Human Lens Thioredoxin: Molecular Cloning and Functional Characterization

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PURPOSE. To molecularly clone the human lens thioredoxin (TXN) gene, characterize the recombinant protein (rTrx1) and study the regulation expression of thioredoxin (Trx1) in human lens epithelial cells under oxidative stress.

METHODS. The human TXN gene was cloned from a human lens cDNA library. Trx1 activity was measured by insulin reduction assay. For study of the upregulation of Trx1, 1.6 million human lens epithelial cells (HLE B3) were exposed to H2O2 (0.1 mM) for 0, 5, 10, 15, 20, and 30 minutes. The cells were lysed with lysis buffer and used for Trx1 activity assay, Western blot analysis, and real-time PCR.

RESULTS. The sequence of the human lens TXN gene was identical with that of other human tissues. Recombinant Trx1 was sensitive to iodoacetic acid but showed strong resistance to oxidation (0.1 mM H2O2) at its approximate physiological protein level. Western blot analysis and assay of Trx activity revealed that Trx1 was expressed in HLE B3 cells and localized in epithelial, cortical, and nuclear regions of human and porcine lenses. In vivo oxidative stress of HLE B3 cells resulted in a 35% upregulation of the level of Trx1 protein after 10 minutes of H2O2 treatment. Real-time PCR analysis showed an increase of approximately 50% in the level of Trx1 mRNA under the same oxidative stress conditions.

CONCLUSIONS. The upregulation of Trx1 in HLE B3 cells under oxidative stress and the presence of Trx1 in the lens suggest that the thioredoxin system may be an effective defense system against oxidative stress and the presence of Trx1 in the lens suggest that the thioredoxin system may be an effective defense system against oxidative stress.

The upregulation of Trx1 in HLE B3 cells under oxidative stress and the presence of Trx1 in the lens suggest that the thioredoxin system may be an effective defense system against oxidative stress. (Invest Ophthalmol Vis Sci. 2003;44:3263–3271) DOI:10.1167/iovs.02-1322

Thioredoxin has been demonstrated to be a multifunctional protein implicated in a wide variety of biochemical functions. It is a source of reducing equivalent for ribonucleotide reductase, methionine sulfoxide reductase, and sulfate reductase1 and for enzymes directly inactivated under oxidative stress conditions, including glyceraldehyde-3-phosphate dehydrogenase, ornithine decarboxylase, and iodothyronine 5'-deiodinase. Thioredoxin selectively activates the DNA-binding of several transcription factors, such as AP-1, NF-kB, glucocorticoid receptor, and other factors. It can act as a catalyst to facilitate refolding of disulfide-containing proteins and also as a growth factor for a variety of cell types. There is evidence suggesting that thioredoxin may have a neuroprotective function through the activation of AP-1 and may play a role in resistance to the cell-killing effect of anticancer drugs. Furthermore, thioredoxin can act as an antioxidant to protect cells against oxidative stress by removing H2O2 or scavenging free radicals. Thus, accumulating evidence suggests that thioredoxin regulates an increasing number of cell processes, such as cell division, radical scavenging, nonspecific defense, transcription, DNA replication, immune response, detoxification, meliosis, and embryogenesis, and has emerged as a regulatory protein that plays a fundamental role in plants and animals.

