Nipradilol and Timolol Induce Foxo3a and Peroxiredoxin 2 Expression and Protect Trabecular Meshwork Cells from Oxidative Stress

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PURPOSE. Oxidative stress plays an important role in pathogenesis of glaucoma. The purpose of this study is to investigate the novel effect of antiglaucoma drugs on the expression of antioxidant peroxiredoxins of trabecular meshwork (TM) cells.

METHODS. The expression of the peroxiredoxin family was investigated using immortalized TM cell lines. Cells were treated with antiglaucoma drugs and analyzed for the expression of peroxiredoxin, and cellular sensitivity to oxidative stress. Furthermore, the effect of antiglaucoma drugs on the molecular regulation of the expression of peroxiredoxin was examined using a reporter assay and siRNA strategy.

RESULTS. Glaucomatous TM cells highly express peroxiredoxin 2 but not when compared with normal TM cells. Nipradilol and timolol, but not latanoprost, induce the expression of peroxiredoxin 2 through the activation of the Foxo3a transcription factor. TM cells showed reduced sensitivity to H2O2 when cells were treated with either nipradilol or timolol, but not with latanoprost. In addition, both Foxo3a and PRDX2 expression were enhanced by drug-induced signal transduction through its receptor.

CONCLUSIONS. These results indicate that both nipradilol and timolol possess a novel mechanism of action and function as potent protective agents against oxidative stress. (Invest Ophthal Vis Sci. 2009;50:2777–2784) DOI:10.1167/iovs.08-3061

Glaucoma is an irreversible chronic progressive disease that affects approximately 2% to 4% of the population older than age 40 years.1,2 Intraocular pressure (IOP) is a major factor affecting the development of glaucoma and the progression of glaucomatous damage to the optic nerve. This elevated IOP is due to an increase in aqueous humor outflow resistance and may be associated with morphologic and biochemical changes in the trabecular meshwork.7,8 The major investigated factors, such as elevated IOP,9,10 reduced ocular blood flow (OBF),11,12 ocular vascular dysregulation,13 blood pressure (BP) alteration,14–16 and oxidative stress,17 have been suggested as contributing factors to the pathogenesis of glaucomatous damage.18 Recently, growing evidence has been accumulating that shows that oxidative stress, which can be defined as an imbalance between the production and removal of reactive oxygen species (ROS), plays an important role in the pathogenesis of various ocular diseases, including glaucoma.19–22 The eye is protected against oxidative stress by several antioxidant enzymes and low-molecular-weight antioxidants. Peroxiredoxins (PRDXs) are a ubiquitous family of multifunctional antioxidant thioredoxin-dependent peroxidases that scavenge reactive oxygen species (ROS). PRDXs are also involved in the cellular response to ROS23,24 and function as a cellular defense system against ROS. PRDXs possess redox-active cysteines to reduce peroxides. PRDXs are often expressed in the same cell in various intracellular locations.25–27 Nipradilol (3,4-dihydroxy-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran (molecular weight of 326.35 Da) is a novel antiglaucoma agent that has nonselective β-receptor and selective α1-receptor blocking activities25–27 and reduces intraocular pressure by decreasing aqueous production and by increasing uveoscleral outflows in both rabbits and humans.28,29 It has been reported recently that nipradilol has neuroprotective activities.30–34 However, the protective cellular effect of nipradilol against oxidative stress has not been reported, and little is known about the role and significance of PRDXs in TM cells. In the present study, we show that Foxo3a regulates the expression of PRDX2 in TM cells and both nipradilol and timolol (another antiglaucoma agent with nonselective β-receptor blocking activity) can induce the expression of both Foxo3a and PRDX2, as novel mode of action of these drugs. These results provide new insights into the significance of redox regulation in TM cells and therapeutic strategies for glaucoma treatment.

