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Regulation of Thioredoxin by Ceramide in Retinal Pigment Epithelial Cells

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Abstract

The purpose of this study was to determine the expression, regulation and signaling of a key redoxin family member thioredoxin 1 (Trx1) in normal, oxidant-stimulated and growth factor-pretreated RPE cells. Trx1 is expressed in early passage, human RPE cell cultures. RPE cells exposed to C₂-ceramide for 24 h showed no significant change in expression of Trx1 vs. controls with and without pretreatment for 24 h with hepatocyte growth factor (HGF). Neither hypoxia from 1% O₂ or from CoCl₂ exposure resulted in any alteration in Trx1 expression in RPE cells. C₂-ceramide treatment caused translocation of Trx1 from cytosol to the nucleus, which was abolished by pre-treatment of cells with a p38 MAPK-specific inhibitor. Furthermore, the gene and protein expression of thioredoxin interacting protein (Txnip) increased with ceramide treatment and was significantly ($p < 0.001$) elevated with HGF preincubation vs. untreated controls. Prominent protection from ceramide-induced RPE cell death by exogenous rTrx1 was demonstrated. Although Trx1 directly interacts with its inhibitor, Txnip, p38 inhibition does not appear to have a role in this interaction. We found no direct interaction between apoptosis signal regulating kinase (ASK-1) and Txnip under the same experimental conditions. In summary, our data demonstrate the expression of Trx1 and Txnip in human RPE cells. Ceramide treatment results in translocation of Trx1 to the nucleus, and upregulation of Txnip expression; exogenous rTrx1 protects from ceramide-induced cell death. These results suggest that Trx1 and Txnip play an important role in the response of RPE to ceramide toxicity.

Keywords

Oxidant injury; thioredoxin; thioredoxin interacting protein; hepatocyte growth factor; nuclear translocation

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1. Introduction

Ceramide is an endogenous membrane sphingolipid molecule that plays a critical role in apoptosis, proliferation, cellular senescence, and gene regulation through the activation of transcription factors such as NF κ B (Obeid et al., 1993; Wiegmann et al., 1994; Kajimoto et al., 2004). Ceramide influences gene regulation by modulating a variety of phosphatases and protein kinases. Our previous studies have shown that ceramide induces apoptosis in retinal pigment epithelial (RPE) cells and decreases catalase activity (Kannan et al., 2004).

Pretreatment of early passage human RPE cells with hepatocyte growth factor (HGF) inhibits ceramide-induced apoptosis and prevents the reduction in catalase activity and expression (Kannan et al., 2004). Our laboratory has previously shown that RPE cells secrete HGF and express functional c-Met, making the HGF/HGF-receptor system a potential autocrine loop for RPE (He et al., 1998). HGF plays an important role in epithelial and endothelial cell proliferation and survival (Matsumoto and Nakamura, 1996). Attenuation of ceramide induced apoptosis by HGF via catalase was partial, suggesting a role for endogenous antioxidants in protection of RPE from oxidative injury.

Thioredoxin-1 (Trx1), a ubiquitous redoxin family member, is involved in an array of cellular functions, including proliferation, survival, and activation of transcription factors such as p53, NF κ B, and activation protein-1 (AP-1) (Nakamura et al. 1997; Nishiyama et al., 2001; Tanaka et al. 2002). The Trx1 system regulates cellular balance by reversible oxidation of its redox active cysteine residues (Holmgren, 1985). Targeted disruption of the Trx1 gene causes early embryonic lethality in the mouse (Matsui et al., 1996), showing its significance for early differentiation and morphogenesis. The Trx1 inhibitor Txnip is a negative regulator of Trx1 and functions as an oxidative stress mediator, inducing apoptosis by inhibiting Trx1 activity through the interaction between Trx1 and apoptosis signal regulating kinase (ASK)-1, which combines with the active site of Trx1 to inhibit thioredoxin activity (Junn et al., 2000). It also prevents the nuclear translocation of Trx1 and regulates Trx-dependent transcriptional mechanisms (Schulze et al., 2002).

Protection against reactive oxygen species (ROS) and free radicals in the retina is mediated by superoxide dismutase, the glutathione (GSH) system, and the Trx system (Yamamoto et al., 1999). Trx1 is upregulated in response to an array of stresses, such as viral infections, ultraviolet and x-ray irradiation, and ischemia-reperfusion injury. Evidence suggests that imbalances in the tissue or cellular redox status are associated with pathological conditions and that normalization of redox status through manipulation of endogenous and exogenous levels of thiol antioxidants, for example Trx1, is an effective therapeutic strategy for various diseases, including ischemia-reperfusion injury in the lung and brain (Okubo et al., 1997; Takagi et al., 1999). Overexpression of Trx1 protects against neuronal damage associated with oxidative stress (Takagi et al., 1999; Hattori et al., 2004) and suppresses retinal photooxidative injury in Trx1 transgenic mice (Tanito et al. 2002a). Thioredoxin effectively inhibits retinal damage, including light-induced photic injury and ischemia/reperfusion (Shibuki et al., 1998; Tanito et al. 2002a; Tanito et al., 2002b). In other studies, intravitreal injection of Trx1 effectively attenuated *N*-methyl-D-aspartic acid-induced retinal cell damage by preventing apoptosis in rat (Inomata et al., 2006). To our knowledge, however, the regulation of the redoxin family member Trx1 in RPE by oxidative stimuli and the effect of growth factors in arresting injury to RPE have not been addressed. In the present study, we have examined the role, expression, and regulation, cell signaling and protein interaction of Trx1 in ceramide-induced oxidative injury and protection by HGF in RPE cells.