Oxygen metabolism occurring in the cells of all aerobic organisms continuously generates reactive oxygen species (ROS) within the cells, such as superoxide anion (O2•−), hydroperoxide (H2O2) and hydroxyl radical (·OH). Cells also can be exposed to environmental oxidants such as UV and ionizing radiation, heavy metals, redox active chemicals, anoxia, and hyperoxia, which increase ROS production. Aerobic organisms have widely adapted oxidation-reduction reactions to function in key metabolic and regulatory pathways necessary for normal cellular function. The primary antioxidants, which include nonenzymatic (e.g., glutathione [GSH], vitamin C, vitamin E, and carotenoids) and enzymatic systems (e.g., glutathione peroxidase, thioredoxin peroxidase, superoxide dismutase, and catalase), act as ROS scavengers to prevent their accumulation and possible deleterious effect. When the level of ROS exceeds the primary cellular antioxidant defenses, the repair enzyme systems are used to repair the oxidatively damaged proteins and to regulate redox homeostasis in the cells. Such repair systems include NADPH-dependent thioredoxin and thioredoxin reductase and glutathione-dependent thioredoxin transferase (TTase; glutaredoxin). Oxidative stress is considered one of the major risk factors for human age-related cataract formation. The lens is rich in oxidant-sensitive SH-containing proteins. Oxidation of the sulfhydryl groups (S-thiolation) of the lens proteins can lead to formation of protein-thiol mixed disulfide conjugates, such as protein-GSH and protein-cysteine, as well as intra- and intermolecular protein–protein disulfides. The altered redox status of cysteine residues can affect both the structure and the function of proteins and lead to decreased protein solubility, formation of high molecular weight aggregates, and eventual opacification of the lens (cataracts). Findings in one a study have suggested that the epithelial cell layer is an initial target of oxidative...
stress. It has been shown that epithelial cell damage precedes the loss of lens transparency in the eye. Most biochemical activities in the lens are concentrated in the epithelial cell layer, whereas fiber cells contain a high concentration of structural proteins that have a lower level of defense against oxidative stress. Therefore, the defense systems that protect the lens against oxidation are also concentrated in the epithelium. The lens has an unusually high level of GSH and of the complete primary antioxidants and oxidation defense enzymes. The oxidation damage-repair enzyme, GSH-dependent thioltransferase, has been identified in the lens, and the gene has been cloned from human lens epithelial cells. The second repair system in the lens, NADPH-dependent Trx/TrxR, is not well-studied. Although cytosolic Trx1 has been found in the human lens and in the Emory mouse lens, this lens protein has neither been purified nor characterized. Herein, we report the cloning of the human lens Trx1 gene and the functional characterization and tissue distribution of Trx1, to study the role of Trx1 in the control of physiological processes in the human lens.

Materials and Methods
Cloning of Human Trx1 cDNA
To clone human Trx1 cDNA, we performed a polymerase chain reaction with a human lens cDNA library as a template, the forward primer 5'-CATATGGTTAGCACAGATCGAG-3', the reverse primer-1 5'-TGT-CACGACGATCGACACACTG-3' and the reverse primer-2 5'-CGGTGATTAATTCTAAATGCGAGGTCTC-3'. The three primers were designed based on the known nucleotide sequence gene of human lymphocyte thioredoxin (GenBank accession numbers X70286, X70286, and X70288, respectively; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Reverse primer-1 and primer-2 are designed to clone Trx1, with or without a His-tag sequence in an Escherichia coli expression system. The forward primer was used with reverse primer-1 or reverse primer-2 to generate 439- and 342-bp PCR products, respectively. The conditions of the PCR were as follows: 30 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. The obtained PCR fragments were purified and ligated into the pET23a(+) expression vector. The recombinant plasmids designated as pCR-Trx were analyzed for the presence and orientation of the insert by electrophoresis. The recombinant plasmids were used to clone Trx1 into the top10F (CMV) promoter into a vector (TA pCR 3.1-Uni Vector; Invitrogen, San Diego, CA) and used to transform TOP10F (Novagen, Madison, WI) driven by the T7 promoter. The purified PCR fragment of 518 bp was cloned into the NdeI-EcoRI sites of the pET23a(+) expression vector (Novagen, Madison, WI) driven by the T7 promoter. The purified PCR fragment of 518 bp was cloned into the NdeI-EcoRI sites of the pET23a(+) vector directly and was designated pET-Trx(His). The expression plasmids pET-Trx and pET-Trx(His) were transformed into the BL21 (DE3) cells (Novagen). For the expression of recombinant proteins, transformed BL21 (DE3) cells were grown at 37°C in LB medium with 50 μg/ml ampicillin until an optical density at 600-nm absorbance (OD600) of 0.4 to 0.6 was reached. Then, expression of the fusion proteins was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested after 3 to 4 hours by centrifugation, and the pellet was resuspended in 1:25 culture volume of reagent (BugBuster with Benzonase Nuclease; Novagen). The supernatant of the lysed cells was cleared by centrifugation and loaded onto a resin column (His-Bind; Novagen) equilibrated with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). The column was washed with binding buffer followed by washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), and then recombinant Trx was eluted with a linear gradient from 5 to 1000 mM imidazole in binding buffer. Fractions containing Trx1 activity were combined, dialyzed against TE buffer (50 mM Tris-HCl, 1 mM EDTA, [pH 7.5]), concentrated, and applied to a gel filtration column (Sephadex G-50; Amersham Biosciences, Piscataway, NJ) equilibrated with TE buffer. Fractions containing Trx1 activity were pooled, concentrated, and stored at -20°C. The size and purity of recombinant Trx1 was confirmed by SDS-PAGE. Protein concentration was determined using the bicinchoninic acid (BCA) microprotein assay with bovine serum albumin (BSA) as the standard.

