Mitochondria-Targeted Peptide MTP-131 Alleviates Mitochondrial Dysfunction and Oxidative Damage in Human Trabecular Meshwork Cells

Min Chen,1,2 Bingqian Liu,1,2 Qianying Gao,1 Yebong Zhuo,1 and Jian Ge1

PURPOSE. To investigate the antioxidative ability of a novel mitochondria-targeted peptide MTP-131 in immortalized human trabecular meshwork (HTM) and glaucomatous human trabecular meshwork (GTM) cell lines.

METHODS. Cultured HTM and GTM cells were pretreated with MTP-131 for 1 hour, and sustained oxidative stress was induced by subjecting TM cells to 200 μM hydrogen peroxide (H2O2) for 24 hours. Untreated cells and cells incubated with H2O2 alone were used as controls. Lactate dehydrogenase (LDH) assay was used to determine cell viability. Changes of mitochondrial membrane potential (ΔΨm) and generation of intracellular reactive oxygen species (ROS) were analyzed by flow cytometry and confocal microscopy. Activation of caspase 3 was quantified by Western blotting, and apoptosis was measured by flow cytometry. Release of cytochrome c and changes in cytoskeleton were analyzed by confocal microscopy. Data were analyzed with commercial data analysis software and P < 0.05 was considered to be statistically significant.

RESULTS. In both HTM and GTM cells, decrease of ΔΨm and elevation of intracellular ROS were detected after sustained oxidative stress induced by H2O2. When cells were pretreated with MTP-131, the H2O2-induced mitochondrial depolarization was prevented; intracellular ROS, LDH release, and apoptosis were significantly decreased; release of cytochrome c from mitochondria to cytoplasm and activation of caspase 3 were inhibited. In addition, cytoskeleton changes caused by H2O2 were also alleviated by MTP-131.

CONCLUSIONS. Mitochondria-targeted peptide MTP-131 could prevent both HTM and GTM cells from sustained oxidative stress induced by H2O2. (Invest Ophthalmol Vis Sci. 2011;52: 7027–7037) DOI:10.1167/iovs.11-7524

Trabecular meshwork (TM) is the main site of aqueous humor (AH) outflow in the eye and plays a key role in regulating normal intraocular pressure (IOP).1 Specific morphologic and biochemical changes in the TM, such as loss of TM cells, accumulation of extracellular matrix (ECM), and accelerated senescence, have been reported to increase AH outflow resistance and induce subsequent elevation of IOP.2,3 IOP elevation and associated progressive optic neuropathy are the most important features of glaucoma, which is one of the leading causes of irreversible blindness. Primary open-angle glaucoma (POAG) is the most common form.4 Various factors may contribute to the pathogenesis of POAG, such as oxidative stress, genetic factors, vascular dysregulation, and mechanical injury.4–6 Recently, mounting evidences indicate that oxidative stress may trigger degeneration in human trabecular meshwork (HTM), which causes abnormal accumulation of protein and affects cytoskeleton and adhesive properties in HTM cells, subsequently leading to increased IOP.7–9 In addition, it has been reported that the antioxidative capacity in the AH is markedly reduced in patients with POAG compared with age-matched controls.10,11

Mitochondria plays an important role in oxidative stress, acting as the main source of ROS generation as well as the target of ROS.12,13 Li et al.14 demonstrated that sustained oxidative stress induced by H2O2 resulted in an increase of ROS in porcine TM cells. Our previous studies have proved that mitochondria dysfunction due to mitochondrial complex I defect is involved in the pathogenesis of POAG, which can induce release of ROS, decrease of mitochondrial membrane potential (ΔΨm), and the degeneration of glaucomatous trabecular meshwork (GTM) cells.15,16 Recently, Izzotti et al.17 reported that mitochondrial DNA deletion was dramatically increased in the TM of glaucoma patients compared with controls, and a significant decrease in the number of mitochondria per cell was also revealed. Therefore, mitochondria-targeted protection may be beneficial in prevention and treatment of glaucoma.

