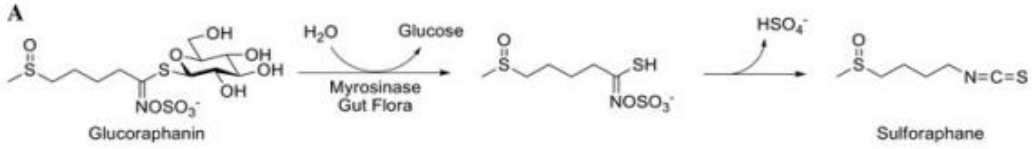

Figure 1.10 Activation of SFN. The inactive glucoraphanin is converted into SFN by myrosinase which is activated by chewing and the microflora of the gut (Keum 2011).

SFN has been found to have a broad spectrum of protective properties in several tissues, including anti-oxidant, anti-inflammatory, anti apoptotic, and chemopreventive effects (Juge et al 2007). It also activates the Nrf2 pathway, although the exact mechanism leading to this activation is unclear. SFN may disrupt the Nrf2 and Keap 1 interaction by modifying Cys151 on Keap1, allowing

newly synthesised Nrf2 to accumulate and translocate into the nucleus. SFN may also interact with other reactive thiol groups on Keap 1, including Cys257, Cys273, Cys28, which then leads to activation of Nrf2 (Zhang and Hannink 2003). However kinases upstream of Nrf2 have also been implicated in Nrf2 regulation (Keum et al 2006).

SFN has generally been shown to be a strong activator of Nrf2- regulated phase II genes, including NQO1 and GST in a wide variety of tissues in mammals, including kidney, prostrate, colon, pancreas, liver and eye. In relation to the eye, SFN has been shown to activate the Nrf2 pathway and specific downstream target genes to provide cytoprotection. Microglial activation in the retina is associated with several disease processes. Yang *et al*. reported that SFN inhibited the effects of LPS-induced retinal microglial activation through a p38 MAPK-dependent mechanism. p38 mitogen-activated protein kinase is a part of the MAPK family. p38-MAPK is a part of a pathway which aids the relaying of extracellular signals and stimulations, often cytokines and cellular stress, to intracellular responses through phosphorylation of MAPK (Roux and Blenis 2004, Yang et al 2007). SFN has also been reported to protect RPE cells from a lipofuscin pigment via NQO1 and GST (Zhou et al 2006). SFN induced GSH and the enzymes that regulate it in the human ARPE-19 cell line, including GCL, GS and GR. Wang et al demonstrated that SFN-induced glutathione and GCL were regulated by Phosphoinositide 3-Kinase (PI3K) (Shapiro et al 2001, Wang et al 2008). PI3K is a family of kinases which Talalay *et al* showed that in the presence of UV stress SFN upregulated GCL and NQO1 expression in ARPE-19 cells (Gao and Talalay 2004). Interestingly, whilst HO-1 has been

shown to be upregulated by SFN in the liver, kidneys and skin, HO-1 has not been induced by SFN within the retina.

The Mueller cells are of key interest in the progression of retinal diseases. Further understanding into their role and function in the diseased and healthy retina is essential. How oxidative stress and antioxidants affect these cells and other cell types, including the RPE, and by which mechanisms needs to be fully understood. The Nrf2 pathway is a good candidate by which neuroprotection can be provided within these cells.

1.7 Aims of this study

To investigate

(A) The effects of oxidative stress inducers on anti-oxidant gene expression in Mueller cells.

(B) The effects of the antioxidant SFN on the Nrf2 pathway in MIO-M1 cells and examine the mechanism involved.

Chapter 2

Material and Methods

2.1 General Reagents

LY 294002, Bisindolymaleimide 1, Lipopolysaccharide *E.coli* O111:B4 (LPS) and MG-132 were all obtained from Calbiochem, (Nottingham UK). All plastic consumables were obtained from Fisher (Loughborough UK). The 96® AQ AQ_{ueous} One Solution Cell Proliferation Assay was from Promega Southampton, H_2O_2, and BSA were all obtained from Sigma-Aldrich, (Poole, UK) Sulforaphane was purchased from LKT Laboratories, Inc, Germany. D-Glucose was obtained from Fisher Scientific (Loughborough, UK) Human TNF-α was purchased from R&D systems, Abingdon. Unless otherwise stated, all other reagents were obtained from Invitrogen (Paisley UK).

2.2 Cell culture

2.2.1 Reagents

DMEM/F-12, RPMI 1640, DMEM glucose free medium, DMEM + GlutaMAX, L-Glutamine 2mM, penicillin 10000 units/mL, streptomycin 10000µg/mL, 0.25% Trypsin-ethylenediaminetetraacetic (EDTA) were all from Invitrogen. 10% fetal calf serum (FCS) was obtained from Biosera, (S.America Origin). 0.25% Trypsin-ethylenediaminetetraacetic (EDTA), Eagle's minimum essential medium (EMEM), 50 µg/mL gentamycin, 0.4% Trypan blue solution and sodium bicarbonate were all obtained from Sigma-Aldrich, (Poole, UK).

2.2.2 Heat inactivation of FCS

FCS was heated to 56°C for 45 min in a water bath and then aliquoted into 50 mL aliquots and stored at -20°C.

2.2.3 Cell Lines

2.2.3.1 THP-1

THP-1 cells are a human monocytic leukemic cell line derived from the blood of a boy with acute monocytic leukaemia (Tsuchiya et al 1980). They were obtained from the European Collection of Cell Cultures (ECACC). They have been previously validated in the laboratory as a model of human monocytes. These cells were cultured in RPMI 1640 media with penicillin (100 units/mL) and streptomycin (100µg/mL), 2 mM L-glutamine and 10% FCS. The cells were passaged biweekly at 3.5 days apart by counting the cells, removing the appropriate cells and media and adding media so that they were kept in the range of 2.5 and 6×10^5 cells/mL and were used between passages 3-30. Cells were maintained in a humidified atmosphere at 37°C, 5% CO_2.

2.2.3.2 MIO-M1

The Moorfields/Institute of Ophthalmology-Müller 1 cell line (MIO-M1) is a spontaneously immortalised human Mueller cell line, which was a kind donation from Dr A. Limb at the University College London's Institute of

Ophthalmology (Limb et al. 2002). These cells were cultured in DMEM medium with Glutamax, 10% FCS, penicillin (100 units/mL) and streptomycin (100 µg/mL).

Confluent cells were passaged by removing the complete media and washing with 5 mL of warmed PBS. This was removed and 5 mL of warmed 0.25% trypsin EDTA was added for 3 to 4 min at room temperature to detach the cells from the flask. The trypsin was neutralised with complete media and the cells were counted. Cells were then centrifuged at 800 rpm for 8 min. The supernatant was removed and the cell pellets were resuspended in 10 mL of complete medium. Cells were passaged 1/5 every seven days, fed every 3.5 days and maintained between $5x10^4$ and $2.5x10^5$ at 37°C , 5% CO_2.

2.2.3.3 ARPE-19

ARPE-19 is a human retinal pigment epithelial (RPE) cell line which arose spontaneously from a primary culture of RPE cells from a male donor (Dunn et al. 1996).The cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM/F12 with 10% FCS, 0.15% Sodium Bicarbonate 2 mM L-Glutamine and penicillin (100 units/ mL) and streptomycin (100 µg/mL). The cells were trypsinised as above and split 1/10, fed every 3.5 days and maintained between $5x10^4$ and $2.5x10^5$ at 37°C, 5% CO_2.

2.2.4 Counting Cells

Cells were counted using a Neubauer haemacytometer (Fisher). 20 µL of cells was mixed with 80 µL of 0.4% Trypan blue solution to make a 1/5 dilution. The cells were counted using the grid on the haemocytometer. The mean number of cells was calculated and this was multiplied by the dilution factor and then by 10^4 to scale the volume of the haemocytometer (0.1 mm^3) to cells/mL. This calculation is summarised in the figure below.

mean x dilution factor x 10^4 = number of cells/mL

2.2.5 Cryopreservation

The ARPE-19 were frozen down by trypsinising the cells as described above, centrifuging at 400 rpm for 5 min and resuspending in freezing media consisting of 10% cell culture grade DMSO and 90% filter sterilised FCS. The MIO-M1 cells were trypsinised as described above and were resuspended in 1 mL of freezing media which consisted of 10% cell culture grade DMSO, 50% GlutaMax Media and 40% filter sterilised FCS. Approximately 2.5 million cells were put into one cryovial in 1 mL of freezing media. This was placed on ice and then frozen at -80°C before being stored long term in liquid nitrogen.

Cells were thawed by defrosting the cells, which were then transferred to eppendorfs and centrifuged into a pellet at 400 rpm for 5 min. The freezing medium was removed and the cells were resuspended in 10 mL of complete medium, placed into a T75 flask and maintained at 37°C, 5% CO_2. For adherent cells, the media was changed the next day once cells had attached.

2.3. Stimulations

Cells and tissues were treated with a range of stimuli and inhibitors for various times.

2.3.1 LPS

A stock concentration of 5 mg/mL of Lipopolysaccharide *E.coli* O111:B4 (LPS) (Calbiochem) was made by adding 1 mL of 37°C RPMI media to 5 mg LPS powder and was vortexed for 5 min before being heated for 5 min in a 37°C water bath and then vortexing again for 2 min. The heating and vortexing was repeated 4 times and then the LPS were aliquoted into 4 labelled eppendorfs, wrapped with parafilm (Parafilm® M, Sigma Aldrich) and stored at -20°C.

2.3.2 TNF-α

Human recombinant TNF-α (R&D systems) was made into a stock concentration of 10 µg/mL. Human TNF-α was used at a final concentration of 10 ng/mL.

2.3.4 Sulforaphane

Sulforaphane (LKT Laboratiories, Inc) was made into a 100 mM stock by adding 1410 µL cell culture grade DMSO to 25 mg of Sulforaphane (SFN) and mixed well. A final concentration of 1-10 µM of SFN was added to the cells for various amounts of time, depending on the experiment.

2.3.4 H_2O_2

A stock concentration of 200 mM H_2O_2 (Fisher) was made with 21.3 µL 9.4 M H_2O_2 into 978.7 µL sterile dPBS, which was stored at 2-8°C. A final concentration of 200 µM was used for various periods of time depending on the experiment.

2.3.5 Glucose

A stock concentration of 25 M was made with 450.4 mg of D-Glucose (Fisher) in 1mL of warmed sterile dPBS. This was vortexed and heated to ensure that the glucose was fully dissolved. The glucose was used within 7 days of being made. Glucose was used at varying times at a final concentration of 25 mM.

2.3.6 Oxygen and Glucose Deprivation

Cells were seeded in 35 mm dishes at 10000 cells per well and left for 4 days to grow 90% confluent. Cells were then serum starved for 24 h with DMEM medium with Glutamax, penicillin (100 units/mL) and streptomycin (100 µg/mL). To initiate the experiment ,the cells which were going into the ischemia chamber had their media changed into DMEM glucose free medium supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL) which had been starved of oxygen by bubbling 95% N_2 and 5% CO_2, from a cylinder, through the medium for 10 min. The Modular Incubator chamber (Billups-rothenberg Inc)

(Wolf Laboratories Limited, York UK) was used to recreate ischemic conditions. Once the cells had been sealed into the chamber using the attached metal ring, it was filled with 95% N_2 and 5% CO_2 by connecting directly to a cylinder via a tube for 10 min to flush out the atmospheric oxygen and create anaerobic conditions. The tubes were then sealed off and the chamber was incubated at 37°C for either 50 min or 1 h and 50 min, depending on the experiment. The cells were then removed from the chamber and the anaerobic media was replaced by the serum starved medium. The cells were then reperfused for varying amounts of time. The controls to this experiment were subjected to the media changes as in the test conditions but instead of anaerobic media, the controls had DMEM glucose free medium supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL) and they were not placed within the chamber but incubated at 37°C with 5% CO_2 for either 50 min or 1 h and 50 min.

2.4 Inhibitors

2.4.1 LY 294002

LY 294002 (Calbiochem) was made into a stock concentration of 50 mM in DMSO. This inhibitor was incubated with the cells at various concentrations between 1 and 25 µM for 30 min preincubation before the adding of another stimulus.

2.4.2 Bisindolymaleimide 1

Bisindolymaleimide 1 (BIS1) (Calbiochem) was made into a stock concentration of 50 mM in DMSO. This inhibitor was incubated with the cells at various concentrations between 1 and 10 µM for 30 min pre- incubation before the adding of another stimulus.

2.5 MTS cell viability assay

The MTS assay used the 96® AQ_{ueous} One Solution Cell Proliferation Assay. This is a colourimetric method used to quantify the number of viable cells within a sample. This reagent contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, which is a MTS tetrazolium compound, and an electron coupling reagent (phenazine ethosulfate; PES) which increases the stability of the solution. The MTS

tetrazolium compound is converted into a coloured formazan product by cells that are viable. The viable cells achieve this conversion by NADPH or NADH produced by dehydrogenase enzymes in the mitochondria. The level of mitochondrial activity is an early indicator of cell death of damage. The amount of formazan produced is directly proportional to the amount of viable cells. Formazan compound can be measured by an absorbance at 490nm.

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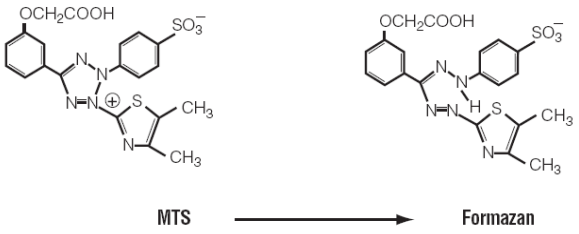

Figure 2.1 Reaction in the MTS assay. The MTS tetrazolium compound is converted into the formazan product in viable cells. The amount of formazan that is produced is directly proportional to the amount of viable cells within the sample which can be read at the absorbance of 490nm. (www.promega.com/tbs/tb245/tb245.pdf)

Cells were seeded in a 96 well plate in 100 µL of media at varying seeding concentrations incubated at 37°C with 5% CO_2 for 4 days to achieve confluent cells. The outer wells of the plate were never used for cell seeding but were filled with 200 µL of water to prevent evaporation. Cells were then serum starved for 24 h. Following various stimulations and incubations after the relative stimulations and incubations, 10 µL of Cell titer 96 AQ_{ueous} One Solution Reagent (Promega) was added to each well and incubated in darkness at 37°C with 5% CO_2 for 2.5 h. The plates were then read on a BMG Labtech FLUOstar OPTIMA microplate reader at 492 nm. All controls and test conditions were carried out in triplicate on a 96 well plate. Controls included untreated cells,

serum starved cells and vector controls of compounds added to the cells. A blank consisting of media was taken away from the measured absorbances and the mean of the triplicates was calculated. By comparing the measurement to controls in the same test conditions the percentage cell viability was calculated.

2.6 Real-Time (RT) Quantitative PCR

2.6.1 Reagents

RT reagents were from Applied Biosystems, the TriReagant Solution from Ambion and the designed primers from Invitrogen. All other reagents were obtained from Sigma Aldrich.

2.6.2 Cell Lysis

Adherent cells were seeded in 35 mm dishes in 2 mL of media at varying seeding concentrations incubated at 37°C with 5% CO_2 for 4 days to achieve confluent cells. Cells were then serum starved and after the required experiments the cells were lysed using 1 mL TriReagant Solution (Ambion). After 5 min cells were then scraped and solution placed into labelled eppendorfs. The samples were then frozen at -80°C until RNA extraction was performed.

Suspended cells were seeded 16 h prior to the first stimulation in 24 well plates at 5×10^5 cells/mL in 2 mL of media and left to rest for 16 h over night prior to the first stimulation. After the required experiments the cells were placed in

labelled eppendorfs and centrifuged at 200 rpm for 5 min to pellet the cells. The medium was then removed and the cells were resuspended in 1 mL of TriReagant Solution. The mixtures were pipetted up and down 10 times to ensure the cells were lysed and left at room temperature for 5 min. The samples were then frozen at -80°C until RNA extraction was performed.