Purification of Mammalian TrxR
Thioredoxin reductase was purified from porcine liver according to the method of Arner et al. with modification. The procedure included anion-exchange (DEAE-Sepharose), affinity (ADP-Sepharose), and gel-filtration (Sephadex G-75) chromatographic steps (Amersham Biosciences). The enzyme was usually electrophoretically pure after the Q-Sepharose step. Otherwise, an additional gel filtration step (Sephadex G-75) was used to remove traces of contaminating proteins.

Trx and TrxR Assay
The activity of Trx1 was determined by insulin and 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) assays. The insulin assay is based on the ability of Trx to reduce insulin with NADPH in the presence of an excess of TrxR. Briefly, aliquots of Trx1 were preincubated at 37°C for 15 minutes with 2 μL buffer containing 50 mM HEPES (pH 7.6), 100 μg/ml BSA, and 2 mM diithiothreitol (DTT) in a total volume of 70 μL. Then, 40 μL of a reaction mixture composed of 250 mM HEPES (pH 7.6), 2.4 mM NADPH, 10 mM EDTA, and 6.4 mg/ml insulin was added. The reaction started with the addition of 10 μL of TrxR from porcine liver (3.0 A412 unit), and incubation was continued for 20 minutes at 37°C and in a final volume of 120 μL. The reaction was stopped by the addition of 0.5 mL of 6 M guanidine-HCl and 1 mM DTNB, and the absorbency at 412 nm (A412) was measured. The rate of DTNB reduction was calculated from the increase in A412, using a molar extinction coefficient of 27,200 M⁻¹ cm⁻¹ (reduction of DTNB by 1 mole of Trx(SH)₂ yields 2 moles of TNB, with a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹). One unit of activity was calculated by A412 × 0.62/(13.6 × 2) as the micromoles of NAPDH oxidized, because 1 mole of NADPH corresponds to 2 moles of sulfhydryl groups. The activity of porcine liver TrxR was determined by DTNB assay, as described by Holmgren and Bjornstedt.

Hydrogen Peroxide and Iodoacetamide Treatment of Trx
To ensure that the rTrx1 was fully reduced before exposure to H₂O₂, the protein was treated with DTT activation buffer composed of 50 mM HEPES (pH 7.5), 10 mM EDTA, and 2 mM DTT at room temperature for 20 minutes. Then an aliquot (5 μL) of the 2 or 20 μM rTrx1 solution was added to an equal volume of H₂O₂ solution to achieve a final concentration of H₂O₂ at 0.05, 0.1, 0.5, or 1 mM. The final concentration of DTT after the addition of hydrogen peroxide was 28 μM. The reaction mixture was incubated for 20 minutes at 30°C. Catalase (2 μg) was added for an additional 5 minutes to detoxify the unused H₂O₂. An aliquot of each reaction mixture was used to measure Trx1 activity. Hydrogen peroxide mixed with catalase before incubation with rTrx1 was used as a control. For iodoacetamide (IAA) studies, an aliquot of 20 μM rTrx1 was added to 5 μL solution containing 12 μM IAA, and the complete reaction mixture was incubated for 0, 1, 2, and
5 minutes. An aliquot of each reaction mixture was used for the Trx1 activity assay. RTrx1 pretreatment without IAA was used as a control.

**Preparation of Porcine and Human Lens Homogenate**

Prefrozen normal human lenses (19, 21, and 37 years) were obtained from the Lion’s Eye Bank of Omaha, Nebraska. The whole porcine eyes were delivered fresh on ice from Farmland, Inc. (Crete, NE). The lenses were removed surgically, and the capsule epithelial layer was peeled off and immediately frozen on dry ice. The decapsulated lens was also placed on dry ice until half frozen before further dissection. A cork borer (3 mm) was used to remove the center cone of the lens, and both ends (1 mm) were removed with a razor blade. This center piece was considered the nucleus (25%–30% of the whole lens weight), and the remaining portion was considered the cortex. Each lens portion was homogenized in 200 µL (epithelium) or 500 µL (cortex or nucleus) ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 µg/mL aprotinin, and 100 µg/mL PMSF. The homogenate was centrifuged at 13,000 g for 25 minutes at 4°C, and the supernatant was used for determination of the protein content, Trx assay, and immunoblot analysis. Human lens was processed similarly.

