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Neuroligin-3 protects retinal cells from H_2O_2-induced cell death via activation of Nrf2 signaling

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ABSTRACT

Intensified oxidative stress can cause severe damage to human retinal pigment epithelium (RPE) cells and retinal ganglion cells (RGCs). The potential effect of neuroligin-3 (NLGN3) against the process is studied here. Our results show that NLGN3 efficiently inhibited hydrogen peroxide (H_2O_2)-induced death and apoptosis in human RPE cells and RGCs. H_2O_2-induced reactive oxygen species (ROS) production, lipid peroxidation and DNA damage in retinal cells were alleviated by NLGN3. NLGN3 activated nuclear-factor-E2-related factor 2 (Nrf2) signaling, enabling Nrf2 protein stabilization, nuclear translocation and expression of key anti-oxidant enzymes (*HO1*, *NOQ1* and *GCLC*) in RPE cells and RGCs. Further results demonstrate that NLGN3 activated Akt-mTORC1 signaling in retinal cells. Conversely, Akt-mTORC1 inhibitors (RAD001 and LY294002) reduced NLGN3-induced *HO1*, *NOQ1* and *GCLC* mRNA expression. Significantly, Nrf2 silencing by targeted shRNAs reversed NLGN3-induced retinal cytoprotection against H_2O_2. We conclude that NLGN3 activates Nrf2 signaling to protect human retinal cells from H_2O_2. NLGN3 could be further tested as a valuable retinal protection agent.

1. Introduction

Reactive oxygen species (ROS) over-production induces oxidative damage to resident human retinal pigment epithelium (RPE) cells and retinal ganglion cells (RGCs) [1–3], serving as a main contributor of retinal degeneration [1–3]. Oxidative stress further induces lipid peroxidation, calcium overload and DNA damage, eventually causing retinal cell death and apoptosis [4–8]. Reversely, ROS scavenging efficiently protects retinal cells from oxidative stress [4–8]. Our group has been testing novel agents to protect human retinal cells from oxidative stress [4–8].

Neuroligin-3 (NLGN3), a member of NLGN family protein [9,10] and a type I membrane protein, is also a cell adhesion protein [11]. NLGN3 locates at neuronal postsynaptic membrane, which is essential for synapse formation [12,13]. Studies have recently shown that NLGN3 is a novel and key mitogen, which is sufficient and necessary to promote cell survival and growth [12]. Further studies demonstrate that NLGN3 activates several receptor tyrosine kinases (RTKs) and downstream signalings (*i.e.* PI3K-Akt-mTOR) to promote cell survival [12–14]. The potential activity of NLGN3 in human retinal cells has not been studied thus far.

Nuclear-factor-E2-related factor 2 (Nrf2) signaling is a key cellular defense mechanism against oxidative stress [15–17]. Once activated, Nrf2, a transcription factor, enters cell nuclei to promote transcription and expression of multiple anti-oxidative enzymes [15–17]. The Nrf2-dependent genes, including *heme oxygenase-1* (*HO1*), *γ-glutamyl cysteine ligase catalytic subunit* (*GCLC*) and *NAD(P)H quinone oxidoreductase 1* (*NQO1*) [15], have potent anti-oxidant activity [15–17].

Nrf2 activation is tightly regulated by its suppresser protein Keap1, the latter is an adaptor protein of the Cul3-ubiquitin ligase complex, dictating Nrf2 cytosol ubiquitination and proteasomal degradation [15–17]. Activated Nrf2 disassociates with Keap1, enabling Nrf2 stabilization, accumulation and nuclear translocation [15–17]. This will then lead to Nrf2-ARE (anti-oxidant response element) binding and transcription of Nrf2-dependent genes [15–17]. Here, we show that NLGN3 treatment in RPE cells and

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RGCs induces Nrf2 signaling activation, which inhibits oxidative stress by hydrogen peroxide (H_2O_2).

2. Materials and methods

2.1. Reagents, chemicals and antibodies

Hydrogen peroxide (H_2O_2), NLGN3 and puromycin were purchased from Sigma-Aldrich (St. Louis, Mo). All the antibodies were purchased from Cell Signaling Tech (Beverly, MA). Carboxy-H2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), a cell-permeable fluorescence ROS probe, was obtained from Invitrogen (Carlsbad, CA). Cell culture reagents were purchased from Gibco BRL (Rockville, MD). Akt-mTOR inhibitors LY294002 and rapamycin were obtained from Selleck (Beijing, China). Trizol reagents were from Invitrogen (Carlsbad, CA). All the *mRNA primers* were reported previously [4,18–20].