2.6.3 RNA extraction

Samples were fully defrosted and 100 µL of 1-bromo-3-chloropropane (BCP) was added to each sample and inverted for 10 seconds and then left for 10 min at room temperature. The samples were then centrifuged at 12000 -g for 20 min at 4°C. The upper phase was removed into a fresh eppendorf tube and mixed with 500 µL of isopropanol. This was vortexed for 10 seconds and rested for 10 min at room temperature. The sample was centrifuged for 15 min at 12000 -g at 4°C. The supernatant was then removed and the pellet was washed in 1 mL of ethanol and spun at 7500 -g for 12 min at 4°C. The ethanol was then removed and the pellets left to air dry at room temperature. The RNA was then dissolved in RNA free water and stored at -80°C.

2.6.4 RNA quantification

The RNA samples were fully defrosted and 1 µL of RNA solution was placed on to a Thermoscientific Nanodrop machine which had been blanked with RNA-free water (Fisher Scientific). The quantified samples were diluted so that the range of RNA was between 100 and 200 ng/mL. RNA gives an

absorbance reading at 260 nm. The Nanodrop also gives a reading which indicates the purity of the RNA sample. The ratio of absorbance at 260/280 indicates if there is any DNA contamination. This ratio ideally should be 2.0 with RNA samples. This research aimed for a ratio between 1.8 and 2.0, as varying compositions of nucleotides can affect this ratio. Lower values than this could indicate phenol or protein contamination which absorbs at 280 nm. The 260/230 ratio is a secondary measure of RNA purity, ideally the ratio should be 2.0, however although lower ratios can indicate contamination this ratio is not an accurate measure of contamination as the 260/280 ratio.

2.6.5 cDNA synthesis

Quantified RNA was diluted with RNA-free water to make equivalent amounts of cDNA in each sample. The total volume was made up to 4.5 µL. This was achieved by adding the same amount of RNA per sample to 5.5 µL of Master mix (Applied Biosystems, Roche) which included mixture of 2.5 µL $MgCl_2$ (25µM), 1 µL 10× random hexamers, 0.25 µL Reverse Transcriptase buffer, 0.25 µL of RNAase Inhibitor and 1 µL of 2.5 µM dNTPs and 4 µL H_2O. This 10 µL mixture was placed in a thermocycler PCR for 40 min which had the repeated cycle of 10 min at 21°C, 15 min at 42°C, 5 min at 99°C and 5 min at 4°C. This allows for the RNA with the Master Mixture to be converted into cDNA ready to be used for qRT-PCR. A control without the Master Mix, (replaced with water), and a control with the Master Mix without the Reverse Transcriptase, (replaced with water), were added to each run to ensure no amplification of RNA contamination. cDNA samples were stored at -80°C.

2.6.6 qRT-PCR

qRT-PCR is a system which uses fluorescent quantification of specific mRNA transcripts which have been converted into cDNA as described in 2.7.5. qRT-PCR was executed with guidance taken from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines outlined in Bustin et al 2009 which specify the best options for standardising qRT-PCR data (Bustin et al 2009).

Primers were designed using the Universal Probe Library (Roche). For a list of primers see Table 2.1. When designing the primers, the following considerations were taken into account; the primers were approximately 20 nucleotides in length; the nucleotides content consisted of 50% GC; the melting points for the forward and reverse primers were similar; the primers did not form unwanted secondary structures on themselves; no primer-dimers greater than 6 base pairs between each primer and itself and also between the two primers; the forward and reverse primers were about 500 base pairs apart; and the 3' end of the primer should be a G or a C (Qiagen).

Table 2.1 Forward and reverse primers used

Name	Forward Primer	Reverse Primer
Nrf2	5'-AAC CAC CCT GAA AGC ACA GC-3'	5'-TGA AAT GCC GGA GTC AGA ATC-3'
hNQO1	5'-CGC AGA CCT TGT GAT ATT CCA G-3'	5'-TTC TAT GAA CAC TCG CTC AAA CC-3'
hHO-1	5'-ATG GCC TCC CTG TAC CAC ATC-3'	5'-TGT TGC GCT CAA TCT CCT CCT-3'
Heavy Chain Ferritin	5'-AAC AAC GAG GTG GTG GCC-3'	5'-TTC AGC CCG CTC TCC CAG T-3'
GAPDH	5'-AAC AGC CTC AAG ATC ATC AGC A-3'	5'-TGC TAA GCA GTT GGT GGT GC-3'
Thioredoxin reductase	5'-GGG CAA TTT ATT GGT CCT CA-3'	5'-GGT CCT TCA CCA GTG GCA AT-3'
β-actin	5'-GCT CTT TTC CAG CCT TCC T-3'	5'-CGG ATG TCA ACG TCA CAC TT-3'

GAPDH was used as a housekeeping gene to normalise the gene of interest, as it is not affected by the conditions of the experiment. For each sample tested, for the gene of interest and the housekeeping gene, 5 µL of the cDNA sample was placed into a tube and mixed with 10 µL SYBR Green, 4 µL water and 1 µL of a mix of front and reversed primers at 5 µM that related to the specific gene of interest to make a total solution of 20 µL per sample. The SYBR green consists of 20 mM Tris-HCl pH 8.3, 100mM KCl, 7 mM $MgCl_2$, 0.4

mM dNTP (dATP, dCTP, dGTP, and cTTP), stabilisers, 0.05unit/µL Taq DNA polymerase, Jumpstart Antibody and SYBR green 1. Five standards for each gene used were made from a mix of the total cDNA for analysis purposes; this was carried out by doing a serial dilution with RNAase free water, giving known relative concentrations of 1, 0.5, 0.25, 0.125 and 0.0625. The samples were run on the Qiagen Rotorgene Q machine for 40 cycles that consisted of 15 seconds at 95°C, 60 seconds at 40°C. A melting curve was also run for each primer in each experiment.

2.6.7 Analysis

Rotor-Gene Q series Software 1.7 was used to record the data from the Qiagen PCR machine. From this, CT values used were taken from the exponential region of the amplification and by using the standard curve the concentrations of the RNA of the experiment were calculated. The standard curve efficiency ideally should be 1 and a range of 0.85 to 1.15 was generally accepted, the R and R^2 values also had to be within 0.99 and 1. The gene of interest calculated concentrations were normalised against the housekeeping genes calculated concentrations. The house keeping gene CT values were within 1 standard deviation of each other. These normalised results were then produced as fold inductions of the experimental control, to show the gene expression of interest as normalised fold difference to the control.

2.7. Western Blots

2.7.1 Reagents

Unless otherwise stated below all other reagents were obtained from Invitrogen. Prestained SDS Page Standards Broadrange Molecular Weight Marker and PVDF Membrane was from BIO RAD and the dried milk powder was from Marvel. The Methanol, Tris base, NaCl, 5M HCL, Tween-20, Trisbase, Triton X-100, NaCl, 5M HCL, Tween-20, chemiluminescent reagent ECL, SuperSignal chemiluminescent reagent and CL-XPosure^{TM} Film Clear blue X-Ray film were all obtained from Fisher.

2.7.2 Cell Lysis

Adherent cells were seeded in 35 mm dishes in 2 mL of media at varying seeding concentrations and incubated at 37°C with 5% CO_2 for 4 days to achieve confluent cells. Cells were then serum starved for 24 h and after the required experiments, media was removed from the cells and the samples were lysed using 100 µL 1× Tris-Glycine SDS sample reducing buffer, diluted with dPBS, mixed well and by using a cell scraper were put into labelled eppendorfs. The samples were then wrapped in parafilm, and heated to 95°C for 5 min and frozen at -20 until required.

Suspended cells were seeded 16 h prior to the first stimulation in 24 well plates at 5×10^5 cells/mL in 2 mL of media and left to rest for 16 h over night prior to the first stimulation. After the required experiments the cells were placed in

labelled eppendorfs and centrifuged at 200 rpm for 5 min so the cells were in a pellet. The medium was then removed and the cells were resuspended in 100 µL 1× Tris-Glycine SDS sample reducing buffer and mixed well. The samples were then wrapped in parafilm, boiled for 5 min and frozen at -20°C until required.

2.7.3 Poly Acrylamide Gel Electrophoresis

Samples were defrosted and 9 µL or 13.5 µL (for MIO-M1 cells only) was mixed with 1 µL or 1.5 µL of NuPAGE Reducing Agent respectively at a 1:10 ratio. The samples were then vortexed, pulse centrifuged to bring down reagents to the bottom of the eppendorfs, wrapped in parafilm and boiled in water for 5 min to ensure that the samples were not viscous. The samples were then pulse centrifuged again.

The preset 4-12% Bis-Tris Gel was loaded into an Invitrogen Xcell II Gel rig with a Surelock system. 200 mL of running buffer with 500 µL NuPAGE Antioxidant was added to the Upper Buffer Chamber. After ensuring no leakages occurred, 400 mL of running buffer was added to the lower chamber. Before the samples were loaded the lanes were rinsed out with the running buffer. 10 µL of each sample was loaded into the lanes of the gel and 15 µL was loaded when using MIO-M1 samples. The gel was loaded from left to right using gel loading tips with 5 µL of prestained SDS PAGE Standard Broad Range Molecular Marker (Biorad) loaded in the first lane of the gel. Any empty lanes were filled with the same ratio and volume of lysis buffer mixed with PBS. The rig was attached to a power 330 Fisher Scientific powerpack and run at

200V for 50 min when the gel loading dye had ran all the way to the foot of the gel.

2.7.4 Transfer

The polyvinylidene fluoride (PVDF) membrane was soaked in methanol for 30 seconds and washed in the 1× transfer buffer on a shaker for at least 15 min. 6 blotting pads were saturated in the 1× transfer buffer for at least 15 min. Once the gel electrophoresis had completed it was released from the rig and the gel was released from the gel plates. A piece of filter paper the same size as the gel soaked in the 1 × transfer buffer was placed directly on top of the gel and any air bubbles were removed. The foot and tops of the wells were cut away. The gel was then flipped over on to a glass plate and the pre-soaked PVDF membrane was placed on the gel. Another pre-soaked filter paper was then placed on top of the membrane. Any remaining air bubbles were removed. The assembled western sandwich was placed between pre-soaked blotting pads and placed into the Invitrogen Blot Module with the gel closest to the cathode core. The blot module was then placed into the rinsed Invitrogen X cell II. The blot module was filled with the 1× NuPAGE Transfer buffer until the gel/membrane assembly was just covered. The outer chamber was filled with 650 mL ultrapure deionised water. The transfer was run at 30V for 1 h using a Power 350 Fisher Scientific powerpack.

2.7.5 Staining the Gel

After the transfer the gel was stained to check how well the gel had run. The gel was washed three times for 5 min in ultrapure water at room temperature on a shaker. The gel was then stained with 20 mL SimplyBlue SafeStain for 1 h, this was then poured off and three more 15 min washes with deionised water were performed.

2.7.6 Immunoblotting

Once the transfer was complete the PVDF membrane was placed in blocker solution for 1 h at room temperature or overnight at 4°C. The primary antibody was diluted as stated in Table 2.2 and applied to the PVDF membrane for 1 h at room temperature. The membrane was then quickly rinsed in the 1× TBST. The membrane was washed in blocker solution three times for 5 min each and this was followed by three 5 min TBST washes. Next, the appropriate secondary antibody, as shown in Table 2.3, was put into blocker buffer and applied to the membrane for 30 min at room temperature. This was followed by a quick rinse in 1× TBST buffer then three 5 min washes in blocker and three 5 min washes in 1× TBST buffer.

Table 2.2 Primary Antibodies

Antigen	Type	Species	Dilution	Supplier
HO-1	Monoclonal antibody	Mouse	1:1000	Stressgen
HO-1	Polycolonal antibody,	Goat	1:2000	RnD
Nrf2	Polycolonal antibody	Rabbit	1:1000	Abcam
β-actin	Monoclonal antibody	Mouse	1:100000	Sigma Aldrich

Table 2.3 Secondary antibodies

Antigen	Type	Dilution	Supplier
Goat - Anti Mouse	Monoclonal antibody,	1:1000	Dako
Goat Anti Rabbit	Polycolonal antibody	1:1000	Santa Cruz
Donkey Anti Goat	Polycolonal antibody	1:1000	Santa Cruz

2.7.7 Developing

2.7.7.1 ECL Reagents

Various methods of development were used throughout this research as equipment and resources changed. First, the membrane was developed using the chemiluminescent reagent ECL (Pierce) which consisted of two chemiluminescent reagents; 1 and 2, 2 mL of each reagent were pipetted into a foil covered centrifuge tube, mixed well and kept in subdued light. This was then added to the semi-dried membrane for 1 min and the excess was blotted off. The membrane was placed between two sheets of acetate membrane and kept in the dark.

SuperSignal chemiluminescent reagent (Pierce) was used using the same method as described above as it gave more stable signal for a longer period of time. This was applied by spraying the reagent evenly over the membrane.

In later experiments an ECL rapid step spray (Calbiochem) was used. This was applied in a light, even covering to the membrane which was then put into an acetate sleeve and kept in subdued light.

2.7.7.2 Imaging

Throughout the research two different imaging methods were used as different equipment became available. The first involved placing the membrane in an acetate envelope into a film cassette. CL-XPosure^{TM} Film Clear blue X-

Ray film (Thermoscientific) was place into the cassette in total darkness for various amounts of time varying from 2 sec to 30 min depending on the level of protein expression. This was then place in an X-Ograph X4 imager machine to be developed. When developing by the second method, a G box SynGene Machine was used by placing the membrane in an acetate envelope into the machine and using GeneTools and GeneSnap software to develop and analyse the membrane. In all developing methods the molecular weight marker was later added to the developed image of the expressed protein of interest.

2.7.8 Stripping Membrane for reuse

The membrane was kept in a fridge in blocking solution until being stripped for reuse. 18 mL ddH_20 with 2 mL re-blot was mixed together and put on the membrane for 30 min on a shaker at room temperature. This was followed by three 5 min PBS-T washes. The membrane was then ready for the Immunoblotting protocol.

2.7.9 Buffers for Western Blot Analysis

20× TBST buffer stock solution

48.4g Tris base,

160g NaCl,

62 mL 5M HCL

20 mL Tween-20

1 L of distilled water.

The pH was adjusted to 7.6.

1× TBST buffer solution

950 mL deionised water

50 mL 20 × TBST buffer stock solution

Blocker solution

20g of dried milk

400 mL 1X TBST buffer solution and kept at 4°C.

Running Buffer.1×

1× running buffer consisted of

760 mL ultrapure deionised water,

40 mL 20× MOPS SDS

Transfer buffer

680 mL deionised water,

80 mL methanol, 40 mL

20× Transfer Buffer

1 mL NuPAGE Antioxidant.

2.8. Immunocytochemistry for Cell Cultures

2.8.1 Fixation and Preparation

Adherent cells were grown on sterilised glass slides placed in 35 mm dishes. Different cell concentrations were seeded into the dishes and left to grow for 4 days. All cell types were fixed in 4% paraformaldehyde at room temperature for 30 min. This was followed by three 10 min washes in PBS. The fixed samples were stored at 4°C.

2.8.2 Immunoblotting

The fixed cells on slides were blocked in 100 µL blocking solution with 0.2% Triton X-100 for 90 min at room temperature. The blocking solution was gently removed and 100 µL primary antibody diluted in blocking solution (see Table 2.3 for dilutions) was added to each sample for 90 min at 37°C. The samples were then washed three times for 10 min with blocking solution with 0.2% Triton X-100. All steps from this stage were done in subdued light conditions. 100 µL of the secondary antibody diluted in blocking solution without Triton X100 as stated in Table 2.4 was put on the samples for 1 h at 37°C . Three 10 min PBS washes were performed before excess moisture on the slides was removed and 50 µL PBS + DAPI (1:100 dilution) was placed on each tissue sample for 10 min. Three 10 min washes were then performed before the excess moisture was removed again with blue tissue. A drop of hydromount was added to a glass slide and the cover slip was placed on to the glass slide

with the cells facing down on to the glass. The samples were then wrapped in foil and left in dark conditions overnight at room temperature before being stored at 4°C in the dark.