**Cell Culture and H2O2 Treatment of HLE B3 Cells**

HLE B3 cells were cultured in MEM containing 20% FBS and 50 µg/mL gentamicin in a humidified 5% CO2 atmosphere at 37°C. Cells were seeded and grown to a density of 4 x 10^6 cells per 100 mm tissue in a culture dish. The cells were weaned from the serum by first incubating overnight in 2% FBS and then in serum-free medium for 30 minutes at 4°C, and the supernatant was considered the cortex. The remaining portion was considered the nucleus (25%–30% of the whole lens weight), and the supernatant was considered the cortex. Each lens was homogenized in 200 µL (epithelium) or 500 µL (cortex or nucleus) ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 µg/mL aprotinin, and 100 µg/mL PMSF. The homogenate was centrifuged at 13,000 g for 25 minutes at 4°C, and the supernatant was used for determination of the protein content, Trx assay, and immunoblot analysis. Human lens was processed similarly.

**Western Blot Analysis**

Cell lysates were separated by 12% SDS-PAGE and transferred to a membrane (TransBlot; Bio-Rad). The membrane was incubated with goat anti-human Trx IgG (American Diagnostica Inc., Greenwich, CT) diluted 1:1000 in TBST buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% [vol/vol] NP-40, 1 mM Na3VO4, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM NaF, and 1 µg/mL each of aprotinin, pepstatin, and leupeptin). The lysates were used for Trx analysis by immunoblot analysis.

**RESULTS**

**Cloning of Human Trx1**

The known nucleotide sequence of the human lymphocyte Trx was used to design primers to amplify Trx1 from a human lens cDNA library. An open reading frame (ORF) encoding Trx1 was isolated by PCR amplification, purified, cloned into the PBR 3.1-Umi vector, analyzed by restriction analysis for the presence and orientation of the insert, and sequenced. The complete composite sequence of the ORF consisted of 318 bp (Fig. 1) encoding a protein of 104 amino acids with an estimated molecular mass of 12 kDa. The nucleotide sequence for the human lens TXN gene has been deposited in the GenBank database under GenBank accession number AY004872.

**Analysis of the Nucleotide and Predicted Amino Acid Sequences**

The multiple sequence alignment obtained by the GCG program showed that the coding region of the human lens TXN gene sequence was identical with the coding region of the TXN sequences of the human fibrosarcoma cell line HT-1080 (GenBank accession number D28576), human HTLV-I-transformed T-cell line (GenBank accession number X77584), and human lymphocyte (GenBank accession numbers X70286, X70207, X70280). Trx1 possessed the active site with the characteristic thiol motif, a conserved amino acid sequence of Trp-Cys-Gly-Pro-Cys found in all Trxs (Fig. 2). The 104 amino acids of Trx1 share 100% sequence identity with the previously identified human ATL-derived factor/Trx (GenBank accession number CA54687) and 94.2%, 88.5%, and 35.5% sequence identity with the previously identified porcine Trx1 (GenBank accession number AF382821_1), rat Trx1 (GenBank accession number SO4352), and human lens Trx2 (GenBank accession number
ber AF276920_1), respectively. Trx1 has a higher homology with the porcine Trx than with the rat Trx and has insignificant homology with human Trx2 (mitochondrial Trx). One important common feature of all mammalian Trx1 is the presence of structural cysteine residues (Cys), Cys-62, Cys-69, and Cys-73 (all positions are for human Trx1). These Cys residues may impart unique biological properties to mammalian Trx1.

Expression and Purification of rTrx1

rTrx1 was overexpressed in E. coli and purified to homogeneity by immobilized metal affinity (HisBind resin; Novagen), followed by gel filtration (Sephadex G-50; Amersham) chromatographic procedures. The purified protein appeared as a single band with an apparent molecular mass of 12 kDa on SDS-PAGE under reducing conditions (Fig. 3A). It also reacted positively with anti-human Trx1 polyclonal antibody (data not shown).

