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EXPRESSION AND DISTRIBUTION OF THIOL-REGULATING ENZYME GLUTAREDOXIN 2 (GRX2) IN PORCINE OCULAR TISSUES*

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Abstract

Glutaredoxin2 (Grx2) is a mitochondrial isozyme of the cytosolic glutaredoxin1 (thioltransferase or TTase). Both belong to the large oxidoreductase family and play an important role in maintaining thiol/disulfide redox homeostasis in the cells. Grx2 is recently found in the lens where its activities of disulfide reductase and peroxidase, similar to TTase, can protect the lens against oxidative stress. Since other eye tissues are also highly sensitive to oxidative stress, and TTase's distribution in the eye is known, we focused on this study by investigating the Grx2 distribution in the ocular tissues in comparison to the lens. Fresh porcine eyes were dissected into cornea, iris, ciliary body, the lens, vitreous humor, retina, and optic nerve. Each tissue (pooled from three eyes) was homogenized and processed for mitochondrial isolation. The mitochondrial fraction was analyzed for Grx2 protein using Western blotting with anti-Grx2 antibody, and Grx2 activity using the published procedure. The eye tissues were also measured for Grx2 mRNA expression by RT-PCR with GAPDH as the control. Grx2-rich mouse liver and purified recombinant mouse Grx2 were used as positive controls for the above analyses. It was found that Grx2 was present in all the tested ocular tissues, except vitreous humor. In comparison with the mouse liver, the protein levels of Grx2 in porcine ciliary body and the lens were 27-fold and 0.75-fold, respectively. Comparing to the lens, Grx2 protein was highest in the ciliary body (13.5-fold), followed by retina (9.2-fold), iris and optic nerve (2-fold), and cornea (1.2-fold). Enzyme activity assays showed that the retina

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#Equal contribution

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had the highest Grx2 specific activity (3.9 mU/mg protein), followed by ciliary body (3.1 mU/mg), the lens (0.58 mU/mg), and optic nerve (0.32 mU/mg). Grx2 gene expression in these ocular tissues was further confirmed by RT-PCR analysis. Grx2 mRNA expression showed the highest in ciliary body, followed by retina, optic nerve, cornea, iris, and the lens. No Grx2 mRNA, protein or enzyme activity could be found in the vitreous humor. The results indicate that Grx2 level was higher in eye tissues rich in vasculature and mitochondria (i.e. ciliary body and retina), corroborating with the levels of mRNA expression and Grx2 activity. The rich presence of Grx2 in these tissues is also consistent with their known sensitivity to oxidative stress.

Keywords

Glutaredoxin2; thiol/disulfide regulation; oxidative stress; protein-S-S-glutathione; mitochondria; disulfide reductase; ocular tissues

1. 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 (Harding and Crabbe, 1984; 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, glutaredoxin1 (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/enzymes that form protein-S-S-glutathione mixed disulfide bonds (or glutathionylation) during oxidation, and restores their functions (Holmgren, 1989; Mieyal et al., 1991; Raghavachari and Lou, 1996). Glutaredoxin 2 (Grx2) is a recently identified mitochondrial isozyme of Grx1 that has a molecular weight of 16 kDa and a motif of vicinyl cysteine, Cys-X-X-Cys, at the active site, similar to other oxidoreductases, and reduces protein mixed disulfides (Gladyshev et al., 2001; Lundberg et al., 2001).

Grx1 has been studied extensively in the lens (Xing and Lou, 2003; Lou, 2003) and its distribution in the ocular tissues has also been examined (Wu et al., 1998). However, Grx2, being a more recently discovered enzyme, is less known for its physiological functions. Grx2 has catalytic properties similar to that of the cytosolic Grx1, but it mainly functions in the mitochondria and the nuclei (Gladyshev et al., 2001; Lundberg et al., 2001; Fernando et al., 2006; Pai et al., 2007). Previous reports have shown that Grx2 not only protects human lens epithelial cells against oxidative damages but also its knockdown sensitizes primary lens epithelial cells to H₂O₂- induced cell injury, which could be prevented by imported

