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Hydroxyl radicals cause fluctuation in intracellular ferrous ion levels upon light exposure during photoreceptor cell death

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ABSTRACT

Iron accumulation is a potential pathogenic event often seen in age-related macular degeneration (AMD) patients. In this study, we focused on the relationship between AMD pathology and concentrations of ferrous ion, which is a highly reactive oxygen generator in biological systems. Murine cone-cells-derived 661W cells were exposed to white florescence light at 2500 lx for 1, 3, 6, or 12 h. Levels of ferrous ions, reactive oxygen species (ROS), and hydroxyl radicals were detected by RhoNox-1, a novel fluorescent probe for the selective detection of ferrous ion, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), and 3′-*p*-(aminophenyl) fluorescein, respectively. Reduced glutathione, total iron levels and photoreceptor cell death were also measured. Two genes related to iron metabolism, transferrin receptor 1 (*TfR*1) and H ferritin (*HFt*), were quantified by RT-PCR. The effects of ferrous ion on cell death and hydroxyl radical production were determined by treatment with a ferrous ion chelating agent, 2,2′-bipyridyl. We found that the ferrous ion level decreased with light exposure in the short time frame, whereas it was upregulated during a 6-h light exposure. Total iron, ROS, cell death rate, and expression of *TfR* and *HFt* genes were significantly increased in a time-dependent manner in 661W cells exposed to light. Chelation with 2,2′-bipyridyl reduced the level of hydroxyl radicals and protected against light-induced cell death. These results suggest that light exposure decreases ferrous ion levels and enhances iron uptake in photoreceptor cells. Ferrous ion may be involved in light-induced photoreceptor cell death through production of hydroxyl radicals.

1. Introduction

Iron plays critical roles in diverse biological processes in living cells, such as Fe/S clusters, erythropoietic function (Hegde et al., 2011), oxidative metabolism (Khandakar et al., 2013) and cellular immune response (Kortman et al., 2012; Munoz et al., 2011). To maintain iron homeostasis, the dynamics of cellular iron metabolism are modified by iron-regulatory proteins through the regulation mRNA stability for Tfr and translation for ferritin (Casarrubea et al., 2013). However, some factors such as aging, hypoxia, and genetic polymorphisms might trigger the dysregulation of homeostasis of intracellular iron (Synowiec et al., 2012), and the resulting excess iron can produce hydroxyl radicals (˙OH) in the presence of hydrogen peroxide via Fenton chemistry Equation (1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-} \quad (1)$$

The hydroxyl radical is the most harmful reactive oxygen species (ROS), which can cause oxidative damages to various cellular macromolecules, including carbohydrates, lipids, proteins, and nucleic acids, that can lead to cell death and diseases (Kell, 2010; Valavanidis et al., 2009). Local dysregulation of iron homeostasis appears to be associated with several neurodegenerations, such as Parkinson's disease (Jellinger et al., 1993; Sofic et al., 1991) and Alzheimer's disease (Rolston et al., 2009). In addition, iron accumulation has been reported in the retina of age-related macular degeneration (AMD) patients (Song and Dunaief, 2013), and genetic polymorphism of the iron-regulatory protein-1 and -2 genes increase risk of dry type of AMD (Synowiec et al., 2012). Previously, we and another group have reported that some anti-oxidant agents (Imai et al., 2010; Yamauchi et al., 2011) and deferiprone (DFP) (Song et al., 2012), an iron chelation agent, showed significant protective effects against light-induced retinal degeneration, which

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is widely used as a model for dry type AMD (Nakanishi et al., 2013). However, the physiological and pathophysiological roles of the ferrous ion have not been sufficiently explored, partially due to a lack of appropriate methods for visualizing intracellular ferrous ion.

Recently, we have presented a novel turn-on fluorescent probe (RhoNox-1) for the selective detection of ferrous ion based on *N*-oxide chemistry (Hirayama et al., 2013): RhoNox-1 shows high selectivity for ferrous ion over other metal species such as alkali, alkali earth, and first row transition metal ions including ferric and zinc ions. We also confirmed that RhoNox-1 was inert against biologically abundant reductants and ROS as well, indicating its high selectivity for ferrous ion. In the present study, we investigated the involvements of intracellular ferrous ion and hydroxyl radical in light-induced photoreceptor cell death using the new ferrous ion detecting chemical tool, RhoNox-1, along with the highly reactive oxygen species (hROS) probe, 3′-p-(aminophenyl) fluorescein (APF).

2. Material and methods

2.1. Cell culture

A transformed mouse cone-cell line (661W cells) derived from mouse retinal tumors was a kind gift from Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA) which is widely used as *in vitro* model for the study of cone photoreceptor cell biology and associated diseases because it expressed SV40 T antigen, blue and green cone pigments, transducin, and cone arrestin and blue and green opsins (Tan et al., 2004). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika) under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were passed by trypsinization every 2 or 3 days.