Natural antioxidants such as coenzyme Q and vitamin E have shown protective effects on oxidative damages.18,19 However, they may not reach the relevant sites of ROS generation and may fail to achieve significant intracellular concentrations, resulting in poor efficiency.20 A novel, water-soluble peptide was recently reported (originally designed by Hazel H. Szeto and Peter W. Schiller), named MTP-131 or SS-31.21 With a unique alternating aromatic cationic motif (d-Arg-Dmt-Lys-Phe-NH2), MTP-131 has a highly favorable structure for membrane penetration, targeting to mitochondria in a potential-independent manner (Fig. 1). This is a major advantage when dealing with abnormal mitochondria with compromised ΔΨm.25 The fraction of MTP-131 partitioned to mitochondria was estimated to be 1000- to 5000-fold compared with extramitochondrial concentration, and localized to the inner mitochondrial membrane (IMM) rather than in the matrix.21 It was reported that MTP-131 could reduce mitochondrial ROS accumulation in a...
dose-dependent manner and was potent in preventing oxidative damage in several cell types in vitro.24–26

In this study, we aimed to investigate the antioxidant effect of MTP-131 on GTM³ and iHTM, and the different responses to mitochondria-targeted antioxidant were compared between the two cell lines.

MATERIALS AND METHODS

Drugs and Chemicals

MTP-131 was kindly provided by Stealth Peptides International Inc. (Newton Centre, MA). All cell culture reagents were obtained from Gibco BRL (Gaithersburg, MD). TMRM (tetramethylrhodamine methyl ester), H₂DCFDA (2′,7′-dichlorofluorescein diacetate), and an intense red-fluorescent dye (MitoTracker Deep Red; Molecular Probes/Invitrogen, Carlsbad, CA) and prepared as 50 μM, 1 mM, and 1 mM stock solutions in dimethyl sulfoxide, respectively. A cytotoxicity detection kit was obtained from Roche Diagnostics (Mannheim, Germany). Annexin V and propidium iodide (PI) apoptosis assay kits were acquired from Multisciences (Hangzhou, China). Unless otherwise specified, all other reagents were supplied by Sigma-Aldrich (St. Louis, MO).

Cell Culture

iHTM was kindly provided by Dr. Vincent Raymond (Laboratory of Ocular Genetics and Genomics, Québec City, Canada) and GTM³ was obtained as a gift from Prof. Yuhao Peng (glaucoma research; Alcon Laboratory, Fort Worth, TX). Cells were grown in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 (DMEM/F12; Gibco) containing 15% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (100 U/mL penicillin and 100 ng/mL streptomycin) at 37°C and 5% CO₂.

Mitochondria-Targeted Pretreatment and Chronic Oxidative Stress

TM cells at approximately 70–80% confluence were pretreated with MTP-131 (1, 0.1, and 0.01 μM) and incubated at 37°C for 1 hour.24 Chronic oxidative stress was induced by subjecting TM cells to 200 μM H₂O₂ in a serum-free medium for 24 hours. Untreated cells and cells incubated with H₂O₂ alone were used as normal control and H₂O₂ control, respectively.

Evaluation of Cell Viability

Cell viability was evaluated by measuring LDH released from the cells with the a calorimetric assay (Cytotoxicity Detection KitPLUS, Roche Diagnostics) according to the manufacturer’s instructions.15 Both iHTM and GTM³ cells were plated in 96-well plates at a density of 1 × 10⁵/well for 24 hours. After being pretreated with MTP-131 for 1 hour, cells were incubated with 200 μM H₂O₂ for 24 hours. Culture supernatant of each well (50 μL) was transferred to a fresh 96-well plate and 100 μL reaction mixture solution was added to each well and incubated at room temperature for another 30 minutes in the dark. Termination was achieved by adding 50 μL stop solution to each well. The microplate was read under the absorbance at 490 nm (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Cell viability was calculated as follows: cytotoxicity (%) = [experimental value – low control]/(high control – low control) × 100%.

Measurement of ΔΨm

ΔΨm was evaluated with fluorescent probe TMRM, as described previously.24 To quantify ΔΨm by flow cytometry, iHTM and GTM³ cultures (1 × 10⁵ cells/well in a six-well plate) were pretreated with MTP-131 for 1 hour and then incubated with 200 μM H₂O₂ for 2, 4, and 8 hours. At subsequent time points, cells were harvested and suspended in freshly prepared TMRM (100 nM) in phenol and serum-free DMEM/F12 for 30 minutes at 37°C in the dark. All samples were rinsed twice with PBS and analyzed immediately by flow cytometry (excitation/emission [ex/em] = 548/573 nm, FASC Aria; BD Bioscience, San Jose, CA). Ten thousand cells were routinely collected, and the mean fluorescence intensity (MFI) in arbitrary units (AU) from at least three separate experiments was analyzed.