2. Materials and methods

2.1. Cell culture and treatment

The Institutional Review Board of the University of Southern California approved the use of cultured human RPE cells. RPE cells isolated from human eyes (Advanced Bioscience Resources, Inc., Alameda, CA) were cultured in Dulbecco's minimal Eagle's medium (DMEM; Fisher Scientific, Pittsburgh, PA) with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA). Third to fourth passage cells grown to confluence were used for the present study. Cells were changed to DMEM containing 1% FBS for 24 h, pretreated for overnight with recombinant human HGF (20 ng/ml; R&D Systems, Minneapolis, MN) in 1% FBS, and then treated continuously with the C₂ analog of ceramide (Biomol, Plymouth Meeting, PA) for specified periods of time. Time dependency studies were carried out with 25 µM ceramide while ceramide induced cell signaling was performed using 10 µM. To study the regulation of thioredoxin in a hypoxic state, we exposed serum starved RPE cells either in 1% Oxygen, 94% N₂ and 5% CO₂ (hypoxia) in a commercially available gas-control chamber (Billups-Rothenberg, Del Mar, CA) for 1 – 4h or to 100 µM of a known hypoxia mimetic cobalt chloride (CoCl₂). For the latter, cells were starved in 0% serum overnight and treated with 100 µM CoCl₂ for 1, 2, 3 and 4 h. Cell viability was determined using Trypan Blue exclusion method.

2.2. Western blot analysis

Cells were harvested after the specified treatment period and washed with phosphate-buffered saline (PBS). Protein was extracted from the cells using mammalian protein extraction reagent with protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL) and quantified with a protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Equal amounts of protein (25–50µg) were resolved on Tris-HCl polyacrylamide gels (120 V, Ready Gel; Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF blotting membranes (Millipore, Billerica, MA). Membranes were probed with rabbit polyclonal anti-Trx1 (1:1000, Santa Cruz Biotechnology, CA), anti-Trx2 (1:500, Santa Cruz Biotechnology), anti-Grx1 (1:1000, GeneTex Inc, San Antonio, TX), anti-Grx2 (1:1000, Abcam, Cambridge, MA), mouse anti-VDUP-1/Txnip (1:1000, MBL Medical & Biological Laboratories, Japan) mouse anti-HIF 1-α (1:500, Novus Biologicals, CA) and Phospho p38 MAP Kinase and p38 MAP Kinase (1:1000, Cell Signaling Technology, MA) overnight at 4° C. For facilitating the analysis of Trx2, an enriched mitochondrial fraction was prepared as described (Sreekumar et al., 2005). We used HeLa whole cell lysate (Santa Cruz Biotechnology, Santa Cruz, CA) as positive control for Trx and K-562 whole cell lysate for Txnip. After incubation with the corresponding secondary antibody tagged with horseradish peroxidase, signals were detected by enhanced chemiluminescence system (Amersham, Cleveland, OH). Membranes were then stripped and reprobed for GAPDH (Chemicon, Temecula, CA) or Beta actin (Sigma, St. Louis, MO). Protein band intensity was measured by Scion Image Densitometer Software (Scion, Frederick, MD).

2.3. Immunoprecipitation

RPE cells after various treatments were washed in PBS and total cellular protein was extracted using mammalian protein extraction buffer (Pierce Biotechnology, Rockford, IL) with protease inhibitor cocktail. For immunoprecipitation assay, 500µg total- cell lysates were precleared with 50µl beads 1h at 4°C. The lysates were then incubated with 2µg of the first protein-specific antiserum (anti-Txnip, MBL Medical & Biological Laboratories, Japan) overnight at 4°C. 20µl of protein G agarose (Sigma) was added and incubated for 2h at 4°C and the immunocomplexes were collected by centrifugation followed by 4 washes with lysis buffer. The immunocomplexes were subjected to immunoblot analyses with the second protein of

interest Trx1 or ASK-1, (Santa Cruz Biotechnology, CA) and visualized with chemiluminescence system.

2.4. Immunofluorescence cell staining

Cells were grown on 4-well chamber slides and preincubated with 25 μ M specific p38 MAP Kinase inhibitor (SB203580) for 2h and then coincubated 10 μ M C₂ ceramide for 24h. After treatment cells were fixed with methanol for 20 min at -20 °C, washed three times, and blocked in 10% normal goat serum in PBS for 20 min. After incubation with primary antibody (anti-Trx1, 1:50, overnight at 4° C in 1.5% normal goat serum), slides were incubated with FITC conjugated secondary antibody (Vector laboratories, Burlingame, CA) at 1:100 dilution for 30 min. room temperature in dark. Cells were mounted with fluorescent mounting medium (DAPI) and examined using a laser scanning confocal microscope (LSM510, Zeiss, Thornwood, NY).

