σ-1 receptor stimulation protects against pressure-induced damage through InsR-MAPK signaling in human trabecular meshwork cells

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Abstract. The purpose of the present study was to investigate the protective effect of the σ-1 receptor (Sig-1R) agonist (+)-pentazocin (PTZ) on pressure-induced apoptosis and death of human trabecular meshwork cells (hTMCs). The expression levels of Sig-1R and insulin receptor (InsR) were examined in hTMCs. Cells were cultured under a pressure of 0, 20, 40, 60 and 80 mmHg for 48 h, and under 80 mmHg for 44 h, after which the cells were treated with (+)-PTZ (20 µM), N-(2-(3,4-dichlorophenyl)ethyl)-N-methyl-2-(dimethylamino)ethyamine (BD-1063; 20 µM) administered 30 min prior to (+)-PTZ, or BD-1063 (20 µM) and then exposed to 80 mmHg again until the 48 h time-point. The changes of the cells were observed by optical and electron microscopy, the apoptosis and death of hTMCs were detected by ethidium bromide/acridine orange dual staining assay and the expression of Sig-1R and InsR by reverse transcription-quantitative polymerase chain reaction and western blot analysis. The phosphorylation of extracellular signal-regulated kinase (ERK), an important downstream protein of the InsR-mitogen-activated protein kinases (MAPK) signaling pathway, was also detected by western blot analysis when (+)-PTZ and BD-1063 were added to the 80 mmHg-treated cells. Sig-1Rs and InsRs were expressed in hTMCs. The apoptosis and death of hTMCs increased from 40 mmHg with 50% cell death when the pressure was at 80 mmHg and the structure of the cells noticeably changed. The expression of Sig-1R and InsR increased along with the elevation of pressure. (+)-PTZ decreased the apoptosis and death of hTMCs and increased the expression of Sig-1R and InsR, and the phosphorylation of ERK. Such effects were blocked by BD-1063. The present study suggested that Sig-1R agonist (+)-PTZ can protect hTMCs from pressure-induced apoptosis and death by activating InsR and the MAPK signal pathway.

Introduction

Primary open angle glaucoma (POAG) is a progressive optic neuropathy often associated with an abnormal elevation of intraocular pressure (IOP) and progressive death of retinal ganglion cells (RGCs) (1,2). The major risk factor for the elevation of IOP is the resistance of aqueous humor (AH) outflow through the trabecular meshwork (TM) (3). On this basis, the preferable treatment for POAG is essentially aimed at maintaining TM function, lowering IOP and protecting RGCs.

Large population-based prevalence and incidence studies (4–6) have identified a positive association between diabetes and POAG. Sato and Roy reported that high glucose levels in the AH of patients with diabetes may increase fibronectin syntheses and accumulation in TM, and accelerate the depletion of TM cells (7). Basic studies (8–11) have also demonstrated that diabetes, not only affects vascular tissues, but also compromises neuronal and glial functions and metabolism in the retina, which ultimately leads to the apoptotic death of retinal neurons including RGCs. This impaired metabolism of neurons and glial by diabetes may render RGCs susceptible to additional stresses including elevated IOP (8). Although there have been a number of explanations (12–15) for this association, more evidence is required to confirm how diabetes influences IOP and glaucoma. In view of this, a previous study detected the change of the insulin receptors (InsRs) in the high air-pressure cell model employed by this current study (16). Insulin works by binding to the cell surface receptor InsR. There are two main intracellular pathways that are activated by InsRs: The InsR substrate (IRS)-phosphatidylinositol 3-kinase (PI3K) pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway (17–19).
The α-1 receptor (Sig-1R), which has been studied thoroughly in the central nervous system (CNS), is also recognized to be overabundant in the eye, including the lacrimal glands, retina, iris-ciliary body, cornea and lens (20). Modulating Sig-1R activity can lower IOP and protect RGCs (21-23). Sig-1R is a 26-kDa protein that is categorized as a unique non-opioid receptor. It is located at the endoplasmic reticulum (ER)-mitochondrion membrane (MAM) (24) and is a modulator of a variety of receptors and ion channels, acting as an amplifier in signal transduction cascades (25). It functions by binding to various types of exogenous and endogenous ligands, including (+)-pentazocin (PTZ), pregnenolone and N-[2-(3,4-dichlorophenyl)ethyl]-N,N,N'-trimethylamine-1,2-diamine (26).

