Expression and Distribution of Thiol- regulating Enzyme, Glutaredoxin 2 in Porcine Ocular Tissues

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EXPRESSION AND DISTRIBUTION OF THIOL-REGULATING ENZYME, GLUTAREDOXIN 2 IN PORCINE OCULAR TISSUES

by

Bijaya Prasad Upadhyaya

A THESIS

Presented to the Faculty of
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For the Degree of Master of Science

Major: Veterinary Science

Under the Supervision of Professor Marjorie F. Lou

Lincoln, Nebraska
August, 2012
EXPRESSION AND DISTRIBUTION OF THIOL-REGULATING ENZYME, GLUTAREDOXIN 2 IN PORCINE OCULAR TISSUES

Bijaya Prasad Upadhyaya, M.S.
University of Nebraska, 2012

Advisor: Marjorie F. Lou

Glutaredoxin 2 (Grx2), a thiol-regulating enzyme of oxidoreductase family and a mitochondrial isozyme of glutaredoxin 1, was discovered 11 years ago in our laboratory. Grx2 is present in the lens where it shows dethiolase, peroxidase, and ascorbate recycling activities. Recently, Grx2 has also been identified to protect the mitochondrial electron transport system with anti-apoptotic function. Since other eye tissues besides the lens are rich in mitochondria and are very sensitive to oxidative stress, we speculate the presence of Grx2 therein as an important redox regulator. This study is to investigate the expression and distribution of Grx2 in ocular tissues using porcine eye as a model.

Fresh enucleated porcine eyes obtained from a local abattoir were immediately dissected into cornea, iris, the lens, vitreous humor, ciliary body, retina, and optic nerve; frozen in dry ice; and stored at -80°C. Each sample with 3 tissues pooled was homogenized in 1.0 ml of ice-cold 10 mM HEPES buffer (pH 7.2) containing 225 mM D-mannitol, 65 mM sucrose and 1mM EGTA in a glass-to-glass homogenizer, followed by a series of centrifugations to remove tissue debris while isolating mitochondrial fraction. Mitochondrial sample proteins of each sample were separated by 12% SDS-PAGE, followed by Western blot analysis to detect Grx2 by a specific Grx2 antibody. The same tissue sample with 5mg/ml mitochondrial protein was completely dissolved in 1% lauryl maltoside, and measured for Grx2 enzyme activity following the published procedure.

Western blots showed the expression of Grx2 in all the tested ocular tissues, except vitreous humor. Relative expression of Grx2 to the positive control, mouse mitochondrial liver homogenate, revealed that the ciliary body had the highest expression ratio (26.64), followed by the retina (11.92), and optic nerve (8.60). The lens had the lowest expression.
ratio of 0.75, while the vitreous humor did not show any Grx2 positive band. Enzyme activity assays showed that the retina had the highest Grx2 specific activity (3.89 mU/mg protein), closely followed by ciliary body (3.10 mU/mg), lens (0.58 mU/mg), and optic nerve (0.32 mU/mg). Vitreous humor had no Grx2 activity.

In conclusion, Grx2 was found in all porcine ocular tissues except for vitreous humor. The Grx2 protein expression level was higher in eye tissues rich in mitochondria (ciliary body and retina), corroborating with their high Grx2 activity. The rich presence of Grx2 in these tissues is consistent with their known sensitivity to oxidative stress.
ACKNOWLEDGMENTS

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Finally, I would like to thank especially to my parents, brother, my wife (Sushma Dhungana), and my friends-Nabaraj, Siroj, Sunita, Pravat, and roshani for their endless support and inspiration.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper, zinc superoxide dismutase</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>DHA</td>
<td>Dihydroascorbic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetracetic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>G3PD</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione, reduced form</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HED</td>
<td>β-Hydroxyethyl disulphide</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HLE</td>
<td>Human lens epithelium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat dry milk</td>
</tr>
<tr>
<td>PICOT</td>
<td>Protein kinase C-interacting cousin of thioredoxin</td>
</tr>
<tr>
<td>Prx</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>PSH</td>
<td>Protein thiols, reduced form</td>
</tr>
<tr>
<td>PSSP</td>
<td>Protein-protein disulfide</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Trisbuffered saline</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TTase</td>
<td>Thioltransferase</td>
</tr>
<tr>
<td>VDAC</td>
<td>Volatage-dependent anion channel</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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</tbody>
</table>
# TABLE OF CONTENTS

**TITLE**

**ABSTRACT**

**ACKNOWLEDGMENTS**

**ABBREVIATIONS**

**TABLE OF CONTENTS**

**LIST OF FIGURES**

**CHAPTER I: INTRODUCTION**

**CHAPTER II: LITERATURE REVIEW**

- Anatomy of Eye
- Reactive Oxygen Species in the Eye
- Oxidative Stress in the Eye and Cataract Formation
- Antioxidants and Defense Mechanisms in the Eye
- Glutathione and Glutathionylation
- Enzymatic Antioxidants
- Thioredoxin System
- Glutaredoxin System
- Mammalian Glutaredoxin Isoforms
- Glutaredoxin 2
- Biological Functions of Grx2

- Dethiolase Activity
- Ascorbate Recycling Activity
- Peroxidase Activity
- Anti-apoptotic Function
Iron-sulfur Clustering ................................................................. 20
Other Functions ........................................................................... 20

CHAPTER III: PURPOSE OF STUDY ................................................. 21

CHAPTER IV: MATERIALS AND METHODS ..................................... 22

Materials ....................................................................................... 22
Methods .......................................................................................... 23
Amino Acid Sequence Analyses ..................................................... 23
Dissection of Porcine Ocular Tissues ................................................. 23
Isolation of Mouse Liver Mitochondrial Fraction ............................... 23
Preparation of Porcine Eye Tissue Homogenate and Mitochondrial-
Isolation ......................................................................................... 24
Protein Concentration Determination ................................................. 24
Western Blot Analysis ...................................................................... 25
Glutaredoxin 2 Activity Assay .......................................................... 26

CHAPTER V: RESULTS .................................................................... 27

CHAPTER VI: DISCUSSION ........................................................... 38

CHAPTER VII: CONCLUSIONS ....................................................... 41

CHAPTER VIII: FUTURE DIRECTIONS ......................................... 42

REFERENCES .................................................................................. 43
LIST OF FIGURES

Figure 1. Diagrammatic cross-section through the mammalian eye..........................3

Figure 2. One-electron reduced reactive intermediates of molecular oxygen.............7

Figure 3. Structure of glutathione.................................................................10

Figure 4. The monothiol and dithiol mechanism of Grx..................................13

Figure 5. Sharing of biological functions between cytosolic Grx1 and mito-

chondrial Grx2 isozymes of Grx...............................................................14

Figure 6. Three dimensional structure of mouse Grx2.....................................16

Figure 7. Dethiolase activity of Grx2.............................................................17

Figure 8. Ascorbate recycling property of Grx2............................................17

Figure 9. Grx2 has peroxidase activity..........................................................18

Figure 10. Anti-apoptotic property of Grx2....................................................19

(A) Mitochondrial respiratory chain and the sites for ROS generation.........19

(B) Reduction of mitochondrial Complex I by Grx2 and prevention

of Apoptosis..............................................................................................19