Purification of TrxR from Porcine Liver

Porcine liver TrxR showed a single protein band at 55 kDa on SDS-PAGE after purification by a series of column chromatographic procedures (Fig. 3B). The enzyme purity was more than 90%. The specific activity of pure TrxR was approximately 11 U/mg. Approximately 0.3 to 0.5 mg of pure TrxR was obtained from 100 g of porcine liver. This purified TrxR was used for Trx1 activity assays.

Properties of rTrx1

To test the activity of the recombinant protein, we examined the ability of rTrx1 to reduce insulin in an insulin reduction

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assay. The insulin disulfides were effectively reduced by rTrx1 with NADPH in the presence of porcine liver TrxR (Fig. 4A). The recombinant enzyme showed an activity of 68 U/mg. rTrx1 activity was enhanced after DTT reduction (Fig. 4B), suggesting that the noncatalytic half-cysteine residues Cys-62, Cys-69, and Cys-73 in human Trx1 can undergo spontaneous oxidation, which occurs during long storage in the absence of reducing agents by chemical oxidation or on storage at high concentrations. This may lead to dimerization and inactivation of the enzyme.

The purified rTrx1 showed strong resistance to heat treatment. The activity of the rTrx1 remained intact after a 5-minute incubation at a temperature increasing from 37°C to 80°C (data not shown). rTrx1 displayed optimal activity at a pH range of 7.6 to 7.9 (data not shown). We used the Trx-dependent DTNB reduction assay to determine the affinity of porcine liver TrxR for rTrx1. To obtain saturation Michaelis-Menten kinetics, we used the Trx-dependent DTNB reduction assay to determine the affinity of porcine liver TrxR for rTrx1. To obtain saturation Michaelis-Menten kinetics, we used a low concentration of TrxR (10 nM). The $K_m$ of TrxR for recombinant Trx1 at pH 7.0 and 25°C was $1.8 \pm 0.18 \mu M$. TrxR showed a slightly lower $K_m$ for human rTrx1 than the rat liver TrxR for its homologous Trx1 (2.5 $\mu M$ for rat liver Trx1). Sensitivity of rTrx1 to Deactivation by H$_2$O$_2$ and IAA

Proteins may be differently sensitive to oxidation according to their conformation, their content of critical cysteine residues, their cellular levels or the intensity of oxidative stress. Because Trx has vicinal dithiol moieties at its active site, the protein may be vulnerable to oxidative damage. We compared the inactivation of rTrx1 by H$_2$O$_2$ at a low concentration (1 $\mu M$) of Trx1 with the inactivation at the approximate physiological concentration of the Trx1 (10 $\mu M$). rTrx1 at low concentration was sensitive to H$_2$O$_2$ and showed a 20% activity loss after treatment with 0.05 mM H$_2$O$_2$ for 20 minutes and a 40% loss after treatment with 1 mM H$_2$O$_2$. In contrast, at the physiological level, rTrx1 was resistant to H$_2$O$_2$ in which approximately 80% activity remained even when the protein was exposed to 1 mM H$_2$O$_2$. The data are summarized in Fig. 5A.

The redox status at the active center of rTrx1 was very sensitive to IAA alkylation, even at a low concentration of IAA. As shown on Fig. 5B, rTrx1 lost nearly 50% of its activity within 5 minutes of IAA (6 $\mu M$) treatment.

**Trx1 Activity and Expression in the Porcine and Human Lens Tissues**

Trx1 activity was detected in epithelial, cortical, and nuclear regions of the porcine lens. To decrease the background in the

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**Figure 4.** Insulin reduction coupled to the protein-disulfide reductase activity of rTrx1 in the presence of the TrxR and effect of DTT on rTrx1 activity. (A) Trx was assayed for its ability to catalyze disulfide reduction of insulin with NADPH in the presence of mammalian TrxR. Data are the average of two measurements. (B) Trx was assayed for its ability to catalyze disulfide reduction of insulin in the presence or absence of DTT. (△) rTrx1 alone; Δ rTrx1 preincubated with DTT. Data represent the mean ± SD of the results of three independent experiments.