A previous study demonstrated that a Sig-1R agonist decreased IOP and protected against retinal damage in a rat model of chronic ocular hypertension glaucoma (27). Nevertheless, the mechanism of this IOP-lowering effect in vitro remains to be elucidated. It is assumed that this effect may be mediated by the increasing outflow of the AH through TM. In the present study, human trabecular meshwork cells (hTMCs) were cultured under different air pressures and Sig-1R agonist (+)-PTZ and antagonist N-(2-(3,4-dichlorophenyl)ethyl)-N-methylyl-2-(dimethylamino) ethylamine (BD-1063) were administered to the cells at high pressure. It was identified that (+)-PTZ can protect hTMCs from pressure-induced apoptosis and death and that the protection was associated with the activation of InsR and its MAPK pathway. The effects of Sig-1Rs on IOP lowering and RGCs protection may make it an efficient therapy for POAG.

Materials and methods

Drugs. The (+)-PTZ (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in warm 0.1 N HCl and then diluted with pH 7.0 Dulbecco's modified Eagle's medium (DMEM); the BD-1063 (Tocris Cookson Inc., Ellisville, MO, USA) was dissolved in warm 0.1 N HCl and then diluted with pH 7.0 Dulbecco's modified Eagle's medium (DMEM); a mixed-gas (5% CO₂ and 95% air) cylinder (Liming Gas Group Co. Ltd., Harbin, China) was dissolved in distilled water and then diluted with DMEM.

Cell culture. Cells from the immortalized hTMC line were kindly provided as a gift by the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center of Sun Yat-sen University (Guangzhou, Guangdong, China). The cells were grown in culture flasks in Dulbecco’s modified Eagle’s medium with 100 β/ml penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 20 mM/l HEPES buffer and 10% fetal bovine serum (GE Healthcare Life Sciences) in an atmosphere containing 5% CO₂ and 95% air at 37°C. The cells were passed every 3 days using 0.125% trypsinogen and washed twice with 1X PBS, then incubated with a 5% EB and 5% AO mixture for 5 min at room temperature. The cells were then placed onto slides and visualized using a fluorescence microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan). The number of EB- and AO-stained cells were counted and expressed as the ratio of orange-red cells to total cells for late apoptosis and death rate and then averaged over three different fields.