Figure 11. Alignment of the amino acid sequences of porcine and mouse Grx2.......31
Figure 12. Mouse liver acts as a positive control……………………………………..32

Figure 13. Vitreous humor acts as a negative control……………………………..33

(A) Western blot analysis of Grx2 in porcine vitreous humor…………………33

(B) Enzyme activity assay of Grx2 in porcine vitreous humor…………………33

Figure 14. Expression of Grx2 in porcine ocular tissues in relation to mouse liver……34

(A) Western blot analysis of Grx2 in ocular tissues……………………………34

(B) Relative expression of Grx2 in ocular tissues……………………………..35

(C) Enzyme activity assay of Grx2 in porcine ocular tissues…………………..35

Figure 15. Expression of Grx2 in porcine ocular tissues in relation to porcine lens……36

(A) Western blot analysis of Grx2 in ocular tissues……………………………36

(B) Relative expression of Grx2 in ocular tissues……………………………..37
CHAPTER I: INTRODUCTION

The eye is constantly subjected to oxidative stress due to daily exposure to sunlight (UV light), high metabolic activities, and oxygen tension. Furthermore, oxidative stress can induce age-related macular degeneration and cataract formation, which are leading causes of blindness worldwide (WHO, 2010). The lens of the eye is very sensitive to oxidative stress, and the combination of redox imbalance and protein modification can easily cause structural crystallin proteins to aggregate and deflect light, resulting in loss of transparency, known as a cataract (Lou, 2003). Moreover, retina is also prone to oxidative damage because it utilizes more oxygen than any other tissue in the body (Santosa and Jones, 2005).

Antioxidant enzymes which are present in the eyes are important to maintain redox homeostasis of ocular tissues. Among these antioxidant enzymes, glutaredoxin 1 (Grx1), also known as thioltransferase (TTase), is a member of the thiol-disulfide oxidoreductase enzyme family. Grx1 is a small cytosolic protein with a molecular weight of 11.8 kDa and it specifically catalyzes the reduction of proteins that form protein-thiol mixed disulfide bonds during oxidation (Holmgren, 1989; Raghavachari and Lou, 1996). Glutaredoxin 2 (Grx2) is a recently identified mitochondrial isozyme of Grx that has a molecular weight of 18 kDa and a motif of Cys-Ser-Tyr-Cys at the active site, similar to other oxidoreductases (Gladyshev et al., 2001; Lundberg et al., 2001).

Various reports have shown that Grx2 protects human lens cells against oxidative damages and the knockdown of Grx2 sensitizes primary lens epithelial cells to H$_2$O$_2$-induced cell damage (Wu et al., 2010). These results suggest that Grx2 has an important
role in two ways: cytoprotection and maintaining transparency in the lens (Wu et al., 2010). However, the distribution of Grx2 in other ocular tissues rich in mitochondria remains unclear. Therefore, the current study was designed to investigate Grx2 distribution and detect its enzyme activity in porcine ocular tissues. Our results show that Grx2 was found in most porcine ocular tissues, but was absent in the vitreous humor. Highest Grx2 protein expression level was found in ciliary body of the anterior part and retina of the posterior part of the eye.
CHAPTER II: LITERATURE REVIEW

Anatomy of Eye

Mammalian eye, a ball-shaped tissue located in a boney orbit in the skull, is a complex optical system whose function is to focus the light from the surrounding environment onto the retina, translate the light into the nerve impulses and then transmit these electrical signals to the brain through neural pathways (Saude and Fletcher, 1993). Various components of the eyeball, from the anterior to the posterior, include cornea, iris, ciliary body, lens, vitreous humor, retina, and optic nerve (Figure 1).

Figure 1. Diagrammatic cross-section through the mammalian eye. Adopted from Berman, 1991.
The eyeball is composed of three layers: a protective outer fibrous coat consisting of sclera, cornea, and corneal limbus; a vascular middle (uveal) coat comprising choroid, ciliary body, and iris; and the innermost neural (retinal) coat. Sclera is the white part of the eye that protects the eye surface. At the anterior part of the eyeball is the limbus where the sclera and cornea meet. (Saude and Fletcher, 1993).

The cornea is a transparent tissue that covers the front surface of the eye and is responsible for the protection against mechanical injuries. This serves as the main refractive medium of the eye as it supplies two-thirds of the 60D refractive power of the eye. Cornea is composed of five anatomically distinct layers: (1) an outermost epithelial layer accounting for about 8-10% of the total thickness; (2) Bowman’s layer of about 10 µm thickness; (3) the stroma that makes up 90% of corneal thickness and is 0.5mm thick; (4) Descemet’s membrane, a thin (8-10 µm) elastic lamina that serves as a basement membrane for the endothelium; and (5) a monolayer of innermost endothelial cells that comprises about 1% of the total thickness. Since the cornea is avascular, its nutritional needs are met by the limbal arcades, aqueous humor (source of glucose) and tear film (source of oxygen) (Maurice, 1957; Saude and Fletcher, 1993).

The colored part seen in the anterior surface of the eye is the iris. The color of the iris could be brown, grey, blue or green depending upon the level of melanin pigment it contains. The primary function of the iris is to control the pupil size in response to varying amount of light entering the eye (Saude and Fletcher, 1993).

Ciliary body is roughly a triangular part of the uvea that lies in between the anterior end of the choroid and the root of the iris. Ciliary body is structurally made up of the ciliary
epithelium, the stroma, and the ciliary muscle. Ciliary epithelium along with solutes and fluids from the plasma constitute the aqueous humor, which is normally a clear, colorless liquid filling the anterior and posterior cavities. The stroma is rich in pigment and blood vessels, whereas the ciliary muscle moves centripetally so that the lens can focus on both the distant and nearby objects. Aqueous humor is responsible not only for supplying nutrients and oxygen to the lens epithelial cells and corneal endothelial cells but also for maintaining shape of the eyeball by stabilizing intraocular pressure (IOP) (Saude and Fletcher, 1993).

The lens is an avascular, biconvex, transparent tissue enveloped in the elastic capsule which is located behind the iris. It separates the eyeball into two chambers: anterior aqueous humor and posterior vitreous humor. It contributes roughly one-third of the eye’s 60D power. The crystalline lens consists of three layers: the capsule, the epithelium, and fiber cells that occupy the rest of the lens. The epithelial cells contain nuclei, mitochondria and other cytoplasmic organelles and are metabolically active (Chylack, 1994). At the equator region the epithelial cells undergo continuous mitosis throughout the life and differentiate into elongated fiber cells which lose all the organelles during the process. Such new fiber cells form concentric layers and hence the oldest cells are found in the nucleus, and the newest ones in the cortex (Berman, 1991). A suspensory ligament called zonule holds the lens in its proper place. Therefore, the contraction and relaxation of the zonule is responsible for adjusting the curvature of the lens thereby enabling the lens to focus the objects accordingly.

There is a high level of proteins found in the lens at around 30-40% of the total wet weight of the tissue (Zigler, 1994). Conversely, the water content is lower than that of the
most other tissues (Anderson, 1993). Among the major structural and soluble proteins, crystallins account for 90-95% of the total lens protein. Furthermore, there are three major classes of crystallins, namely α, β, and γ crystallins. Out of those three types of crystallins, α-crystallin has a chaperone-like activity (Horwitz, 1992) and can prevent insolublization and nonspecific aggregation of lens proteins.

