Neuroprotective Effects of Methyl 3,4-dihydroxybenzoate Against H$_2$O$_2$-Induced Apoptosis in RGC-5 Cells

Xing Zhou$^{1,2}$, Chao-Fen Su$^1$, Zheng Zhang$^3$, Chen-Yu Wang$^4$, Jin-Qi Luo$^4$, Xiao-Wen Zhou$^5$, Liang Cai$^1$, Li Yan$^1$, Wei Zhang$^1$, and Huan-Min Luo$^{1,6,7,*}$

$^1$Department of Pharmacology, School of Medicine, $^2$Department of Clinical Medicine, School of Medicine, $^3$Institute of Brain Sciences, Jinan University, Guangzhou 510632, China
$^4$The Ministry of Basic Medicine, Yichun Vocational and Technical College, Yichun 336000, China
$^5$The First Affiliated Hospital of Jinan University, Guangzhou 510632, China
$^6$Pharmacy Department of GuangZhou Chest Hospital, Guangzhou 510632, China
$^7$The Joint Laboratory of Brain Function and Health, Jinan University and The University of Hong Kong, Jinan University, Guangzhou 510632, China

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Abstract. In the present study, we investigated the protective effect of methyl 3,4-dihydroxybenzoate (MDHB) against H$_2$O$_2$-induced apoptosis in RGC-5 cells. The RGC-5 cells were cultured in plates for 24 h, which were then pretreated with dimethyl sulfoxide, different concentrations of MDHB, or probucol for 12 h prior to addition of 300 µM H$_2$O$_2$ for 24 h. The cell viability was detected by MTT assay. The rate of apoptosis, level of lipid peroxidation, and mitochondrial membrane potential (MMP) were detected by flow cytometry. Western blot analysis was also used to measure the expression level of Bcl-2, Bax, caspase 9, and caspase 3 proteins in H$_2$O$_2$-treated RGC-5 cells. Our study showed that the cell viability of RGC-5 cells significantly decreased after treatment with 300 µM H$_2$O$_2$ for 24 h, but MDHB (8, 16, 32 µM) increased RGC-5 cell survival, suppressed the rate of apoptosis, scavenged reactive oxygen species, and restored MMP. MDHB also obstructed H$_2$O$_2$-induced apoptosis by regulating the expression of Bcl-2 and Bax, as well as suppressing the activation of caspase 9 and caspase 3. Our results showed that MDHB is an effective neuroprotective compound that mitigates oxidative stress and inhibits apoptosis in RGC-5 cells.

Keywords: methyl 3,4-dihydroxybenzoate, retinal ganglion cell, neuroprotection, oxidative stress, apoptosis

Introduction

Retinal degenerative diseases, such as glaucoma or diabetic retinopathy, are characterized by loss of retinal ganglion cells (RGCs) (1). These neurons play key roles in integrating visual information and transmitting the information to the visual center of the brain through the optic nerve. Apoptosis of RGCs results in progressive loss of optic vision, and ultimately blindness. Multiple harmful factors are involved in RGCs apoptosis, including excitotoxicity, hypoxia, oxidative stress, and neurotrophin withdrawal (2–4). Oxidative stress usually resulted from either increased levels of ROS or mitochondrial dysfunction (4). ROS was produced by mitochondria through electron leakage of complex I and III of the oxidative phosphorylation (OXPHOS) pathway (5). Overproduction of ROS would cause mitochondrial dysfunction, which can reciprocally aggravate ROS production, and this vicious circle may cause further deterioration of the disease. Under normal conditions, there was a balance between ROS generation and clearance, and ROS are essential participants in the regulation of the cell signaling pathway (6). When their cellular production, however, overwhelms intrinsic antioxidant...
capacity, damage to normal cells and tissues would occur.

Oxidative stress has been implicated as a causative factor in several clinical conditions including neurodegenerative disorders (7–9). It can increase neuronal susceptibility and lead to glial dysfunction (4). Studies have suggested that oxidative stress is one of the most significant factors inducing pathogenesis of glaucomatous neurodegeneration (11).