EXPERIMENTAL PROCEDURES

Cell Culture

Two immortalized TM cell lines, NTM5 cells and GTM3 cells, derived from normal and glaucomatous trabecular meshwork, respectively, were used and cultured in Dulbecco’s modified Eagle medium (Nissui Seiyaku Co., Tokyo, Japan). These cell lines were kindly provided by Abott F. Clark (Glaucoma Research, Alcon Research, Ltd., Fort Worth, TX). The detailed information of these cell lines has been described previously.35 Briefly, cells isolated from the trabecular meshwork (TM) of a normal and glaucoma patient were transformed by transfection with an origin defective mutant of SV40 virus. The glaucoma patient had glaucomatous visual field and was treated with β-blocker therapy.35
Antibodies and Drugs

Antibodies against FKHR1 (Foxo3a and sc-20), β2-AR (sc-9042), PGF2αR (sc-33364), PRDX2 (sc-23967), and PRDX4 (sc-23974) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PRDX antibody (AC-15) was purchased from Sigma-Aldrich Co. (St. Louis, MO). To generate anti-PRDX1 and anti-PRDX5 antibodies, the synthetic peptides PDVQKSKEYFSKQ and GLTCSLAPNIISQL were used as immunogens, respectively, and the antisera obtained from immunized rabbits were affinity purified using the synthetic peptide antigens. The anti-PRDX3 antibody was a kind gift from Hiroki Nanri (Seinan Jogakuin University, Fukuoka, Japan).36 Nipradilol was kindly provided by Kowa Ltd. (Tokyo, Japan). Timolol maleate (a nonselective β-adrenoceptor antagonist) and latanoprost acid (a selective FP prostanoid receptor agonist) were purchased from Sigma-Aldrich. Drug concentrations in this study correspond with those used in clinical practice.

Plasmid Construction

To obtain the full-length complementary DNA (cDNAs) for human Foxo3a, PCR was carried out on a cDNA library (Invitrogen Life Technologies, Carlsbad, CA) using the following primer pairs: ATGGCAGAGGCAACCGGCCGTCCGCG and TGCAAGCTTGGAAAGGGCGGTTCAGCGG. This PCR product was cloned (pGEM-T Easy Vector; Promega, Madison, WI). To construct a plasmid expressing HA-tagged Foxo3a, the pcDNA3-HA vector was prepared by ligation of the HI-fragment including HA into a pcDNA3 vector (Invitrogen). pcDNA3-HA Foxo3a was obtained by ligation of the NotI fragment including the Foxo3a cDNA into a pcDNA3-HA vector. The construct PRDX2-Luc (~402 to +68) was made using the following primer pairs: 5'-AGATCTTTGACATCGCCTAGGCGG5'- and 5'-AAGCTTTGCAAGCTTAGACGCGG-5' for PRDX2-Luc. This PCR product was cloned and ligated into the BglII-HindIII sites of the pGL3-basic vector (Promega).

Western Blotting Analysis

Whole-cell lysates were prepared as described previously.37,38 The indicated amounts of whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) microporous membranes (Millipore, Billerica, MA) using a semidry blotter. The blotted membranes were treated

**Figure 1.** (A) Expression of PRDXs in NTM5 and GTM3 cells. Whole-cell extracts (50 μg) were subjected to SDS-PAGE, and Western blotting was performed with the indicated antibodies. Relative intensity is shown under each blot. (B) The sensitivity of NTM5 and GTM3 cells to H2O2. NTM5 cells or GTM3 (2.5 × 10^4) cells were seeded into 96-well plates. The following day, to induce oxidative stress, cells were incubated with the indicated concentrations of H2O2 in the serum-free medium for 40 minutes. Then the medium was changed and added to the fresh culture medium. After 72 hours, cell survival was analyzed by a WST-8 assay. All values are the means of at least three independent experiments. Bars indicate the SD. (C, D) Downregulation of PRDX2 sensitizes cells to oxidative stress. Upper panel: NTM5 cells or GTM3 cells were transiently transfected with 100 pmol of control or PRDX2 siRNAs. After 72 hours, whole-cell extracts (50 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibody. Immunoblotting of β-actin is shown as a loading control. Relative intensity is shown at the bottom of the panel. Lower panel: 2.5 × 10^4 NTM5 cells or GTM3 cells were transfected with 40 nM of control siRNA (black square), PRDX2 siRNA (white triangle). The following day, to induce oxidative stress, cells were incubated with the indicated concentrations of H2O2 in the serum-free medium for 40 minutes. Then the medium was changed and fresh culture medium was added. After 48 hours, cell survival was analyzed by a WST-8 assay. All values are the means of at least three independent experiments. Bars indicate the SD. CBB, Coomassie Brilliant Blue.
FIGURE 2. (A) Schematic representation of the promoter region of the human PRDX2 gene. Shown below the figure are the human and mouse promoter sequences for comparison. (B) Expression of Foxo3a in NTM5 and GTM3 cells. Whole-cell extracts (50 μg) were subjected to SDS-PAGE, and Western blotting was performed with the indicated antibodies. Relative intensity is shown under each blot. (C) Reporter assay using PRDX2 promoter in TM cells. PRDX2-Luc. plasmid (0.5 μg) was transiently transfected into NTM5 cells or GTM3 cells. pGL3-P.V. indicates the pGL3 promoter vector in which the luciferase gene is driven by the SV-40 promoter. The results shown are normalized to protein concentrations measured using the Bradford method, and are representative of at least three independent experiments. Bars represent the SD. The luciferase activity of PRDX2-Luc or pGL3-P.V. for NTM5 cells was set to 100.