2.8.3 Imaging

The samples were imaged using a Leica Laser Microscope CMS GmbH. Images were processed and analysed using the Leica Application Suite and compared to the secondary Antibody controls and controls from the experimental procedures.

Table 2.4 Secondary Antibodies for Immunocytochemistry

Antigen	Type	Dilution	Supplier
Goat Anti Mouse IgG (H+L)	AlexaFluor 488	1:1000	Invitrogen, Paisley, UK
Goat Anti Mouse IgG (H+L)	AlexaFluor 568	1:1000	Invitrogen, Paisley, UK
Goat-Anti Rabbit IgG (H+L)	AlexaFluor 568	1:1000	Invitrogen, Paisley, UK

2.9 Statistical Analysis

One way ANOVA and the secondary Tukey test were applied to all results and occasionally an unpaired T- test was applied. All data were calculated and analysed using GraphPad Prism Version 5.00 (GraphPad Software, Inc) and Microsoft Office Excel 2007.

Chapter 3

Effect of oxidative stress on antioxidant gene expression in human retinal cells

3.1 Introduction

Mueller and RPE cells play an extremely important role in the retina, providing support, growth factors and immunomodulators to the surrounding cell types. Mueller cells have a key role in regulating ion and water homeostasis, protecting and maintaining the inner blood-retinal barrier (Bringmann et al 2009b). They interact closely with neurons in the retina, providing them with trophic substances and removing their metabolic waste (Limb and Jayaram 2010). They also aid the survival of photoreceptor and neuronal cells both structurally and by modulating immune and inflammatory responses (Bringmann et al 2006). These cells have been reported to become activated by a range of pathological stimuli, including excess glutamate, oxidative stress, inflammation and damage to the retina. This activation is termed gliosis.

When Mueller cells undergo gliosis, morphological, biochemical and physiological changes occur to protect the retina from incurring damage. The upregulation of the Mueller marker GFAP is an early sign of gliosis. Another early sign is cell size increase and proliferation with de-differentiation into progenitor-like cells (Bringmann et al 2009a). The aim of gliosis is to maintain and protect the retina when it is under stress. Whilst low level gliosis activation is cytoprotective, high levels of activation have toxic effects which can lead to neuronal degradation (Bringman et al 2006). In addition, Mueller cells have been reported to activate the immune system when the retina is subjected to retinal injury. They can up-regulate inflammatory factors and aid the recruitment of immune cells to the affected area of retina which can release cytotoxic cytokines and produce ROS, which have a significant role in neuronal degeneration after ischemia-reperfusion (Wang et al 2010).

Oxidative stress is a major contributor in the pathogenesis of retinal neurodegenerative eye diseases (Tezel 2006, Jarrett and Boulton 2012). Exposure to an array of different stimuli can result in oxidative stress, increasing ROS within the eye. Potential sources of oxidative stress within the retina include oxygen and glucose deprivation (OGD) reperfusion from restricted or changed blood flow. Decreased blood flow is proposed to be part of the underlying pathophysiological mechanisms of glaucoma (Flammer et al 2002). In addition, the high glucose concentrations found in patients with uncontrolled diabetes can induce ROS in the retina. The oxidising agent, H_2O_2, is a by-product of oxidative metabolism and has also been shown to be cytotoxic in the retina (Liang and Godley 2003). TNF-α and LPS are pro-inflammatory mediators which can induce ROS. LPS is an endotoxin from the outer membrane of gram negative bacteria and has been shown to be involved in uveitis in the eye (Kalariya et al 2012). TNF-α is a cytokine which induces apoptotic cell death through the TNF-α receptor-1 in a capase mediated pathway and has been shown to be produced by Mueller cells during gliosis, which can cause oxidative stress within the retina (Wang et al 2010). It has been reported that Mueller cells are more resilient to oxidative stress compared to other retinal cell types. Moorfields/Institute of Ophthalmology-Müller 1 cell line (MIO-M1) (Figure 3.1A) is a spontaneously immortalised human Mueller cell line (Limb et al 2002).

Nrf2 is a transcription factor found ubiquitously in all tissues. Nrf2 responds to oxidative stress by activating an array of cellular anti-oxidants and other cytoprotective proteins (Kaspar et al 2009). HO-1 is a downstream target of Nrf2 key in providing cytoprotection and has been shown to be upregulated

as a result of oxidative stress in mice retinas and the RPE (Castilho et al 2012). It is the rate-limiting enzyme in heme catabolism which metabolises cytotoxic heme to bilirubin and CO. This leads to release of free iron which can lead to the ROS, OH′ (the hydroxyl radical) being released in the cell through the Fenton reaction (He et al 2007). There is evidence to suggest that patients with AMD have excessive iron accumulation within the retina (Hahn et al 2004). Ferritin, another Nrf2-driven gene, is an iron sequestering protein that works with an ATPase pump to remove free cytosolic iron from the cell. Ferritin has been reported to be in the inner nuclear layer, the choroid, RGCs and RPE (Gnana-Prakasam et al 2010).

NQO1 is a Phase II detoxifying enzyme, which is regulated by the Nrf2 pathway. It reduces ROS intermediates as a result of redox cycling via NADP and NADPH (Dinkova-Kostova and Talalay 2010). NQO1 has been reported to be expressed in RPE cells and pro-oxidative chemicals from cigarette smoke activate NQO1 expression in ARPE-19 cells (Cano et al 2010).

Thioredoxin (Trx1) is another cytoprotective protein that helps maintain the cell in a reduced state by having two redox-active Cys residues (Bertini et al 1999). Thioredoxin reductase is the enzyme responsible for taking Trx1 back into a reduced state; both Trx1 and thioredoxin reductase are Nrf2 regulated genes. Trx1 has been reported to be within the eye and can provide cytoprotection against light damage. Trx1 expression has been detected in RPE cells and photoreceptor cells (Tanito et al 2005).

The Nrf2 pathway has not been reported previously in Mueller cells and it is unclear whether this pathway plays a role in the response of these cells to oxidative stress. In this study, the effects of OGD and other oxidative stress

inducers on anti-oxidant gene expression were investigated in the human Mueller cell line MIO-M1. Comparisons were also made to the ARPE-19 human RPE cell line.

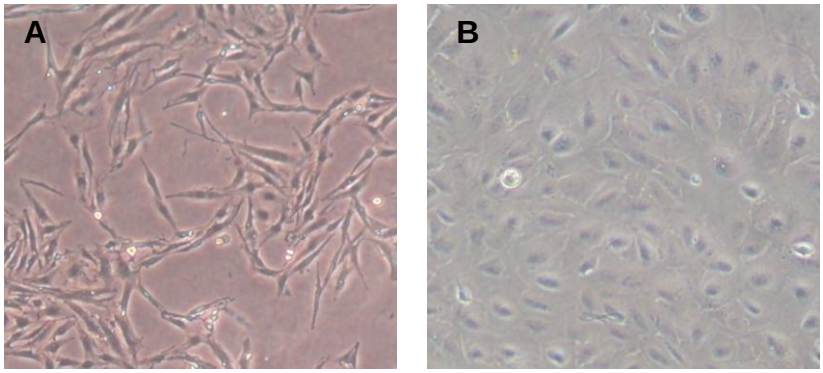

Figure 3.1 Morphology of MIO-M1 and ARPE-19 cells. (A) MIO-M1cells and **(B)** ARPE-19. 20 × magnification was used.

3.2 Aim

To investigate the effects of oxidative stress inducers on anti-oxidant gene expression in Mueller cells.

3.3 Results

3.3.1 The Effects of OGD on Nrf2 driven genes in MIO-M1 cells

OGD is one of the major inducers of oxidative stress in the retina and can affect the viability of retinal cells. Mueller cells were subjected to 1 h OGD using a modular incubator chamber followed by 24 h reperfusion. Oxygen levels in the medium were measured using an oxygen probe to ensure that they were maintained at a very low level during the experiments (Figure 3.2A). The results demonstrate that the oxygen levels were reduced from normal saturation levels (100%) to none (0%) by bubbling 95% N_2 and 5% CO_2 gas through the media and that the chamber maintained low levels of oxygen throughout 1 h of OGD. Cell viability was measured by MTS assay. OGD with 24 h reperfusion induced a small but significant proliferation of MIO-M1 cells compared to the control (Figure 3.2B), suggesting that MIO-M1 cells are not adversely affected by OGD.

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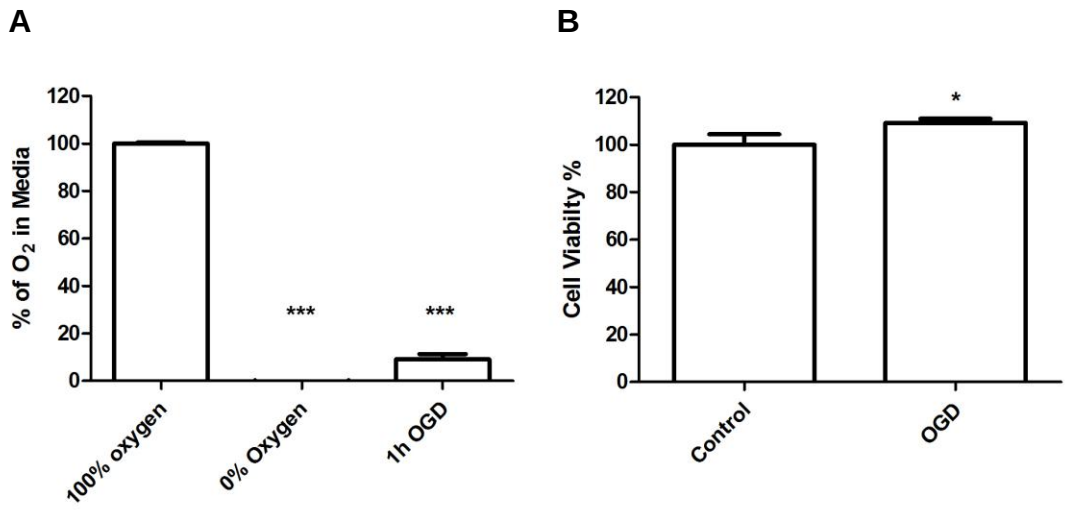

Figure 3.2 Effect of OGD on viability of MIO-M1 cells. (A) Verification of OGD chamber function. Levels of oxygen in the media, 100% is the normal saturated level, 0% is when the media has been depleted of oxygen and 1 h OGD is the levels of oxygen in the media after 1 hr OGD. (B) Serum starved MIO-M1 cells were subjected to 1h OGD with 24 h reperfusion. After the experimental conditions MTS was added for 3 h. Absorbance was measured at 490nm. Mean ± SEM (n=3) p*<0.05, p**<0.01.

Ischemia reperfusion has previously been reported to induce HO-1 via the Nrf2 pathway in liver and kidney cell lines and *in vitro* in rat and mouse models. The effects of OGD on Nrf2-dependent gene expression were investigated. Figure 3.3 (A-D) demonstrates that serum-starved cells subjected to OGD with reperfusion for various times (0-16 h) had no significant effect on the mRNA levels of Nrf2, NQO1, ferritin or HO-1. However the results do show some trends, specifically, NQO1 did increase slightly with OGD induction. The effect of OGD on HO-1 protein expression in MIO-M1 cells was also investigated. Figure 3.4 demonstrates that OGD had no effect on HO-1 protein expression

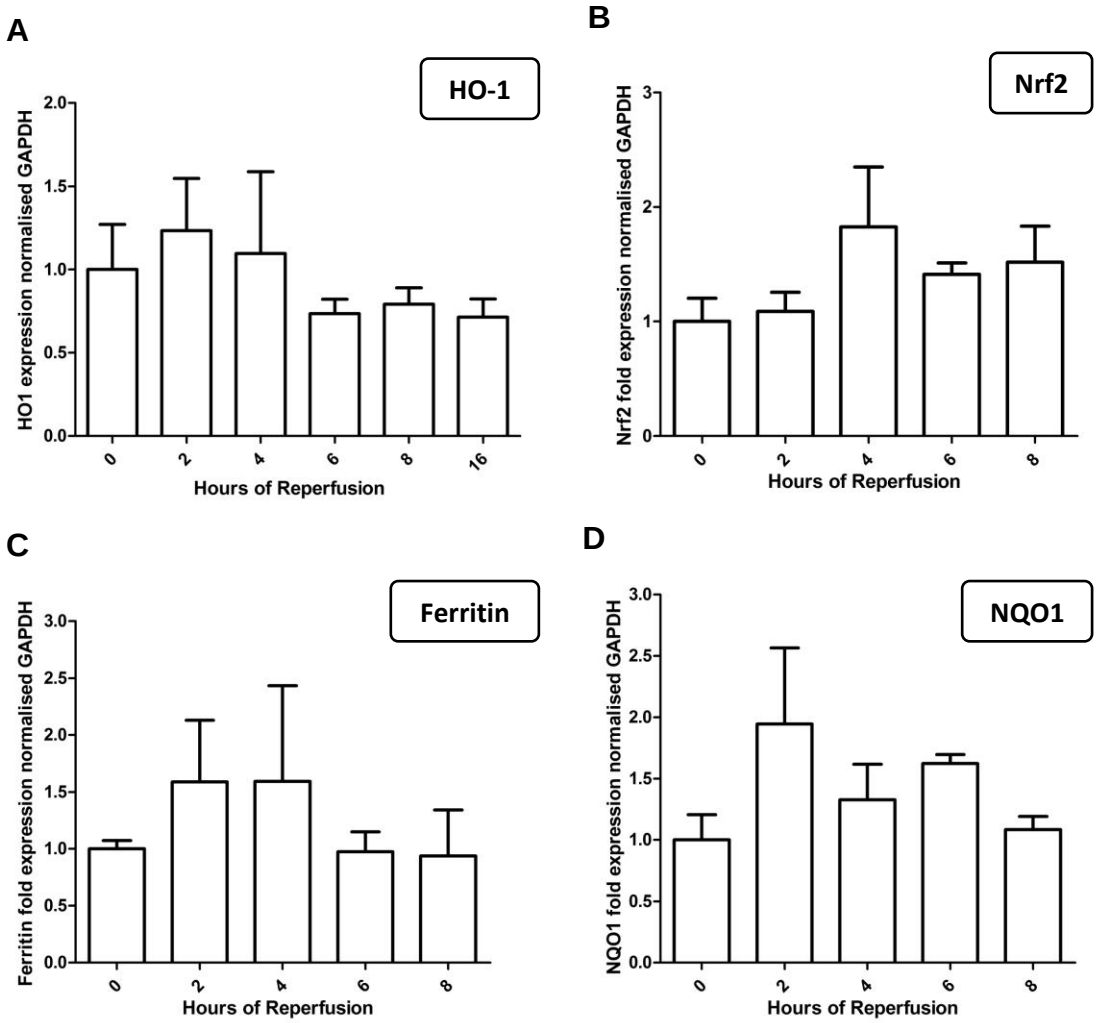

Figure 3.3. Effects of OGD and reperfusion on Nrf2-dependent gene expression in MIO-M1 cells. MIO-M1 cells had 1 h OGD and 0-16 h reperfusion. **(A)** HO-1, **(B)** Nrf2, **(C)** Ferritin and **(D)** NQO1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3-4).