To avoid difficulties in the purification of the RGCs, the rat retinal cell line RGC-5 was used. The RGC-5 cell line was established by transforming retinal cells from postnatal day 1 rats with the β2E1A virus (12, 13). This cell line is positive for RGCs markers, such as Thy-1 and Brn-3c, and negative for the Müller glial cell marker glial fibrillary acidic protein (GFAP). This cell line does not recapitulate all electrophysiological features of normal RGCs, but it is sensitive to excitotoxicity and neurotrophin withdrawal (14). Therefore, RGC-5 cells, which are neuronal precursor-like cells, provide a valuable tool to study molecular/biochemical mechanisms underlying the pathogenesis of retinal neurodegenerative diseases (15, 16).

Recently, many efforts have been made to find natural compounds with neuroprotective effects, which are able to scavenge free radicals and protect RGC-5 cells against oxidative stress (1, 17, 18). MDHB is a kind of phenolic acid compounds, and it is extracted from plants such as Kalimeris indica. Our previous study showed that MDHB has a neurotrophic effect on rat primary cortical neurons, indicating neurite-promoting action in vitro (19). Here, we investigated the anti-apoptosis effect of MDHB against H2O2-induced cytotoxicity in RGC-5 cells and the possible underlying mechanism, which would contribute to future drug developments of retinal degenerative diseases.

Materials and Methods

Reagents

RGC-5 cell line was kindly provided by Dr. Mengfei Chen (Zhonghan Ophthalmic Center, Guangzhou, China). High-glucose Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FBS) were purchased from HyClone (Logan, UT, USA). Cell culture plates were from Jet Biofil (Guangzhou, Guangdong, China). MDHB (> 97%; lot No. 10131708) was obtained from the Alfa Aesar Co. (Ward Hill, MA, USA). MTT, probucol, and DMSO were purchased from Sigma (St. Louis, MO, USA). Malondialdehyde (MDA) assay kit and superoxide dismutase (SOD) assay kit were obtained from Nanjing Jiancheng Bioengineering Insti-

Culture of the RGC-5 cells

The RGC-5 cells were maintained in DMEM (HyClone) containing 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were passaged by trypsinization every 2 to 3 days. RGC-5 cells were seeded into 96-well (6.5 × 103 cells/cm²) or 6-well culture plates (8.5 × 104 cells/cm²) and cultured for 24 h. They were then pretreated with DMSO (Sigma), different concentrations (8, 16, 32 μM) of MDHB (Alfa Aesar) or probucol (Sigma) for 12 h prior to addition of 300 μM H2O2 for 24 h.

MTT assay

Cell viability was evaluated by MTT assay. The RGC-5 cells were cultured in a 96-well culture plate at a density of 6.5 × 104 cells/cm² and 300 μM H2O2 was added to the medium for 24 h. Then the supernatant was discarded, and 100 μl DMEM containing 10% MTT (0.5 mg/mL, dissolved in sterile water and filtered through a 0.22 μm membrane; Sigma) was added. After 4 h at 37°C, the formazan crystals were dissolved in 150 μl DMSO (Sigma), and the absorption at 570 nm was measured using a Bio-Rad 400 microplate reader (Bio-Rad, Hercules, CA, USA). Experiments were repeated at least 3 times and compared with control experiments. The results were compared by one-way analysis of variance followed by Dunnett’s t-test.

Flow cytometry was used to detect cell apoptosis

Cell apoptosis was detected by the Annexin V-FITC/PI apoptosis detection kit. The RGC-5 cells were seeded into 6-well culture plates (8.5 × 104 cells/cm²), and cultured for 24 h. Then, the cells were pretreated with DMSO, MDHB (8, 16, 32 μM) or probucol (40 μM) and then treated with 300 μM H2O2 as described above. After 24 h, the cells were collected by trypsinization and stained with the Annexin V-FITC/PI apoptosis detection kit. After staining, the cells were analyzed by flow cytometry.