with 5% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl and 0.2% (v/v) Tween 20, and incubated for 1 hour at room temperature with primary antibody. The following antibodies and dilutions were used: a 1:10,000 dilution of anti-PRDX1, a 1:5000 dilution of anti-PRDX2, a 1:5000 dilution of anti-PGFr2α. Membranes were then incubated for 40 minutes at room temperature with a peroxidase-conjugated secondary antibody and were visualized using an ECL kit (GE Healthcare Life Sciences, Piscataway, NJ). For the correlation assay, the intensity of each band was quantified using a NIH imaging program, version 1.62 (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Knockdown Analysis Using siRNAs

The following double-stranded RNA 25-base-pair oligonucleotides were commercially generated (Invitrogen): 5′-UGGCAAGGGAUAGCAUGGACUGCA-3′ (sense) and 5′-UCAGACUAGCGCUCAUAGUUCGA-3′ (antisense); Foxo3a#1 siRNA, 5′-UGCAGGGACUGCUCAUAGGUGA-3′ (sense) and 5′-UCAACUCUUCCUGGCAAGUGA-3′ (antisense); Foxo3a#2 siRNA, 5′-AAGGCAUCACUGGACAUUGGUA-3′ (sense) and 5′-UCCGACUAUGCAUGUAUGUCCU-3′ (antisense); Foxo3a#3 siRNA, 5′-UUACCCACGAGGGUGCCUAGAGU-3′ (sense) and 5′-ACGUAUGUAUGCAUGCCUAUGAU-3′ (antisense); ADRB2#1 siRNA, 5′-AAGCCCAACAUUGAAUUGGACGU-3′ (sense) and 5′-GGACCCCAAGAUUGGUGCCU-3′ (antisense); ADRB2#2 siRNA, 5′-AAUGAACGAGGUGUGCCUAGGUA-3′ (sense) and 5′-GUGUGUCCAGUUUGGUGGUGGU-3′ (antisense); and ADRB2#3 siRNA, 5′-AAUGGACGGGUGUGGUGGUGGUA-3′ (sense) and 5′-CAGCAGAGGGUGUGGUGGUGU-3′ (antisense).

siRNA transfections were performed as described previously.37,38 Ten microliters of reagent (Lipofectamine 2000; Invitrogen) was diluted in 250 μL of medium (Opti-MEM I; Invitrogen) and incubated for 5 minutes at room temperature. Next, 250 pmol of indicated siRNA or inverted control duplex buffer ( Stealth RNAs; Invitrogen), diluted in 250 μL of medium (Opti-MEM I; Invitrogen), was added gently and incubated for 20 minutes at room temperature. Oligomer-lipofectamine complexes and aliquots of 1 × 10^6 cells in 500 μL of culture medium were combined and incubated for 10 minutes at room temperature. Aliquots of 1.5 × 10^5 NTM5 cells were used in luciferase assays and aliquots of 2.5 × 10^5 TM cells or GTM3 cells were used in the WST-8 assay, as described below. The remaining cells were seeded into 35 mm dishes with 2 mL of culture medium and harvested after 72 hours of culture for Western blotting analysis, as described above.