2.5. Quantitative real-time PCR

Total RNA was isolated from RPE cells using TRIzol reagent, (Invitrogen Life Technologies, Carlsbad, CA), the contaminating genomic DNA was removed (DNA-free, Ambion, Austin, TX) and RNA quantified with a spectrophotometer. First strand cDNA synthesis by reverse transcription was achieved with oligo (dT) primer and 1 μ g total RNA in a 20 μ l reaction volume, as per the manufacturer's protocol (Reverse Transcription System, Promega, Madison, WI). The PCR experiments were performed using SYBR Green (Applied Biosystems, Foster City, CA). Each 25 μ l PCR contained cDNA template, SYBR Green PCR master mix, and 1.5 μ M each gene-specific primer. Reaction conditions were as follows: 6 min at 95°C, followed by 40 cycles of 3 s at 95°C, 15 s at 55°C, and 15 s at 72°C. Quantification analysis of Txnip mRNA was normalized using β -actin as the internal standard. The sequences of primers used were Trx1 forward: 5'-TGTGGGCCTTGCAAAATGA-3', reverse: 5'-GGAATATCACGTTGGAATACTTTTCA-3' and Txnip, forward: 5'-ACCTGCCCTGGTAATTGG-3' and reverse: 5'-TTCGGCTGGCCATGCT-3'. The specificity of PCR amplification products was checked by performing dissociation melting curve analysis and/or by electrophoresis through 1% agarose gels containing ethidium bromide. Relative multiples of change in mRNA expression was determined by calculating $2^{-\Delta\Delta C_T}$. Results are reported as mean difference in relative multiples of change in mRNA expression $\pm$ SD.

2.6. TUNEL staining

RPE cells grown in chamber slides were co-incubated with 5 μ g/ml recombinant Trx1 (US Biological) and 25 μ M ceramide for 24h. Apoptosis (DNA fragmentation) was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method following the manufacturer's protocol (Apoptag peroxidase *in situ* apoptosis detection kit (Chemicon, Temecula, CA). In short, RPE cells in chamber slides were fixed in 1% paraformaldehyde solution, followed with treatment with 3% H₂O₂ at room temperature for and the cells were incubated with TdT enzyme for 1 h at 37°C. The Dig-labeled nucleotides incorporated into DNA breaks were detected by applying anti-Digoxigenin conjugate and peroxidase substrate. The number of TUNEL positive cells was counted from all the experiments under a microscope and was expressed as mean $\pm$ SD.

2.7. Statistical analysis

All experiments were performed three times. Results are expressed as means $\pm$ SD. One-way ANOVA with Tukey-Kramer post test was performed using Graphpad InStat version 3.00 (Graphpad software, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Cell viability with ceramide treatment

Initial studies were performed to assess the cell toxicity of varying doses of C₂ ceramide using the Trypan Blue exclusion method. Percent viability of experimental groups was compared to that of untreated control RPE cells. Percent viability of cells treated with varying doses of ceramide for 24 h showed a gradual decrease from 96 ± 1.8 in control to 56.7 ± 4.7 with 30 μ M ceramide. The cell viability with 25 μ M ceramide, a dose that we had used in our previous studies on examining apoptotic mechanisms, was $75.3 \pm 2.6\%$.

3.2. Expression of Trx1 in RPE cells

Western blot analysis shows that RPE cells expressed Trx1 (Fig. 1). The molecular weight of Trx1 was found to be 12 kDa in comparison with molecular weight standards. In order to determine whether other redoxin family members were expressed by RPE, we also performed western blot analysis using specific antibodies to Trx2, Grx1 and Grx2. RPE also express the cytosolic Trx family member Grx1, and the mitochondrial Trx family members Trx2 and Grx2 (see supplemental figure 1).

3.3. Trx1 protein expression is not significantly altered with oxidative stress

Our previous work on the generation of ROS with synthetic ceramide and the mitochondrial pathway of apoptosis was performed with C₂ ceramide at a concentration of 25 μ M. We used the same ceramide concentration to study regulation of Trx1 by RPE cells. We conducted time course (0, 2, 4, 6, 18, 24 h) experiments with 25 μ M ceramide and determined Trx1 protein expression. These studies showed no apparent alteration of Trx1 protein expression for up to 24 h (Fig. 2). Therefore, all subsequent experiments were performed with 25 μ M ceramide for 24 h. Human RPE cells treated with 25 μ M C₂ ceramide for 24 h showed no significant increase in Trx1 gene expression as compared to untreated controls. A significant ($p < 0.01$) increase in mRNA expression of Trx1 was observed in cells with HGF pretreatment followed by ceramide as compared to ceramide treatment alone (Figs. 3A). Human RPE cells treated with 25 μ M C₂ ceramide for 24 h showed insignificant change at the protein level for Trx1 vs untreated controls irrespective of HGF pretreatment (Fig. 3B,C).