Transmission electron microscopy of hTMCs. Cells cultured under the pressure of 0 and 80 mmHg were respectively dissociated with 0.125% trypsinogen and washed twice with 1X PBS, then incubated with a 5% EB and 5% AO mixture for 5 min at room temperature. The cells were then placed onto slides and visualized using a fluorescence microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan). The number of EB- and AO-stained cells were counted and expressed as the ratio of orange-red cells to total cells for late apoptosis and death rate and then averaged over three different fields.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of Sig-1R and InsR in experimental hTMCs. Experiments were performed to detect the mRNA expression of Sig-1R and InsR in hTMCs which had been cultured under the pressure of 0, 20, 40, 60 and 80 mmHg, and the effects of agonist and antagonist on Sig-1R and InsR.
expression under the pressure of 80 mmHg. Total RNA was isolated using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was reverse transcribed from 1 µg total RNA in 20 µl RT reaction mix using the manufacturer’s protocol (Promega Corporation, Madison, WI, USA). Primers and Taqman probes (Shanghai Chaoshi Biotechnology Co. Ltd., Shanghai, China) for human Sig-1R (NM_005866.2) were 5’-AGCTCACCACCT ACCTCTTGG-3’ (forward primer), 5’-CATGGGCTCTCA GCAGTG-3’ (reverse primer) and 5’-FAM-CTTACAGGA GCGACCCTGAAGG-BHQ1 (Probe); for human InsR (NM_000208.2) were 5’-CAAGAGGAGCTCATCAGCATA-3’ (forward primer), 5’-TCCACCCACTGTGAAGGAGAG-3’ (reverse primer) and 5’-FAM-TAAATGGATGTCCTGTAG TCCAGTGCT-BHQ1 (Probe); for internal control human β-actin (NM_001101.3) were 5’-CCAGCACAATGAA GATCAAGATCAT-3’ (forward primer), 5’-ATCTGCTGGAAG GTGGACACCGA-3’ (reverse primer) and 5’-FAM-TGACAA GTACTCCGTGTTGATCGGCCG-BHQ1 (Probe); qPCR was performed in an ABI Prism 7900 DNA Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cycling variables were: 2 min at 50˚C, 10 min at 95˚C and then 40 cycles of 30 sec at 95˚C and 30 sec at 55˚C. Cycle threshold values of Sig-1R and InsR were normalized to β-actin for each sample and calculated by comparison of 2^ΔΔCq (28).

Western blot analysis of Sig-1R, InsR and phosphorylated and total (p/t) ERK in experimental hTMCs. Experiments were performed to detect the protein expression of Sig-1R and InsR in hTMCs which had been cultured under the pressure of 0, 20, 40, 60 and 80 mmHg, and the effects of agonist and antagonist on Sig-1R, InsR and p/ERK expression under the pressure of 80 mmHg. Cells were lysed on ice in RIPA lysis buffer (Beyotime Institute of Biotechnology) plus 1 protease inhibitor cocktail tablets (Complete and PhosSTOP; Roche Applied Science, Penzberg, Germany) per 10 ml lysis. Lysates were centrifuged at 15,000 x g for 5 min at 4˚C. The protein content of each sample was determined by a spectrophotometer (SmartSpec 3000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent amounts of protein from each sample was boiled with protein loading buffer at 95˚C for 5 min, loaded onto SDS-PAGE gels for electrophoresis and then transferred onto nitrocellulose membrane with a semi-dry transfer cell (Trans-Blot; Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% skimmed milk for 1 h, incubated with anti-Sig-1R monoclonal antibody (cat. no. sc-166392; 1:100), anti-ERK polyclonal antibody (cat. no. sc-710; 1:200), anti-pERK polyclonal antibody (cat. no. sc-16982R; 1:200), anti-InsR polyclonal antibody (cat. no. sc-514302; 1:200; all Santa Cruz Biotechnology Inc., Dallas, TX, USA) and anti-GAPDH polyclonal antibody (cat. no. KC-5G4; 1:1,000; Kangcheng Biotechnology Co., Ltd., Shanghai, China) respectively at 4˚C overnight, and GAPDH (34 kDa) acted as an internal control. Western blotting was performed to detect the protein expression of Sig-1R and InsR in hTMCs (Fig. 1).

Statistical analysis. Statistical analyses were performed with the SAS statistical package for Microsoft Windows version 9.1.3 (SAS Institute, Cary, NC, USA). Data were expressed as mean ± standard deviation for all the measurements. Analysis of variance was used for comparison of experimental groups with control group in EB/AO dual staining assay, RT-qPCR and western blot analysis. The q-test was the post hoc statistical test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Sig-1R and InsR in hTMCs. RT-qPCR and western blot analysis were used to detect the expression of Sig-1R and InsR and demonstrated moderate amounts of Sig-1R and InsR in hTMCs (Fig. 1).