For confocal microscopy, cells were plated in Petri dishes. After various treatments, cells were rinsed twice and loaded with 500 nm TMRM for 30 minutes at 37°C in the dark followed by a 5-minute incubation with Hoechst 33342 (1:1000; Molecular Probes/Invitrogen). After being rinsed twice with PBS, cells were imaged by confocal microscopy using Z-stack mode (LSM 510; Carl Zeiss, Germany; ex/em = 548/573 nm).


FIGURE 2. MTP-131 reduced H₂O₂-induced LDH release in (A) iHTM and (B) GTM³. Cells were pretreated with MTP-131 for 1 hour and then incubated in the absence or the presence of H₂O₂ for 24 hours. Data were expressed as means ± SEM of three independent experiments performed in triplicate (*P < 0.05, ***P < 0.001).
**Measurement of Intracellular ROS**

Intracellular ROS was evaluated with the fluorescent probe H$_2$DCFDA, as described previously.$^{15}$ Briefly, cells were pretreated with MTP-131 for 1 hour and incubated with 200 µM H$_2$O$_2$ at 37°C for 24 hours. Cells were harvested and suspended in freshly prepared 1 µM H$_2$DCFDA at 37°C for 30 minutes in the dark. After being rinsed twice with PBS, cells were analyzed immediately by flow cytometry (ex/em = 488/530 nm). Ten thousand cells were routinely collected, and the MFI in AU from at least three independent experiments was analyzed.

For visualization by confocal microscopy, cells were plated in Petri dishes. After pretreatment with MTP-131 and incubation with H$_2$O$_2$, cells were washed and loaded with 5 µM H$_2$DCFDA at 37°C for 30 minutes in the dark. After being rinsed twice with PBS, cells were imaged by confocal microscopy (ex/em = 488/530 nm).

**Detection of Apoptosis by Annexin V and PI**

Apoptosis was quantified by flow cytometry. Briefly, cells were pretreated with MTP-131 for 1 hour and then incubated with 200 µM H$_2$O$_2$. At the subsequent 4-, 8-, 12-, and 24-hour time points, cells were collected and incubated with 5 µL Annexin V and 10 µL PI according to the manufacturer’s instructions. Annexin V-FITC was analyzed by flow cytometry (ex/em = 488/530 nm) using the FITC channel (FL1).

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/)
and PI staining was analyzed by the PE channel (FL2). Cells were distinguished as viable (Annexin V−/PI−, Q3), early apoptotic (Annexin V+/PI−, Q4), late apoptotic (Annexin V+/PI+, Q2), or dead (Annexin V−/PI+, Q1) cells. The apoptotic rate was calculated as the percentage of early apoptotic cells (Q4%) plus the percentage of late apoptotic cells (Q2%).

**Measurement of Cytochrome c Release**

Release of cytochrome c from mitochondria to cytoplasm was measured by confocal microscopy as previously described. Cells were seeded onto growth cover glasses (Fisherbrand Coverglass; Thermo Fisher Scientific, Waltham, MA) at a density of 2000 cells/chamber. After being pretreated with MTP-131 for 1 hour, cells were incubated with 200 μM H₂O₂ for 6 hours. Freshly prepared red fluorescence dye (200 nm) was loaded into cells in advance and incubated at 37°C for another 30 minutes. Then the cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes and permeabilized in methanol for 5 minutes on ice. After being blocked with 5% BSA for 30 minutes, cells were incubated with mouse monoclonal anti-cytochrome c (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After being rinsed with PBS three times, cells were incubated with conjugated goat anti-mouse IgG (Dylight 488, 1:500; Multisciences) for 30 minutes at 37°C in the dark, followed by Hoechst 33342 for 5 minutes, then washed and mounted with antifade fluorescence mounting medium (Applygen Technologies, Beijing, China). Translocation of cytochrome c from mitochondria to cytoplasm was analyzed by overlapping of costained cytochrome c and highly intense red fluorescent dye.