3.4. Ceramide treatment causes nuclear translocation of Trx

To analyze the involvement of Trx1 in ceramide induced signaling cascade, we studied subcellular localization of Trx1. For this purpose, we used a ceramide dose that resulted in less than 10% cell death which enabled us to examine the effect of inhibition of phosphorylation (see below). RPE cells grown to confluence on chamber slides and treated with ceramide and HGF were examined by confocal microscopy to study Trx1 translocation. In cells starved in DMEM containing 1% serum, a predominant cytoplasmic distribution of Trx1 was observed with only punctate nuclear localization (Fig. 4, panel A). Ceramide (10 μ M) treatment resulted in extensive translocation of Trx1 to the nucleus (panel D). Nuclear expression of Trx1 was $74.4 \pm 3.7\%$ in ceramide treated cells, whereas it was absent in untreated control cells. Further, we found that inhibition of p38 MAP kinase with a two hour preincubation with 25 μ M of a specific inhibitor (SB203580), followed by coincubation with ceramide, blocked nuclear translocation of Trx1 (panel G). When RPE cells were incubated overnight with 20 ng/ml HGF alone, no Trx1 nuclear translocation could be detected (panel J) suggesting that this growth factor by itself does not produce any changes in Trx1 localization. Additional evidence for the involvement of p38 MAP kinase in ceramide action was obtained by western blot analysis. Western blot analysis showed significant phosphorylation of p38 MAP Kinase with 10 μ M ceramide treatment while the total p38 MAP Kinase remained unaffected in compared to untreated controls (Fig. 5 panel A). A significant, more than three fold increase in p38 MAP

Kinase phosphorylation was observed with 25 μ M ceramide as compared to controls. HGF pretreatment partially reduced the extent of phosphorylation of p38 MAP Kinase from ceramide (Fig. 5 panel B).

3.5. Interaction of Txnip with Trx1, but not with ASK-1

RPE cells exposed to either 10 or 25 μ M ceramide with or without preincubation with p38 inhibitor were subjected to co-immunoprecipitation with Txnip. Only Trx1 bound with Txnip under these conditions (Fig. 6A) and p38 inhibition did not exert any appreciable effect on Txnip-Trx1 interaction. No binding was evident between Txnip and ASK-1 under the same experimental conditions (Fig. 6B).

3.6. Upregulation of Txnip gene expression by ceramide

The regulation of Trx function is reported to be controlled by its endogenous inhibitor Txnip. We tested the effect of ceramide on Txnip gene expression and its potential modulation by HGF. Expression of Txnip mRNA was strongly stimulated by treatment with exogenous 25 μ M C₂ ceramide for 24 h (Fig. 7A). There was also a significant increase of Txnip mRNA in ceramide treated RPE that had been pretreated with HGF as compared to control RPE ($p < 0.001$). The same phenomenon held true for Txnip protein expression, in which ceramide exposure after HGF pretreatment resulted in a 72% ($p < 0.01$) increase in Txnip expression (Figs. 7B,C).

3.7. Exogenous Trx1 protects RPE cells from ceramide induced cell death

We wished to examine whether exogenous rTrx1 elicits protective function under conditions of elevated Txnip from ceramide exposure of RPE. RPE cells, when incubated with 10 μ M or 25 μ M ceramide for 24h caused about 8% and 30% cell death respectively. The higher dose of ceramide significantly upregulates Txnip expression (*cf* Fig. 7). To understand whether exogenously added recombinant Trx1 has a protective role under these conditions, we preincubated RPE cells with 5 μ g/ml recombinant Trx1 with 25 μ M ceramide. Cell death was significantly ($p < 0.01$) decreased with Trx1 incubation suggesting that Trx1 has cytoprotective properties. (Fig. 8, panels A–D).

4. Discussion

In this study, we established the expression of Trx1 and other redoxin family members Trx2, Grx1 and Grx2, and then examined in detail the regulation Trx1 by oxidative stress. Our data show exposure of RPE cells to short chain C₂ ceramide either alone or after HGF pretreatment had no significant effect on protein expression of Trx1. Physiological or chemically induced hypoxia did not result in any apparent change in Trx1 expression. However, treatment with ceramide induced gene and protein expression of Txnip significantly and preincubation with HGF further enhanced its expression. Additionally, our studies revealed that treatment with ceramide resulted in the translocation of Trx1 from the cytosol to the nucleus together with increased phosphorylation of p38. Our studies also revealed that Txnip directly interacts with Trx1, but not with ASK-1 and inhibition of p38 does not influence this interaction. Further, exogenously added recombinant Trx1 protected RPE cells from ceramide induced cell death.

Cobalt treatment and hypoxia regulate a similar group of genes (Vengellur et al., 2003). CoCl₂ stimulates the hypoxia responsive pathways and has been shown to induce oxidative stress and apoptosis by mitochondrial pathways and hypoxia inducible factor 1- α (HIF-1 α) dependent and independent mechanisms (Badr et al., 1999). CoCl₂'s ability to stabilize HIF-1 α allows HIF-1 α to translocate into the nucleus and enhance transcription of hypoxia-responsive genes. In the present study, hypoxia induced by CoCl₂ or incubation of

RPE with 1% O₂ did not produce any significant change in Trx1 expression (supplemental figure 2).