Pressure-induced abnormalities of hTMCs. Pressure-induced changes in hTMCs were significant. The EB/AO staining assay demonstrated that treatment with pressure increased the number of EB-positive cells considerably, and the rates of EB-positive cells to total cells of the former five groups (cells cultured under 0, 20, 40, 60 or 80 mmHg of pressure for 48 h, respectively) were 2.63±1.25, 2.80±1.25, 24.50±2.78, 39.17±1.76 and 57.67±1.04 (P<0.0001 with the exception of the 0 and 20 mmHg groups, Fig. 2Aa-e). (+)-PTZ reduced the pressure-induced cell apoptosis and death (36.00±2.16, P<0.0001; Fig. 2A-f) compared with the 80 mmHg-treated cells without (+)-PTZ. To confirm that cell protection was
Figure 2. Pressure-induced apoptosis and death of hTMCs by EB/AO dual staining assay. Fluorescence images of cells captured under the microscope (x200 magnification), normal cells were stained green (AO positive), advanced apoptotic and dead cells were stained orange-red (EB positive). (Aa-e) Pressure of 0, 20, 40, 60 and 80 mmHg, respectively, for 48 h. (A-f) 80 mmHg plus (+)-PTZ (20 µM) at 44 h. (A-g) 80 mmHg plus BD-1063 (20 µM) 30 min prior to (+)-PTZ at 44 h. (A-h) 80 mmHg plus BD-1063 (20 µM) at 44 h treated cells. (B) Summary of pressure-induced cell apoptosis and death. The quantitative data collected from the fluorescence images are expressed as the mean percentage ± standard error of the mean of the ratio of apoptotic and dead cells to total cells of three different fields of cells, where each field contained 6-150 cells. *P<0.0001 vs. control (0 mmHg pressure); #P<0.0001 vs. each other group. hTMCs, human trabecular meshwork cells; EB/AO, ethidium bromide/acridine orange; PTZ, pentazocin; BD-1063, N-(2-(3,4-dichlorophenyl)ethyl)-N-methyl-2-(dimethylamino)ethylamine.

Figure 3. Pressure-induced morphologic changes of hTMCs by transmission electron microscopy. (A-C) Feature of normal hTMCs is shown. (A) Arrows indicate numerous pinosomes in hTMCs. (B) Arrows indicate abundant organelles (including mitochondria, rough endoplasmic reticulum and Golgi bodies) and a prominent spiked band of heterochromatin present along the nuclear circumference of hTMCs. (C) Arrows indicate multiple cell villous projections at cell surfaces. (D-H) Changes of 80 mmHg pressure treated hTMCs are shown. (D) Advanced stage of apoptosis; arrows indicate abundant apoptotic bodies. (E) Swelling and disappearance of mitochondrial cristae and reduction of endoplasmic reticulum and pinosomes indicated by arrows. Arrows indicate (F) the disappearance of villous projections, reduction of pinosomes and formation of apoptotic bodies, (G) the non-age apoptosis of hTMCs; the formation of apoptotic bodies and (H) indicates another form of non-age apoptosis; aggregation of chromatin and karyopyknosis. hTMCs, human trabecular meshwork cells.
mediated by Sig-1R activation, the 80 mmHg-treated cells were exposed to BD-1063 prior to (+)-PTZ, which apparently increased cell apoptosis and death (65.30±1.48, P<0.0001; Fig. 2A-g) compared with the (+)-PTZ group. These data suggested that BD-1063 was able to block the protective effect of (+)-PTZ. The 80 mmHg-treated cells that were exposed only to BD-1063 experienced almost total apoptosis and death (99.07±1.07, P<0.0001; Fig. 2A-h) compared with the 80 mmHg-treated cells, which suggested that antagonism of Sig-1R can cause pressure associated with cell death. In Fig. 2B, data are presented as the mean percentage ± standard error of the mean in three fields of cells, where each field contained 60–150 cells (P<0.0001 between each two groups with the exception of the 0 and 20 mmHg groups).