**Measurement of Caspase 3 Activity by Western Blotting**

Whole cell lysates were prepared for Western blotting of caspase 3 as previously described. Briefly, cells at a density of 2 × 10⁵ cells/mL were plated onto 100-mm plates and incubated at 37°C for 48 hours. After being pretreated with MTP-131 for 1 hour, cells were incubated with 200 μM H₂O₂ for another 12 hours. Then, cells were rinsed and lysated in mammalian cell lysis reagent (Sigma-Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich). Samples (20 μg) were loaded onto a 10% SDS-PAGE gel and electrophoresed at 150 V for 1 hour. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad). After being blocked with 5% nonfat milk for 1 hour, anti-caspase 3 monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA) and anti-β-actin antibody (1:1000; Multiscience Biotech, Hangzhou, China) were applied at 4°C overnight. Bands were visualized by incubation with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:10,000; Multiscience Biotech) and chemiluminescence substrates (ECL Plus; PerkinElmer Inc, Covina, CA). Blots were developed and analyzed with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The protein levels were normalized by β-actin.

**RESULTS**

**MTP-131 Protected iHTM and GTM₃ Cells against H₂O₂-Induced Cytotoxicity**

Incubation with 200 μM H₂O₂ for 24 hours resulted in a significant increase in LDH release in iHTM and GTM₃ cells, which was dose-dependently inhibited by pretreatment with 1, 0.1, and 0.01 μM MTP-131 (Fig. 2).

**MTP-131 Prevented H₂O₂-Induced Mitochondrial Depolarization**

Mitochondrial depolarization is an early event in oxidative stress. Confocal microscopy showed that there was a loss of ΔΨm after incubation with 200 μM H₂O₂ for 2 hours, which was inhibited by pretreatment with 1 μM MTP-131, in both iHTM and GTM₃ cells.

### Table 1. Changes of Mitochondrial Membrane Potential

<table>
<thead>
<tr>
<th>Cell/Time Points (h)</th>
<th>Ctrl</th>
<th>H₂O₂ (200 μM)</th>
<th>MTP-131 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>iHTM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>54.3 ± 0.64</td>
<td>90.7 ± 0.67*</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>34.25 ± 2.46</td>
<td>55.87 ± 1.36*</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>29.01 ± 0.95</td>
<td>53.19 ± 1.29*</td>
</tr>
<tr>
<td>GTM₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>60.3 ± 2.33</td>
<td>75.67 ± 4.18†</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>48.67 ± 1.33</td>
<td>61.00 ± 2.52†</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>38.67 ± 3.48</td>
<td>51.00 ± 2.31†</td>
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* P < 0.001, † P < 0.01, ‡ P < 0.05, compared with cells treated with H₂O₂ alone, one-way ANOVA.
(Fig. 3A). As quantified by flow cytometry, pretreatment with MTP-131 dose-dependently protected iHTM and GTM₃ cells from H₂O₂-induced mitochondrial depolarization (Table 1; Figs. 3B–E).

**MTP-131 Inhibited H₂O₂-Induced Increase in Intracellular ROS**

As illustrated by confocal microscopy, incubation with 200 μM H₂O₂ for 24 hours resulted in a significant increase in ROS, which was significantly decreased by pretreatment with 1 μM MTP-131 (Fig. 4A). Flow cytometry analysis further confirmed that MTP-131 pretreatment dose-dependently reduced ROS production, in both iHTM and GTM₃ cells (Figs. 4B–E).

**MTP-131 Protected against H₂O₂-Induced Apoptosis**

Compared with untreated cells, the percentage of viable cells (Q3%) in H₂O₂-treated cells decreased with the increase of incubation time, accompanied by an increase in the percentage of apoptotic cells (Q2+Q4%), in both iHTM and GTM₃ cells (Table 2; Fig. 5). Pretreatment with MTP-131 dose-dependently inhibited apoptosis, especially early apoptosis, in iHTM cells, and the percentage of apoptotic cells (Q2+Q4%) was significantly lower than that of H₂O₂-treated cells at 4, 8, 12, and 24 hours after incubation. With respect to GTM₃ cells, MTP-131 pretreatment could also alleviate apoptosis at the subsequent 4, 8, 12, and 24 hours. The effect, however, was not as good as that on iHTM cells, and 0.01 μM MTP-131 showed little anti-
apoptotic effect at the 24-hour time point (Table 2), which indicated that MTP-131 pretreatment could partly inhibit apoptosis in GTM3 cells.