Vitreous humor is a colorless, avascular and gelatinous extracellular matrix that fills the space between the lens, ciliary body and the retina. It comprises approximately 80% of the eyeball’s volume and weight. Vitreous gel is composed of 98-99% water while remaining 1-2% accounts for proteins like collagen fibrils and mucopolysaccharides. The function of the vitreous humor is not only to maintain the shape of the eyeball but also to serve as a shock absorber against mechanical impact (Denlinger et al., 1980).

Retina is a thin, semitransparent and innermost tissue that covers the posterior two-thirds of the eyeball and extends up to ciliary body anteriorly. Retina has 10 distinct layers of cells, including non-neural retinal pigment epithelium (RPE) and neural retina. A monolayer of RPE comprises cubical cells near the fovea while flattened cells toward the periphery. RPE cells have a high metabolic activity; however, they do not undergo mitosis. Physiologically, neural retina can be divided into two parts: the outer light-detecting photoreceptor cells and the inner signal-processing layers. Among photoreceptors the rod cells are specialized for night vision and cone cells for colorful photopic vision. These photoreceptors are responsible for receiving and transferring the visual excitation to optic nerve (Saude and Fletcher, 1993).
The optic nerve is a part of visual complex that transfers the visual sensation from the retina to the brain. The brain, in turn perceives the visual information and then the image of the object will form in the visual cortex. Hence, the brain, not the eyes, can read the image formed in the visual system.

**Reactive Oxygen Species in the Eye**

Oxygen is the basis of all aerobic life forms. Most of the aerobic cells can reduce oxygen to water through several pathways in order to generate adenosine triphosphate (ATP). However, not all the molecular oxygen goes through the complete tetravalent reduction in this process. 0.1-2% of total oxygen consumed by the aerobic cells is reduced in univalent steps to produce the reactive intermediates as follows (Murphy, 2009):

![Diagram](image)

Figure 2. One-electron reduced reactive intermediates of molecular oxygen: (1) superoxide anion; (2) hydrogen peroxide; and (3) hydroxyl radical. Adopted from Berman, 1991.

These reactive intermediates like superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$) are collectively considered as reactive oxygen species (ROS) (Gardner and Fridovich, 1991). Intracellular sources of ROS mainly include electron leakages at complex I (NADH dehydrogenase) and complex III (cytochrome $bc_1$ complex) of mitochondrial electron transport chain (Melov, 2000). Several other enzymes
can also produce ROS and include NADPH oxidase, lipoxygenase, xanthine oxidase, cytochrome P-450, etc. (Rao et al., 1999; Bayir, 2005). Cytokines and growth factors such as interleukin-1 and tumor necrosis factor-α (TNF-α) can also generate ROS (Meier et al., 1989). Increased amount of ROS has also been detected in phagocytic cells (Weiss et al., 1978). On the other hand, exogenous sources of ROS include ionizing radiation, ultraviolet light, chemicals or drugs, and smoke or environmental pollutants.

**Oxidative Stress in the Eye and Cataract Formation**

When ROS levels increase excessively and the detoxification mechanisms cannot work efficiently, it leads to a condition known as oxidative stress. In other words, oxidative stress is a condition that occurs when there is a significant imbalance between production and accumulation of ROS and antioxidant defenses, thereby causing a damage of deoxyribonucleic acid (DNA), oxidation of lipids and structural proteins, and inactivation of enzymes. Hence, oxidative stress not only has an impact on cells and cellular components but also on organ systems and immune status leading to aging and diseases. That is why oxidative stress is considered as one of the most important risk factors for many diseases in humans, including cancer, diabetics, Alzheimer disease- the most common form of dementia, Parkinson’s disease- a degenerative disorder of the central nervous system, macular degeneration- a loss of vision due to retinal damage, and cataract (Spector, 1995; Andley et al 2000).

The function of the lens is to maintain its transparency in order to let the light pass through and get focused on the retina. Cataract is a diseased state of cloudiness or opacity in the lens of the eye, which is otherwise normally transparent (Spector, 1985). This
Cloudiness can cause vision-impairment, fuzziness or blurriness, and may eventually lead to blindness. In the worldwide scenario about 51% of blindness affecting 20 million people is due to age-related cataract (WHO, 2010). In the United States, senile lens changes have been reported to 42% at the age between 52 and 64, and up to 91% at the age between 75 and 85 (Sperduto et al. 1980).

Cataractogenesis or cataract formation is a multifactorial process. However, oxidative stress has been considered as one of the major risk factors. Therefore, the lens depends on redox equilibrium to maintain its transparency. When redox equilibrium shifted towards an excessive generation of ROS, then it can result into oxidative stress. For example, hydrogen peroxide that is present in aqueous humor at normal level of 20-30 µM was found increased in cataract patients (Spector et al., 1988). Oxidative stress leads to the formation of disulfide bonds between two proteins thiols (Truscott and Augusteyn, 1977) or between protein thiol and non-protein thiol molecules like glutathione or cysteine (Lou, et al., 1990). Furthermore, the lens protein thiols can directly be oxidized to sulfenic acid or sulfinic acid (reversible changes) and sulfonic acid (irreversible change). Thus, protein-protein cross-linkage results in protein aggregation that eventually leads to lose their characteristics features like mobility, solubility, and enzyme activity. When the lens protein has this kind of structural modification, it causes the scattering of light leading to loss of the lens transparency known as cataract (Spector, 1995).

**Antioxidants and Defense Mechanisms in the Eye**

In order to maintain the intracellular redox homeostasis, mammalian tissues, including the eyes have well-designed systems of defense against the reactive oxygen species
(Augusteyn, 1981). Primarily, protective molecules against the oxidants stress include small antioxidants such as glutathione, vitamin C, vitamin A, vitamin E, and flavonoids. Similarly, oxidation defense enzymes such as catalase, superoxide dismutase (SOD), peroxiredoxins, thioredoxin (Trx), thioredoxin reductase (TR), glutathione peroxidase (Gpx), glutathione reductase (GR), and glutaredoxin (Grx) are essential in the antioxidation defense processes.

**Glutathione and Glutathionylation**

Among the small, antioxidant molecules, glutathione (GSH) is the most abundant non-enzymatic thiol in cells. GSH level is highest in the lens, followed by red blood cells, and kidney and liver (Dickinson and Forman, 2002). Within the cell, about 90% GSH is found in the cytoplasm, and remaining GSH found in mitochondria, nucleus and endoplasmic reticulum. Glutamate, cysteine, and glycine together form this tripeptide where the free sulfhydryl group of cysteine is the most sensitive to oxidation. The primary function of GSH is to maintain redox homeostasis by protecting the cells against ROS and xenobiotics. To have this function, GSH is required to be in its reduced form (Dickinson and Forman, 2002).