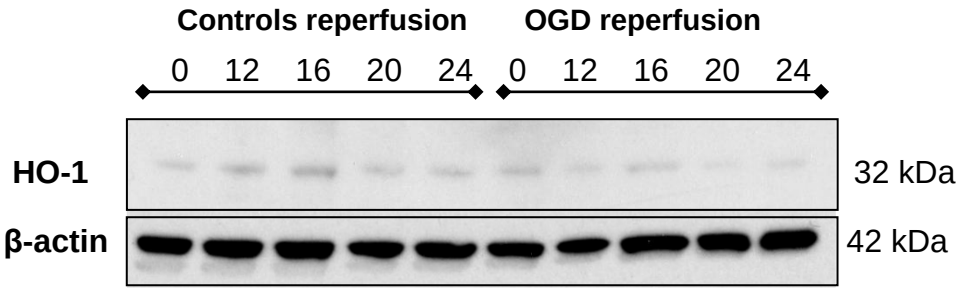

Figure 3.4. OGD does not increase HO-1 protein expression in MIO-M1 cells. Cells were untreated or treated with 1 h of OGD and reperfused for 0-24 h and protein extracts were prepared. HO-1 protein expression was assessed by western blot analysis. Figure is a representative of n=3**.**

3.3.2 Effects of oxidative stress on HO-1 expression in MIO-M1 and ARPE-19 cells

In retinal cells it has been shown that production of oxidative stress stimuli can induce expression of HO-1 in human retinal pigment epithelial cells (Bertram et al 2009). As there were no major changes with OGD, the effects of a range of oxidative stimuli on HO-1 expression in ARPE-19 and MIO-M1 cells was investigated. Figure 3.5 displays the effect of different oxidative stimuli on expression of HO-1 mRNA in MIO-M1 cells. Serum starved cells were treated with either 10 ng/mL TNF-α**,** 10 µg/mL LPS, 25 mM high glucose or 100 µM H_2O_2 for 0-12 h. Results were normalised to the level of GAPDH mRNA in the cells and the fold induction compared to the control. The TNF-α**,** LPS and H_2O_2 experiments were repeated 4 times and the glucose experiments repeated 3 times. The result show that TNF-α**,** LPS, glucose and H_2O_2 gave no significant fold increase induction in HO-1 mRNA expression during the 12 h time course (Figure 3.5). However, again, certain trends are noticeable within these results. Notably, whilst HO-1 does not give a significant fold increase after treatment

with H_2O_2 by a one way ANOVA with a post-hoc Tukey Test, using a student's t test there was a significant difference between 0 and 3 h ($p<0.05$).

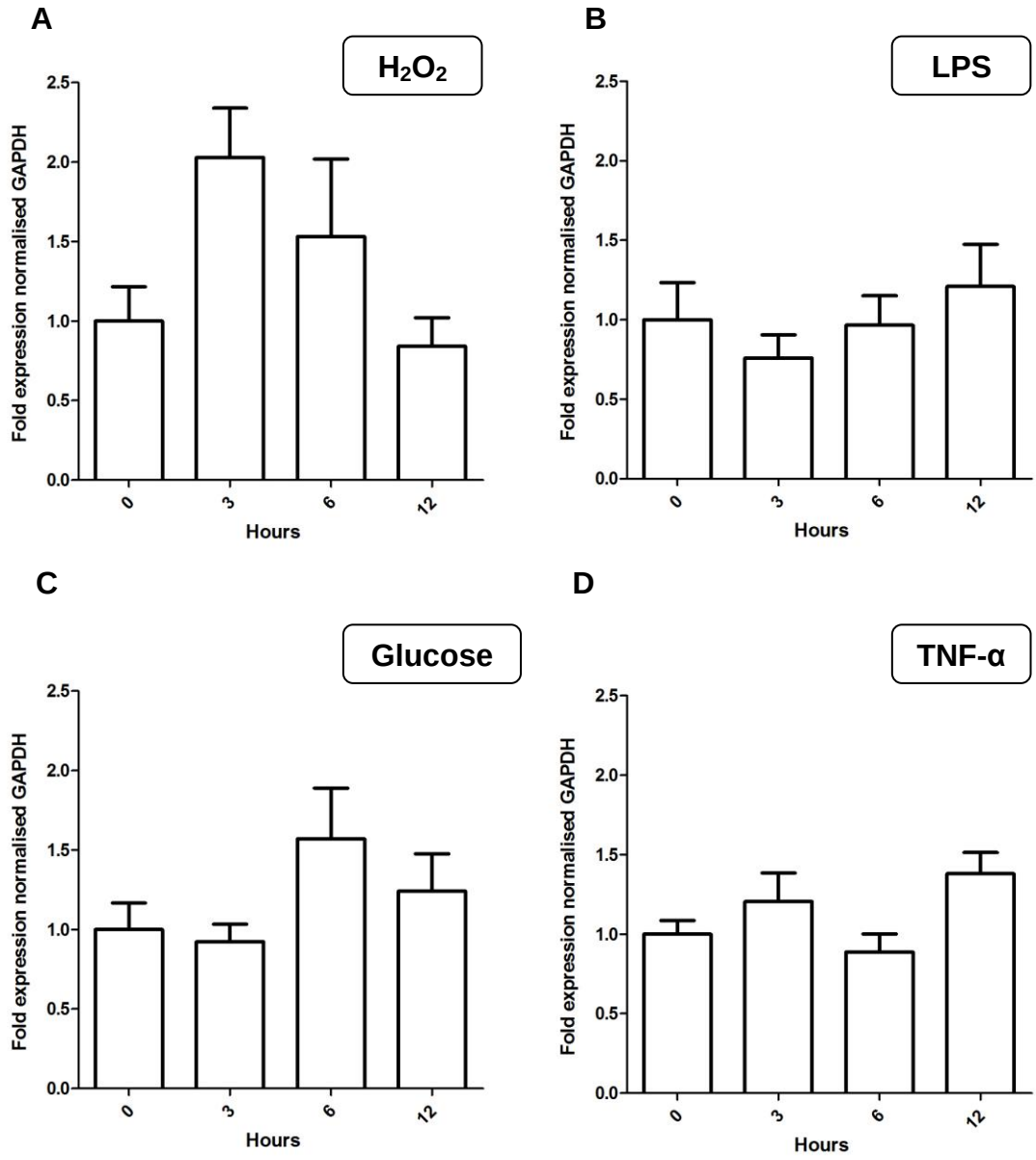

Figure 3.5 Effect of oxidative stimuli on HO-1 mRNA expression in MIO-M1 cells. Serum Starved MIO-M1 cells were treated with 100 µM H_2O_2 (A)**,** 10 µg/mL LPS (B), 25 mM Glucose (C) and 10 ng/mL TNF-α (D) for 0-12 h and RNA extracts were prepared. HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3/4).

Sources of oxidative stress have been suggested to increase HO-1 expression in ARPE-19 cells. To compare the results from the MIO-M1 oxidative stimuli experiments, the same set of oxidative stimuli were used. The effect of different oxidative stress stimuli on HO-1 expression in ARPE-19 cells is shown in Figure 3.6. The serum starved cells were treated with either 10 ng/mL TNF-α**,** 10 µg/mL LPS, 25 mM Glucose or 100 µM H_2O_2 for 0-12 h. All results were normalised to GAPDH and the fold induction compared to the control. The result show that TNF-α**,** LPS and Glucose gave no fold induction or trends in HO-1 expression during the 12 h time course. However, H_2O_2 did result in a significant fold induction in the expression of HO-1 mRNA. At 3 h the induction was at 21-fold, which continued to increase to 55-fold at 6 h before reducing to 13-fold by 12 h.

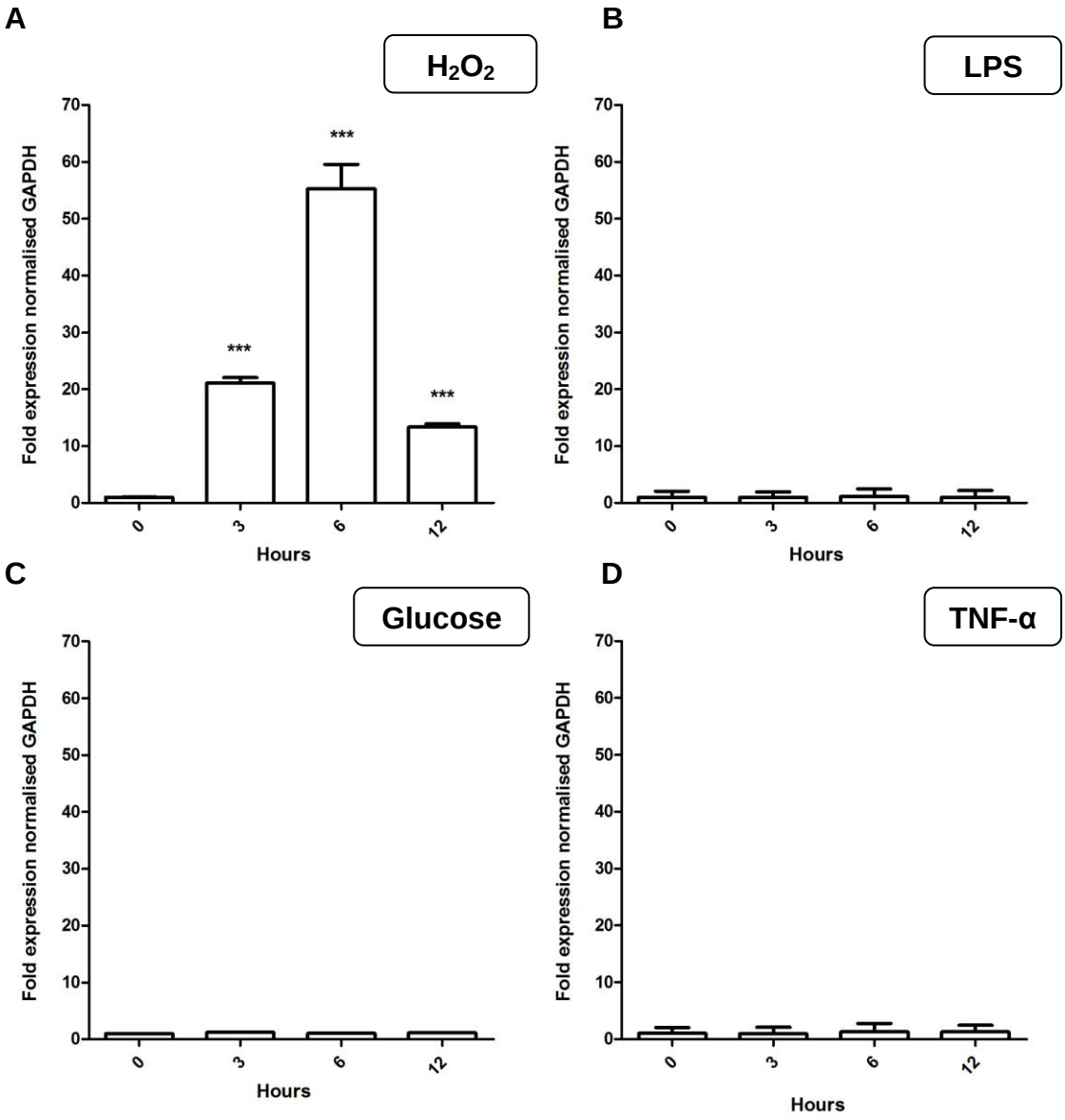

Figure 3.6. Effects of proinflammatory stimuli on HO-1 mRNA in ARPE-19 cells. Serum starved ARPE-19 cells were treated with either**,** 100 µM H_2O_2 (A), 10 µg/mL LPS (B), 25 mM Glucose (C) or 10 ng/mL TNF-α (D) for 0-12 h and RNA extracts prepared. HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3/4) p***<0.001.

The induction by H_2O_2 in ARPE-19 cells clearly showed that that methodology was working. Further positive controls for the other stresses have also been carried out within the lab using different models. A positive control carried out as part of this research (Figure 3.7), demonstrates that LPS does induce HO-1 in THP-1 cells, a human monocytic leukemic cell line which is a well established model for studying the Nrf2 pathway (Tsuchiya et al 1980, Ogborne 2005).

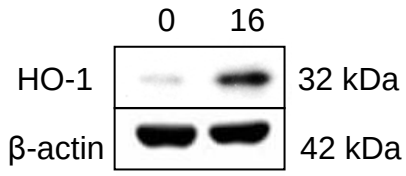

Figure 3.7. LPS induction of HO-1 protein expression in THP-1 cells. Cells were incubated with 10 µg/mL LPS. Total protein extracts were prepared and HO-1 protein expression measured by western blot analysis. Representative experiment of n=3.

3.4 Discussion

Oxidative stress plays a key role in retinal diseases and in the activation of Mueller cells (Bringmann et al 2009b). This novel data found that that whilst other cell types respond to oxidative stress stimuli and invoke Nrf2 driven genes, the Mueller cell line, MIO-M1, had little response to oxidative stress and did not cause a significant upregulation in Nrf2 regulated genes. It is known that Mueller cells have an important role in the protection of neuronal cells (Bringmann et al 2009b). The activated state of gliosis is stimulated by pathological stresses and can provide damage limitation in the retina. However, it can also inadvertently have more toxic effects in the retina, depending on the level of activation (Bringmann et al 2009a). It may be by this mechanism and not Nrf2 that oxidative stress is combated and cytoprotection given.

No work has been performed previously on the effect of OGD on the MIO-M1 cell line. One study investigating hypoxia on primary rat mueller cells for 12 h found that they showed a transient proliferation and it was only when they performed hypoxia for 24 h that cytotoxicity occurred (Zheng et al 2010). This is comparable to the results seen here with OGD, where exposure resulted in a small but significant increase in MIO-M1 cell number. Other research has increased the intraocular pressure of rat eyes for 60 min to simulate ischemic conditions and did find that HO-1 expression was increased in Mueller cells which were found to be more resistant to the insult. However, although ischemic conditions were confirmed, the increase in IOP could have also triggered other mechanisms in the retina that contributed to the increase of HO-1 (Arai-Gaun et al 2004). Both these studies commented on the resilience of Mueller cells. OGD

has resulted in cytotoxicity in other retinal cell types (RGC, neuronal and RPE cells) which seem to be less resistant to cytotoxic effects (Osborne et al 1997, Osborne et al 1999). Other models of OGD in the liver and kidney have shown that HO-1 is upregulated in the reperfusion period (Tanaka et al 2007). Upregulation was not observed in MIO-M1 cells.

The presented results suggest that whilst OGD in the MIO-M1 causes a slight but significant increase in cell viability at 1 h, which could be a sign of gliosis, it was not related to a significant induction of Nrf2, NQO1, ferritin or HO-1. However, a slight trend for NQO1 and ferritin expression was noted as a result of OGD with reperfusion. This suggests the Nrf2 pathway could be present in these cells but that it is not the main mechanism by which oxidative stress is managed. However, the resistance to the cytotoxic effects of OGD in the MIO-M1 cells is unlikely to be due to the induction of the Nrf2 pathway.

The Nrf2 pathway can also be activated by an array of different oxidative stresses including TNF-α and LPS in THP-1 cells as shown in Figure 3.16, (White et al 2010, Rushworth et al 2011). Saviranta et al demonstrated that HO-1 can be induced by H_2O_2. They showed that 1.1 µM H_2O_2 after 24 h caused over 20-fold induction of HO-1 expression in ARPE-19 cells (Saviranta et al 2011). LPS, TNF-α, Glucose, and H_2O_2 did not significantly induce HO-1 expression in the MIO-M1 cells. However LPS did show a potential trend to increase HO-1 which may have become significant with a longer time course and glucose, a factor in diabetic retinopathy, may have induced a small increase of HO-1 expression at 6 h, but this is marginal. LPS, TNF-α and Glucose, did not induce HO-1 expression in the ARPE-19. However, a significance increase in HO-1 in the MIO-M1 cells was seen in response to

H_2O_2 at 3 h (Student's t-test; $p<0.05$). However, analysis by one way anova, with a post-hoc Tukey Test, did not show significance. This suggests that H_2O_2 can increase HO-1 expression in the MIO-M1 cells. It would be important to carry out further experiments using different concentrations and time points to confirm these results. The effect of H_2O_2 on HO-1 expression in MIO-M1 cells was compared to that in ARPE-19 cells. The response was much reduced in the MIO-M1 cells with 100 µM H_2O_2 at 6 h giving a 55-fold induction in the ARPE-19 cells. This highlights the differences between the MIO-M1 and the ARPE-19 cells, despite both having similar roles within the retina in aiding the immune response in the retina and producing cytokines. This suggests that the MIO-M1 cells have another mechanism by which they are able to provide themselves with cytoprotection and deal with oxidative stress.