Measurement of MMP

The MMP was evaluated using JC-1, a cell-permeable cationic dye that penetrates into mitochondria based on the highly negative MMP. At the end of the experiment, 1 × 10⁶ cells were harvested by trypsinization, plates were washed 3 times with PBS, and then centrifugation
at 800 × g for 5 min; subsequently, cells were incubated with JC-1 for 15 min at 37°C in the dark. The MMP was analyzed by flow cytometry.

**Measurement of ROS, MDA, and SOD**

The level of ROS was evaluated by flow cytometric analysis with the fluorescent dye CM-H$_2$DCFDA. MDA, which is one of the most abundant lipid peroxidation productions of RGC-5 cells, was measured by the thiobarbituric acid colorimetric assay. SOD activity was estimated using the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay. After the treatment as described above, cells were collected by centrifugation, washed twice with phosphate-buffered saline (PBS, pH 7.0), and resuspended in 300 μl PBS. They were lysed by sonication, and the suspension was used to measure the levels of MDA and SOD according to the manufacturer’s instructions (Nanjing Jiancheng Co.). All experiments were performed at least 3 times.

**Western blotting analysis**

Cells were lysed in 60 μl ice-cold lysis buffer (Beyotime, Haimen, Jiangsu, China). Lysates were collected by centrifugation at 12,000 × g for 5 min, and protein concentration was determined by a BCA protein assay kit (Beyotime). Samples were stored at −80°C until they were used for western blotting analysis. After adjustment for protein concentration, samples were then fractionated by 12% SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was blocked with 4% skim milk in tris-buffered saline containing 0.1% Tween (TBST) for 2 h at room temperature. Then they were incubated overnight at 4°C with the appropriate primary antibodies, which were diluted at 1:1,000 in 5% BSA (DingGuo, Beijing, China). After washing 3 times with TBST, the PVDF membranes were then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing 3 times with TBST, the blots were then visualized with an ECL reaction kit (Beyotime) and chemiluminescence film (Beyotime). In the western blot analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

**Statistical analyses**

All data are expressed as the mean ± S.D. Statistical analyses were performed by using one-way analysis of variance (ANOVA) followed by the SNK-test and Dunnett’s t-test using SPSS 16.0. A P-value of < 0.05 was considered statistically significance.

**Results**

**MDHB protects RGC-5 cells against H$_2$O$_2$-induced cell viability decrease**

Viability of cultured RGC-5 cells was evaluated using the MTT assay. First, we investigated the effects of MDHB on the viability of RGC-5 cells cultured for 24 h. At the concentration range from 4 to 64 μM, MDHB has no toxicity to RGC-5 cells (Fig. 1A). We therefore used MDHB at concentrations of 8 – 32 μM in our experiments. We then studied the protective effects of MDHB on neurotoxicity induced by H$_2$O$_2$ on RGC-5 cells. In our study, we used proculoc as a positive control, which is a powerful antioxidant. Our study showed that the cell viability of RGC-5 cells significantly decreased after they were treated with 300 μM H$_2$O$_2$ for 24 h, and pretreatment with MDHB (8, 16, 32 μM) for 12 h could enhance cell viability (Fig. 1B). We also investigated the protective effect of MDHB against tert-buthylhydroperoxide (TBHP)-induced oxidative stress in RGC-5 cells. The cell viability was detected by using the MTT assay. The study showed that MDHB (4, 8, 16, 32 μM) could enhance cell viability in a dose-dependent manner (Fig. 1C), which provided more evidence to prove that MDHB can exert protective effects against H$_2$O$_2$-induced oxidative stress.