**MTP-131 Inhibited H$_2$O$_2$-Induced Cytochrome c Release**

As illustrated by confocal microscopy in Figure 6, cytochrome c was observed in the cytoplasm in iHTM and GTM3 cells after treatment with 200 $\mu$M H$_2$O$_2$ for 6 hours, which was more obvious in GTM3 cells. Pretreatment with 1 $\mu$M MTP-131 for 1 hour could inhibit H$_2$O$_2$-induced cytochrome c release from mitochondria to cytoplasm, in both iHTM and GTM3 cells.

**MTP-131 Inhibited H$_2$O$_2$-Induced Caspase 3 Activation**

Western blot analysis was performed to evaluate the expression of caspase 3. Compared with untreated cell control, caspase 3 was upregulated after a 12-hour incubation with 200 $\mu$M H$_2$O$_2$, whereas pretreatment with 1 $\mu$M MTP-131 for 1 hour could inhibit activation of caspase 3, in both iHTM and GTM3 cells, with a better effect in iHTM cells (Fig. 7).

**MTP-131 Protected TM Cells against H$_2$O$_2$-Induced Morphologic Changes**

As demonstrated by phase-contrast microscopy, most cells showed a shrinking or vacuolar appearance after treatment with 200 $\mu$M H$_2$O$_2$ for 24 hours, with a considerable portion of cells detached from culture dishes. However, cells pretreated with MTP-131 still maintained a fairly healthy appearance, in both iHTM and GTM3 cells (Fig. 8).

**MTP-131 Protected TM Cells from Collapse of Cytoskeleton**

As illustrated by confocal microscopy (Fig. 9), both iHTM and GTM3 cells were immunopositive to F-actin (red) and vinculin (green). Treatment with 200 $\mu$M H$_2$O$_2$ for 24 hours resulted in a significant collapse in cytoskeleton organization and a reduction in the distribution of vinculin. Cells pretreated with MTP-

### Table 2. Summary of Flow Cytometry Data of Apoptosis Measured by Annexin V and PI

<table>
<thead>
<tr>
<th>Cell/Time Points (h)/%</th>
<th>Ctrl</th>
<th>H$_2$O$_2$ (200 $\mu$M)</th>
<th>MTP-131 (1 $\mu$M)</th>
<th>MTP-131 (0.1 $\mu$M)</th>
<th>MTP-131 (0.01 $\mu$M)</th>
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<tbody>
<tr>
<td>iHTM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>97.83 ± 0.12</td>
<td>94.03 ± 0.30</td>
<td>97.97 ± 0.20*</td>
<td>98.73 ± 0.03*</td>
<td>97.40 ± 0.15*</td>
</tr>
<tr>
<td>Q4</td>
<td>0.77 ± 0.17</td>
<td>4.77 ± 0.59</td>
<td>0.97 ± 0.22*</td>
<td>1.17 ± 0.22*</td>
<td>1.37 ± 0.32*</td>
</tr>
<tr>
<td>Q2</td>
<td>1.27 ± 0.26</td>
<td>1.20 ± 0.35</td>
<td>1.05 ± 0.26</td>
<td>1.00 ± 0.25</td>
<td>1.23 ± 0.17</td>
</tr>
<tr>
<td>Q2 + Q4</td>
<td>2.05 ± 0.31</td>
<td>5.97 ± 0.30</td>
<td>2.00 ± 0.17*</td>
<td>2.17 ± 0.05*</td>
<td>2.60 ± 0.15*</td>
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<tr>
<td>GTM3</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Q3</td>
<td>94.07 ± 0.52</td>
<td>82.93 ± 1.66</td>
<td>94.17 ± 1.37*</td>
<td>91.94 ± 1.07*</td>
<td>90.00 ± 1.62*</td>
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<td>Q4</td>
<td>1.27 ± 0.29</td>
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<td>Q2</td>
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<td>Q2 + Q4</td>
<td>5.07 ± 0.62</td>
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<td>6.03 ± 1.48*</td>
<td>7.30 ± 1.04*</td>
<td>9.23 ± 0.59*</td>
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</table>