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**Figure 5.** Effect of H$_2$O$_2$ and IAA on rTrx1 activity. (A) Trx was incubated with various amounts of hydrogen peroxide and then analyzed for its activity: (△) 10 $\mu M$ rTrx1 solution after H$_2$O$_2$ treatment. (●) 1 $\mu M$ rTrx1 solution after H$_2$O$_2$ treatment. After incubating rTrx1 with various doses of hydrogen peroxide and after incubation with catalase, an aliquot of each reaction mixture was transferred to an assay mixture to measure the remaining enzyme activity. Data represent the mean ± SD of results of three independent experiments. (B) Trx was pretreated with DTT and then incubated with 6 $\mu M$ IAA (final concentration) for various time intervals. After incubation, rTrx1 was immediately assayed for its activity: (●) 10 $\mu M$ rTrx1 solution without IAA treatment (control). (△) 10 $\mu M$ rTrx1 solution after IAA treatment. Data represent the mean ± SD of results of three independent experiments.
insulin assay due to interaction of DTNB with −SH groups, we used heated lens extracts (70°C, 10 minutes). The lens epithelial layer showed the highest activity at 151.9 ± 10.5 mU/mg of total soluble protein, whereas, the lens cortical and nuclear regions displayed a lower activity at 59.4 ± 4.5 mU/mg and 65.8 ± 4.4 mU/mg, respectively (Fig. 6A). Total soluble fractions prepared from various porcine and human lens tissues were subjected to immunoblot analysis with human antibody specific to Trx1 (Figs. 6B, 6C). Comparison of the blot intensities of proteins with various amounts of purified rTrx1 provided an estimation of the amount of Trx1 in the crude extracts of various regions of the porcine and human lenses (Figs. 6D, 6E). The amounts of Trx1 in the epithelial layer and the cortical and nuclear regions of porcine lenses were estimated by a fluorescence imaging system (Fluor-S MAX MultiImager; Bio-Rad) analysis to be 2.2 ± 0.07, 0.8 ± 0.05, and 0.9 ± 0.06 μg/mg of total soluble protein, respectively (Fig. 6D). Thus, the levels of Trx1 in various regions of porcine lens calculated from immunoblot analysis were in agreement with the calculated values in the Trx assays. The amounts of Trx1 in epithelial, cortical, and nuclear regions of the human lens were similar to the level found in porcine lens and estimated to be 1.60 ± 0.32, 1.12 ± 0.15, and 0.93 ± 0.14 μg/mg of total soluble protein, respectively (Fig. 6E). However, studies with fresh human lenses are needed to confirm these data.

**Upregulation of Trx1 in HLE B3 Cells Treated with H2O2**

Trx1 activity in HLE B3 cells (255 ± 6.6 mU/mg of total soluble protein) was increased after a 5-minute treatment with a bolus of 0.1 mM H2O2 in serum-free medium. This increased activity reached its maximum over the control at 10 minutes before gradually returning to its basal level at 30 minutes (Fig. 7A). H2O2 in the medium decayed rapidly and was mostly detoxified after 60 minutes (data not shown). Studies of Trx1 expression by using immunoblot analysis showed (Fig. 7B) that the enzyme increased 35% after a 10 minutes of H2O2 treatment and gradually returned to its basal level at 30 minutes (Fig. 7C). Analysis of GAPDH expression as an internal control confirmed that all samples used in these experiments contained equal amounts of total protein.

The upregulation of TXN gene expression in HLE B3 cells under oxidative stress (0.1 mM H2O2) was further confirmed by using real-time PCR and amplification plots (change in fluorescent signal versus cycle number) were obtained (Fig. 8A). After 27 cycles, a significant increase in fluorescence was observed in samples treated for 10 minutes with H2O2. However, in the control sample (no H2O2 treatment), fluorescence increased only after cycle 29. The standard curve showed a linear range across at least six logs of DNA concentrations with a correlation coefficient of 0.999 (Fig. 8B). Melt curve analysis...
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of the reactions revealed (with the exception of the zero template control) a single amplified product at a melting temperature (T_m) of 81°C of the intended product (data not shown). From the standard curve, these fluorescence increases were observed after 27 and 29 cycles in samples containing 4.5 and 7.5 × 10^5 copies of plasmids (containing 104-bp PCR fragment), respectively. Thus, the data of real-time PCR revealed an approximately 50% increase in TXN gene expression in HLE B3 cells after exposure to H_2O_2 for 10 minutes (Fig. 8C). Analysis of GAPDH gene expression as an internal control confirmed that all samples used in these experiments contained equal amounts of cDNA (data not shown).