Nuclear translocation is considered to be an important mechanism of activation of transcription factors. Nuclear translocation of Trx has been shown previously in a variety of cells under varying stress conditions (Hirota et al., 1997., Wei et al., 2000., Bai et al., 2003., Arai et al., 2006, Malik et al., 2006). Our study shows such a translocation of Trx1 also occurs in RPE subjected to oxidative stress induced by exogenous C₂ ceramide. It is believed that Trx becomes reduced under oxidative stress and is translocated to the nucleus, and indirectly modulates AP-1 DNA binding activity through the intranuclear redox factor ref-1 (Schenk et al., 1994). Ischemic preconditioning triggers nuclear translocation of thioredoxin and its interaction with Ref-1 followed by their association (Malik et al., 2006). In this context, we studied the regulation of ref-1; while ceramide-induced stress does not alter its expression, p38 inhibition significantly downregulated ref-1 expression (data not shown). The role of p38 and ref-1 in relation to Trx1 nuclear translocation is unclear at the present time and needs further studies. Wei et al. (2000) reported that ionizing radiation changes the redox status of Trx, resulting in regulation of the DNA binding activity of AP-1 transcription factor through the interaction between redox factor-1 and Trx. Only the reduced form of Trx activates AP-1 DNA binding activity, and it is suggested that before nuclear translocation, an upstream factor such as Trx reductase might reduce Trx (Wei et al., 2000). Many additional possible mechanisms by which Trx will translocate into the nucleus exist including the direct activation of the nuclear translocation signal on the Trx and activation of the sequences of signaling cascades occurring during ROS formation that activate cytoplasmic kinases.

The p38 mitogen-activated protein kinase (MAPK) pathway is an important mediator of cellular responses to environmental stress (Rouse et al., 1994). We observed that Trx1 nuclear translocation was prevented by pretreatment of cells with p38 MAPK-specific inhibitor SB203580 suggesting that stress-activated p38 kinase is involved in ceramide-mediated Trx1 nuclear translocation. Similar to previous observations, we believe that generation of phosphorylated form of p38 MAP Kinase could cause the translocation of Trx to the nucleus in RPE cells exposed to ceramide. While Trx1 interacted with Txnip under these conditions, p38 inhibition did not inhibit this association, suggesting that role of p38 in this nuclear translocation is not direct. Although we have not performed studies on the role of p38 MAP Kinase on other redoxin members, it is of interest that the induction of Prx I expression by arsenate was associated with the activation of different MAPK pathways and p38 activation was required for PrxI expression in osteoblasts (Li et al., 2002).

Endogenous Trx has been shown to be expressed throughout the retinal layers and is upregulated by oxidative stress, such as photooxidative stress, indicating that the increased Trx level in retina is likely to be a defense mechanism against retinal pathology (Tanito et al., 2005). The Trx system protects against H₂O₂ and tumor necrosis factor- α -mediated toxicity, in which ROS play an important role (Kang et al., 1998). Yegorova et al. (2003) found a transient upregulation of Trx1 in human lens epithelial cells treated with 0.1 mM H₂O₂, and these changes occurred in both catalytic activity and protein levels. In the present study using human cultured RPE cells, oxidative stress from ceramide treatment did not result in significant change in Trx1 cellular protein expression. This was further supported by the finding that secretion of Trx1 from RPE with ceramide treatment was not different from that of control (data not shown). This lack of translational regulation in the face of gene upregulation has also been observed in some recent studies in other cell types treated with C₂ ceramide, such as human Jurkat T cells and mouse hybridoma T cell lines (Chen et al., 2008) and in the hearts of resveratrol-fed rats (Das et al., 2008). Inefficient translation due to mRNA silencing, decreased proteosomal activity, and/or activation of other compensatory redox mechanisms may be some of the factors that are operative (Freeman and Neuzil, 2006).

We hypothesized that the interaction of redoxins with Txnip, a Trx inhibitor, may be involved in the regulation of the oxidative stress-induced apoptosis in RPE. Txnip gene expression was significantly regulated by ceramide in RPE cells, suggesting the active role of Txnip in ceramide-induced oxidative stress. It is of interest that a very recent publication on ceramide induced signaling and apoptosis in human Jurkat cells (Chen et al. 2008) reported similar findings, namely that Txnip was upregulated both at the transcriptional and translational levels with corresponding decrease in Trx activity. Our findings are also consistent with previous studies with UV irradiation (Cheng et al., 2004), H₂O₂, TGF- β 1 and heat shock (Junn et al. 2000). Txnip gene regulation seems to be readily regulated by a variety of stresses, suggesting that it may be actively involved in stress response. In favor of this view, Nishiyama et al. (1999) reported that Txnip inhibits the reducing activity of Trx by interacting through the catalytic active site of Trx. Txnip is reported to compete with other Trx-binding proteins, such as ASK-1 and Prx1 for the active site of Trx (Junn et al., 2000). Thus, from the current study it may be proposed that Txnip upregulation from ceramide-induced oxidative stress allows Txnip to compete with other ligands in binding to Trx, thereby reducing Trx activity. It was reported that Txnip induced stress response by inducing the dissociation of ASK-1 from Trx and by increasing the gene expression of Txnip more than that of Trx (Saitoh et al., 1998; Junn et al., 2000).

Trx has a neuroprotective role in different cell types and retina. In the present study, when RPE cells were challenged with 25 μ M ceramide we observed about 25% cell death. Cell death was almost completely blocked when RPE were coincubated with human recombinant Trx1. These results indicate that Trx in the cell environment promotes cell survival. Trx has been shown to induce ASK-1 ubiquitination and degradation, resulting in the inhibition of ASK-1-induced apoptosis (Liu and Min, 2002) and the same mechanism could work in our experimental conditions because we found activation of ASK-1 at the protein level with 25 μ M ceramide (supplemental figure 3). Inomata et al (2006) found that the intravitreal injection of Trx effectively attenuated NMDA-induced retinal cell damage and that suppression of oxidative stress and inhibition of apoptotic signaling pathways were involved in this neuroprotection. Intraocular injection of rTrx suppresses photo-oxidative stress and protects photoreceptors from cell death (Tanito et al, 2002b).