* $p < 0.001$, † $p < 0.01$, compared with cells treated with H$_2$O$_2$ alone, one-way ANOVA.
Mitochondria-Targeted Protection of POAG

Cell–based glaucoma models in vitro. H2O2 is a key oxidant. Hyperoxia (40% oxygen) is one of the common methods in TM. Virus 40. Both cells have very similar morphologic appearances, profiles of extracellular matrix and cytoskeleton components, and pharmacologic and signal transduction properties compared with such properties of primary untransfected TM cells, suggesting that the transformed cells may be a valid substitute for certain pharmacologic studies of TM. Primary TM cells are usually obtained from donor human eyes, and culturing of GTM cells from glaucomatous TM is more demanding. Since primary cells grow very slowly and stop proliferating after a few passages, only limited amounts of cells are available. Besides, it was reported that TM cells in primary culture could be “activated” in vitro with an increasing amount of mitochondria, compared with normal TM cells in vivo. There were no relevant reports about changes of mitochondria in iHTM or GTM. We focus on mitochondria-targeted protection in this study, so two cell lines were applied instead of primary cell cultures.

It has been reported that diets rich in antioxidants may reduce the risk for age-related disease such as glaucoma. Vitamin E, N-acetylcysteine (NAC), and resveratrol have been reported to be effective in reducing endogenous ROS production in GTM cells. However, vitamin E is lipophilic and tends to be retained in cell membrane and failed to achieve significant intracellular concentrations. NAC is effective in reducing oxidative damage but must be given in millimolar concentration in vitro. To improve efficacy and reduce side effects, a number of approaches have been used to selectively target molecules to mitochondria, including mitochondrial potential dependent and independent methods. For example, lipophilic antioxidants were delivered to mitochondria through potential gradient across the IMM by conjugating with the TPP+ (triphenylalkylphosphonium) cation. However, with the accumulation of these lipophilic cations in the mitochondrial matrix, ΔΨm was disrupted. As a result, the therapeutic index of these molecules is rather low. Another method is mitochondrial potential independent. For example, gramicidin S was used as a peptide vector to improve the targeting of nitroxides to mitochondria. This approach provides an alternative to the lipophilic cation approach for targeted mitochondrial delivery.

In the present study, we examined the mitochondria-targeted protection of MTP-131 against H2O2-induced sustained oxidative stress in TM cells. MTP-131 was proved to have no cytotoxicity in either iHTM or GTM cells, even at the highest concentration (1 mM, stock solution; data not shown). Our results showed that pretreatment with MTP-131 could prevent mitochondrial depolarization, decrease intracellular ROS level, and alleviate apoptosis induced by oxidative stress. We also demonstrated that mitochondria-targeted prevention could inhibit release of cytochrome c from mitochondria to cytoplasm and downregulate the activation of caspase-3. Additionally, cytoskeleton changes caused by chronic oxidative stress were also alleviated after MTP-131 pretreatment.

H2O2 is widely used to induce oxidative stress in experimental studies, which can cause ROS generation and apoptosis. Chronic oxidative stress induced by H2O2 treatment or hyperoxia (40% oxygen) is one of the common methods in TM cell–based glaucoma models in vitro. H2O2 is a key oxidant and a normal constituent that presents in approximately 25–70 μM in the aqueous humor. It has been reported that TM cells were exposed to high concentration of ROS and H2O2 in patients with POAG. Therefore, we used H2O2 to induce chronic oxidative stress in this study. H2O2 is relatively unstable in vitro; thus, to avoid decomposition of H2O2, the stock solution was divided into each tube and stored at 4°C to avoid formation of light; each tube was then discarded. To avoid possible physical interaction between MTP-131 and H2O2, the cells were first pretreated with MTP-131 and then incubated with H2O2. Our results confirmed that sustained oxidative stress resulted in apoptosis of TM cells in vitro, accompanied by disarrangement of cytoskeleton and compromise in TM integrity, which was consistent with previous studies.

iHTM and GTM cell lines used in our study were transformed from normal and glaucomatous TM, by transfecting primary TM cells with an origin-defective mutant of simian virus 40. Both cells have very similar morphologic appearances, profiles of extracellular matrix and cytoskeleton components, and pharmacologic and signal transduction properties compared with such properties of primary untransfected TM cells, suggesting that the transformed cells