recombinant Grx2 protein (Wu et al., 2010). Evidence from recent studies also suggests that Grx2 may control mitochondrial redox balance, and the capability of Grx2 to prevent glutathionylation-induced inactivation of mitochondrial complex I plays a key role in regulating mitochondrial functions in the electron transport system, ATP production, and anti-apoptosis (Beer, et al., 2004; Enoksson, et al., 2005; Wu, et al., 2010). Furthermore, Grx2 gene knockout mice exhibited early and faster progression of cataract as a function of age in comparison with the age-matched wild type mice (Lou, et al., 2012). These results suggest that Grx2 has an important role in two ways: cytoprotection, and maintaining transparency in the lens. However, the distribution of Grx2 in other ocular tissues that are oxidation sensitive and rich in mitochondria remains unclear. Therefore, the current study was designed to investigate Grx2 distribution and detect its enzyme activity in ocular tissues using porcine eyes. Our results showed that Grx2 was found in most porcine ocular tissues, but was absent in the vitreous humor. Highest Grx2 protein expression level or activity was found in ciliary body of the anterior part and in retina of the posterior part of the eye.

2. Materials and methods

2.1. Materials

Trizma base, NADPH, glutathione and glutathione reductase were all purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyethyl disulfide (HEDS) was purchased from Aldrich Chemical Company (Milwaukee, WI). Bicinchoninic acid (BCA) protein assay reagent kit was from Pierce Biotechnology Inc. (Rockford, IL). Anti-glyceraldehydes-3 phosphate dehydrogenase (G3PD) antibody was purchased from Santa CruzTM Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Grx2 antibody was from Abcam Co. (Cambridge, MA) while rabbit polyclonal voltage-dependent anion channel (VDAC) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Purified mouse recombinant Grx2 was prepared in our laboratory following the procedure of Gladyshev et al. (2001). Bovine serum albumin standard and enhanced chemiluminescence system (ECL) were from Thermo Fisher Scientific Inc. (Waltham, MA). Precision plus proteinTM Western CTM standards, Sodium dodecyl sulfate (SDS), Tween 20, bis N'N'-methylene bis-acrylamide were all purchased from BioRad Co. (Hercules, CA). Page rulerTM prestained protein ladder was obtained from Fermentas (Hanover, MD). Polyvinylidene difluoride (PVDF) membrane was purchased from GE Healthcare (Boulder, CO). TRIzol RNA reagent was from Ambion (Carlsbad, CA). Ultra-pure DEPC-treated water, oligo dT, RNase OUT, Prime pfx reaction kit and DNA polymerase were from Invitrogen (Carlsbad, CA). AMV buffer, dNTP and AMV RT were from Promega (Madison, WI). All the chemicals and reagents were of analytical grade.

2.2. Comparison of the amino acid sequence analyses of Grx2 between porcine and mouse

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

2.3. Dissection of porcine ocular tissues

Porcine eyeballs from 5–6 month-old pigs were obtained from a local abattoir (Farmland Foods, Inc., Crete, NE) and kept on ice during transportation and dissection. All the dissection procedures were performed under the dissecting microscope using microsurgery instruments. Seven fresh ocular tissues, namely cornea, iris, the lens, vitreous humor, ciliary body, retina, and optic nerve were taken from each eye. Three samples of each eye tissue were pooled and frozen on dry ice and kept at -80°C until further use.

2.4. Isolation of mitochondrial fraction from mouse liver and ocular tissues

Mitochondrial fraction of tissue homogenates was isolated according to the modified protocol described by Rehncrona, et al. (1979). Fresh mouse liver was cut into small pieces and homogenized with a glass-to-glass homogenizer (Kontes Glass Co, Vineland, NJ) in 1 ml homogenizing buffer (pH 7.2) containing 225 mM mannitol, 65 mM sucrose, 1 mM ethylene glycol tetra-acetic 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 was centrifuged again at 16,000g for 10 minutes at 4°C and the final mitochondrial pellet was saved and re-suspended in 1 ml homogenizing buffer. The purified pellet re-suspension was added 1% lauryl maltoside to solubilize the mitochondrial proteins and allowed to stand for 30 minutes on ice before centrifuging at 10,000g for 30 minutes at 4°C . Then the supernatant containing soluble mitochondrial fraction was collected and saved for the designed experiments.