Pressure-induced morphologic changes of hTMCs were observed by transmission electron microscopy. Normal hTMCs exhibited multiple cell villous projections at cell surfaces, abundant organelles (including mitochondria, rough ER and Golgi bodies), numerous pinosomes, clear nucleoli and prominent spiked bands of heterochromatin along the nuclear circumference (Fig. 3A-C). The ultra-microstructural changes observed in the 80 mmHg-treated group were the formation of apoptotic bodies, swelling and disappearance of mitochondrial cristae, reduction of ER and pinosomes, aggregation of chromatin and karyopyknosis (Fig. 3D-H). The images indicated that high pressure-treated cells were undergoing an apoptotic procedure.

Pressure-induced change of Sig-1R and InsR mRNA expression in hTMCs. RT-qPCR analysis of Sig-1R mRNA expression of seven groups of cells (0, 20, 40, 60 and 80 mmHg for 48 h, respectively, 80 mmHg plus (+)-PTZ (20 µM) at the 44 h, 80 mmHg plus BD-1063 (20 µM) 30 min prior to (+)-PTZ at the 44 h) demonstrated that the amount of Sig-1R under pressures of 60 and 80 mmHg were 11- to 12-fold higher compared with the 0, 20 and 40 mmHg groups; (+)-PTZ upregulated the Sig-1R mRNA levels of 80 mmHg group, while the upregulation was reversed by BD-1063 (P<0.0001; Fig. 4). The results suggested that short-term exposure to high pressure can cause upregulation of Sig-1R mRNA expression in vitro.

The expression of InsR mRNA in the seven groups of cells was consistent with that of Sig-1R; the amount of InsR under pressures of 60 and 80 mmHg were 3- to 4-fold higher compared with the 0, 20 and 40 mmHg groups. Furthermore, the change in InsR mRNA levels coincided with those of Sig-1R when administering (+)-PTZ/BD-1063 (P<0.0001; Fig. 4).

Pressure-induced change of Sig-1R, InsR and p/t ERK protein expression in hTMCs. Western blot analysis of Sig-1R and InsR protein (the seven groups of cells as with RT-qPCR analysis above) were consistent with the result of RT-qPCR (P<0.05; Fig. 5A). This indicated that high pressure can also cause the upregulations of Sig-1R and InsR protein expression in vitro. To explore the association between Sig-1R and InsR signaling, the phosphorylation of ERK (an important downstream protein of the InsR-MAPK signal pathway) was analyzed by western blotting. The data demonstrated that (+)-PTZ caused an increase of Sig-1R itself, InsR and pERK protein expression (P<0.05; Fig. 6; tERK was unchanged). To confirm whether this effect was mediated by Sig-1R, BD-1063 was used 30 min prior to (+)-PTZ and the results demonstrated that the effect was attenuated by BD-1063 (P<0.05; Fig. 6). It was notable that...
Sig-1R ligands modulated InsR and pERK expression, which indicated that Sig-1R served its functions partly by activating InsR and its MAPK signal pathway.

Discussion

A previous study demonstrated that Sig-1R agonist can decrease IOP and protect against retinal damage in a rat model of chronic ocular hypertension glaucoma (26). Numerous researchers (29-31) have performed experiments to study the anti-apoptotic mechanism of Sig-1R agonist on RGCs, however, the mechanism of IOP lowering remains to be fully elucidated. The present study identified that the Sig-1R agonist (+)-PTZ protects hTMCs from pressure-induced apoptosis and death by activating InsR and its MAPK pathway.

Elevated IOP is the main risk factor for the development and progression of glaucomatous damage (32,33). It is hypothesized that elevated IOP results from increased aqueous outflow resistance and is due to several morphologic changes in the TM, including loss of TMCs (34,35). As a result, it was considered useful to observe what TMCs experience under the high air-pressure directly. In the present study, hTMCs were cultured under high pressure (80 mmHg) and it was observed that the cells underwent an apoptotic process, with 50% cell death at 48 h. A loss of the hTMCs, followed by substitution with extracellular matrix (ECM), may contribute to an increased resistance to AH outflow (36,37). Morphologic changes were also observed in hTMCs cultured under high pressure. Among the abnormalities, the reduction of pinosomes was notable and possibly meant reduced phagocytic activity in glaucomatous TMCs, which would induce increased deposition of ECM material in the outflow pathway (38). Previous studies also suggested that these structural changes in the TM and a decrease in TMCs are also triggered by apoptosis (35,39,40).