Luciferase Assay

Transient transfection and a luciferase assay were performed as described previously.37,38 NTM5 cells or GTM3 cells (1 × 10^5) were seeded into 12-well plates. The next day, cells were co-transfected with the indicated amounts of PRDX2 reporter plasmid and expression plasmids using a reagent (Superfect; Qiagen, Valencia, CA). Cells were then transfected with siRNA as described above, followed by transfection with the indicated amounts of reporter plasmid at intervals of 12 hours. Forty-eight hours post-transfection, cells were lysed with reporter lysis buffer (Promega) and luciferase activity was detected using a reagent kit (PicoGene; Toyo Inki, Tokyo, Japan). The light intensity was measured using a luminometer (Luminescence JNII RAB-2300; Atto Corporation, Tokyo, Japan). The results shown are normalized to the protein concentrations measured using the Bradford method, and are representative of at least three independent experiments.
Cytotoxicity Analysis

The water-soluble tetrazolium salt (WST-8) assay was performed as described previously.37,38 Briefly, 2.5 × 10^3 NTM5 cells or GTM3 cells transfected with the indicated amounts of siRNA were seeded into 96-well plates. The next day, to induce oxidative stress, cells were incubated with the indicated concentrations of H2O2 in the serum-free medium for 40 minutes. Then the medium was changed and added to the culture medium. After 72 h, surviving cells were stained with a cell proliferation assay (TetraColor ONE; Seikagaku Corporation, Tokyo, Japan) for 90 minutes at 37°C. The absorbance was then measured at 450 nm.

Statistical Analysis

Expression levels of protein assayed by Western blotting were assessed numerically with the NIH image program. The Pearson correlation was used for statistical analysis, and significance was set at 5%.

RESULTS

It is difficult to employ primary human TM cells, because human TM tissue is not frequently available under Japanese law. We used two immortalized TM cell lines, NTM5 cells and GTM3 cells, derived from normal and glaucoma tissues, respectively. Initially, we investigated the expression of five 2-Cys peroxiredoxin family. As shown in Figure 1A, PRDX2 to 5 were found to be expressed, but PRDX1 was not expressed in both NTM5 and GTM3 cells. PRDX2 is highly expressed in GTM3 cells in comparison with NTM5 cells.

Next we examined the sensitivity of these cell lines to H2O2. GTM3 cells were more resistant to H2O2 than NTM5 cells (Fig. 1B). Downregulation of PRDX2 sensitized both cell lines against the acute oxidative stress of H2O2 (Fig. 1C, D). To investigate the transcriptional regulation of the PRDX2 gene, we compared the nucleotide sequences of this gene promoter.
between human and mouse to identify the conserved elements. We found one conserved Foxo3a-binding site in the core promoter region (Fig. 2A). Then we examined Foxo3a expression in both NTM5 and GTM3 cells. As expected, Foxo3a was highly expressed in GTM3 cells when compared with NTM5 cells (Fig. 2B). The reporter assays also showed that the promoter activity of PRDX2 was higher in GTM3 than in NTM5 cells (Fig. 2C). PRDX2 expression was significantly reduced by downregulation of Foxo3a expression by siRNA transfection (Fig. 3A). The reporter assay showed that PRDX2 promoter activity is enhanced by the co-transfection of Foxo3a expression plasmids (Fig. 3B). Furthermore, the promoter activity of the PRDX2 gene was downregulated by the transfection of Foxo3a specific siRNA (Fig. 3C). As a result, downregulation of Foxo3a expression conferred cell sensitivity to H2O2 (Fig. 3D).