All the ocular tissues previously dissected and stored at -80°C were thawed in ice before preparing the tissue homogenates. Mitochondrial fraction from each tissue was processed following the same procedure as that of the liver mitochondria described above. The purified mitochondrial pellet was re-suspended in 50 μL of the same buffer. To the pigmented tissue like iris and ciliary body, 1% maltoside was added to each, allowed to stand for 30 minutes in ice, and centrifuged at 10,000g for 30 minutes at 4°C . Finally, the supernatant containing soluble mitochondrial fraction was collected and saved for further experiments.

2.5. Western Blot (WB) analysis

According to the method of Laemmli (1970), mitochondrial proteins from different segments of the eye were denatured and subjected to 12% SDS-PAGE gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Boulder, CO, USA). Anti-mouse Grx2 antibody was used for the mouse and porcine tissue samples. Immunodetection of bands was done using the enhanced chemiluminescence (ECL) Western Blotting Detection System (Thermo Scientific, Rockford, IL). The immunoblot was analyzed with an imaging system (Versadoc 5000 MP Imaging System, Bio-Rad, Richmond, CA).

2.6. Glutaredoxin 2 activity assay

Grx2 assay for its disulfide reductase activity was carried out following the method for Grx1 assay given by Mieyal, et al. (1991) and modified by Raghavachari and Lou (1996), in which the lens Grx1 reduced both the synthetic substrate (hydroxyethyl disulfide) and protein-thiol mixed disulfides. A reaction mixture was made with 0.2 mM NADPH, 0.5 mM

glutathione, 0.2 M phosphate buffer (pH 7.4), 0.4U glutathione reductase, and 50 μ L of sample containing up to 250 μ g 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. Although Grx1 and Grx2 catalyze the same synthetic substrate for the disulfide reduction, the assays conducted in this study only used the isolated and purified mitochondria from the liver or ocular tissues. Therefore, the disulfide reductase activity was contributed mostly if not all from the mitochondrial Grx2, and little or none from the cytosolic Grx1.

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.

2.7. Total RNA isolation from porcine ocular tissues and mouse liver

Total RNA was isolated using TRIzol isolation kit (Ambion, Carlsbad, CA), according to the manufacturer's instructions. Porcine ocular tissue or mouse liver of 100 mg was each homogenized in 1ml TRIzol Reagent by glass-to glass homogenizer. The homogenate was then mixed with 0.2 ml chloroform for 2–3 min at room temperature before centrifuging at 12,000g for 15 min at 4°C. The total RNA in the supernatant was saved and mixed with 0.5 ml isopropyl alcohol, centrifuged at 12,000 g for another 10 min at 4°C. The RNA containing pellet was washed three times with 1 ml 75% ethanol, centrifuged to collect the final pellet, which was then re-suspended in RNase-free, or DEPC-treated water (Ambion). The isolated RNA was further treated with DNase inhibitor, quantified and stored at –80°C until use. All laboratory glasswares used in RNA preparation were pretreated with RNase denaturing agent.

2.8. RT-PCR analysis for Grx2 gene expression

RT-PCR was conducted following Yegorova et al. (2006) with modifications. cDNA was prepared using 2 μ g total RNA isolated from mouse liver or porcine ocular tissue, mixed with 0.5 μ g oligo dT (Invitrogen, USA) and DEPC treated water to a total volume of 11 μ L. The reaction mixture was heated at 70°C for 5 min in a thermo cycler (TEchne, FTGENE2D, United Kingdom), quickly incubated on ice for 5 min and then added 5 μ L of AMV buffer (Promega, USA), 2.5 μ L with 1 mM each of dNTP Mix (Promega, USA), 1 μ L of RNase OUT (40 units/ μ L), 3 μ L of AMV RT (Pomega), and DEPC treated water to make a final volume of 25 μ L. The mixture was incubated at 42°C for 60 min in the thermo cycler, stored at –80°C until use.

The primer sets for detecting Grx2 (forward 5'-CCTCTGGGATGGGGAACCAC-3' and reverse 5'-TGTCCATTGCGCCTCCAATA-3') or GADPH, the internal control (forward 5'-CCCTGAGACACGATGGTGAA-3' and reverse 5'-GGTTCACGCCCATCACAAAC-3') were used. Both primers were designed by using Primer 3 software of NCBI. The sizes of the PCR product for Grx2 and GADPH were 297bp and 415bp, respectively.