It is to be noted that in all of the above-described studies, we found that treatment of RPE with HGF alone did not elicit a significant response in redoxin gene expression as compared to untreated controls. This fact, together with the observed effects of ceramide and ceramide plus HGF, suggests that a stimulus such as oxidative stress is needed for regulation of redoxin genes by HGF.

In summary, we have characterized the expression of Trx1 and its inhibitor in RPE cells and their regulation with ceramide treatment. In addition, we demonstrate nuclear translocation of Trx1 with ceramide treatment; a response that is blocked by p38 phosphorylation inhibitor. Therefore, differential subcellular localization in response to the extracellular stimuli may constitute one of the mechanisms of the pleiotropic action of Trx1 in the cell. Further, Txnip was upregulated at the transcriptional and translational levels which could explain the partial protection provided by HGF with ceramide treatment observed in our previous study (Kannan et al., 2004). Understanding the regulation and role of redoxin in RPE would be of value in the development of novel therapeutics agents for retinal injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Arai RJ, Masutani H, Yodoi J, Debbas V, Laurindo FR, Stern A, Monteiro HP. Nitric oxide induces thioredoxin-1 nuclear translocation: possible association with the p21Ras survival pathway. *Biochem Biophys Res Commun* 2006;348:1254–1260. [PubMed: 16914115]
- Badr GA, Zhang JZ, Tang T, Kern TS, Ismail-Beigi F. Glut1 and glut3 expression, but not capillary density, is increased by cobalt chloride in rat cerebrum and retina. *Mol Brain Res* 1999;64:24–33. [PubMed: 9889305]
- Bai J, Nakamura H, Kwon YW, Hattori I, Yamaguchi Y, Kim YC, Kondo N, Oka S, Ueda S, Masutani H, Yodoi J. Critical roles of thioredoxin in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells. *J Neurosci* 2003;23:503–509. [PubMed: 12533610]
- Chen CL, Lin CF, Chang WT, Huang WC, Teng CF, Lin YS. Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. *Blood* 2008;111:4365–4374. [PubMed: 18270325]
- Cheng GC, Schulze PC, Lee RT, Sylvan J, Zetter BR, Huang H. Oxidative stress and thioredoxin-interacting protein promote intravasation of melanoma cells. *Exp Cell Res* 2004;300:297–307. [PubMed: 15474995]
- Das S, Khan N, Mukherjee S, Bagchi D, Gurusamy N, Swartz H, Das DK. Redox regulation of resveratrol-mediated switching of death signal into survival signal. *Free Radic Biol Med* 2008;44:82–90. [PubMed: 18045550]
- Freeman RE, Neuzil J. Role of thioredoxin-1 in apoptosis induction by α -tocopheryl succinate and TNF-related apoptosis-inducing ligand in mesothelioma cells. *FEBS Lett* 2006;580:2671–2676. [PubMed: 16647070]
- Hattori I, Takagi Y, Nakamura H, Nozaki K, Bai J, Kondo N, Sugino T, Nishimura M, Hashimoto N, Yodoi J. Intravenous administration of thioredoxin decreases brain damage following transient focal cerebral ischemia in mice. *Antioxid Redox Signal* 2004;6:81–87. [PubMed: 14713338]
- He PM, He S, Garner JA, Ryan SJ, Hinton DR. Retinal pigment epithelial cells secrete and respond to hepatocyte growth factor. *Biochem Biophys Res Commun* 1998;249:253–257. [PubMed: 9705867]
- Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A* 1997;94:3633–3638. [PubMed: 9108029]
- Holmgren A. Glutaredoxin from *Escherichia coli* and calf thymus. *Methods Enzymol* 1985;113:525–540. [PubMed: 3911009]
- Inomata Y, Nakamura H, Tanito M, Teratani A, Kawaji T, Kondo N, Yodoi J, Tanihara H. Thioredoxin inhibits NMDA-induced neurotoxicity in the rat retina. *J Neurochem* 2006;98:372–385. [PubMed: 16805832]
- Junn E, Han SH, Im JY, Yang Y, Cho EW, Um HD, Kim DK, Lee KW, Han PL, Rhee SG, Choi I. Vitamin D₃ up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* 2000;164:6287–6295. [PubMed: 10843682]
- Kajimoto T, Shirai Y, Sakai N, Yamamoto T, Matzuzaki H, Kikkawa U, Saito N. Ceramide-induced apoptosis by translocation, phosphorylation and activation of protein kinase Cdelta at Golgi complex. *J Biol Chem* 2004;279:12668–12676. [PubMed: 14715667]
- Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor- α . *J Biol Chem* 1998;273:6297–6302. [PubMed: 9497357]
- Kannan R, Jin M, Gamulescu MA, Hinton DR. Ceramide-induced apoptosis: role of catalase and hepatocyte growth factor. *Free Radic Biol Med* 2004;37:166–175. [PubMed: 15203188]