These hypotheses, together with the findings of the present study, indicate that TMCs serve a key role in maintaining the
balance of drainage pathways, that air-pressure over 40 mmHg can cause a decrease in hTMCs and a disequilibrium between the cells and ECM and that such disequilibrium can concomitantly cause increased IOP. As a result, finding a way to protect hTMCs from apoptosis and death is important.

Sig-1R has been studied thoroughly in CNS, where it acts as a neuroprotector. In the eye, Sig-1Rs are located in the lacrimal glands, retina, iris-ciliary body, cornea and lens, and the effects of modulating their activities include lowering IOP and protecting RGCs against stress (15-17). Bucolo et al (22) demonstrated that topical agonists [(+)-PTZ and flunarizine ] caused a significant dose-related reduction of IOP in ocular normotensive and hypertensive albino rabbits. A previous study also demonstrated that intraperitoneal injection of a Sig-1R agonist (pregnenolone) reduced IOP in a chronic ocular hypertension rat model (27). It was hypothesized that this may be caused by the increasing AH outflow through the other quadrants of TM with normal episcleral vein pressure. Thus, it was required to perform in vitro experiments to explain the phenomenon. The present study demonstrated that air-pressure over 40 mmHg may contribute to the apoptosis and death of hTMCs. The Sig-1R agonist (+)-PTZ can prevent the process of apoptosis and maintain the quantity and structure of these cells, which is beneficial for the homeostasis of TM. A short time exposure to high air-pressure of hTMCs caused an increase of Sig-1Rs, which may be beneficial to the survival of the cells. This result corresponds to the hypothesis that, besides ligands, Sig-1Rs can also respond for cellular stress, including deprivation of glucose, depletion of calcium in ER and oxidation (24); high pressure being a type of stress.

For years, an increasing number of epidemiological studies demonstrated that patients with diabetes exhibit an increased risk of developing POAG (15,41-44). A possible reason for the impairment of InsRs in patients with diabetes inducing InsR signaling dysfunction, may result in decreased phosphorylation of protein kinase B and ERK1/2. This decrease may cause apoptosis and death of hTMCs, unbalanced homeostasis of TM and an increase in the resistance of AH outflows. The present study also observed that the expression of InsR was increased with the upregulation of ERK1/2, a protein kinase B and ERK1/2. This decrease may cause apoptosis and maintain the quantity and structure of these cells, which is beneficial for the homeostasis of TM. A short time exposure to high air-pressure of hTMCs caused an increase of Sig-1Rs, which may be beneficial to the survival of the cells. This result corresponds to the hypothesis that, besides ligands, Sig-1Rs can also respond for cellular stress, including deprivation of glucose, depletion of calcium in ER and oxidation (24); high pressure being a type of stress.

Previous studies have demonstrated that the Sig-1R is primarily located at the MAM (46-48). Activation and cellular stress promote redistribution of the receptors from MAM to other subcellular locations, including the periphery of endoplasmic membranes, the vicinity of the cell membrane or nuclear envelopes. This relocation possibly increases the number or types of proteins with which the Sig-1R can interact. The results from the current study may provide a new protein, InsR, interacting with the Sig-1R, a hypothesis which requires further elucidation.

In conclusion, the present study demonstrates that high pressure can induce hTMCs apoptosis and death, which is one of the causes of the progression of POAG. The finding that the Sig-1R agonist (+)-PTZ protects hTMCs from pressure-induced apoptosis and death by activating InsR and the MAPK signal pathway, together with the retina protecting effect of the receptor, may make the Sig-1R agonist a superior therapeutic for POAG in the near future.

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