PCR was performed on 1µL aliquots from each cDNA reaction, mixed with 2 µL of 10×AccuPrime pfx reaction mix (Invitrogen, USA), 1 µL of the primer set (8 µM each), 0.5 µL of DNA polymerase (Invitrogen, USA) and DEPC-treated water to make a final volume of 20µL. Conditions for PCR reaction were initial cycle at 94°C for 5 min (denaturation), 30 cycles at 94°C for 30 sec (denaturation), followed by 63°C for 1min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 10 min. RT-PCR products were analyzed by agarose gel electrophoresis (1.0%) followed by digital imaging of the ethidium bromide-stained gel. DEPC-treated water was used as the negative control.

2.9. Protein concentration determination and statistical analysis

Protein concentration was determined by microanalysis of the BCA method (Smith, et al., 1985). Statistical data were analyzed using the t-student test. Multiple comparisons toward a control group were performed with Dunnett's test. For all tests, *p* value < 0.05 was considered significant. The error bars in all the graphs represent standard deviations.

3. Results

3.1. Conservation of amino acid sequences 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 used porcine eye as the model due to its local availability. Therefore, we first wanted to clarify if porcine Grx2 has a similar amino acid sequence as that of the mouse Grx2, and if there is an acceptable sequence homology between the two. Grx2 sequence was carried out using a BLAST sequence alignment program from NCBI. Figure 1 shows the homology sequence between porcine Grx2 and mouse Grx2. Porcine Grx2 has a sequence of 155 amino acids while mouse Grx2 has 154 amino acids. BLAST bioinformatics analysis showed there was 77% homology in between the two. This high homology served our purpose to use the porcine eye for the Grx2 distribution study.

3.2. Determination of Grx2 presence in mouse liver

Another control-study we carried out was 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 mitochondria and Grx2-rich liver for such purpose since we planned to use the anti- mouse Grx2 antibody for the study. The purified mitochondrial fraction from mouse liver was validated using Western blot analysis with anti-mouse Grx2 antibody and the antibody of a mitochondria-specific protein, VDAC, as a loading control. Purified recombinant mouse Grx2 protein was also used to ensure that the Grx2 antibody is functional. Grx2 antibody showed several nonspecific positive bands with the proteins in the mitochondrial fraction of mouse liver (data not shown). However, the major protein reacted

with Grx2 antibody was at 16 kDa, the same position as that of the pure recombinant Grx2 protein (10 ng) (Fig. 2A). The intensity of this Grx2 positive band was proportionally enhanced when increment of 50, 100, and 150 μ g of the liver mitochondrial preparations were applied on the gel (Fig. 2B). The relative pixel densities between VDAC and Grx2 bands are shown in Figure 2C, in which 100 μ g and 150 μ g liver samples were calculated to be 3.8 and 6.2 folds, respectively over the 50 μ g liver sample that was standardized to 1.0 unit pixel density. Therefore, mouse liver is a suitable positive control for studying the porcine eye tissues described below.

3.3. Comparison of the Grx2 protein level and activity in porcine lens, vitreous humor and ciliary body to mouse liver

Since the lens tissue is known to contain Grx2 while ciliary body is known to have high level of mitochondria, we first chose to compare the status of Grx2 in those two ocular tissues with that of mouse liver, using 50 μ g mitochondrial proteins from each for the immunoblot analysis with VDAC as the loading control. The mitochondrial preparation of vitreous humor (50 μ g proteins) was also used for comparison of the potential Grx2 protein expression. As shown in the Western blot in Figure 3A, both the lens and ciliary body (CB) have a clear Grx2 positive band at the 16 kDa position where the intensity is low for the lens but high for ciliary body. However, the vitreous humor has none. Results of the pixel density of the lens and CB after normalized to liver are shown in Figure 3B, in which the lens is 0.75 fold while ciliary body is 27-fold over the liver (1.0). Mouse liver of 50 μ g mitochondrial preparation had 1.70 ± 0.01 mU/mg of Grx2 activity (Fig. 3C). Both ocular tissues each with 50 μ g of mitochondrial proteins also displayed Grx2 enzyme activity, and the relative activity in each tissue was corroborated with the respective level of its Grx2 protein (Fig. 3C). Vitreous humor was analyzed and found no Grx2 activity (data not shown).