**MDHB protects RGC-5 cells against apoptosis**

RGC-5 cells were pretreated with MDHB for 12 h, prior to addition of 300 μM H$_2$O$_2$ to the medium for 24 h. To assess that 300 μM H$_2$O$_2$ induced RGC-5 cells apoptosis, Annexin V/PI staining and MMP were detected by flow cytometry. Treatment of RGC-5 cells with H$_2$O$_2$ induced apoptosis, accompanied with mitochondrial depolarization, which was restored to normal values after MDHB (8, 16, 32 μM) administration. Annexin V/PI staining showed that MDHB (8, 16, 32 μM) reduced RGC-5 cell apoptosis compared with H$_2$O$_2$-treated cells (Fig. 2). Furthermore, quantification of the red/green fluorescence ratio showed a significant decrease in H$_2$O$_2$-treated cells relative to that in control cells, and the different concentrations of MDHB could dramatically increase that ratio (Fig. 3). Both of the results showed that MDHB protected RGC-5 cells against apoptosis.

**MDHB protects RGC-5 cells against ROS production and lipid peroxidation**

The level of ROS was analysed by flow cytometry by incubating RGC-5 cells with the fluorescent dye CM-H$_2$DCFDA. H$_2$O$_2$ significantly increased the fluorescent intensity in RGC-5 cells, 1.4-fold relative to the control cells, and that could be suppressed by MDHB.
Lipid peroxide and antioxidant substances were detected by measuring intracellular levels of MDA and SOD. Incubation with MDHB (8, 16, 32 μM) decreased the intracellular level of MDA induced by H₂O₂ (Fig. 4B) and improved the activity of intracellular SOD (Fig. 4C), which catalyzes the dismutation of the superoxide anion into H₂O₂ and molecular oxygen.

**MDHB regulates the expression of Bcl-2, Bax, caspase 9, and caspase 3 in RGC-5 cells**

As the MTT and Annexin V/PI staining results indicated that MDHB prevented apoptosis induced by H₂O₂, we investigated the effects of MDHB on the expression of the Bcl-2 family of apoptosis regulators upon H₂O₂ exposure. In our study, the relative level of Bcl-2, Bax, caspase 9, and caspase 3 proteins were detected by western blotting. In treated RGC-5 cells, MDHB (8, 16, 32 μM) was able to reverse the H₂O₂-induced reduction of Bcl-2 expression (Fig. 5A) and increase the level of Bax expression (Fig. 5B). The level of cleaved-caspase 9 (Fig. 5C) and cleaved-caspase 3 (Fig. 5D) were also detected, and results showed that MDHB suppressed the activation of these proteins.

**Discussion**

Physiological features of the retina, such as a high content of polyunsaturated fatty acids and high oxygen consumption, indicate that retina has a high risk of exposure to oxygen radicals, such as H₂O₂, hydroxyl radicals, and superoxide anions. In turn, the retina contains very high quantities of antioxidants, such as SOD, catalase, glutathione peroxidase, and thioredoxin (20 – 23). In this study, we used H₂O₂ as an exogenous inducer of oxidative stress, which has been used in several studies (24 – 26). Mitochondria are rich in cardiolipin and ROS are produced by mitochondria through the electron leakage pathway. Mitochondria may be the most sensitive primary targets of oxidative injury in neuronal cells (27, 28). Mitochondrial dysfunction, due to excessive production of ROS or the decrease of MMP, has long been implicated in apoptosis (29 – 31). The literature shows that mitochondrion is not only the main place of ROS production but also the main target of ROS damage, so we mainly focus our study on the mitochondria apoptotic pathway. Excessive ROS may lead to the loss of MMP by opening MPTP, then cause the release of Cyt-C from the mitochondrion into the cytosol, and the release of Cyt-C promotes the activation of caspase 9, which in turn causes activation of caspase 3 and subsequent cell apoptosis. That is also the reason we paid attention to caspase 9 and caspase 3.