This research clearly demonstrated that the Nrf2 pathway is not the main mechanism by which the MIO-M1 cells respond to oxidative stress, however it does not conclude by which mechanism MIO-M1 cells do respond. This work was all carried out in human cell lines and not animal models in which a lot of retinal research is carried out. However, a future direction available may be to work directly from human retinal tissue samples. Another line of inquiry could include a micro-array approach, scanning the expression of many different genes. This technique could be employed to investigate which pathways may be involved in providing cytoprotection to MIO-M1 cells when subjected to oxidative stress. Another direction of research would be to look at the activation of GFAP, which is an early sign of gliosis to investigate if it is by this mechanism that Mueller cells cope with oxidative stress.

It was novel to find that the MIO-M1 cell line was resistant to many oxidative stresses and that the Nrf2 pathway was not majorly involved in providing a level of cytoprotection when these cells were subjected to them. This meant that investigating the Nrf2 pathway when using oxidative stress as a stimulus was not possible and so it was decided not to pursue this direction of research further. Instead, it was decided investigate whether the Nrf2 pathway could be induced in these cells by stimulating the cells with antioxidants.

Chapter 4

Effects of sulforaphane on Nrf2-mediated gene expression in retinal cells

4.1 Introduction

The eye is constantly subjected to oxidative stress caused by UV light and other factors (Varma et al 2011). Oxidative stress can lead to retinal damage and is associated with the development of diseases such as cataracts, age-related macular degeneration, diabetic retinopathy and glaucoma (Liang and Godley 2003) Anti-oxidants are thought to combat oxidative stress and protect the eye from damage. Anti-oxidants can be found in the diet and are produced endogenously in cells in response to oxidative stress (Ogborne et al 2004). The transcription factor Nrf2 is a key sensor of oxidative stress and responds by activating cellular anti-oxidants and other cytoprotective proteins, including HO-1, NQO1 and Trx1 to defend the cell from oxidative stress (Motohashi Yamamoto 2004, Kensler et al 2007, Dinkova-Kostova 2010).

Sulforaphane (SFN, 4-methylsufinylbutyl isothiocyanate), an organosulphur compound found in cruciferous vegetables, has been found to have various protective properties in several tissues, including anti-oxidant, anti-inflammatory and chemopreventive effects (Juge et al 2007). SFN exists in vegetables in the precursor form of a thioglucoside, which in broccoli is called glucoraphanin. Once the vegetable is consumed, this is hydrolysed into SFN by the enzyme myrosinase (Keum 2011, Zhang et al 1992).

SFN has been reported to activate Nrf2 or its downstream target genes in various cell types, including work from this laboratory where SFN activated HO-1 and NQO1 expression in primary human monocytes (Rushworth et al 2008). SFN has also been found to induce Nrf2-driven gene expression in retinal cells For example, in the ARPE-19 cell line, SFN induces Trx1, HO-1,

NQO1 and enzymes involved in regulating GSH synthesis including GCL, GS, and GR (Cano et al 2008, Wang et al 2008). SFN has also been reported to protect RPE cells from lipofuscin damage via induction of NQO1 and GST (Zhou et al 2006). In tubby mice, the retinal Trx1 system is impaired which results in photoreceptor degeneration. In this model, SFN can induce Trx1 and delay inherited photoreceptor degeneration via the extracellular signal-regulated kinase (ERK)/Nrf2 signal cascade (Kong et al 2009).

HO-1 has been suggested to have cytoprotective roles within the retina, and is activated by dietary anti-oxidants including curcumin and epigallocatechin via Nrf2 (Rushworth et al 2006, Ogborne et al 2004). However, there have been no reports in the literature of SFN activating HO-1 in retinal cells. In addition, to date, the effects of SFN on retinal cells have been limited to RPE, microglial, astrocytes and photoreceptor cells (Tanito et al 2005, Kong et al 2007, Yang et al 2007, Cano et al 2008). Mueller cells, however, are very important in the retina, transversing all layers, and have the dual function of being both protective and detrimental in the retina (Grierson 2002, Limb et al 2002). They also play a key role in response to oxidative stress and damage within the retina (Bringmann et al 2009a)

4.2 Aim

To investigate the effect of the antioxidant SFN on the Nrf2 pathway in MIO-M1 cells and examine the mechanism involved.

4.3 Results

4.3.1 The effects of SFN on HO-1 expression in ARPE-19 and MIO-M1 cell lines.

MIO-M1 cells were serum-starved for 24 h and then treated with 10 μM SFN or vehicle control (DMSO) for 0, 3, 6 and 12 h. HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH expression. SFN significantly increased HO-1 expression in MIO-M1 cells (Figure 4.1). As SFN has been previously reported to activate Nrf2-driven gene expression in ARPE-19 cells, these were used as a control in this study. SFN significantly increased HO-1 expression in ARPE-19 cells (Figure 4.1). Both cell types showed the same pattern of induction with peaks at 3 h and expression remaining high at 6 h before the levels of HO-1 mRNA expression decreased at 12 h. As 3 h gave the peak of HO-1 expression in both the MIO-M1 and ARPE-19 cells, this time point was used in further experiments.

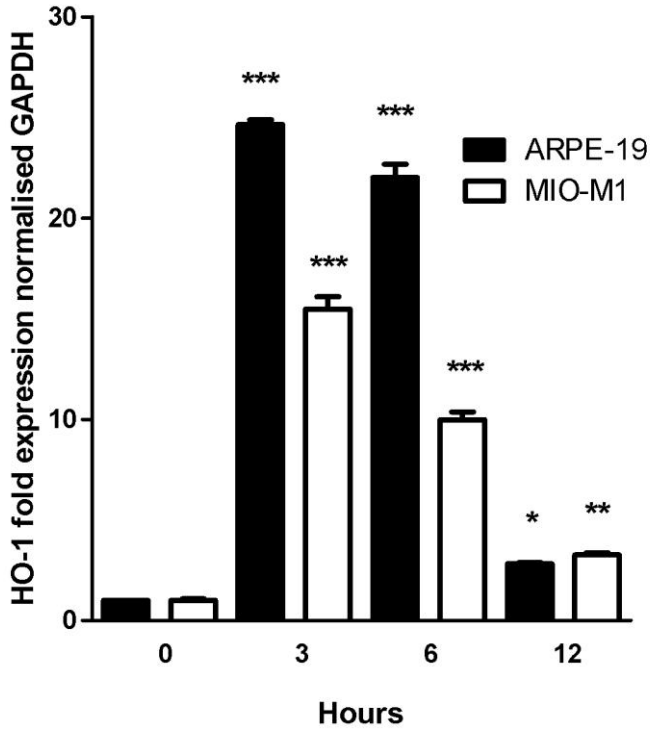

Figure 4.1. SFN induces HO-1 mRNA expression in ARPE-19 and MIO-M1 cells. MIO-M1 and ARPE-19 cells were treated with 10 µM of SFN for 0-12 h and total RNA extracted. HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3), *p<0.05, **p<0.01, ***p<0.001.

To test whether the induction of HO-1 was due to cell death, MIO-M1 cells were subjected to an MTS assay. Cells were serum starved for 24 h, followed by incubation with 1-10 µM SFN for 24 h prior to addition of MTS for 2.5 h. Controls included a blank, cells on their own and serum starved cells. Figure 4.2 demonstrates that SFN did not reduce cell viability at any concentration (1, 2, 5 or 10 µM). This suggests that any effects that may occur due to SFN are not as a result of apoptotic/cell death pathways and that the SFN does not have cytotoxic effects on the MIO-M1 cells.

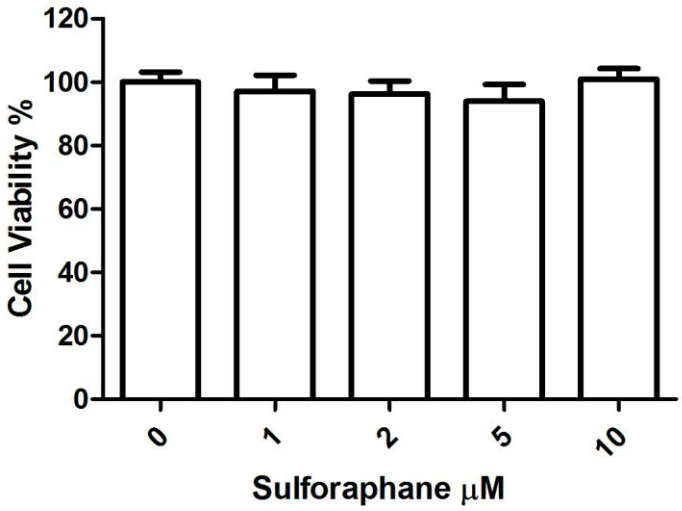

Figure 4.2. SFN (1-10 μM) does not affect MIO-M1 cell viability. MIO-M1 cells were treated with 0-10 μM SFN for 24 h before the addition of MTS for 3 h. Absorbance was measured at 490 nm. Mean ± SEM (n=3).

To determine if SFN-induction of HO-1 expression was dose-dependent, MIO-M1 cells were incubated with 2, 5 or 10 μM SFN for 3 h. Results were normalised to GAPDH and the fold inductions were compared to the vehicle control (DMSO). Figure 4.3 demonstrates that SFN dose dependently increased HO-1 mRNA expression. Whilst 2 μM SFN slightly increased HO-1 expression, this was not significant. However, 5 μM and, as shown previously, 10 μM SFN significantly increased levels of HO-1.

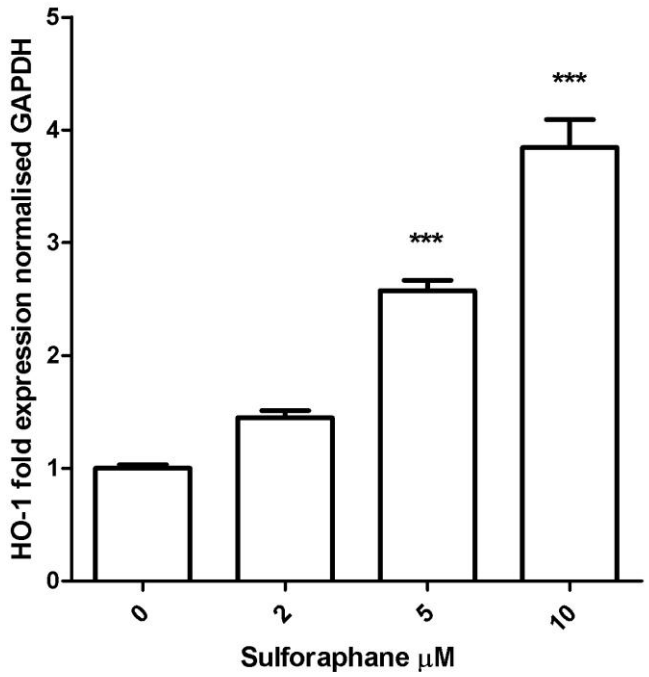

Figure 4.3. Induction of HO-1 mRNA expression in MIO-M1 cells is concentration-dependent. Cells were treated with 0 – 10 μM SFN for 3 h and total RNA extracted. HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3), p***<0.001.

4.3.2 SFN induction of Nrf2 driven genes.

As HO-1 is regulated by the transcription factor Nrf2, other ARE/Nrf2 regulated genes were investigated to discover if they are also upregulated with SFN in the MIO-M1 cell line. The genes investigated were Nrf2 (in case SFN induced *de novo* synthesis), NQO1, ferritin (heavy chain) and Trx1 (1). All results were normalised to GAPDH and the fold induction compared to the control which included the vehicle control DMSO without SFN. Nrf2 mRNA is basally expressed in MIO-M1 cells, Figure 4.4. (A) demonstrates that SFN did not induce Nrf2 mRNA expression in these cells by 12 h. This was not

surprising as not many stimuli have been shown to induce Nrf2 *de novo* synthesis, with an exception being LPS in THP-1 cells (Rushworth et al 2008). SFN also did not increase ferritin mRNA expression (Figure 4.4). In fact, ferritin mRNA decreased slightly at all time points compared to the control. However, the relative CT values were extremely high, which indicated that there was a high amount of basal ferritin mRNA expressed, suggesting that ferritin may be providing a level of cytoprotection by another means. SFN induced NQO1 mRNA expression as time progressed, with the 12 h time point showing a significant increase in NQO1 levels. The Trx1 CT levels were very low, on the border of detection using qRT-PCR, demonstrating very low basal expression of this antioxidant enzyme. This made the results more variable. There was no significant increase in Trx1 mRNA expression following SFN stimulation (Figure 4.4).

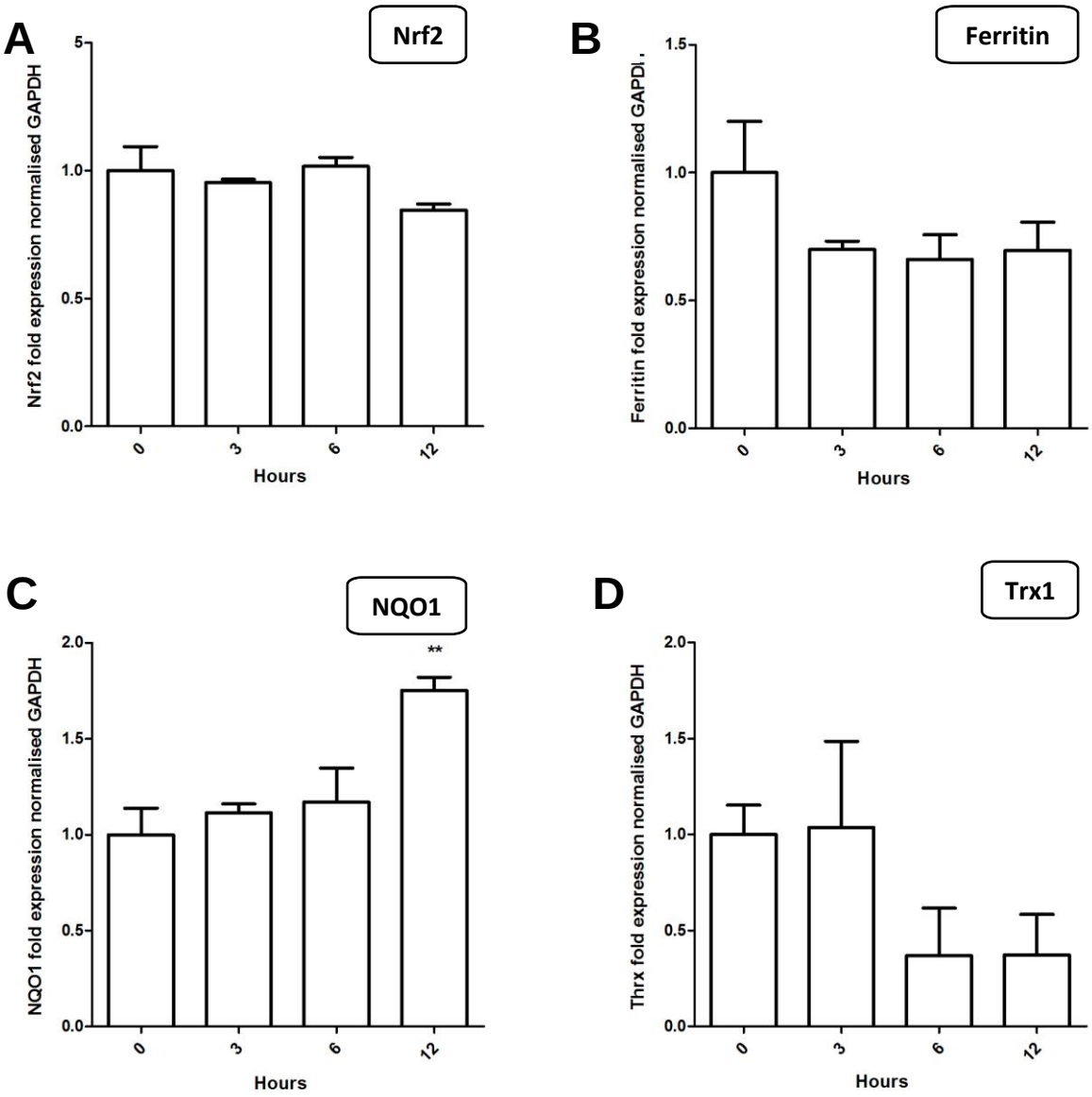

Figure 4.4. Effects of SFN on Nrf2-mediated gene expression in MIO-M1 cells. Cells were treated with 10 μM SFN for 0-12 h and total RNA extracted. Nrf2 (A), Ferritin (B), NQO1 (C) and Trx1 (D) mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3), p**<0.01.