3.4. Relative Grx2 protein content and activity of porcine Grx2 in ocular tissues

All the porcine ocular tissues, including cornea, iris, the lens, ciliary body, retina and optic nerve except vitreous humor were examined for the presence of Grx2 protein by immunoblot analysis against anti- mouse Grx2 antibody. Equal amounts of protein (50 μ g) were loaded in a single gel and VDAC was also probed in each tissue to ensure equal loading. The result of Western blot is shown in Figure 4A, in which all tissues display a 16 kDa Grx2 positive band with different intensity. Of all the tissues, ciliary body and retina had higher while cornea, iris, optic nerve and the lens had lower Grx2 content. The relative abundance of Grx2 is shown in the pixel density graph in Figure 4B, in which the relative protein content of Grx2 in different ocular tissues were standardized with that of the lens (1.0 unit). Overall, ciliary body had the highest expression of Grx2 (13.5 fold) followed by retina (9.2 fold), iris (2.1 fold), optic nerve (2.0 fold), and cornea (1.1 fold) over the lens (1.0).

The enzyme activity in each tissue was also compared with that of the lens (Fig. 4C). Retina was found to have the highest Grx2 activity (3.89 ± 0.34 mU/mg) closely followed by ciliary body (3.10 ± 0.26 mU/mg), the lens (0.58 ± 0.07 mU/mg) and optic nerve (0.32 ± 0.03 mU/mg). The normalized value of lens/CB/Retina/ON is 1/5.3/6.9/0.55. Cornea and iris were not

included in this study as the mitochondrial samples were insufficient to conduct the activity assay in addition to the WB analysis. Because of the hard surface of cornea, we had difficulty in processing it and using the minced tissue for the multistep-mitochondrial isolation, resulting in low yield of corneal mitochondria. The pigmented iris was also troublesome. It was necessary to use many washing steps to remove the pigment totally. Therefore, we had low yield of iris mitochondrial preparation.

3.5. Relative mRNA expression of Grx2 in porcine ocular tissues

RT-PCR was used to study the expression level of Grx2 gene in all the ocular tissues in comparison to that of mouse liver. GAPDH was used as the internal control (415bp) and DEPC-treated (RNase-free) water as the negative control. As shown in Figure 5A, a clear band of Grx2 mRNA at 297bp is visible in every ocular tissue, with highest presence in the ciliary body followed by retina and optic nerve while the cornea, iris and lens are low. Vitreous humor contained neither Grx2 nor GAPDH (data not shown). The level of Grx2 mRNA in the mouse liver was similar to that of the optic nerve. The equal intensity of GAPDH on the gel indicated equal loading of the samples. The relative Grx2 to GAPDH gene expression in pixel density is shown in Figure 5B. When normalized each tissue against the lens (1.0 unit), ciliary body was shown to be 5.4-fold, retina at 3.2 fold, optic nerve at 2.6 fold, cornea at 1.5 fold, iris at 1.0 fold and the mouse liver at 2.5 fold over the mRNA expression in the lens.

4. Discussion

This is the first qualitative examination of Grx2 in the ocular tissues using porcine eye as a model. Grx2 has been found in all ocular tissues, namely cornea, iris, the lens, ciliary body, retina, and optic nerve except the vitreous humor. The high gene sequence homology between mouse and porcine Grx2, as well as the positive control of mouse liver allowed us to use anti-mouse Grx2 antibody to carry out this ocular distribution study.

The absence of mitochondrial Grx2 in the vitreous humor is reasonable because the mature vitreous body does not have many cells and contains mostly collagen fibers and hyaluronic acid (Swann and Sotman, 1990). We did a mock mitochondrial Grx2 isolation just to ensure that vitreous humor was compared with liver and other ocular tissues under the same conditions. In contrast, the cellular-rich ciliary body in the eye contains high anti-oxidative defense activities by having high antioxidant enzymes of superoxide dismutase, catalase, and glutathione peroxidase (Bhuyan and Bhuyan, 1977). This active oxidation-defense system is also contributed from its rich glutathione content, which is a key antioxidant and cofactor for Grx1, Grx2 (Holmgren, 1989; Gladyshev, et al., 2001) and glutathione peroxidase (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 (Kiel et al., 2011; Strauss, 2005). Hence, the more of vasculature, the more mitochondria-rich cells are needed to combat with the oxidative stress through a protective mechanism of Grx2. It is thus not surprising to find a relatively higher Grx2 gene expression (Fig. 5), protein content and activity in ciliary body (Figs. 3–4). In

fact, the abundance of Grx2 in the ciliary body was much higher than that of the liver (Figs. 3 and 5).