- Li B, Ishii T, Tan CP, Soh JW, Goff SP. Pathways of induction of peroxiredoxin I expression in osteoblasts: roles of p38 mitogen-activated protein kinase and protein kinase C. *J Biol Chem* 2002;277:12418–12422. [PubMed: 11796722]
- Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res* 2002;90:1259–1266. [PubMed: 12089063]
- Malik G, Gorbounov N, Das S, Gurusamy N, Otani H, Maulik N, Goswami S, Das DK. Ischemic preconditioning triggers nuclear translocation of thioredoxin and its interaction with Ref-1 potentiating a survival signal through the PI-3-kinase-Akt pathway. *Antioxid Redox Signal* 2006;8:2101–2109. [PubMed: 17034353]
- Matsui M, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 1996;178:179–185. [PubMed: 8812119]
- Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. *J Biochem (Tokyo)* 1996;119:591–600. [PubMed: 8743556]
- Nakamura H, Nakamura K, Yodoi J. Redox regulation of cellular activation. *Ann Rev Immunol* 1997;15:351–369. [PubMed: 9143692]
- Nishiyama A, Masutani H, Nakamura H, Nishinaka Y, Yodoi J. Redox regulation by thioredoxin and thioredoxin-binding proteins. *IUBMB Life* 2001;52:29–33. [PubMed: 11795589]
- Nishiyama A, Matsui M, Iwata S, Hirota K, Masutani H, Nakamura H, Takagi Y, Sono H, Gon Y, Yodoi J. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* 1999;274:21645–21650. [PubMed: 10419473]
- Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. *Science* 1993;259:1769–1771. [PubMed: 8456305]
- Okubo K, Kosaka S, Isowa N, Hirata T, Hitomi S, Yodoi J, Nakano M, Wada H. Amelioration of ischemia-reperfusion injury by human thioredoxin in rabbit lung. *J Thorac Cardiovasc Surg* 1997;113:1–9. [PubMed: 9011676]
- Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* 1994;78:1027–1037. [PubMed: 7923353]
- Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK1). *EMBO J* 1998;17:2596–2606. [PubMed: 9564042]
- Schenk H, Klein M, Erdbrügger W, Dröge W, Schulze-Osthoff K. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci U S A* 1994;91:1672–1676. [PubMed: 8127864]
- Schulze PC, De Keulenaer GW, Yoshioka J, Kassik KA, Lee RT. Vitamin D3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin. *Circ Res* 2002;91:689–695. [PubMed: 12386145]
- Shibuki H, Katai N, Kuroiwa S, Kurokawa T, Yodoi J, Yoshimura N. Protective effect of adult T-cell leukemia-derived factor on retinal ischemia-reperfusion injury in the rat. *Invest Ophthalmol Vis Sci* 1998;39:1470–1477. [PubMed: 9660496]
- Sreekumar PG, Kannan R, Yaung J, Spee CK, Ryan SJ, Hinton DR. Protection from oxidative stress by methionine sulfoxide reductases in RPE cells. *Biochem Biophys Res Commun* 2005;334:245–253. [PubMed: 15993845]
- Takagi Y, Mitsui A, Nishiyama A, Nozaki K, Sono H, Gon Y, Hashimoto N, Yodoi J. Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci U S A* 1999;96:4131–4136. [PubMed: 10097175]
- Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G, Yodoi J. Thioredoxin-2 (*TRX-2*) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J* 2002;21:1695 – 1703. [PubMed: 11927553]

- Tanito M, Kwon YW, Kondo N, Bai J, Masutani H, Nakamura H, Fujii J, Ohira A, Yodoi J. Cytoprotective effects of geranylgeranylacetone against retinal photooxidative damage. *J Neurosci* 2005;25:2396–2404. [PubMed: 15745966]
- Tanito M, Masutani H, Nakamura H, Ohira A, Yodoi J. Cytoprotective effect of thioredoxin against retinal photic injury in mice. *Invest Ophthalmol Vis Sci* 2002b;43:1162–1167. [PubMed: 11923261]
- Tanito M, Masutani H, Nakamura H, Oka S, Ohira A, Yodoi J. Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. *Neurosci Lett* 2002a;326:142–146. [PubMed: 12057848]
- Vengellur S, Woods BJ, Ryan HE, Johnson RS, LaPres JJ. Gene expression profiling of the hypoxia signaling pathway in hypoxia-inducible factor 1alpha null mouse embryonic fibroblasts. *Gene Exp* 2003;11:187–197.
- Wei SJ, Botero A, Hirota K, Bradbury CM, Markovina S, Laszlo A, Spitz DR, Goswami PC, Yodoi J, Gius D. Thioredoxin nuclear translocation and interaction with redox factor-1 activates the activator protein-1 transcription factor in response to ionizing radiation. *Cancer Res* 2000;60:6688–6695. [PubMed: 11118054]
- Wiegmann K, Schutze S, Machleidt T, Witte D, Kronke M. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* 1994;78:1005–1015. [PubMed: 7923351]
- Yamamoto M, Lidia K, Gong H, Onitsuka S, Kotani T, Ohira A. Changes in manganese superoxide dismutase expression after exposure of the retina to intense light. *Histochem J* 1999;31:81–87. [PubMed: 10416679]
- Yegorova S, Liu A, Lou MF. Human lens thioredoxin: molecular cloning and functional characterization. *Invest Ophthalmol Vis Sci* 2003;44:3263–3271. [PubMed: 12882768]

Fig. 1

Fig. 1.

Expression of Trx1 in cultured human RPE cells.