As HO-1 mRNA expression was highly upregulated by SFN, the expression of SFN-induced Nrf2 and HO-1 protein expression was investigated. MIO-M1 cells, serum starved for 24 h, were treated with SFN for 0, 1, 2, or 4 h and total protein extracted. Proteins were separated by SDS PAGE and western blot analysis was performed. The 0 control was also the vehicle control (Figure 4.5). The results suggested that SFN induced Nrf2 protein expression in a time dependent manner. By 1 h Nrf2 protein was present and this continued to increase at 2 and 4 h.

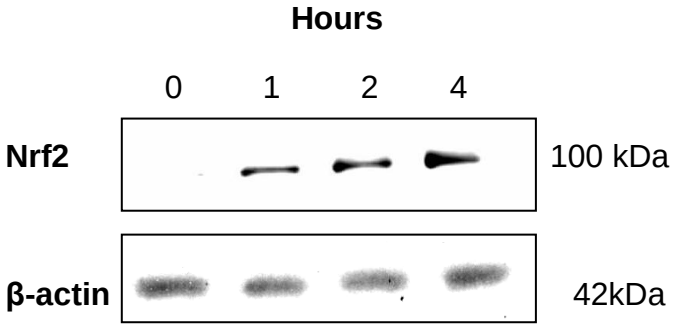

Figure 4.5. SFN induces Nrf2 protein expression in MIO-M1 cells. Cells were untreated or treated with 10 µM SFN for 1-4 h and protein extracts prepared. Nrf2 protein expression was measured by western blot analysis. Figure is a representative of n=3

As HO-1 mRNA expression peaked at 3 h, serum starved MIO-M1 cells were incubated with 10 μM SFN for 0-8 h, total protein was extracted and proteins separated by SDS PAGE. Western blot analysis demonstrated that SFN increased HO-1 protein expression in a time dependent manner. At 4 h HO-1 expression was present and by 6 and 8 h the expression was strong.

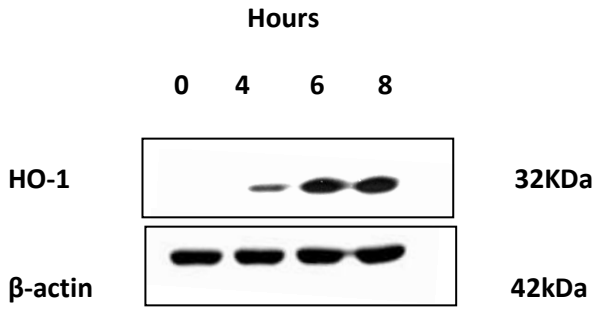

Figure 4.6 SFN induces HO-1 protein expression in MIO-M1 cells. Cells were untreated or treated with 10 μM SFN for 1-8 h and total protein extracted. HO-1 protein expression was measured by western blot analysis. Representative of n=3.

Nrf2 needs to translocate into the nucleus for the upregulation of antioxidant genes. Immunocytochemistry was carried out in order to visualise the translocation of the Nrf2 protein in MIO-M1 following SFN stimulation. Figure 4.7 demonstrates that Nrf2, shown in red, is present following 4 h induction with 10 μM SFN; however, translocation in to the nucleus (stained blue with DAPI) is not clear.

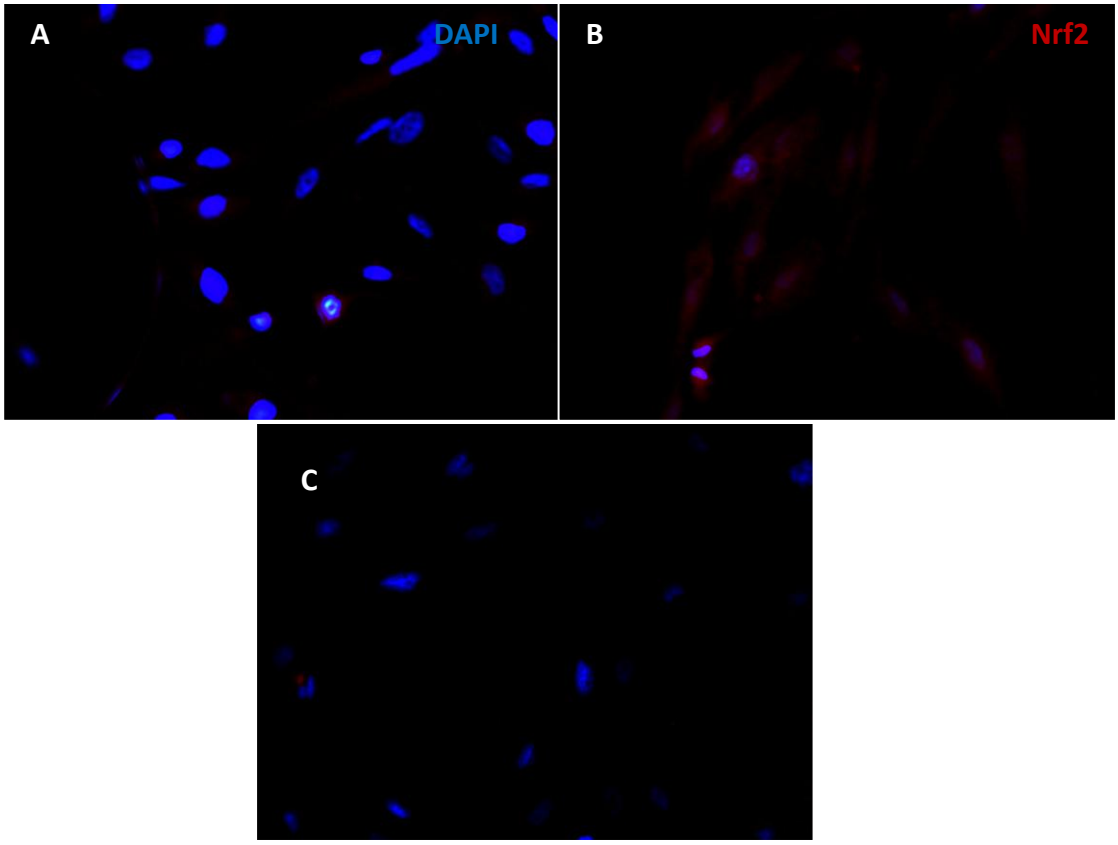

Figure 4.7 Nrf2 expression (Red) in MIO-M1 SFN stimulation. DAPI is shown in blue in all images. **A.** Sulforaphane 0hrs (no stimulation), **B.** 10 µM Sulforaphane 4h, **C.** Secondary Antibody Control (Rabbit anti goat)

4.3.3 PKC and PI3K regulation of the Nrf2 pathway induced by SFN

As discussed in Chapter 1, there are several signalling kinases that have been associated with the regulation of ARE-driven genes including p38, MAPK, PERK, JNK, PI3K and PKC (Ogborne et al 2004, Keum et al 2006). Two key kinase pathways that have been reported to regulate both sulforaphane-induced bioactivity and Nrf2-driven HO-1 expression are PKC and PI3K and these were investigated to see if they were involved in the mechanism of action of SFN inducing HO-1 in MIO-M1 cells. This was performed using the pan-PKC inhibitor, Bisindolylmaleimide I (BIS I) and the PI3K inhibitor LY294002. MIO-

M1 serum starved cells were either preincubated with 25 µM LY294002 or 10 µM BIS I for 30 min followed by 10 µM SFN for 3 h. The 0 control was the DMSO vehicle control. Figure 4.8 demonstrates that both LY294002 and BIS I significantly inhibited SFN induced HO-1 mRNA expression ($p^*<0.05$), whilst having minimal effect on reducing the basal levels of HO-1.This indicates that PKC and PI3K play a role in the induction of HO-1 by SFN in MIO-M1 cells.

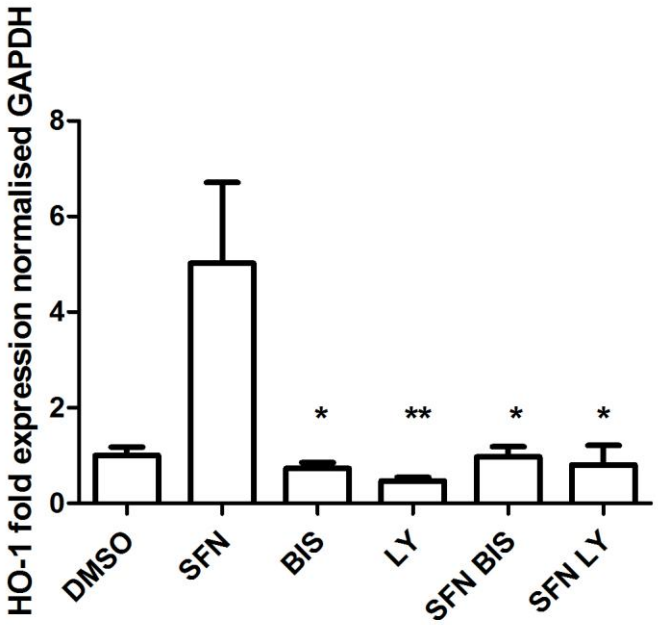

Figure 4.8. Role of PI3K and PKC in SFN-induced HO-1 expression in MIO-M1 cells. Cells were pre-treated with 25 µM LY294002 (LY) or 10 µM Bisindolylmaleimide I (BIS) for 30 min prior to SFN treatment for 3 h. Total RNA was extracted and HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean $\pm$ SEM (n=3) $p^*<0.05$ $p^{**}<0.01$.

To determine if the effect of BIS I on HO-1 expression was dose-dependent, serum starved cells were preincubated with 1, 5 or 10 µM BIS I for 30 min and then 10 µM SFN was added to the cells for 3 h. Controls without SFN added were performed to compare the effect that the SFN had on the cells with the inhibitors. The 0 control was the DMSO vehicle control. Figure 4.9 demonstrates that BIS I alone at any concentration had no significant effect on basal HO-1 mRNA expression in MIO-M1 cells. However, BIS I significantly inhibited SFN-induced HO-1 expression in a dose dependent manner. 1 µM BIS I actually enhanced induction of HO-1 mRNA by SFN.

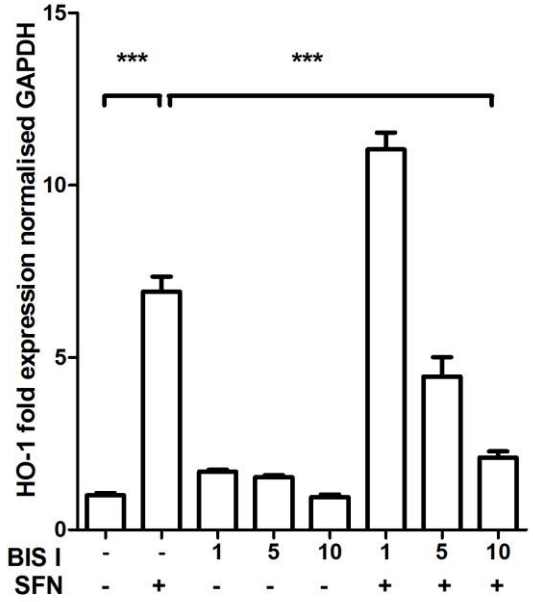

Figure 4.9 Bisindolylmaleimide I dose-dependently inhibits SFN-induced HO-1 mRNA expression in MIO-M1 cells. Cells were pre-treated with 1, 5 or 10 µM Bisindolylmaleimide I (BIS) for 30 min prior to 10 µM SFN treatment for 3 h. Extracts were prepared and HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3), p***<0.001.

Figure 4.10 shows the effect of preincubation with lower concentrations of the PI3K inhibitor LY 294002 on SFN-stimulated HO-1 mRNA expression in MIO-M1 cells. Serum starved cells were preincubated with 1, 5, 10 or 25 µM LY 294002 for 30 min and then 10 µM of SFN was added to the cells for 3 h. At all concentrations, LY294002 alone did not significantly affect the basal expression of HO-1 mRNA. LY294002 significantly reduced SFN-induced HO-1 mRNA expression at all concentrations but not in a dose-dependent manner, suggesting that it is an on/off switch effect. As 10 µM had the same effect as 25 µM, 10 µM will be used in future experiments as using 25 µM would not be specific for PI3K inhibition.

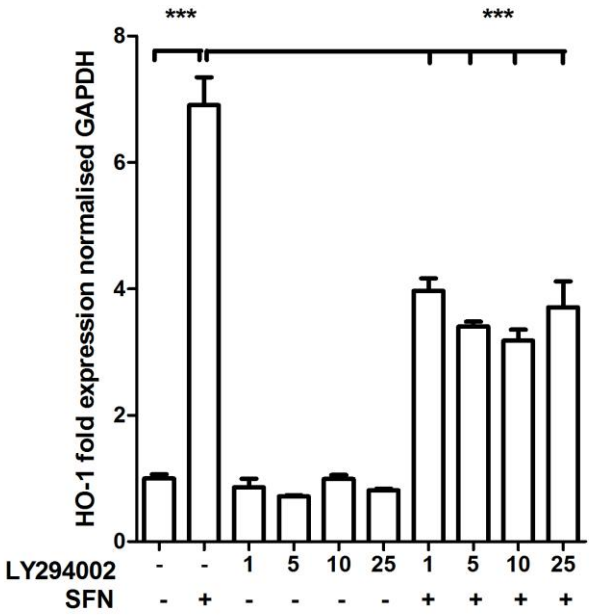

Figure 4.10 LY294002 dose-dependently inhibits SFN-induced HO-1 mRNA expression in MIO-M1 cells. Cells were pre-treated with 1, 5, 10 or 25 µM LY294002 for 30 min prior to 10 µM SFN treatment for 3 h. Extracts were prepared and HO-1 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Mean ± SEM (n=3), p***<0.001.

As inhibition of PI3K and PKC resulted in prevention of SFN induced HO-1 mRNA expression, the effect of LY294002 and Bisindolylmaleimide I on Nrf2 and HO-1 protein expression was also investigated. Serum starved cells were preincubated with 10 µM BIS or 10 µM LY294002 for 30 min and then cells were treated with 10 µM SFN 2 h (for Nrf2 protein) or 8 h (for HO-1 protein). Total protein was extracted and separated by SDS PAGE and western blot analysis performed. SFN-induced Nrf2 protein expression was highly inhibited by both BIS I and LY294002 (Figure 4.11). SFN-induced HO-1 protein expression was also highly inhibited by LY294002 and BIS I (Figure 4.12). These results suggest that PKC and PI3K regulate SFN-induced HO-1 expression, upstream of Nrf2.

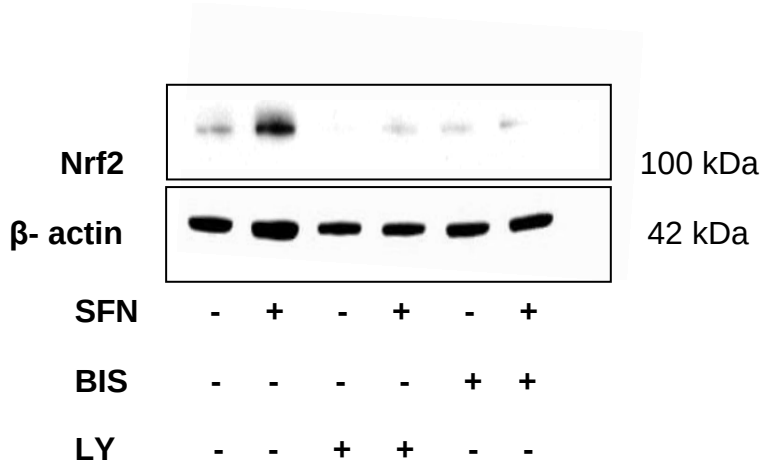

Figure 4.11 Bisindolylmaleimide I and LY294002 inhibit SFN-induced Nrf2 protein expression in MIO-M1 cells. Cells were pre-incubated with 10 µM LY294002 or 10 µM BIS I for 30 min prior to 10 µM SFN treatment for 2 h Total protein extracts were prepared and HO-1 protein expression measured by western blot analysis. Representative experiment of n=3.