Similar to ciliary body, retina is also a highly vascular and cellular tissue. Moreover, retina is prone to oxidative stress because of the larger surface area exposed where the light gets focused (Handelman and Dratz, 1986). Retina has a high amount of unsaturated fatty acids in the membrane layers and is susceptible to oxidative damage (Winkler et al., 1999). Hence, it is expected to find highly expressed and active Grx2 in the retina (Figs. 4–5). The rich presence of Grx2 may play some role in protecting the retina against oxidative stress, and potentially the age-related macular degeneration (Handelman and Dratz, 1986). In this study, only the neural retina was peeled off and used while retina pigmented epithelial (RPE) layer was not collected. However, according to our most recent data, Grx2 was highly expressed in RPE layer isolated from human donor eyes, where it protects RPE cells against oxidative stress-induced apoptosis via preventing protein thiol oxidation to protein-S-S-glutathione conjugates (Wu et al., 2014). Further studies characterizing the mechanism of how Grx2 prevents or repairs oxidative stress damage in the retina are likely to provide insight into the etiology of retinal disorders and may lead to new treatments for retinal degenerative diseases.

It is known that the lens contains a single layer of epithelial cells with the remaining tissue filled with fiber cells, which lost nuclei and other organelles during the differentiation process (Harding and Crabbe, 1986). Therefore, it is understandable to observe a relatively weak expression and low specific activity of Grx2 in the whole lens relative to that in the ciliary body, retina and the liver. Further study on Grx2 in the lens warrants focusing on using lens epithelium layer. However, isolating adequate amount of mitochondria from epithelial layers for such study is quite challenging.

Other ocular tissues studied, namely cornea and iris, showed weak expression of Grx2 (Figs. 4–5). Because of the low mitochondrial yield of both tissues, we chose to conduct protein analysis over the enzyme assay to establish if Grx2 is present in these two tissues relative to other ocular tissues. Although the enzyme activity assay was not performed on these two tissues, but based on the result of RT-PCR, cornea and iris may have Grx2 activity in the same range as that of the lens. Grx2 expression in optic nerve was 2-fold higher than that of the lens, but the specific activity was only half as much. This may due to the low solubility of this tissue.

Previous studies have shown that the distribution of cytosolic Grx1 (TTase) isozyme is concentrated more in the anterior segment of the bovine eye (Wu et al., 1998). The highest specific activity was present in the iris, conjunctiva and corneal epithelial and endothelial cells. In contrast, retina and ciliary body had moderate activity, similar to the lens epithelial cells. The whole lens was very low and the vitreous humor had no Grx1 activity. Such difference in the ocular distribution between Grx1 and Grx2 implies the close association between the specific mitochondrial regulatory functions of Grx2 with the vascular and mitochondria-rich ocular tissues.