The expression of Trx1 was determined by western blot analysis of lysates from confluent human RPE grown in DMEM as described in Materials and Methods. The molecular weight of Trx1 is 12 kDa.

Fig. 2.

Effect of ceramide treatment on Trx1 protein expression.

RPE cells were treated with 25 μ M of C₂ ceramide for indicated time periods. Immunoblot was performed in 15% Tris-HCl gel with 25 μ g of cell lysate loaded into each lane. A rabbit polyclonal primary antibody (anti-Trx1) at 1:1000 dilution was used and signals were detected using chemiluminescence system. Protein expression is shown as ratio normalized with GAPDH. No significant change in protein expression with ceramide treatment as compared to the untreated controls over time was found.

Fig. 3.

Effect of ceramide with and without HGF preincubation on Trx1 mRNA (A) and protein expression (B and C) in RPE cells. RPE cells were preincubated with 20ng/ml HGF overnight followed by treatment with 25 μ M ceramide for 24 h.

A. mRNA expression of Trx1. HGF preincubation followed by ceramide treatment significantly ($p < 0.01$) increased Trx1 expression over controls. However, no significant change in expression was observed with ceramide or HGF treatment alone.

B. Western blot analysis of the effect of ceramide and HGF on Trx1 protein expression. No significant change in the expression of Trx1 with ceramide alone or after preincubation with HGF could be found as compared to controls.

C. Densitometric analysis of immunoreactive bands from Trx1/GAPDH immunoblots from four independent experiments. Trx1: GAPDH ratios were normalized to control (n=4). No significant change was observed between untreated controls and C₂-ceramide and HGF treated RPE.

Fig. 4.

Evidence for nuclear translocation of Trx1 with C₂ ceramide treatment.

RPE cells treated with 10 μ M ceramide were processed for immunocytochemistry as described in Materials and Methods. Panels A–C describe control untreated cells. RPE cells treated with 10 μ M ceramide for 24 h (D–F), RPE cells preincubated with 25 μ M p38 inhibitor followed by incubation with 10 μ M ceramide for 24h (G–I) and RPE cells treated with 20ng/ml HGF (J–L). Green fluorescence indicates positive immunoreaction for Trx1. Blue fluorescence indicates nuclear staining with DAPI. In the non-stimulated control cells Trx1 showed cytoplasmic localization. Ceramide treatment induced nuclear translocation of Trx1 (D, see arrows), which was blocked by p38 inhibitor (G). HGF treatment alone did not translocate Trx1 (J–L).

Figure 5.

Phosphorylation of p38 MAP Kinase with ceramide treatment of RPE cells.

RPE cells were treated with 10 and 25 µM C₂ ceramide with and without HGF preincubation for 24 h and cell lysate was subjected to immunoblot analysis of phospho p38 MAP Kinase and total p38 MAP Kinase using specific antibodies as described in Materials and Methods. Exposure to ceramide resulted in significant upregulation of phospho p38 MAP Kinase while total p38 MAP Kinase was unaltered (Panels A and B). Protein expression is shown as ratio normalized with GAPDH in B.

Fig. 6.

Txnip-Trx interaction is not influenced by p38 inhibition.

RPE cells treated with 25 µM C₂ ceramide for 24 h with and without pretreatment followed by co-treatment with p38 inhibitor. The cell lysate from the experiment were immunoprecipitated (IP) with anti-Txnip antibody, followed by immunoblotting (IB) using Trx1 (Panel A) or ASK-1 (Panel B) antibodies. Cell lysate without ceramide treatment and immunoprecipitation (WCL- whole-cell lysate) was used as positive control. Mouse IgG was used as the negative control.

Fig. 7.

Regulation of Txnip gene and protein expression by ceramide.

A. Real-time quantitative RT-PCR showing the effect of ceramide and HGF treatment on Txnip gene expression in RPE cells. Results are presented as the difference in fold levels compared to untreated cells. * ($p < 0.05$) and ** ($p < 0.01$) denote significant increase as compared to controls and ceramide-treated RPE, respectively.

B. Immunoblot analysis of Txnip in RPE treated with ceramide for 24 h. Immunoblotting was performed in 15% Tris-HCl gel with 30 μ g of cell lysate loaded into each lane. A monoclonal antibody (anti-Txnip) at 1:1000 dilution was used and bands were detected using a chemiluminescence system. Equal protein loading was confirmed with GAPDH antibody.

C. Txnip expression is shown as ratio normalized to GAPDH ($n=3$). The ratio of 25 μ M ceramide-treated cells and cells preincubated with HGF followed by ceramide treatment differed significantly from untreated controls (* $p < 0.05$, ** $p < 0.01$).

Fig. 8.

Exogenously added Trx1 protects RPE cells from ceramide induced cell death.

A. RPE cells grown in chamber slides were co-incubated with 5 μ g recombinant human Trx1 and 25 μ M ceramide and apoptotic cells were determined by TUNEL assay (Panels a–d). Panel a, Control cells with out any treatment, b, Treated with 10 μ M ceramide, c, treated with 25 μ M ceramide and d, treated with 5 μ g rhTrx1 and 25 μ M ceramide.

B. Semiquantitative measurement of TUNEL positive cells. Apoptosis was significantly ($p < 0.01$) lower in cells coincubated with ceramide and rhTrx1 (5 μ g).