![Figure 3. Structure of glutathione. Adopted from Thomas (1999).](image)
However, oxidative stress induces glutathione oxidation (GSSG) and glutathionylation of proteins (protein-GSH conjugation through disulfide bridge). Hence, intracellular glutathione concentration (GSH: GSSG) can be an indicator of redox status of the cell (30:1-100:1 in cytosol, 10: 1 in mitochondria, and 1:1 in endoplasmic reticulum) (Pietro et al., 2005). Protein glutathionylation is a principal mechanism to regulate glutathione redox status and hence the enzyme activity (Sies, 1999). Mixed disulfides formed between GSH and cysteine thiols of proteins (PSSG) affect the activity of many metabolic enzymes such as glyceraldehydes 3 phosphate dehydrogenase (G3PD), creatine kinase, cyclic adenosine monophosphate (cAMP) dependent protein kinase, and α-keto glutarate dehydrogenase. Hence, oxidant-induced protein modifications can also affect protein functions (Lodish et al., 2000).

**Enzymatic Antioxidants**

The enzymatic antioxidants play a second line of defense against the oxidative stress in the body, including the eye. The ocular tissues have SOD to eliminate free oxygen radicals, superoxides. Similarly, catalase and glutathione peroxidase are capable of dissociating hydrogen peroxides. Among protein/enzyme repair systems, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin/thioredoxin reductase system (Holmgren, 1989) and GSH-dependent glutaredoxin system (Holmgren, 1989; Raghavachari and Lou, 1996) are the primary and most important ones.
Thioredoxin System

Thioredoxin system is composed of thioredoxin (Trx), thioredoxin reductase (TR), and NADPH, and is effective in reducing protein-protein disulfide bonds (PSSP). Trx reduces PSSP to PSH, and the oxidized Trx in turn is reduced by TR with reducing equivalents from NADPH, generated by pentose shunt pathway (Holmgren, 1989). Virtually all prokaryotes and eukaryotes including mammalian tissues such as the lens contain Trx as a vital redox regulator (Liyanage, 2005). This protein was first discovered and identified as a hydrogen donor for ribonucleotide reductase in E. coli (Laurent et al., 1964). It is a 12 kDa protein that consists of 105 amino acids having a conserved sequence of Trp-Cys-Gly-Pro-Cys at the active site, five central β strands, four α helices, and a helical turn (Gromer et al., 2004).

Glutaredoxin System

Glutaredoxin (Grx) or thioltransferase (TTase) system consists of GSH, Grx, and glutathione reductase (GR) (Holmgren, 1989) and is critical in cleaving protein-thiol mixed disulfide bonds formed by the oxidation of the cellular proteins/enzymes. Glutaredoxin was first described in the rat liver (Askelöf et al., 1974) and in E. coli as an alternate hydrogen donor for ribonucleoside-diphosphate reductase (Holmgren, 1976). Likewise, TTase was first reported in the lens having the similar structural and functional properties (Raghavachari and Lou, 1996). In many ways, both the Trx and Grx systems share the functions but also exhibit their specific properties (Aslund and Beckwith, 1999). Glutaredoxin differs from thioredoxin in such a way that it requires reduced GSH as a substrate along with GSH regenerating enzyme, GR (Holmgren, 1989).
Glutaredoxin is a member of oxidoreductase family, and hence a strong reductant, that has N-terminal redox center motif with two cysteines separated by two amino acid residues in its active site (CXXC). Hence, Grx can utilize either one or two of its cysteine to reduce different kinds of disulfides (Lonn, 2008) as shown in Figure 4.

![Figure 4](image)

Figure 4. The monothiol and dithiol mechanism of Grx. Adopted from Mieyal et al, 2008.

Monothiol reaction requires more of N- terminal cysteine residue generating a Grx-GSH mixed disulfide, requiring second GSH as a rate-limiting step to form disulfides (Srinivasan et al., 1997). On the other hand, a Grx-protein disulfide is formed when more of C-terminal active site thiol is also attacked in case of dithiol reactions (Lillig et al., 2008). Glutaredoxin system is also considered to be a vital redox regulator that can protect against the oxidative stress in cellular level (Mieyal et al., 1991).
Mammalian Glutaredoxin Isoforms

Dithiol glutaredoxins have a conserved active site (CPYC) and a glutathione binding site (Höög et al., 1983), whereas monothiol glutaredoxins have active site (CGFS) that lacks the C terminal cysteine. So far two dithiol glutaredoxins have been reported in humans; they are cytosolic Grx1 (Mieyal et al., 1991) and mitochondrial/nuclear Grx2 having active site (CSYC) (Gladyshev et al., 2001; Lundberg et al., 2001) that have the similar functions. Grx1 works against oxidative stress in cytosolic fraction, while Grx2 works in mitochondrial compartment as shown in Figure 5.

Figure 5. Sharing of biological functions between cytosolic Grx1 and mitochondrial Grx2 isozymes of Grx. Adopted and modified from Trotter and Grant, 2005.
Similarly, two monothiol glutaredoxins, namely Grx5 (Molina-Navarro et al., 2006) and Grx3 or protein kinase C-interacting cousin of thioredoxin (PICOT) (Witte et al., 2000) have been found in the mitochondria and cytosol of the cell, respectively. Among monothiol glutaredoxins, either it can have a single Grx domain or a Trx domain with multiple Grx domains (Herrero and De La Torre-Ruiz, 2007).

**Glutaredoxin 2**

Cytosolic Grx1 is a 11.8 kDa protein, which has been the well-characterized mammalian glutaredoxin. Grx2 (GLRX2) is the isozyme of Grx1 with molecular mass of 18kDa that was discovered concurrently in two laboratories in 2001 (Gladyshev et al., 2001; Lundberg et al., 2001). Out of five exons in GLRX2 gene, the difference in first exon leads to two different Grx2 isoforms: mitochondrial Grx2a and nuclear Grx2b (Lundberg et al., 2001). Mitochondrial Grx2 and cytosolic Grx1 share only 36% sequence homology (Gladyshev et al., 2001). However, Grx2 possesses a Trx-fold with GSH binding site and a Cys-Ser-Tyr-Cys active site sequence, unlike Grx1 where the active site sequence is Cys-Pro-Tyr-Cys. (Gladyshev et al., 2001; Lundberg et al., 2001). This replacement of proline with serine in the active site of Grx2 results in more affinity towards the reduction of glutathionylated proteins (Johansson et al., 2004).

Grx2 belongs to the Trx fold family of protein and it consists of a four stranded β-sheets surrounded by three α-helices (Eklund et al., 1984; Martin, 1995). The active site motif (Cys-Ser-Tyr-Cys) is located in between β-sheet 1 and α-helix 1, as shown in Figure 6, where two active cysteine residues (Cys$^{70}$ and Cys$^{73}$) are denoted.
Biological Functions of Grx2

A) Dethioloase Activity

Grx2 protects cells against oxidative stress. Previous studies have shown that Grx2 has dethiolase activity in the cells, including the lens epithelial cells (Gladyshev et al., 2001; Wu, et al., 2011). Protein deglutathionylation is a principal mechanism to regulate protein redox status and hence the enzyme activity or protein function (Sies, 1999). Mixed disulfides formed between GSH and cysteine thiols of proteins (PSSG) are cleaved off with the help of reduced Grx2, utilizing GSH as a cofactor. GR together with NADPH can reduce oxidized GSSG to GSH, and is responsible to maintain the GSH pool in its reduced form as shown in Figure 7 (Wu et al., 1998).
B) Ascorbate Recycling Activity

It has also been found that Grx1 in human lens epithelial (HLE-B3) cells can recycle dihydroascorbate (DHA), which is otherwise toxic, to its reduced form (ascorbate) in a GSH dependent manner. Reduced ascorbic acid (Vitamin C) can then directly detoxify the reactive oxygen species as shown in Figure 8 (Fernando et al., 2004). Grx2 has the similar property as Grx1 (Lou, unpublished results).