It is interesting to note that a discrepancy between the protein content and the disulfide reductase activity of Grx2 was found in some ocular tissues. As shown in Figure 3, CB has 25-fold higher Grx2 protein level, but only twice the disulfide reductase activity than the liver tissue. Similarly, CB has higher Grx2 protein content but lower activity than the retina from the same porcine eye (Figure 4). Although the cytosolic Grx1 and the mitochondrial Grx2 catalyze the same synthetic substrate used in the assay, it is unlikely that cytosolic Grx1 contributed much of the activity as only purified mitochondria preparations were used for both WB and activity assays. Furthermore, the chances for cytosolic contamination, if any, only occurred in the liver or retina samples are unlikely as the mitochondrial isolation procedure was done simultaneously with great caution for all tissues. Pai et al (2007) reported that Grx1 is present in the intermembrane space of the mitochondria in the heart and liver tissues. If Grx1 is also present in the mitochondrial intermembrane space of the ocular tissues, according to our calculation, the Grx1 activity in the same mitochondrial preparation would be much lower than that of the Grx2 present in the matrix (unpublished results). Thus, the likely explanation to such disproportional ratio of Grx2 protein content/activity is the unique property of Grx2. Lillig et al. (2005) reported that human Grx2 from HeLa cells is characterized as an iron-sulfur protein and a redox sensor. The enzyme is present as an inactive dimer bridged by a $[2\text{Fe}-2\text{S}]^{2+}$ cluster, and its disulfide reductase activity of the monomer is only fully activated upon oxidative stress when Fe/S cluster is released. Therefore, when intact and untreated mitochondria preparation is used for the activity assay, the level of monomer in the mitochondrial preparation may vary from tissue to tissue, depending on its sensitivity or vulnerability to oxidative stress. In other words, tissues such as the liver, retina or the lens may contain higher level of the active monomer than a tissue like the ciliary body. The immunoblotting assay detects only the reduced Grx2 proteins, which should include the monomer originally present in the intact mitochondria, and the monomer released from the dimer after treatment of the reducing agent used in the WB process. Thus, it is reasonable to observe a high level of Grx2 protein that does not display a full enzyme activity in some of the ocular tissue. Whether Grx1 is present in the intermembrane space of the mitochondria, and whether Grx2 is a Fe/S cluster-containing enzyme in the ocular tissues are certainly the subjects of importance for future investigation.

In conclusion, we have found that mitochondrial Grx2 is present in all porcine ocular tissues except vitreous humor. The gene expression, protein level and enzyme activity were highest in the mitochondrial-rich and vascular-rich tissues of ciliary body and retina, lowest in the tissues that are non-vascular or low mitochondria presence, such as the lens. This study is a survey of the status of Grx2 in normal ocular tissues, using the porcine eye as a model. Our current results may be useful to future research in examining the function and the protective role of Grx2 in normal or diseased ocular tissues.

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Highlights

- Grx2, a redox regulating enzyme, is present in all eye tissues except the vitreous
- Grx2 is abundant in the ocular tissues rich in mitochondria and vasculature
- The distribution of Grx2 may be useful for the studies of degenerative eye diseases


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Porcine Grx2  1  MYWRRALAGRLVRRVSGSTGRF----GGAPGASASGMGNHTSSSLGNTAAAPVNQIQE  56
               M WRRAA  G RLV    ++GR    GA GA+ SGMGN TSS  G +  PVNQIQE
Mouse Grx2   1  MSWRRASVGRRLV-----ASGRILAGRGAAGAAGSGMGNSTSSFWGKSTTTPVNQIQE  55

Porcine Grx2  57  TISNNCVVIFSKTSCSYCTMAKKLFHDMNVSYKVVELDLLEYGSQFQDALCTMTGDRIVP  116
               TISNNCVVIFSKTSCSYC+MAKK+FHDMNV+YK VELD+LEYG+QFQDAL  MTG+RTVP
Mouse Grx2   56  TISNNCVVIFSKTSCSYCSMAKKIFHDMNVNYKAVELDMLEYGNQFQDALHKMTGERTVP  115

Porcine Grx2 117  RIFVNGTFIGGAMDTHRLHQEGKLLPLVHQCYLKKSKRK  155
               RIFVNG FIGGA DTHRLH+EGKLLPLVHQCYLKK + +
Mouse Grx2   116 RIFVNGRFIGGAADTHRLHKEGKLLPLVHQCYLKKQEE  154

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Figure 1. Alignment of the amino acid sequences of porcine and mouse Grx2

NCBI BLAST sequence alignment program was used to compare the known mouse Grx2 amino acid sequence with pig Grx2 amino acid sequence. Conserved amino acids that share their identity are highlighted in yellow.

Figure 2. Examination of the presence of Grx2 in mouse liver

Mitochondrial proteins isolated from mouse liver were used to examine the presence of the mitochondrial Grx2 enzyme in the following studies.

(A) Validation of anti-mouse Grx2 antibody. Western blot analysis was done with purified mouse recombinant Grx2 protein (10 ng) loaded in lane 1 and mouse liver mitochondria (100 µg) loaded in lane 2. Molecular weight (MW) markers were added to locate the target band (see arrow) of Grx2 with 16 kDa mass. The result is a representative of three separate experiments.