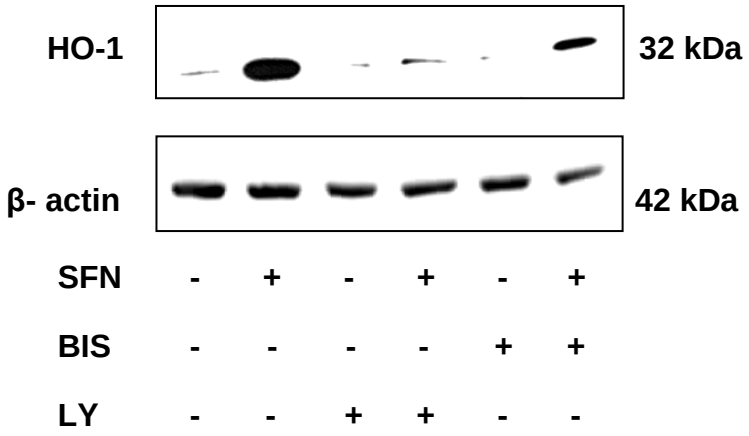

Figure 4.12 Bisindolylmaleimide I and LY294002 inhibit SFN-induced HO-1 protein expression in MIO-M1 cells. Cells were pre-incubated with 10 µM LY294002 or 10 µM BIS I for 30 min prior to 10 µM SFN treatment for 8 h. Total protein extracts were prepared and HO-1 protein expression measured by western blot analysis. Representative experiment of n=3.

4.4 Discussion

Mueller cells are more resilient to oxidative stress compared to other retinal cell types and, through active gliosis, can have protective and cytotoxic effects on the surrounding retinal cells (Bringmann et al 2009a). Therefore it is important that these cells are protected and maintained to ensure protection of the other retinal cell types. Any impairment of the protective functions of Mueller cells may contribute to a loss of neurons by increasing their susceptibility to oxidative stress in the diseased retina (Tanito et al 2007, Saviranta et al 2011, Bringmann et al 2006, Bringmann et al 2009a). The Nrf2 pathway is a central co-ordinator of cytoprotective cellular responses. In this study it was shown for the first time that SFN can activate the Nrf2 pathway in Mueller cells. SFN increased the level of Nrf2 protein in cells but not Nrf2 mRNA expression, suggesting that Nrf2 activation was independent of *de novo* synthesis. In addition, SFN activated the Nrf2 target genes, HO-1 and NQO1, but not ferritin or Trx1. Furthermore, both PI3K and PKC were involved in the regulation of SFN-induced Nrf2 and HO-1 activation. These results demonstrate that the Nrf2 pathway is activated in Mueller cells and suggest that SFN may have a protective function in these cells.

SFN has been reported to have numerous beneficial effects *in vitro* and *in vivo* including chemoprotective, anti-inflammatory, anti-oxidative and cytoprotective effects (Tanito et al 2005, Zhang and Tang 2007, Wagner 2011). In relation to the eye, previous studies in retinal microglial cells, RPE cells and photoreceptor cells have shown that SFN is protective in the retina. It protects RPE cells against oxidative stress by upregulating anti-oxidant and Phase II

enzymes (Zhou et al 2006, Cano et al 2008). In addition, SFN delays the progression of photoreceptor degeneration in mice (Kong et al 2007). This lab and others have previously reported that SFN activates HO-1 in monocytes and liver, lung and heart cells (Wu et al 2001; Jeong et al 2005, Rushworth et al 2006, Ping et al 2010, Zhao et al 2010, Wagner et al 2011). However, SFN has not previously been reported to activate HO-1 in any retinal cell type. This research has shown that SFN activates HO-1 in both ARPE-19 and MIO-M1 cell lines, representing retinal pigment epithelial cells and Mueller cells, respectively. This could be occurring through modification of the Cys151 residue on Keap1 where SFN has previously been suggested to activate the Nrf2 pathway (Zhang and Hannink 2003). However, this would not take to into account how PI3K and PKC inhibition also stopped SFN from inducing HO-1 in these cells. These results suggest direct activation of Keap1 by SFN is not the full mechanism of action. Further research is required to fully map the exact mechanism of action.

NQO1 is an enzyme which provides cytoprotection to cells by catalysing the detoxification of electrophiles and reduces ROS intermediates produced by redox cycling. It is a FAD-dependent flavoprotein which reduces, via NADPH or NADH, a range of quinines, quinonemines and nitroaromatics (Dinkova-Kostova and Talalay 2010). NQO1 has been well documented as being activated by SFN, in multiple organs and cell types of mice and humans including the liver, lung and the eye. NQO1 has previously been activated in ARPE-19 by the antioxidant vitamin E and provided cytoprotection against a chemical found in cigarette smoke which has been linked to being a factor in AMD. Also, the citrus flavoniod, eriodictyol, another antioxidant has been shown to elevate Nrf2 driven gene expression in ARPE-19 cells, including NQO1. However, there

have been no reports to date on SFN activating NQO1 in Mueller cells. This study demonstrated that SFN significantly activates NQO1 mRNA expression after 12 h of induction in the Mueller cell line, MIO-M1 cells. Further studies are required to determine if expression is higher at later time points and to examine the mechanisms involved, including involvement of Nrf2 and upstream kinases.

Excess free iron is a source of oxidative stress (He et al 2007). Within the retinal RPE and photoreceptor cells, several iron-containing proteins are expressed (Gnana-Prakasam et al 2010). Ferritin, which binds to the cytotoxic free iron, has been shown to be induced by SFN in intestine of mice, using micro array analysis (Thimmulappa et al 2002, Hintze and Theil 2005). It is expressed in the choroid and inner nuclear layer of the retina (He et al 2007). Hahn and co-workers have demonstrated that it is expressed in RGC and rod bipolar and photoreceptor cells in mice (Hahn et al 2004). No studies to date have reported SFN activation of ferritin expression in Mueller cells**.** Ferritin consists of two proteins, ferritin H and ferritin L, both of which are regulated by Nrf2. Ferritin H usually responds better to Nrf2 stimulation (Hahn et al 2004). In this study, it was found that SFN did not induce ferritin H mRNA expression in MIO-M1 cells. However, basal ferritin mRNA expression was very high in these cells, as evidenced from ferritin CT values in the real-time PCR analysis, therefore ferritin may still have a cytoprotective function within Mueller cells but not via SFN.

Trx1 is a cytoprotective protein that maintains the cells' normal reduced state. Trx1 has been shown to be upregulated by SFN in ARPE-19 cells and research has suggested that SFN induces Trx1 through the ARE and provides cytoprotection against light damage in whole mice retina and the RPE (Tanito et

al 2005). In tubby mice, SFN induces Trx1 expression in photoreceptor cells (Kong et al 2009). However, no studies to date have reported SFN activation of Trx1 in Mueller cells**.** This research showed that SFN did not significantly induce Trx1 mRNA expression in MIO-M1 cells, and found that the basal levels of Trx1 mRNA were very low in these cells. However, Trx1 levels did show a trend of reducing further by 6 and 12 h, but not by a significant level. This highlights that there are potential variations of Nrf2-driven gene expression and induction in the different cell types within the retina. As Trx1 is responsible for thiol redox control within cells it may be that this type of redox is not required with the Mueller cells, but further research would be required to confirm this theory (Zhong et al 2000).

SFN has been suggested to activate the Nrf2 pathway through Keap1 modification. As SFN is an electrophile it can potentially interact with thiol groups to form thionoacyl adducts, which may affect the cysteine residues on Keap1, especially Cys151, but the stability of this within cells is still under review (Hu 2011). SFN has previously been shown to not affect Nrf2 mRNA levels but upregulates Nrf2 protein over a short period of time (Keum 2011). In this study, SFN did not result in Nrf2 *de novo* synthesis; this was as expected as only a few compounds like LPS in THP-1 cells are known to cause Nrf2 *de novo* synthesis (Rushworth et al 2008). This suggests that SFN may be interacting with Keap1, which leads to more Nrf2 protein being available to go into the nucleus and increase HO-1 and NQO1 mRNA expression. The free Nrf2 protein made available following SFN treatment was shown by immunocytochemistry. However, whilst an increase in Nrf2 was seen in the cytoplasm it was not clearly seen to be translocating to the nucleus. This may

be due to the fact that Nrf2 protein levels started to increase at 1 h and the images taken at 4 h missed the translocation but did show that free Nrf2 was still present as it is shown by the protein levels. Further experimentation would include doing a time course on SFN-induced Nrf2 translocation and using confocal microscopy to confirm location in cell.

Many different pathways and intermediates have been reported to lead to activation of Nrf2, including the ERK and p38 MAP kinases, PI3K, PKC, and GSK3β. How these different pathways interact to cause Nrf2 activation is still under investigation. This study demonstrated that PKC and PI3K aid in the regulation of SFN activation of HO-1 and Nrf2 in MIO-M1 cells. PKCδ has been reported to activate Nrf2 through tBHQ and dissociate with Keap1 when stimulated by antioxidants through Ser40 in HepG2 cells (Bloom and Jaiswal 2003). In our laboratory curcumin has shown been to activate Nrf2 through PKCδ in THP-1 cells. In the same cell type LPS induces HO-1 expression via Nrf2 and PKCα but the subtype is still to be determined (Rushworth et al 2005, Rushworth et al 2006). This can lead to the release of Nrf2 from Keap1 leading to the upregulation of Nrf2-driven gene expression, including HO-1. In this study, inhibiting PKC resulted in the down regulation of SFN induced HO-1 expression and total inhibition of Nrf2 protein in the MIO-M1 cells, suggesting that it plays an important role in SFN-induced Nrf2 activation. Further studies would be required to determine which isoform of PKC were involved, however PKCα and PKCδ would be the first two that should be investigated.

PI3K has been identified to be a regulatory factor of the Nrf2 pathway when activated by a range of dietary antioxidants including carnosyl in PC12 cells, resveratrol in neurons and the plant extract oleanolic in smooth muscle rat

cells (Martin et al 2004, Feng et al 2011, Fukui et al 2010). However, unlike PKC no direct phosphorylation of Nrf2 by PI3K has been shown. Wang *et al.* showed that in the ARPE-19 that SFN induced Nrf2 driven genes GCL and GCL via PI3K (Wang et al 2008). In this study, LY294002 suppressed SFN-induced HO-1 mRNA expression, suggesting that PI3K regulates this pathway. The regulation did not appear to be dose-dependent, but was in an on/off switch manner, as varying the concentration of the inhibitor did not affect the level it reduced HO-1 mRNA expression. However reducing the concentration of the inhibitor further may show a dose dependent activation of PI3K. SFN-induced HO-1 expression was only partially inhibited with the inhibition of PI3K and HO-1 expression did not return to basal levels. Lower concentrations of the inhibitor were used (1-10 μM) due to 25 μM having the possibility of not being specific and could affect other kinase pathways. This suggests that the PI3K pathway is only partially responsible for SFN induced HO-1 mRNA expression in MIO-M1 cells. However, at the protein level inhibition of PI3K did completely prevent SFN induced Nrf2 and HO-1 expression. This could mean there are some post translational mechanisms activity that is regulated by PI3K. This data suggests that PKC and PI3K are both involved upstream of Nrf2 in response to SFN stimulation in MIO-M1 cells. More research is required to fully understand the complex role that both PI3K and PKC have in the regulation of SFN-induced Nrf2 and HO-1 expression in Mueller cells. Further studies should confirm that SFN leads to phosphorylation of these two kinases in MIO-M1 cells, and the specific PKC isoform involved could be identified by using a screen of PKC-specific siRNA or small molecule inhibitors and PKC-specific antibodies.

The immunocytochemistry experiments that were conducted in this research were preliminary and need to be expanded further by having a large range of time points or a live video feed to capture Nrf2 translocating into the nucleus. Ideally, confocal microscopy should be used to determine exact locations in the cell. Expression of HO-1 and NQO1 could also be investigated by immunocytochemistry. Further studies could confirm the involvement of Nrf2 in SFN-induced HO-1 and NQO1 expression by looking at the translocation of Nrf2 into the nucleus by using nuclear and cytosolic extracts and examining by western blot analysis, and examining transcriptional activity by reporter assay, using a multicopy ARE reporter. In addition, the role of Nrf2 in this pathway could be investigated using siRNA knockdown of Nrf2 and functional outcome of HO-1 upregulation examined using HO-1 siRNA.

Finally an interesting way to further this research would be to examine this pathway in human organotypic retinal cultures (HORCs), as all reported work on the Nrf2 pathway and the retina has been in rat or mice models and not in human tissue. Whist Nrf2 had previously been thought to be found in the RPE and potentially in the retina no work had looked at Nrf2 expression in Mueller cells within the human eye. Expression of SFN- induced Nrf2, NQO1 and HO-1 would clarify how and where SFN induces Nrf2 driven genes within the retina and within the Mueller cells.

Diet has been linked with age-related retinal associated diseases, including AMD and diabetes. Fruit and vegetables contain a multitude of flavonoids and other bioactive compounds with anti-oxidant activity. Cucurmin, found in the spice turmeric, has been shown to provide cytoprotection against light and oxidant stress via the Nrf2 pathway in murine retinas and RPE and

photoreceptor cell lines (Mandal et al 2009). Alpha lipoid acid, found in broccoli and tomatoes, has been shown to up regulate HO-1 in THP-1 cells (Ogborne et al 2005). tBHQ, an anti-oxidant used as a preservative in food has also been shown to activate Nrf2 and its downstream genes in primary-cultured hepatocytes cells (Keum et al 2006). Further studies could investigate a wide range of antioxidants that have the potential to upregulate Nrf2 driven genes in Mueller cells and human retinal cultures, to determine their effects on the pathway and the functional effects.

In conclusion, this research has shown for the first time that the non-toxic antioxidant SFN does not induce Ferritin or Trx1 but does induce HO-1 and the Nrf2 pathway in human Mueller cells via PKC and PI3K, suggesting that SFN is a promising antioxidant that could have therapeutic potential in keeping Mueller cells protected from oxidative stress in diseased and healthy retinas.

Chapter 5
General Discussion

Oxidative stress is a major contributing factor in retinal diseases, including glaucoma, diabetic retinopathy and AMD (Tezel 2006, Pan et al 2008, Zhao 2011) The Nrf2 pathway is a major sensor of oxidative stress, and HO-1 is upregulated by most inducers of Nrf2 (Arai-Gaun et al 2004). The Nrf2 pathway plays a key role in the eye by protecting retinal cells from damage. Mueller cells are structurally and functionally supportive of the surrounding retinal cells, especially neuronal cells, and play a key role in protecting the retina from many types of damage (Bringmann et al 2009a). However, the role of the Nrf2 pathway in Mueller cells has not previously been reported. The studies presented here indicate for the first time that the Nrf2 pathway is activated in these cells by SFN, a compound from broccoli that has previously been reported to have cytoprotective and anti-inflammatory effects in vitro and in vivo (Juge et al 2007). In addition, several ROS inducers had no effect on this pathway in Mueller cells, suggesting that compared to other cell types in the retina, Mueller cells may be more resistant to oxidative stress.

This study initially looked at the effect of oxidative stress inducers on the Nrf2 pathway in the human MIO-M1 cell line, a validated model for retinal Mueller cells (Limb et al 2002). Five different inducers were tested on the MIO-M1 cells, OGD, LPS, high glucose, TNF-α and H_2O_2, and these have all previously been reported to induce ROS in various cell types (Danilov et al 2009, White et al 2010, Rushworth et al 2011). Further studies should confirm that they do indeed induce intracellular ROS in Mueller cells. These experiments could be carried out by flow cytometry or a 96-well assay using H2DCFDA, a fluorescent dye that detects intracellular ROS. This research found that the classical ROS inducer, H_2O_2, had a minimal effect on HO-1

expression in MIO-M1 cells when compared with the ARPE-19 cells (50 fold less), suggesting that Mueller cells may be more resistant to oxidative stress. Both Mueller cells and RPE have similar roles in the retina, maintaining and providing structural support to other more vulnerable cell types (the photoreceptors for the RPE and neurons for the Mueller cells). However, the current study suggests that the mechanisms involved in performing this role are different. It should be noted that MIO-M1 cells have been reported to have some stem cell-like properties, which may contribute to a higher resistance to oxidative stress and differences between the two cell types.