C) Peroxidase Activity

Grx2 is also found to have a unique peroxidase property (Fernando et al., 2006). It dissociates H_2O_2 and lipid peroxides in a manner that is GSH-dependent as well as TR-
dependent. During high oxidative stress, being an antioxidant, cellular GSH is oxidized and thus its pool size is extensively decreased, whereas TR is known to be up regulated. It is a salient feature of Grx1 and Grx2 to act as a peroxidase, accepting electrons from both GSH and TR to protect the mitochondria from oxidative insults as shown in Figure 9 (Fernando et al, 2006; Lou, unpublished results).

Figure 9. Grx2 has peroxidase activity. Adopted from Fernando et al, 2006.

D) Anti-apoptotic Function

Grx2 is also found to be antiapoptotic in the cells. It has also been noted that all the electrons carried by mitochondrial electron transport chain (ETC) are not totally efficient for generating ATP. About 0.1-2% of electrons passing through ETC causes the
formation of ROS that can oxidize complex I and other mitochondrial and cytosolic proteins to cause cellular damage (Murphy, 2009). Studies have demonstrated that Grx2 has anti-apoptotic function in lens epithelial cells by reducing complex I and restoring its normal function as shown in Figure 10, whereas this property is extensively diminished in lens epithelial cells isolated from Grx2 knockout mice (Wu, et al., 2010).

Figure 10. Anti-apoptotic property of Grx2. (A) Mitochondrial respiratory chain and the sites for ROS generation. (B) Reduction of mitochondrial Complex I by Grx2 and prevention of apoptosis. Adopted from (Nakano et al., 2006; Wu et al., 2010).
E) Iron - Sulfur Clustering

Grx2 has serine instead of proline in its typical active site resulting in to the enough room for the non-covalent binding of GSH and [Fe-S] cluster (Johansson et al., 2007). Two Grx2 molecules can thus form a dimeric holo Grx2 complex that does not have any enzymatic activity until the complex degrades and regenerates Grx2 (Lillig et al., 2005). However, this complex serves as a redox sensor to activate Grx2 during oxidative stress when the reduced form of GSH becomes a limiting factor for the cluster formation (Lillig et al., 2005; Berndt et al., 2007). It has been proposed that iron-sulfur clustered with many monothiol Grxs may have a role in iron metabolism (Picciocchi, et al., 2007; Mesecke et al., 2008).

F) Other Functions

Recently, it has been documented that Grx2 serves as a novel electron donor for mitochondrial 2-Cys peroxiredoxin (Prx3) and plays an important role in cellular redox signaling (Hanschmann et al., 2010). Another function of glutaredoxin 2 is to protect the mitochondria against the aggregation of mutant Cu, Zn superoxide dismutase (CuZnSOD) (Ferri et al., 2010).
CHAPTER III: PURPOSE OF STUDY

Grx2 is a thiol-regulating enzyme that belongs to the oxidoreductase family, and is a mitochondrial isozyme of Grx1 discovered 11 years ago in our laboratory. Grx2 is present in the lens where it shows dethiolase, peroxidase and ascorbate recycling activities. Recently, Grx2 has also been identified to protect the mitochondrial electron transport system with an anti-apoptotic function. Since other eye tissues besides the lens are rich in mitochondria and are very sensitive to oxidative stress, we speculate that Grx2 should be present there as an important redox regulator. This study is done as an initial qualitative survey to investigate the expression and distribution of Grx2 in ocular tissues using porcine eye as a model. Hence, there are two specific aims or questions to be answered from this study:

1. Is glutaredoxin also present in the ocular tissues other than the lens?
2. What are the enzyme activities in the other ocular tissues in comparison to the lens?

By obtaining the above information, it is anticipated that researchers can use it as the bases to examine the function and the protective role of Grx2 in other ocular tissues.
CHAPTER IV: MATERIALS AND METHODS

Materials

Sodium phosphate, potassium phosphate (monobasic and dibasic anhydrous), hydrochloric acid, trizma base, NADPH, glutathione, glutathione reductase were all purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyethyl disulfide (HEDS) was purchased from Aldrich Chemical Company (Milwaukee, WI).

Bicincichoninic acid (BCA) protein assay reagent kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). Non-fat dry milk (NFDM), anti- glyceraldehydes-3 phosphate dehydrogenase (G3PD), horseradish peroxide-conjugated goat polyclonal-anti-rabbit anti-glutaredoxin 2 (Grx2) antibody (IgG2a-HRP) were all purchased from Santa Cruz™ Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Grx2 antibody-C-terminal was purchased from Abcam plc. (Cambridge, MA). Rabbit polyclonal voltage-dependent anion channel (VDAC) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

BCA protein assay reagents B, BSA standard and enhanced chemiluminescence system (ECL) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Precision plus protein™ Western C™ standards, Sodium dodecyl sulfate (SDS), Tween 20, bis N’N’- methylene bis-acrylamide were all purchased from BioRad Co. (Hercules, CA).

Ammonium persulfate and sodium chloride were supplied by Fischer Scientific Co. (Pittsburgh, PA). Page ruler™ prestained protein ladder was purchased from Fermentas (Hanover, MD). Polyvinylidene difluoride (PVDF) membrane was purchased from GE Healthcare (Boulder, CO). All the chemicals and reagents were of analytical grade.

Porcine eyeballs were obtained from a local abattoir, Farmland Foods, Inc. (Crete, NE). Pigs were all around 6 months old.
Methods

Amino Acid Sequence Analyses

BLAST programs were run to identify the homology in amino acid sequences between mouse Grx2 and porcine Grx2 at National Center for Biotechnology Information (NCBI). Homology sequences as well as the predicted molecular weight of porcine Grx2 were calculated.

Dissection of Porcine Ocular Tissues

Porcine whole eyes were obtained from a local abattoir (Farmland Foods Inc., Crete, NE) and kept on ice during dissection. All the dissection procedures were performed under the dissecting microscope using micro-surgery instruments. Seven fresh ocular tissues, namely cornea, iris, the lens, vitreous humor, ciliary body, retina, and optic nerve were taken from each eye. At first, the cornea was extracted followed by the iris. The ciliary body and the lens along with a thin layer of lens epithelium lining were extracted very carefully. A clear jelly like vitreous humor was collected with the help of syringe without a needle. Then after, the inner lining of retinal wall (neural retina) was peeled off very carefully. Finally, the optic nerve from the backside of the eyeball was trimmed. All the dissection procedures were performed under the dissecting microscope using micro-surgery instruments. Three samples of each eye tissue were pooled and frozen on dry ice and kept in eppendorf tubes at -80°C until further use.