2.2. Light irradiation to 661W cells

The cells were seeded at 3×10^4 cells/ml in all assays. After incubating for 24 h, the entire medium was replaced with fresh medium containing 1% FBS, and the cells were exposed to 2500 lx of white fluorescent light (Nikon, Tokyo, Japan, spectral peaks are 403, 435, 546 and 577 nm).

2.3. Intracellular ferrous ion imaging by RhoNox-1

The cells were seeded at 1.5×10^4 cells per well in a slide chamber plate. After incubating for 24 h, the entire medium was replaced with fresh medium containing 1% FBS, and the cells were exposed to 2500 lx of white fluorescent light (Nikon, Tokyo, Japan) for 1, 3, 6, or 12 h at 37 °C. The entire medium was then replaced with Hank's balanced salt solution (HBSS; Gibco, Carlsbad, CA, USA) containing calcium and magnesium without phenol red and 5 μM RhoNox-1 (prepared from a 1 mM stock solution in DMSO; Nacalai Tesque, Kyoto, Japan). After incubation for 30 min at 37 °C, confocal fluorescence images were acquired with a Zeiss LSM 700 laser-scanning microscope system (Carl Zeiss, Oberkochen, Germany). Experiments were performed with a 40× oil-immersion objective lens. Fluorescence intensity was quantified with an Image J system.

2.4. Cell death assay

661W cells were seeded at 3×10^3 cells per well in 96-well plates and then incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS, and the cells were exposed to 2500 lx of white fluorescent light for 1, 3, 6, or 12 h at 37 °C. Nuclear staining assays were carried out at the end of the light-exposure treatment. Hoechst 33342 (λ_{ex} = 360 nm, λ_{em} > 490 nm) and propidium iodide (PI) (λ_{ex} = 535 nm, λ_{em} > 617 nm) were added to the culture medium for 15 min at a final concentrations of 8.1 μM and 1.5 μM, respectively. Hoechst 33342 freely entered living cells and then stained the nuclei of viable cells, as well as those that had undergone apoptosis or necrosis. PI is a membrane-impermeable dye that is generally excluded from viable cells. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells was determined, and the percentage of PI-positive cells was calculated.

2.5. Measurement of reactive oxygen species (ROS) production in 661W cells

Intracellular radical activation in 661W cells was measured using 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen, Carlsbad, CA, USA). After 1, 3, 6, or 12 h of light irradiation, 10 μM of CM-H_2DCFDA was added to the culture medium, giving a final concentration of 10 μM, and incubated at 37 °C for 1 h. The 96-well plate was loaded into a plate holder in a fluorescence spectrophotometer (Varioskan: Thermo Fisher Scientific, Waltham, MA, USA). The reaction was carried out at 37 °C, and fluorescence was measured at 488 nm excitation and 525 nm emission at the end of light irradiation and 1 h after light irradiation. The number of cells was determined by Hoechst 33342 staining and used to calculate ROS production per cell.

2.6. Intracellular hydroxyl radical imaging by 3′-p-(aminophenyl) fluorescein (APF)

661W cells were seeded at 1.5×10^4 cells per well in a slide chamber plate, then incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS, and the cells were exposed to 2500 lx of white fluorescent light (Nikon, Tokyo, Japan) for 1, 3, 6, or 12 h at 37 °C. The entire medium was then replaced with HBSS (Gibco) containing calcium and magnesium without phenol red. Then 5 μM APF (Sekisui Medical Ltd., Tokyo, Japan; prepared from 1 mM stock solution in DMSO) was added. After incubation for 1 h at 37 °C, confocal fluorescence images were acquired with a Zeiss LSM 700 laser-scanning microscope system. Experiments were performed with a 40× oil-immersion objective lens. Fluorescence intensity was detected with an Image J system (Hirayama et al., 2013).

2.7. RNA isolation

The cells were seeded in 24-well plates at a density of 1.5×10^4 cells per well. After the cells had been incubating for 24 h, the entire medium was replaced with fresh medium containing 1% FBS, then they were exposed to 1, 3, 6, or 12 h of light exposure at 2500 lx. Total RNA was extracted by NucleoSpin RNA II (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. RNA quantity and quality were determined using a NanoVue Plus (GE healthcare Japan, Tokyo, Japan). Single-stranded cDNA was synthesized using a PrimeScript RT Master Mix (Takara).

2.8. Real-time PCR

Quantitative real-time PCR was performed using the TAKARA Thermal Cycler Dice® Real Time System TP800 with an SYBR Premix Ex Taq™ II (Takara), according to the manufacturer's protocol. The thermal cycler conditions were as follows: 10 min at 95 °C, followed by two-step PCR for 45 cycles consisting of 95 °C for 15 s followed by 60 °C for 1 min. The results were expressed relative to the *gapdh*