We next investigated whether an antiglaucoma agent, the a/β blocker nipradilol, is involved in the regulation of the Foxo3a/PRDX2 system in TM cells. Interestingly, the expression of both Foxo3a and PRDX2 was increased when cells were treated with nipradilol in a time-dependent manner (Fig. 4A). Both Foxo3a and PRDX2 expression also increased in response to the addition of nipradilol in a concentration-dependent manner (Fig. 4B). The reporter assay also showed that PRDX2 promoter activity was enhanced by the treatment with nipradilol, suggesting that nipradilol activated the PRDX2 gene through Foxo3a expression at the transcriptional level (Fig. 4C). In addition, we observed that nipradilol treatment renders TM cells resistant to H2O2 (Fig. 4D). We also investigated the effect of two other antiglaucoma agents, a β-blocker (timolol) or a prostaglandin derivative (latanoprost), on the Foxo3a/PRDX2 system. The treatment of TM cells with timolol resulted in the upregulation of both Foxo3a and PRDX2 expression (Fig. 5A). In contrast, there were no significant differences in the expression of both Foxo3a and PRDX2 when cells were treated with latanoprost (Fig. 5B). To examine the protective efficiency of these drugs, cells were exposed to H2O2 in the presence of each drug. Timolol showed significant protection against oxidative stress, but latanoprost did not (Fig. 5C). Next, to understand the molecular mechanism by which a Foxo3a/PRDX2 system is upregulated by antiglaucoma agents, we analyzed the involvement of the receptors for the drugs: Western blot analysis identified a significant amount of both the adrenergic β2 receptor and the prostaglandin F (FP) receptor. The expression of both receptors appeared higher in GTM3 cells than in NTM5 cells (Fig. 6A). Both Foxo3a and PRDX2 expression were reproducibly upregulated by nipradilol when control siRNA was used. Notably, neither Foxo3a nor PRDX2 expression was upregulated by nipradilol at all when the ex-

FIGURE 4. (A) Effect of nipradilol on expression of Foxo3a and PRDX2. NTM5 cells were incubated with 0.1 μM nipradilol for the times indicated. Whole-cell extracts (50 μg) were subjected to SDS-PAGE, and Western blotting was performed with the indicated antibodies. Relative intensity is shown under each blot. (B) NTM5 cells were cultured with the control medium or the medium containing the indicated concentrations of nipradilol. The fol-

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pression of adrenergic β2 receptor was downregulated by siRNA.

**DISCUSSION**

One of the major causes of aging is oxidative stress and glaucoma development is often observed in older people. Oxidative stress has been shown to play a pathogenic role in glaucoma. Therefore, we were interested to investigate whether antiglaucoma agents had any antioxidant activity. There are three major antioxidant systems in mammalian cells—superoxide-dismutase/catalase, glutathione, and peroxiredoxin (PRDX). These systems might function as a protective mechanism from oxidative stress in TM cells. Foxo3a is a member of forkhead transcription factors, which modulate the gene expression involved in a variety of cellular processes, including cell cycle, apoptosis, DNA repair, cell differentiation, glucose metabolism, and oxidative stress. It has been shown that Foxo3a plays an important role in the survival response of normal and cancer cells in hypoxic stress.

In the present study, we provide novel evidence that both Foxo3a and PRDX2 are highly expressed in GTM3 cells (Figs. 1 and 2). We further demonstrate that the treatment with the antiglaucoma agents, nipradilol and timolol, enhances the expression of both Foxo3a and PRDX2 and confers cell resistance to H2O2 (Figs. 3, 4, and 5). Our results indicate a novel mode of action for the α/β blocker, nipradilol, and the β blocker, timolol, in which they act to promote expression of a Foxo3a-dependent PRDX2 to produce an antioxidant activity. We also found that PRDX2 expression is upregulated in glaucoma-derived TM cells when compared with normal-derived TM cells. It has been shown that PRDX2 inhibits cellular senescence. The accumulation of ECM was found in glaucoma tissue, where oxidative stress induces cytokines such as TGF-β and PDGF, which can promote the ECM expression. PRDX2 has also been reported to inhibit the cytokine signaling.

It has been shown that both nipradilol and timolol exert a direct antioxidant activity that protects trabecular meshwork cells from oxidative stress. However, the precise mechanism of this antioxidant activity is not well understood. Nipradilol is a potent nitric oxide (NO) donor and NO release from nipradilol exerts either a neuroprotective effect or an anti-apoptotic ability. As shown in Figure 6, our results indicate that the drug-induced activation of the Foxo3a transcription factor is due to a mechanism that involves a receptor-mediated signaling pathway instead of through a NO-related signaling pathway. The forkhead box transcription factor Foxo3a is one of the FOXO family of transcription factors that have a wide range of cellular functions, such as stress resistance and longevity. Foxo3a expression and its function are finely regulated by a complex signaling pathway. It has also been shown that Foxo3a activates the transcription of manganese superoxide dismutase. Taken together, enhancing antioxidant activities through the
Protection of TM Cells from Oxidative Stress by Nipradilol and Timolol