Isolation of Mouse Liver Mitochondrial Fraction

Mitochondrial fraction of tissue homogenates was isolated according to the modified protocol described by Rehncrona, et al., 1979. Fresh mouse liver was cut into smaller pieces and manually homogenized with a glass to glass homogenizer (Kontes Glass Co, Vineland, NJ) in 1ml buffer (pH 7.2) containing 225 mM mannitol, 65 mM sucrose, 1 mM ethylene glycol tetraacetic acid (EGTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Homogenate was centrifuged at 500g for 10 minutes at 4°C. The supernatant, which contained the cytosolic and mitochondrial fractions, was centrifuged at 16,000g for 10 minutes at 4°C. The mitochondrial pellet
was saved and resuspended in 1 ml buffer containing 225 mM mannitol, 65 mM sucrose, and 10 mM HEPES (pH 7.2). The purified mitochondrial pellet was resuspended in 50 
µL of the same buffer. The purified pellet resuspension was added 1 percent lauryl maltoside to solublize the mitochondrial protein and allowed to incubate for 30 minutes on ice before centrifuging at 10,000g for 30 minutes at 4\(^0\)C. Then the supernatant containing solublized mitochondrial fraction was collected and saved for the designed experiment.

**Preparation of Porcine Eye Tissue Homogenate and Mitochondrial Isolation**

All the ocular tissues previously dissected and stored at -80\(^0\)C were thawed in ice before preparing the tissue homogenates. Hard tissues such as cornea, and optic nerve were chopped into smaller pieces with the help of scissors and manually homogenized with a glass to glass homogenizer in 1ml buffer containing 225 mM mannitol, 65 mM sucrose, 1 mM EGTA, and 10 mM HEPES (pH 7.2). Homogenate was centrifuged at 500g for 10 minutes at 4\(^0\)C. The supernatant, which contained the cytosolic and mitochondrial fractions, was centrifuged at 16,000g for 10 minutes at 4\(^0\)C. The mitochondrial pellet was saved and resuspended in 1 ml buffer containing 225 mM mannitol, 65 mM sucrose, and 10 mM HEPES (pH 7.2). The purified pellet was resuspended in 50 
µL of the same buffer. Vitreous humor was manually homogenized similarly in 1ml of buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM ethylenediaminetetraacetic acid (EDTA). Homogenate was centrifuged at 10,000g for 10 minutes at 4\(^0\)C. To the tissues having pigmentation like iris and ciliary body 1 percent lauryl maltoside was added, allowed to incubate for 30 minutes in ice, and centrifuged at 10,000g for 30 minutes at 4\(^0\)C. Finally, the supernatant containing soluble mitochondrial fraction was collected and saved for further experiments.

**Protein Concentration Determination**

Bicinchorinic acid (BCA) assay protocol (Smith et al., 1985) was used to detect the concentration of protein in the pooled ocular tissues. Commercial BCA protein assay kit containing reagent A (BCA detection reagent, sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.2 N NaOH) and reagent B (4% copper sulfate solution) was used
for this assay. However, working reagent was prepared by mixing 50:1 parts of Reagent A and Reagent B. To generate the protein standard curve with the readings of known serum albumin, its concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml were taken in eppendorf tubes and mixed each with 1 ml of working reagent. Likewise, an aliquot (50µl) of each mitochondrial fraction of the ocular tissue diluted in 0.1M phosphate buffer was mixed with 1 ml working reagent in an eppendorf tube. All the tubes were incubated in water bath at 60°C for 30 minutes. The purple colored reaction product of two molecules of BCA from Reagent A and one cuprous ion (Cu+) from Reagent B was water soluble having a strong absorbance at 562 nm. Hence, the tubes were cooled by putting them in ice and the protein concentrations were measured according to the absorbance detected at 562 nm in a spectrophotometer (Beckman DU-640). Distilled water was used as a blank for the protein assay.

**Western Blot (WB) Analysis**

According to the method of Laemmli (1979), mitochondrial proteins from different segments of the eye were denatured and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 percent polyacrylamide gel in 4°C at 80V for two and a half hours. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Boulder, CO) using Western blot apparatus in 4°C at 22V for 16 hours. The immunoblot was blocked in blocking buffer containing 5% nonfat dry milk (NFDM) and 0.05% Tween 20 in Tris-buffered saline (TBS-T) for one hour. Grx2 antibody (Abcam plc., Cambridge, MA) diluted in 1:100 blocking buffer was used to incubate the PVDF membrane overnight at 4°C. The membrane was washed thrice with TBS-T for 15 minutes every time and then incubated with secondary horseradish peroxide (HRP)-conjugated goat polyclonal anti-rabbit IgG antibody (Santa Cruz™ Biotechnology, Santa Cruz, CA). Immunodetection of bands was done using the enhanced chemiluminescence (ECL) Western Blotting Detection System (Thermo Scientific, Rockford, IL). Finally, the immunoblot was analyzed with Versadoc 5000 MP Imaging System (Bio-Rad, Richmond, CA) having optimum exposure of 600 seconds at the interval of 10 seconds for 60 events.
The membrane was stripped and re-probed with voltage-dependent anion channel protein (VDAC) in 1:1000 dilution as a loading control for mitochondrial fraction (Cell Signaling, Danvers, MA). Again, HRP-conjugated goat polyclonal anti-rabbit IgG antibody diluted 1:5000 in blocking buffer was used a secondary antibody. Precision plus protein™ Western C™ standards were used as molecular weight marker (Bio-Rad, Hercules, CA), while Page ruler™ prestained protein ladder (Fermentas, Hanover, MD) was used a control. Precision protein™ StrepTactin- HRP conjugate (Bio-Rad, Hercules, CA) was used as a secondary antibody against molecular weight markers in 1:5000 dilution. Pixel densities of the observed bands were determined with the help of Bio-Rad softwares.

**Glutaredoxin 2 Activity Assay**

Grx2 assay was carried out following the method given by Mieyal *et al.* (1991) and modified by Raghavachari and Lou (1996). A reaction mixture was made with 0.2 mM NADPH, 0.5 mM glutathione, 0.2 M phosphate buffer (pH 7.4), 0.4 U glutathione reductase, and 50 µL of sample containing up to 250 µg of mitochondrial soluble protein. In order to get the soluble protein, 5mg/ml mitochondrial protein was completely dissolved in 1% sodium lauryl maltoside, mixed and incubated in ice for 30 minutes, and centrifuged at 10,000g for 30 minutes at 4°C. Finally, the supernatant containing soluble mitochondrial fraction was collected and used for the Grx2 activity assay.

To initiate the reaction, a synthetic substrate of hydroxyethyl disulfide (HEDS) at 20 mM was added and the decrease in absorbance of NADPH was monitored every 20 seconds for 3 minutes in 30°C via spectrophotometry at 340nm. The control tube contained the distilled water instead of mitochondrial fraction of tissue homogenate. The subtraction of the slope of the linear time course for decrease in absorbance of NADPH at 340 nm for the control from that of the samples was used to examine the Grx2 dependent rates. The rate of NADPH consumption or the decreased optical density at 340 nm per minute per milligram protein was calculated in mU/mg protein as the enzyme activity of Grx2. In another words, one unit of Grx2 activity is 1µmol of NADPH oxidized in one minute (Raghavachari and Lou, 1996). The overall reaction is the same as depicted in dethiolase activity of Grx2 (Figure 7), where PSSG is replaced with synthetic substrate, HEDS.
CHAPTER V: RESULTS

Homology Sequence Between Porcine and Mouse Grx2

Because the current commercial source of Grx2 antibody is limited to mouse Grx2, and that mouse eye is too small to be dissected into various eye segments for our purpose, we explore the possibility of using porcine eye as our model. The benefits of using porcine eye are two: one is the size of the eye, which allows us to collect more abundant tissue for isolating the mitochondrial fraction for the study, and the second is that porcine eyes can be obtained fresh and conveniently from a local slaughterhouse. Therefore, we first need to clarify if porcine Grx2 has similar amino acid sequence as that of the mouse Grx2, and also if porcine Grx2 has an acceptable sequence homology with mouse Grx2. We conducted a Grx2 sequence analysis using BLAST sequence alignment program obtained from NCBI.