**FIGURE 5.** MTP-131 dose-dependently reduced H2O2-induced apoptosis. iHTM and GTM cells were pretreated with MTP-131 for 1 hour and then incubated with H2O2 for 4, 8, 12, and 24 hours. Apoptosis was quantified by flow cytometry measured by Annexin V and PI staining. Q3%: % viable cells; Q4%: early apoptotic cells; Q2%: % late apoptotic cells; Q2+Q4%: % apoptotic cells.
potential in preventing progressive pathogenesis in the TM tissue of POAG. We also found that the oxidative damage in GTM3 cells is more serious than that in iHTM cells under the same conditions. Mitochondria complex I defect might make GTM3 cells more vulnerable to oxidative stress and also contribute to the underreaction of GTM3 cells to antioxidants.

The detailed mechanism of MTP-131 remains unclear. It has been proved that the antioxidant effect of MTP peptides may be attributed to the dimethyltyrosine (Dmt) residue. Dmt bears much structural similarity (methylated phenol) to that of vitamin E. Substitution of Dmt with phenylalanine (Phe) resulted in complete loss of antioxidant activity, suggesting that the phenolic group on tyrosine mediates the...

**Figure 6.** MTP-131 inhibited H$_2$O$_2$-induced cytochrome c release. Cells were pretreated with 1 μM MTP-131 for 1 hour, and then incubated with 200 μM H$_2$O$_2$ for 6 hours. After being loaded with intense red fluorescent dye, cells were fixed with PFA, followed by immunoblotting with cytochrome c antibody. Confocal microscopic analysis showed the release of cytochrome c (green) from mitochondria (red) to cytoplasm in (A) iHTM and (B) GTM3 cells.
scavenging activity. Our previous study suggested that GTM cells were more vulnerable to Ca\(^{2+}\)/H\(_{2}O\(_{2}\) stress, due to mitochondrial dysfunction. Persistent Ca\(^{2+}\)/H\(_{2}O\(_{2}\) overload could induce mitochondrial permeability transition pore (MPTP) opening, which resulted in mitochondrial release of calcium and ROS into the cytosol. By flow cytometry analysis with the cell-permeant dye Fluo-3AM, we found that pretreatment with MTP-131 could inhibit the increase of calcium in GTM cells induced by both acute and chronic oxidative damage (Chen M, unpublished observation, 2010). Since MPTP was composed of cyclophilin D in the matrix, adeninenucleotide translocase in the IMM and the voltage-dependent anion channel in the outer mitochondria membrane, we hypothesized that MTP-131 might interact with the adeninenucleotide translocase in the

**FIGURE 7.** MTP-131 inhibited H\(_{2}O\(_{2}\)-induced caspase 3 activation. Representative Western blotting demonstrated the relative protein abundance of caspase 3 in total cell lysate at 12 hours after H\(_{2}O\(_{2}\) incubation in (A) iHTM and (B) GTM\(_{3}\) cells (mean ± SEM, n = 3, \(\ast P < 0.05, \ast\ast P < 0.01, \ast\ast\ast P < 0.001\)).

**FIGURE 8.** MTP-131 alleviated H\(_{2}O\(_{2}\)-induced morphologic changes. Cells were pretreated with 1 \(\mu\)M MTP-131 for 1 hour, and then incubated with 200 \(\mu\)M H\(_{2}O\(_{2}\) for 24 hours. Phase-contrast light micrographs showed the morphologic changes in iHTM and GTM\(_{3}\) cells (magnification, \(\times\)20 objective).
IMM and regulate the opening of MPTP, which in turn might inhibit calcium overload, mitochondria depolarization, cytochrome c release, caspase 3 activation, and downstream signal pathways. Similar peptide analog should be applied as a parallel control in our future study if it could be provided. Unfortunately, as a novel developed peptide, no antibody, inhibitor, or agonist is currently available. Isotope or fluorescent-labeled peptide might help to find out precisely what is the specific target on the IMM.

In conclusion, MTP-131 showed a significant antioxidant and antiapoptotic effect on TM cells, and could alleviate the disarrangement of cytoskeleton and compromise of cell integrity. Further studies need to be conducted to elucidate the mechanism of MTP-131 and evaluate its protective effect on trabecular meshwork tissues in experimental glaucoma models.

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References