(B) Western blot analysis of Grx2 in mouse liver mitochondria. The proteins loaded on lane 1, 2 and 3 were 50µg, 100µg, and 150µg, respectively. The antibody for the mitochondrial-

specific protein VDAC was also probed with anti-VDAC antibody as a loading control. The WB is a representative result from three separate experiments.

(C) Relative pixel density was calculated from the intensities of the Grx2-positive vs VDAC-positive bands in each of the 50, 100 and 150 μ g proteins loading. Data are expressed as mean $\pm$ SD with n = 3. **P<0.01 vs 50 μ g loading.

Figure 3. Comparison of Grx2 protein and activity in the porcine vitreous humor, the lens and ciliary body with mouse liver

Equal amount of mitochondrial proteins isolated from porcine vitreous humor, the lens, ciliary body (CB) and mouse liver was used for the following studies.

(A) Western blot analysis of Grx2 in mitochondrial proteins from porcine vitreous humor, the lens, ciliary body and mouse liver (50 µg each) was carried out following the same procedure as above using anti-mouse Grx2 antibody. VDAC was used as a loading control.

(B) Pixel density was calculated for the comparison of Grx2 content in porcine lens and ciliary body with mouse liver, which was standardized to 1.0 value. Data are expressed as mean ± SD with n=3. **P<0.01 vs the liver.

(C) Grx2 enzyme activity in mU/mg protein was compared in porcine lens, ciliary body and mouse liver. Data are expressed as mean ± SD with three separate analyses. **P<0.01 vs the liver.

Figure 4. Comparison of the Grx2 protein level and activity in porcine ocular tissues

All ocular tissues except vitreous humor were analyzed for the protein content and enzyme activity of Grx2.

(A) Western blot analysis of Grx2 in ocular tissues was carried out using 50µg of mitochondrial proteins from each of the eye tissues, including cornea (lane 1), iris (lane 2), the lens (lane 3), ciliary body (lane 4), retina (lane 5), and optic nerve (lane 6) against anti-mouse Grx2 antibody. VDAC was used as a loading control.

(B) Comparison of pixel density of the Grx2-positive bands in all porcine ocular tissues used for the Western blot. Each tissue was compared to the lens, which was standardized to 1.0. Data are expressed as mean $\pm$ SD with n=3. * $P < 0.05$, ** $P < 0.01$ vs the lens.

(C) Grx2 enzyme activity was compared in porcine lens, ciliary body, retina and optic nerve. Data are expressed as mean $\pm$ SD with n=3. * $P < 0.05$, ** $P < 0.01$ vs the lens.

Figure 5. RT-PCR analysis of the gene expression of Grx2 in porcine ocular tissues and the mouse liver

Total RNA from each tissue was isolated with TRIzol RNA isolation kit, and each was analyzed by RT-PCR for the expression levels of Grx2. RT-PCR products were analyzed by agarose gel electrophoresis (1.0%), and by digital imaging of the ethidium bromide-stained gel. DEPC-treated water was used as the negative control. For details, see sections 2.7 and 2.8 in Materials and Methods.

(A) RT-PCR analysis of Grx2 gene expression in ocular tissues in comparison with mouse liver was carried out, including liver (lane 1, positive control), cornea (lane 2), iris (lane 3), the lens (lane 4), ciliary body (lane 5), retina (lane 6), optic nerve (lane 7) and water (lane 8, negative control). GAPDH was used as the loading control with the PCR product of 415 bp indicated by an arrow. The arrow at the upper panel indicates Grx2 PCR product of 297bp. M: marker. The RT-PCR blot is a representative from three separate experiments.

(B) Relative gene expression levels from the RT-PCR analysis were calculated for mouse liver (lane 1), porcine cornea (lane 2), iris (lane 3), the lens (lane 4), ciliary body (lane 5), retina (lane 6), optic nerve (lane 7) and water (lane 8). The value of the lens was normalized to 1.0. The data are expressed as mean $\pm$ SD with $n=3$. * $P < 0.05$, ** $P < 0.01$ vs the lens.