Figure 11 shows the homology sequence between porcine Grx2 and mouse Grx2. Porcine Grx2 has a sequence of 155 amino acids and that of mouse Grx2 is 154. Using NCBI BLAST bioinformatics tool, it was observed that there is 77% homology in amino acid sequences between porcine and mouse Grx2 protein. Even though there is 71% homology between porcine Grx2 and human Grx2, the 77% homology between porcine Grx2 and mouse Grx2 serves our purpose of using porcine eye for this thesis study.
Mouse Liver Acts as a Positive Control

Another control study we have to carry out for this research endeavor is to use a mitochondria-rich tissue as a positive control for all the eye tissues to be examined for Grx2 expression and enzyme activity. We chose the liver as it is a tissue rich in mitochondria and Grx2 also. The purified mitochondrial fraction from mouse liver was therefore used to validate with Western blot for the molecular size of liver Grx2 that positively reacted with mouse Grx2 antibody. Increment of liver mitochondrial preparation was also used on Western blot analysis to confirm the proportional increase of Grx2 positive bands.

The Western blot data as shown in Figure 12 indicates the primary band of mouse Grx2 is located at 16 kDa position. For this experiment 50, 100, and 150 µg of mitochondrial isolate from the mouse liver were loaded and run on a 12% SDS-PAGE. Primary antibody against mouse Grx2 was used to visualize the Grx2 positive band. Increased amount of the sample protein resulted into proportionately heavier bands which were further quantified as relative pixel densities with VDAC, a mitochondrial loading marker. Relative pixel densities for 100 µg and 150 µg of liver mitochondrial isolates were calculated to be 3.8 and 6.2 folds, respectively over the pixel density of 50 µg liver mitochondrial isolate, which was already standardized as a unit pixel density. Therefore, mouse liver is a suitable positive control and was used for the successive experiments on the porcine eye tissues described below.
Vitreous Humor Acts as a Negative Control

After seeing the high homology sequence between mouse Grx2 and porcine Grx2, anti-Grx2 antibody raised in mouse was used to probe for porcine Grx2 from various ocular tissues. As vitreous humor is known to be acellular with little presence of major proteins/enzymes, we chose to analyze vitreous humor first to examine if Grx2 is absent in this jelly-like eye tissue. Porcine vitreous humor homogenate (50µg) was loaded and run on 12% SDS-PAGE. As per our expectation, there was no band of Grx2 as well as VDAC (mitochondria specific loading control) on the presumed site of the blot (Figure 13A). Western blot result was consistent with the Grx2 activity assay result (Figure 13B), where mouse liver had 1.70 mU/mg of Grx2 activity and porcine vitreous humor had insignificant Grx2 activity (0.10 mU/mg). Hence, vitreous humor was verified to be Grx2 free and thus can be used as a negative control for the studies of other eye tissues in the following.

Relative Expression and Activity of Porcine Grx2 in Ocular Tissues

The Western blot analysis of five porcine ocular tissues, namely the lens, vitreous humor, ciliary body, retina, and optic nerve with equal amount of protein (50µg) were applied on a gel as shown in Figure 14A, where a Grx2 positive band can be seen in each. The relative pixel density in comparison to mouse liver (Figure 14B) shows that ciliary body has the highest expression of Grx2 over the liver Grx2 (26.64 fold) followed by retina (11.92 fold), optic nerve (8.60 fold), and the lens (0.75 fold). For the Western blot analysis, the mitochondrial specific protein (VDAC) was used as a loading control.
The enzyme activity in each tissue was also compared with that of the Grx2 activity in mouse liver (Figure 14C). Retina was found to have the highest Grx2 activity (3.89 mU/mg) closely followed by ciliary body (3.10 mU/mg), the lens (0.58 mU/mg) and optic nerve (0.32 mU/mg).

**Relative Expression of Grx2 in Ocular Tissues in Comparison to the Lens**

All the seven porcine ocular tissues: cornea, iris, the lens, ciliary body, vitreous humor, retina, and optic nerve with equal amount of protein (50µg) were loaded in a single gel and found a comparable result as shown in Figure 15A and Figure 15B. However, the relative expressions of Grx2 in different ocular tissues were standardized with that of the lens, considering the expression of Grx2 in the lens as a unit. As predicted, ciliary body had the highest expression of Grx2 (13.45 fold) followed by retina (9.15 fold), iris (2.14 fold), optic nerve (1.97 fold), and cornea (1.06 fold). Again, VDAC was probed in each tissue to ensure equal loading of each sample.
Figure 11. Alignment of the amino acid sequences of Porcine and Mouse Grx2.

NCBI BLAST sequence alignment program was used to compare known mouse Grx2 amino acid sequence with pig Grx2 amino acid sequence. Conserved amino acids that share their identity are highlighted in yellow.
**Figure 12. Mouse Liver Acts as a Positive Control.** 50µg, 100µg, and 150µg of mitochondrial proteins from mouse liver tissues were applied on a 12 percent SDS gel for comparison. Proteins were transferred to a PVDF membrane and probed with 1:100 Grx2 antibody diluted in blocking buffer. Membrane was stripped and probed for a mitochondria specific marker, VDAC in 1:1000 dilution. Relative pixel density was calculated with respect to the gel loading control, VDAC. Molecular weight (MW) markers were used to locate the target band. Arrow heads show the location of the target band as compared to the MW markers.
Figure 13. Vitreous Humor Acts as a Negative Control

(A) **Western blot analysis of Grx2 in porcine vitreous humor.** Homogenized protein from porcine vitreous humor was run on a 12 percent SDS gel. Protein was transferred to a PVDF membrane and probed for Grx2 with 1:100 Grx2 antibody diluted in blocking buffer. Membrane was stripped and probed for a mitochondria specific marker with 1:1000 VDAC antibody. MW markers were used to locate the target band based on the molecular weight. Arrow heads show the location of the target band as compared to the MW markers.

(B) **Enzyme activity assay of Grx2 in porcine vitreous humor.** A reaction mixture of 0.2 mM NADPH, 0.5 mM glutathione, 0.2 M phosphate buffer (pH 7.4), 0.4 U glutathione reductase, and 50 µL of sample containing up to 250 µg of mitochondrial protein was made. After the reaction was initiated with 20 mM of HEDS, the reaction was monitored every 20 seconds for 3 minutes in 30°C via spectrophotometry at 340nm.
Figure 14. Expression of Grx2 in Porcine Ocular Tissues in Relation to Mouse Liver

(A) Western blot analysis of Grx2 in ocular tissues. 50µg of mitochondrial protein from the eye tissues, namely the lens, vitreous humor, ciliary body, retina, and optic nerve; and 50µg of mouse liver mitochondrial fraction (positive control) were run on a 12 percent single SDS gel. Proteins were transferred to a PVDF membrane and probed with 1:100 Grx2 antibody diluted in blocking buffer. Membrane was stripped and probed for a mitochondria specific marker, VDAC in 1:1000 dilution. MW markers were used to locate the target band based on the molecular weight. Arrow heads show the location of the target band as compared to the MW markers.
(B) **Relative expression of Grx2 in ocular tissues.** Comparison of pixel density of Western blot of porcine ocular tissues to that of mouse liver was used to determine the relative expression of Grx2. Here, the pixel densities of all the other tissues indicated were compared to pixel density of mouse liver that was standardized to 1.