Previous studies addressed the mechanism of induction of apoptosis in RGC-5 under H₂O₂ treatment. They demonstrated that H₂O₂ induced an increase in MDA, suppressed SOD activity, and induced mitochondrial dysfunction (1, 18, 24). Our data suggest that MDHB has a strong ability to scavenge free radicals. It inhibited protects the retinal cells against H₂O₂-induced cytotoxicity.
ROS production, MDA production, and enhanced the activity of SOD. Evidences showed that SOD activity in the trabecular meshwork decreases in patients with glaucoma and in the retina in experimental ocular hypertension (32, 33). Furthermore exogenous supplementation or overexpression of SOD-1 protects the axotomized RGCs following SOD-1 administration (34, 35).

The Bcl-2 family is the best characterized group of apoptosis-regulating factors, and their role in RGCs survival in acute and chronic models of optic nerve lesion have been well studied (36). Studies suggested that the delicate balance between Bcl-2 and Bax is important for the regulation of cell death or survival (37, 38). An increase of Bcl-2 inhibits the activation of the caspase cascade and apoptosis (39, 40). Our study showed that different concentrations of MDHB significantly increase the expression of Bcl-2 protein and revert the \( \text{H}_2\text{O}_2 \)-induced increase of Bax expression. The balance between Bcl-2 and Bax can alter the permeability of the mitochondrial membrane by regulating the opening of permeability transition pores (PTP). Apoptosis initiators, such as cytochrome C, Smac, or apoptosis-inducing factor (AIF) are released from mitochondria to the cytoplasm, where they trigger the activation of caspases 9 and caspase 3. Activation of these caspases
has a pivotal role in promoting apoptosis (41). Cleaved-caspase 3 is the executor of apoptosis (42). Our results also showed that the levels of cleaved-caspase 9 and cleaved-caspase 3 were decreased after the MDHB treatment, indicating that MDHB can inhibit the activation of pro-caspase 9 and pro-caspase 3.

In our study, our aim was to investigate the neuroprotective effects of MDHB against H$_2$O$_2$-induced apoptosis in RGC-5 cells, and we used several methods to confirm our results. We used the MTT assay to detect the cell viability, flow cytometry to detect the apoptosis of RGC-5 cells, and western blotting to detect the expression of apoptotic proteins. These indicators confirm our experimental results from different aspects. The results demonstrated that MDHB has neuroprotective effects against H$_2$O$_2$-induced apoptosis in RGC-5 cells. MDHB also rescued the decreased MMP and inhibited overproduction of ROS and lipid peroxidation caused by H$_2$O$_2$. Moreover, MDHB suppressed apoptosis by promoting the expression of Bcl-2 while inhibiting that of Bax and caspase-3. Therefore, the neuroprotective mechanism of MDHB may contain two aspects: on the one hand, it acts as an inducer of the anti-apoptotic proteins Bcl-2, and on the other, it acts as a radical scavenger. MDHB is a methyl derivative of protocatechuic acid (PCA), and its lipid solubility is higher than PCA. Previous studies have been concerned about the pharmacokinetics of PCA in rat blood and brain by microdialysis sampling, and their data showed that PCA could be detected in rat brain tissues after oral administration of danshen extract. Those studies indicated that PCA could penetrate the blood–brain barrier (BBB) and exert a neuroprotective effect. From the structural similarity, we speculate that the in vivo bioavailability of MDHB may be similar to or better than that of PCA (43, 44), which is one of the key factors for clinical application of drugs. In our present study, we first found the anti-apoptotic ability of MDHB in RGC-5 cells and its possible mechanisms. It suggested that MDHB may be a useful candidate for developing drugs for the treatment of retinal degenerative diseases.

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**Fig. 5.** Effect of MDHB treatment on the expression of Bax, Bcl-2, cleaved-caspase 9, and cleaved-caspase 3 in H2O2-treated RGC-5 cells. The protein expression was analyzed by western blotting. Results showed that different concentrations of MDHB enhanced the expression of Bcl-2 protein and decreased the expression of Bax, cleaved-caspase 9, and cleaved-caspase 3 proteins. The data are expressed as means ± S.D. *P < 0.05 vs. control cells; **P < 0.01 vs. H2O2-treated cells (n = 4).


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