2.2. Cell culture

Cultures of human retinal pigment epithelial cells ("RPE cells", the ARPE-19 cell line) and primary human RGCs were described in our previous studies [4,18,19,21]. The primary human RGCs at passage 3–7 were utilized for the *in vitro* experiments. Written-informed consent was obtained from the donor patient. The protocols of using human retinal cells were according to Declaration of Helsinki, approved by the Ethics Review Board of Nanjing Medical University.

2.3. Quantitative real-time PCR

Cells with the indicated treatment were incubated with Trizol reagents to obtain the total cellular RNA. The detailed protocol of the quantitative Real-Time PCR ("qRT-PCR") assay of the RNA was described previously [4]. The melt curve analysis was always performed to calculate product melting temperature. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* expression was tested as the internal control. We utilized the $^{-2\Delta\Delta}$Ct method to quantify expression change of targeted-mRNA.

2.4. Cell viability assay and cell death assay

Cells with indicated treatment were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay, and the MTT optical density ("OD") at 490 nm was recorded to reflect cell viability. Cell death was analyzed by a two-step lactate dehydrogenase (LDH) assay [1,3,5]. Cell death ratio was tested by LDH release in the medium (% of total LDH).

2.5. Quantification of cell apoptosis

Quantification of cell apoptosis by the Histone-bound DNA apoptosis ELISA assay was described previously in our studies [22–24]. ELISA OD at 405 nm was recorded.

2.6. Western blotting assay

Western blotting assay protocol was described previously [7,23,25]. The exact same amount of protein lysates (30 μg per sample) was loaded to each lane of SDS-PAGE gels. The same set of lysates were run in sister gels to detect different proteins. For data analysis, the total gray of each band was quantified via the ImageJ software (NIH, Bethesda, MD), which was always normalized to the loading control band [7]. For the detection of nuclear proteins, cell nuclei were isolated by the nuclei isolation kit from Sigma-Aldrich [23].

2.7. ROS assay

The detailed protocol was described previously [22,23]. Briefly, retinal cells with indicated treatment were incubated with 1 μM of carboxy-H2DCFDA fluorescent dye at the room temperature under the dark for 60 min. The DCF fluorescence intensity OD was assayed by a Fluorimeter Fluoroscan Ascent II (Labsystem, Finland) at 550 nm.

2.8. Lipid peroxidation assay

Thiobarbituric acid reactive substances (TBAR) content assay was utilized to quantify cellular lipid peroxidation. Following treatment, the cellular TBAR, a product of toxic aldehyde from oxidative fatty acyl-degradation malondialdehyde (MDA), was tested via the previously described protocol [26,27]. The TBAR absorbance OD was measured at 550 nm.

2.9. DNA damage assay

The phosphorylated-γ-H2AX ("p-γ-H2AX") intensity was tested to reflect cellular DNA damage levels [28]. Briefly, after the indicated treatment, cells were trypsinized and fixed, incubating with FITC-conjugated anti-p-γ-H2AX (Ser139) antibody (Cellular Signaling Tech). p-γ-H2AX percentage was tested by a FACS machine (BD Pharmingen, San Diego, CA) [28]. p-γ-H2AX ratio was gated.

2.10. shRNA-mediated stable knockdown of Nrf2

The two distant lentiviral Nrf2 shRNAs, targeting non-overlapping sequence of human *Nrf2,* were purchased from Santa Cruz Biotech (sc-37030-V/"shNrf2-a" and sc-44332-V/"shNrf2-b", Santa Cruz, CA) [19]. The Nrf2 shRNA lentivirus was added to cultured retinal cells. After 24 h, puromycin (1.0 μg/mL) was included to select the stable cells for three more passages. The knockdown efficiency of Nrf2 shRNA in the stable cells was verified by Western blotting assay/qRT-qPCR assay.

2.11. Statistical analysis

Quantitative results were normalized, data were presented as mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS 18.0, Chicago, IL). To determine significance between two treatment groups, the two-tailed unpaired *t*-test was applied. Significance was chosen as $\boldsymbol{p} < 0.01$.

3. Results

3.1. NLGN3 protects human RPE cells and RGCs from H_2O_2

First, ARPE-19 cells, the established human RPE cells [5,6], were treated with NLGN3. MTT cell viability assay results show that NLGN3 treatment at tested concentrations (1–100 ng/mL) had no significant effect on the viability of APRE-19 cells (Fig. 1A). Further studies show that NLGN3 failed to induce LDH release (the indicator of cell death, Fig. 1B) nor cell apoptosis (tested by Histone DNA ELISA assay, Fig. 1C) in APRE-19 cells. On the other hand, treatment with H_2O_2 (300 μM) induced significant cell death (Fig. 1A and B) and apoptosis (Fig. 1C) in ARPE-19 cells.

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