In the Mueller and ARPE-19 cells LPS, TNF-α and high glucose concentrations had no significant effect on Nrf2, NQO1, ferritin or HO-1 gene expression. It should be noted that these are both cell lines, and therefore primary human cells may not have the same effect as in the human retina. However, this data does contrast with other studies, where LPS has been shown to induce mRNA expression of all of these genes in human monocytes and the THP-1 monocytic cell line (Rushworth et al 2008 and unpublished data from this lab). Injections of LPS into Nrf2 deficient mice eyes to simulate an uveitis model up regulated inflammatory and cytokines. When the experiment was carried out in wild-type mice these cytokines could be reduced with pretreatment of the potent Nrf2 activator 1-(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)imidazole by activation of HO-1 and NQO1 (Nagai et al 2009). This activator, also known as bardoxolone methyl, is currently in Phase III trials for Type II diabetic patients with chronic kidney disease and potentially may be useful for DR. The results of this trial are due in 2013 (NCT01351675 Reata Pharmaceutical).

TNF-α has also been shown to activate HO-1 expression in THP-1 cells (Rushworth et al 2011) and high and varying concentrations of glucose have been associated with the progression of DR. Rat retinas subjected to high levels of glucose underwent apoptosis and could be attenuated by GSH (Emery et al 2011). Although Nrf2 and its downstream target genes are capable of being activated in these cell types, the findings of this research suggest that the pathway may not be the key component to providing a level of cytoprotection from oxidative stress, particularly in the Mueller cells. Additional experiments should investigate whether Nrf2 protein expression is upregulated in response to these inducers, as it is possible that Nrf2 may be acting through different mechanisms, independently of these genes. In addition, it is plausible that although mRNA expression is not affected the expression of HO-1, Trx1, NQO1 and ferritin protein could be upregulated by less degradation occurring and therefore this should also be investigated. Overall, though, in accordance with other research groups, such as that of Bringmann and co workers, this research has found Mueller cells to be less susceptible to and more resilient to oxidative stresses induces like OGD and therefore they may be present more often in damaged and diseased retina (Bringmann et al 2006, Bringman et al 2009a)

OGD is a source of oxidative stress in several retinal diseases (Castilho et al 2012). There have been no reports to date on the effect of OGD on MIO-M1 cells, therefore the findings presented here make an important contribution to the understanding of how Mueller cells respond in the diseased retina. A clearer understanding of the effects of OGD in Mueller cells and within the retina will enable the field to make further advances in understanding the effect that OGD has in states of diseases with vascular components including AMD

and DR. HORCs could be used in the OGD system to investigate the role of Mueller cells in providing cytoprotection. As HORCs can have blood vessels running through them, the way in which the surrounding Mueller cells react could be monitored by looking at HIF-1α and VEGF levels by immunocytochemistry, mRNA and protein expression. Hif1α and VEGF have both been shown to be upregulated in times of OGD. Investigating whether SFN could counteract their effects via the Nrf2 pathway in both MIO-M1 cells and HORCs could be an interesting line of inquiry. If a positive effect is observed it would show how SFN induced Nrf2 activation could be a therapeutic target for vascular diseases like AMD and DR. Also, occasionally HORCs from AMD and glaucoma patients are available. Contrast studies using immunocytochemistry could be used to monitor different morphological differences if enough donors were available. Mueller cells have been reported to release the cytokine TNF-α. Measuring the amount of TNF-α released by using an ELISA when Mueller cells are under the oxidative stresses OGD, LPS and high glucose could be an indicator of gliosis activation.

The antioxidant SFN has been shown to activate the Nrf2 pathway in vitro and in vivo (Juge et al 2007). This is the first study to show that SFN activates Nrf2 and its targets HO-1 and NQO1. Further studies are required to confirm that NQO1 expression is upregulated in Mueller cells, including longer time courses for mRNA expression and ensuring that protein expression is also increased.

The mechanism by which SFN activates the Nrf2 pathway and HO-1 was also studied. Using kinase inhibitors, the results suggest that PKC and PI3K are

upstream regulators of Nrf2 and are involved in the induction of Nrf2 and HO-1 in MIO-M1 cells. However, further studies should be carried out to validate these results. The activation of these kinase pathways by SFN should be confirmed using phospho-specific antibodies. Both PKC and PI3K kinases are actually families of kinases. PKC is a family of Ser/Thr kinases and several have been reported to be involved in the activation of Nrf2, including PKCδ and PKCα have been shown to directly phosphorylate Nrf2 in vitro. It would be interesting to determine which isoforms are involved in this pathway. In addition, further studies should be carried out to confirm the involvement of Nrf2 in HO-1 activation by SFN. DNA-binding assays could be used to confirm that SFN-induced Nrf2 binds to the ARE in the nucleus of activated cells. In addition, a luciferase reporter assay with a HO-1 promoter plasmid could be used to confirm that SFN-induced Nrf2 activation results in transcriptional activation of the HO-1 promoter.

Keap1 is a cysteine-rich protein and some activators of Nrf2 have been reported to modify several of the cysteine residues, including Cys151. SFN has been reported to modify Cys151 (Zhang and Hannink 2003). The involvement of Cys151 in SFN activation in Mueller cells could be confirmed by using a small peptide to inhibit the interaction of SFN and Keap1. It may also be possible to test whether SFN interacts with Keap1 which has had Cys151 deleted out. In addition, Nrf2 has been reported to be phosphorylated on Ser40 upon activation by the anti-oxidant t-BHQ. This has been reported to be important for the dissociation of Nrf2 from Keap1 but not important in its entry into the nucleus (Bloom and Jaiswal 2003). A phospho-specific antibody is available and could

be used in western blot analysis in response to SFN to examine whether Nrf2 phosphorylation at Ser40 is involved in this pathway in MIO-M1 cells.

It is also unclear as to how SFN enters the cells or if it enters the cells at all. There could be a receptor involved that activates the PKC and PI3K pathways upstream of Nrf2 to activate it. If SFN could be labelled in a way that does not affect its function or 3D structure, the exact movements of SFN could be monitored. Different labelling methods that could be considered are fluorescent labelling or radioactive labelling with immunocytochemistry. Figure 1.5 shows all the potential mechanism of action that SFN could active HO-1 through the Nrf2 pathway.

Not all Nrf2 regulated genes were activated by SFN in these cells. Whilst free Nrf2 protein increased with SFN induction, Nrf2 mRNA expression did not change, suggesting that SFN does not induce *de novo* synthesis of Nrf2. This is in agreement with many other activators of Nrf2. In fact, few inducers have been shown to affect Nrf2 mRNA levels, but one example is LPS in human monocytes (Rushworth et al 2008).

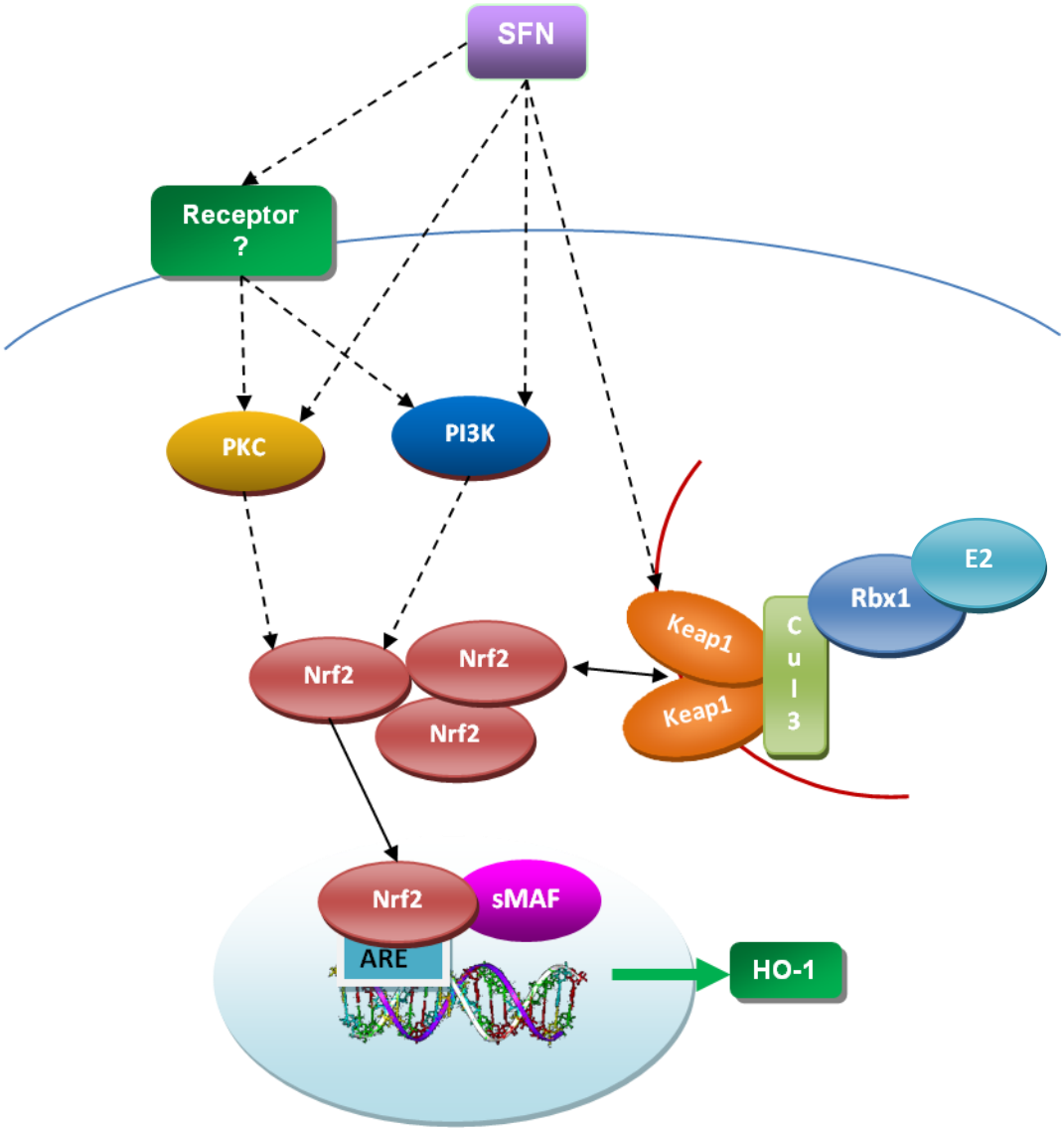

Figure 5.1 Potential mechanism of SFN activating HO-1 expression. Sulforaphane could activate the Nrf2 pathway through Keap1 directly; with or without a receptor activate it through PKC and PI3K.

To aid the determination of the functional effects of SFN in the eye and retina further studies of SFN in vivo are required. Rat models are a good model for performing in vivo studies. SFN could be administered by diet or directly into the eye using drops and injections, then the amount of SFN received in the retina could be monitored. The sequential upregulation of Nrf2 driven genes could be investigated in the different cell types of the rat retina to determine the differences in SFN induced Nrf2 driven genes within a range of retinal cells in vivo.

Nrf2 knockout mice could be utilised to confirm the involvement of Nrf2 in the eye. Research has shown that Nrf2 deprived mice are more susceptible to DR and AMD. Studies have looked at the changes within the eye, especially in relations to the RPE and AMD; however, much research is still required to look at the exact differences between wild type and Nrf2 deficient mice retinal cell types and how they are biochemically effected (Zhao et al 2011). HO-1 deficient mice could also be used to research the exact effects that HO-1 has in the Mueller cells and indeed the other cell types of the retina. Research in this manner may give key ideas to the exact effect that Nrf2 and SFN have on the retina and mueller cells in an in vivo situation.

Trx1 plays an important role in the retina by maintaining a reduced state within the cells. However, SFN did not induce Trx1 mRNA expression in the MIO-M1 cells. This does not mean that Trx1 cannot be induced by other stimuli in these cells. Other research has shown Trx1 can act as a reducing agent lowering the level of H_2O_2 in cells (Zhang et al 1997). H_2O_2 did not induce HO-1 expression to a significant degree. It is possible that H_2O_2 would induce Trx1

expression as Trx1 mops up peroxides. The basal levels of Trx1 were low but further experiments could investigate whether it can be induced by H_2O_2.

Gliosis activation has been reported to be both cytotoxic and cytoprotective. It has been suggested that low activation of gliosis is cytoprotective and higher activation is cytotoxic (Bringmann et al 2009a). The mechanistic action by which antioxidants and oxidative stresses activate gliosis could be investigated. As GFAP is an indicator of gliosis it can be used to measure levels of gliosis activation during stimulation by antioxidant and oxidative stress. This would give an indication of the level of gliosis activation. Coupling this with MTS assays and TUNEL assays to measure the level of cytotoxicity and apoptosis that is occurring in Mueller cells, neurons and RGCs could give a more detailed analysis of how gliosis is activated and the effects that it has on surrounding cell types. These experiments could be performed in HORCs where the effects of antioxidants, oxidative stress, gliosis and the Nrf2 pathway could be investigated by immunocytochemistry, labelling GFAP and Nrf2 and Nrf2 driven genes to see where the Nrf2 pathway is activated.

Nrf2 does not have a key role in cytoprotection against oxidative stress in Mueller cells. This is an important discovery as it must mean that other unknown pathways may be involved. One way to investigate this would be a microarray project. Microarrays have been successfully used to characterise the activation of many cellular processes. This would use different oxidative stimuli on MIO-M1 cells and then screen by microarray for several different genes to monitor how they are up and down regulated. This could be a way to finding out which are the key pathways involved.

Several dietary-derived compounds with anti-oxidant activity have been shown to activate the Nrf2 pathway in various cell types and have functional effects, including anti-inflammatory and cytoprotective effects. These include α-lipoic acid from tomatoes and reseverol derived from grapes and wine (Scapagnini et al 2002, Ogborne et al 2004, Rushworth et al 2006). Curcumin which is derived from turmeric has been shown to activate the Nrf2 pathway in many cell types, including monocytes and RPE cells (Rushworth et al 2006, Mandal et al 2009, Woo et al 2012). As the research presented here shows there are differences as to how the RPE and MIO-M1 react to oxidative stimuli but that both reacted to SFN in a way that caused the induction HO-1 it would be interesting to investigate whether curcumin and other antioxidants also upregulated the Nrf2 pathway and HO-1 in a similar fashion.

SFN is a promising protective agent which has antioxidant, anti-inflammatory and cytoprotective effects *in vivo*. The ability to be able to upregulate the Nrf2 pathway in long term neurodegenerative and inflammatory diseases is a major aspect of current research. With the eye neurodegenerative diseases like DR and glaucoma SFN has the potential to be an effective drug. However before this can be a true possibility the most effective drug delivery system for SFN into the eye needs to be established. The effectiveness of SFN to be able to pass through the retina blood barrier would need to be established, if effective SFN could be given as a dietary supplement. Drops and gels are another potential delivery mechanism as well as injections, which would have a less systematic effect. However, the compliance and ease of the patient to take the administered SFN would also need to be taken into consideration and the

ability of SFN to cross the collagen matrix of the sclera would need to be investigated.

This research has shown that SFN could have the potential to be an exciting new therapeutic for maintaining Mueller cells and the retina. SFN can upregulate Nrf2 pathway within the eye and retina. It could be used in a variety of eye diseases like AMD, glaucoma, and DR, to slow down the progression of these diseases. In addition, this research adds to the knowledge of the nature of Mueller cells which could be important in understanding the function of Mueller cells in the healthy and damaged retina.

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