![Relative pixel density graph](image1)

(C) **Enzyme activity assay of Grx2 in porcine ocular tissues.** A reaction mixture of 0.2 mM NADPH, 0.5 mM glutathione, 0.2 M phosphate buffer (pH 7.4), 0.4 U glutathione reductase, and 50 µL of sample containing up to 250 µg of mitochondrial protein was made. After the reaction was initiated with 20 mM of HEDS, the reaction was monitored every 20 seconds for 3 minutes in 30°C via spectrophotometry at 340nm.

![Grx2 activity graph](image2)
Figure 15. Expression of Grx2 in Porcine Ocular Tissues in Relation to Porcine Lens

(A) Western blot analysis of Grx2 in ocular tissues. Mitochondrial protein fraction (50µg) each from all seven ocular tissues, namely cornea, iris, the lens, vitreous humor, ciliary body, retina, and optic nerve as well as 50µg of mouse liver mitochondrial fraction (positive control) were run on a 12 percent single SDS gel. Proteins were transferred to a PVDF membrane and probed with 1:100 Grx2 antibody diluted in blocking buffer. Membrane was stripped and probed for a mitochondria specific marker, VDAC in 1:1000 dilution. MW markers were used to locate the target band based on the molecular weight. Arrow heads show the location of the target band as compared to the MW markers.
(B) Relative expression of Grx2 in ocular tissues. Comparison of pixel density of Western blot of seven porcine ocular tissues, namely cornea, iris, the lens, vitreous humor, ciliary body, retina, and optic nerve to that the lens was used to determine the relative expression of Grx2. Here, the pixel densities of all the other porcine ocular tissues indicated were compared to pixel density of porcine lens that was standardized to 1.
CHAPTER VI: DISCUSSION

This is the first qualitative examination of Grx2 in the ocular tissues using porcine eye as a model. Glutaredoxin 2 has been found in all ocular tissues, namely cornea, iris, the lens, ciliary body, retina, and optic nerve except the vitreous humor. This is because the mature vitreous body does not have cells and contains only collagen fibres and hyaluronic acid (Swann and Sotman, 1980). As peripheral vitreous body was collected free of any neighboring tissues, it is thus reasonable that neither the Western blot nor activity assay detected Grx2 in vitreous humor. However, the cross-contamination of the loading samples might have produced a very faint band in one of the Western blot results (Figure 15A).

Since a long time it has been known that ciliary body, a component of the eye, contains higher anti-oxidative defense activities resulted through the catalytic mechanism of anti-oxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase (Bhuyan and Bhuyan, 1977). The oxidation-defense system must be active in this tissue because the level of glutathione, a key antioxidant and cofactor for Grx2 and glutathione peroxide, is also high in ciliary body (Shichi, 1990). Furthermore, it is also known that the avascular ocular tissues like the lens and the iris depend on aqueous humor for the nutrients and oxygen (Reitsamer and Kiel, 2003). Ultimately, ciliary circulation is responsible for the production and maintenance of aqueous humor through both the delivery of oxygen and nutrients, and the excretion of metabolic wastes (Reitsamer and Kiel, 2003; Kiel et al., 2011). Hence, the more of vasculature, the more mitochondria-rich cells are needed to combat with the oxidative stress through protective mechanism of
Grx2. It is thus not surprising to find a relatively higher Grx2 expression and activity in ciliary body.

Just like ciliary body, retina also has highly vascular and cellular components. Moreover, retina is prone to oxidative stress because of the larger surface area exposed where the light gets focused (Strauss, 2005). Retina has a high amount of unsaturated fatty acids in the membrane layers and is susceptible to oxidative damage (Handelman and Dratz, 1986). Hence, it is expected to see high expression and ample activity of Grx2 in the retina. Retina is sensitive to oxidative stress, which plays some role in inducing the age-related macular degeneration (Winkler, et al., 1999). In the current study only the neural retina was peeled off and used while RPE layer was not collected. In the future, it would be advised to include RPE along with neural retina to examine the protective role of Grx2 in the retina.

It is known that the lens contains only epithelial cells and fiber cells. The epithelial cells in peripheral region in the polar ends are differentiated to fiber cells losing nuclei and other organelles during the process. The mature fiber cell layers contain a high concentration of structural proteins and hence the epithelial layer and the cells in the polar regions possess the most of the biochemical reactions. Therefore, it is understandable to observe a relatively weak expression and low specific activity of Grx2 in the whole lens sample used in the study. That is why any further studies on the lens redox balance should focus on the lens epithelium layer. However, isolating adequate amount of mitochondria for such study is a matter of challenge.
Other ocular tissues studied, namely cornea and iris showed weak expression of Grx2. However, the expression of Grx2 in optic nerve was higher than the lens, but the specific activity was found to be less mainly due to the low solubility of this tissue.

This study is a survey looking for the expression of Grx2 in ocular tissues. However, it is anticipated that researchers can use this information as the bases to examine the function and the protective role of Grx2 in other ocular tissues besides the lens.
CHAPTER VII: CONCLUSIONS

- Grx2 was found in all porcine ocular tissues, except vitreous humor.
- Grx2 protein expression level as well as enzyme activity assay were higher in eye tissues rich in mitochondria such as ciliary body and retina.
- Rich presence of Grx2 in ocular tissues is consistent with their known sensitivity to oxidative stress.
CHAPTER VIII: FUTURE DIRECTIONS

- For the effective quantification of the data verifying the use mouse antibody against the porcine ocular tissues, experiments involving both mouse liver as well as porcine liver should be carried out to compare if both the livers react to mouse anti-Grx2 antibody and have Grx2 band at the exact location in Western blot analysis.

- Purified Grx2 protein can be used as a standard during Western blotting to separate the target band from non-specific bands.

- In this study HEDS was used as a synthetic source of disulfides while measuring the enzyme activity assay of Grx2. The use of tissue preparations without HEDS could be another effective control for the enzyme activity assay.

- RPE layer of retina are also sensitive to oxidative stress. Hence, RPE cells should be included along with neural retina for the study of oxidative stress in retina.

- To ensure all Grx2 are in reduced form, dithiothreitol (DTT) can be used to reduce the sample prior to the Grx2 activity assay.

- Grx2 in the ocular tissues can be quantified with the level of messenger ribonucleic acid (mRNA) using reverse transcriptase polymerase chain reaction (RT-PCR) so that the presence of Grx2 can be further confirmed in ciliary body, retina, or other ocular tissues that are sensitive to oxidative stress but are rich in mitochondria.
REFERENCES


Lou, M. F. Unpublished results


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