DISCUSSION

The sequence of the human lens TXN gene was identical with that of the TXN gene in other human tissues^36 and had a high level of identity to the TXN gene from other mammalian sources. Recombinant Trx1 showed a high level of expression in an E. coli expression system and was purified to homogeneity. Overexpression of the rTrx1 was confirmed by immunoblot analysis. The specificity of the purified recombinant protein as Trx to catalyze the disulfide reduction of insulin was confirmed by using the insulin reduction assay.

The purified rTrx1 also showed similar properties to the Trx1 from other mammalian tissues, including the enhanced activity on DTT reduction and the nature of heat resistance. The kinetic parameters for rTrx1 were also similar to other mammalian Trxs. Our results showed that the lens possessed approximately three times more Trx1 protein (per milligram soluble protein) in the epithelial cell layer than in the fiber cells (Fig. 6). This seems reasonable considering that lens epithelial cells are known to be constantly exposed to oxidative stress, either from the oxidant in the aqueous humor or in situ through direct photo-oxidation. The cortex and nucleus showed lower but almost equal amounts of Trx1, indicating that the fiber cells maintained their Trx1 level after differentiation and that the embryonic lens (the nucleus) and the younger section of the lens (cortex) may use the enzyme for certain physiological functions. A similar distribution pattern of TTase, another oxidation defense enzyme, has been reported in the lens. It has been demonstrated that TTase in the HLE B3 cells can dethiolate and reactivate the oxidation-modified and inactivated key metabolic enzymes. However, the partially oxidized inactivated recombinant TTase could be reactivated by the Trx-TrxR system in vitro. A recent has shown a possible cross-link between the Trx and glutathione-glutaredoxin (TTase) systems, in which Trx undergoes glutathionylation in T cells exposed to oxidative stress, but the recombinant Trx1 deglutathionylates itself. Also, it is well-known that glutathionylated enzymes-proteins can be reduced by glutaredoxins (TTases). Thus, these two oxidation defense systems may contribute synergistically to repair the damaged protein thiols that occur during oxidative stress. To further understand the possible oxidation and inactivation of the thiol-containing Trx, we determined the sensitivity of rTrx1 to deactivation by subjecting the protein to various concentrations of H_2O_2 in vitro. The fact that the remarkably H_2O_2-resistant nature of rTrx1 at the physiological concentration far exceeded that of TTase further supports our speculation that Trx1 is an important oxidation defense protein in the lens. Knockout studies have shown that the expression of the TXN gene and its protein in the human lens appeared to decline with advanced age and suggested that altered Trx1 at an advanced age may be associated with senile cataractogenesis. Few studies have been published on lens Trx1 as a major regulatory protein with protective functions against oxidative damage. Bhuyan has reported that expression of Trx1, but not Trx2, is specifically upregulated in the lens under oxidative...
In the Emory mouse lens under in vivo oxidative stress, the upregulation of the TXN gene and its protein is a tissue-specific adaptation. We conducted studies on the regulation of Trx1 expression in HLE B3 cells and found a distinct but transient upregulation of Trx1 under oxidative stress conditions. The rapid upregulation of Trx expression, which was followed by downregulation when the oxidant (H2O2) was totally detoxified from the media, may be an adaptive protective response of the cells to restore the altered redox state. Increased levels of the Trx1 protein and Trx1 mRNA under oxidative stress suggest the activation of gene expression and possibly the increase of the Trx1 mRNA half life but not the activation of presynthesized protein. Similar activation of the TXN gene as a possible response to oxidative stress has been shown in yeast cells.45

**CONCLUSIONS**

This report is the first presentation of the distribution of Trx1 in porcine and human lenses and the first study of the regulation of Trx1 expression in cultured HLE B3 cells under oxidative stress. Trx1 was detected and quantified in porcine and human lenses, with a ratio of 3:1:1 in the epithelial layer, cortex, and nucleus, respectively. The TXN gene was upregulated by mild H2O2 stress in human lens epithelial cells, implying a potential physiological function in antioxidation. Further work is needed to clarify the physiological role of Trx1 in the lens.

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References