Redox Biology 8 (2016) 98–109

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

The novel triterpenoid RTA 408 protects human retinal pigment epithelial cells against H_2O_2-induced cell injury via NF-E2-related factor 2 (Nrf2) activation

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ARTICLE INFO

Article history:
Received 29 August 2015
Received in revised form
16 December 2015
Accepted 16 December 2015
Available online 19 December 2015

Keywords:
Retinal pigment epithelial cells
Oxidative stress
RTA 408
Nrf2

ABSTRACT

Oxidative stress-induced retinal pigment epithelial (RPE) cell damage is an important factor in the pathogenesis of age-related macular degeneration (AMD). Previous studies have shown that RTA 408, a synthetic triterpenoid compound, potently activates Nrf2. This study aimed to investigate the protective effects of RTA 408 in cultured RPE cells during oxidative stress and to determine the effects of RTA 408 on Nrf2 and its downstream target genes. Primary human RPE cells were pretreated with RTA 408 and then incubated in 200 μM H_2O_2 for 6 h. Cell viability was measured with the WST-8 assay. Apoptosis was quantitatively measured by annexin V/propidium iodide (PI) double staining and Hoechst 33342 fluorescent staining. Reduced (GSH) and oxidized glutathione (GSSG) were measured using colorimetric assays. Nrf2 activation and its downstream effects on phase II enzymes were examined by Western blot. Treatment of RPE cells with nanomolar ranges (10 and 100 nM) of RTA 408 markedly attenuated H_2O_2-induced viability loss and apoptosis. RTA 408 pretreatment significantly protected cells from oxidative stress-induced GSH loss, GSSG formation and decreased ROS production. RTA 408 activated Nrf2 and increased the expression of its downstream genes, such as HO-1, NQO1, SOD2, catalase, Grx1, and Trx1. Consequently, the enzyme activities of NQO1, Grx1, and Trx1 were fully protected by RTA 408 pretreatment under oxidative stress. Moreover, knockdown of *Nrf2* by siRNA significantly reduced the cytoprotective effects of RTA 408. In conclusion, our data suggest that RTA 408 protect primary human RPE cells from oxidative stress-induced damage by activating Nrf2 and its downstream genes.

1. Introduction

Age related macular degeneration (AMD), a leading cause of blindness in the elderly, is a disease in which there is progressive loss of central vision [1]. AMD occurs in two major forms, the dry form and the wet form [2]. Anti-vascular endothelial growth factor (VEGF) antibody therapy has revolutionized the treatment of wet AMD [3]. However, dry AMD treatment remains a major challenge. Currently, the only treatment for dry AMD is the use of the Age-Related Eye Disease Study (AREDS)-based vitamin formulation, which includes vitamin C, vitamin E, zinc oxide, cupric oxide, lutein, and zeaxanthin [4,5]. However, this formulation does not reverse vision loss but only lowers the risk of developing advanced stages of AMD in certain patients. Therefore, identifying novel therapeutic targets and development of novel therapeutic molecules for AMD are urgently needed.

Oxidative stress-induced retinal pigment epithelial (RPE) cell death is an early event in the development of AMD [6]. The RPE cells remain in a quiescent state throughout life. RPE cells present at birth are constantly exposed to years of oxidative damage before the onset of AMD. Therefore, RPE are very sensitive to oxidative damage, often induced by external sources like UV light and internal sources like reactive oxygen species (ROS) produced by the electron transport chain. Proteins are the main targets of free radicals due to their high abundance and their high reactivity with ROS. As oxidative stress defense systems deteriorate with age, oxidatively modified proteins gradually accumulate underneath the RPE adjacent to the basement membrane and lead to drusen

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http://dx.doi.org/10.1016/j.redox.2015.12.005

formation, which is the hallmark of AMD [7]. Thus, understanding the function of antioxidant pathways in the retina is critical for developing new therapies for AMD.

One of the crucial antioxidant pathways involved is the nuclear factor (erythroid-derived-2)-like 2 (Nrf2) pathway. Nrf2 is a 65 kDa molecule with a basic leucine zipper structure. Normally, Nrf2 in its inactive state is kept in the cytoplasm bound to kelch-like ECH-associated protein 1 (Keap1) [8,9]. With a half-life of only 20 min, Nrf2 is constantly targeted for ubiquitination by Keap1 with consequential degradation via the proteasome. When the cell is in an oxidative stress environment, oxidative stress oxidizes Keap1's active site cysteine residues, preventing Keap1 from interacting with Nrf2. With the accumulation of Nrf2 in the cytoplasm, Nrf2 moves to the nucleus where it binds to the small Maf protein and the antioxidant response element (ARE). Activation of ARE leads to the transcriptional activation of several other antioxidant enzymes and proteins, such as NADPH dehydrogenase (NQO1), heme oxygenase-1 (HO-1), glutaredoxin 1 (Grx1), and thioredoxin 1 (Trx1) [10]. All these enzymes are distinguished by their ability to reverse oxidative damage and stress. NADPH dehydrogenase transforms enzymes and proteins back into their reduced state by the exchange of electrons between NADPH and NADP [11]. HO-1 may be involved indirectly in the antioxidant system by converting heme to other products such as iron (II), carbon monoxide, and biliverdin [12]. Glutaredoxin and thioredoxin are two distinct yet similar systems. Although they are both involved in reducing oxidized protein thiols and allowing proteins to return to their functional state, Grx1 is considered as a vital antioxidant enzyme, considering its essential locations in both the cytoplasm [13,14], the intermembrane space of mitochondria [15], and possibly, the nucleus. Therefore, drugs enabling and amplifying the Nrf2 system are thought to be promising therapies for AMD and other degenerative diseases that rely on the delicate balance of oxidative species in the cell.

RTA 408 represents a novel class of therapeutics that has the potential to increase Nrf2 expression and thereby increase expression of antioxidant enzymes. RTA 408 is a member of the synthetic oleanane triterpenoid compounds. It is currently under clinical investigation for the prevention of cataract surgery-induced loss of corneal endothelial cells, prevention of radiation-induced dermatitis in breast cancer patients undergoing radiotherapy, treatment of solid tumors including melanoma and lung cancer, and treatment of Friedreich's Ataxia and mitochondrial myopathies. Previous studies have demonstrated that RTA 408 has significant cytoprotective effects attributed to the activation of the Nrf2 pathway [16–19]. The present study investigates the connection between RTA 408 and the Nrf2 pathway as well as multiple antioxidant enzymes in RPE cells. This will help determine whether RTA 408 may serve as a potent therapy for AMD and other degenerative eye diseases.

2. Methods

2.1. Materials

The 2-cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic acid (CDDO) derivative RTA 408 was >98% pure (Reata Pharmaceuticals, Inc., Irving, TX, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and 0.05% trypsin and other cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Antibodies listed below were used: anti-Nrf2 (Cell Signaling, Beverly, MA, USA, #12721), anti-NQO1 (Cell Signaling, #3187), anti-Bax (Cell Signaling, #2772), anti-Bcl2 (Cell Signaling, #2876), anti-cleaved caspase 3 (17 kDa) (Cell Signaling, #9664), anti-HO-1 (Cell Signaling, #5061), anti-Grx1 (Abcam, Cambridge, MA, USA, ab45953), anti-Trx1 (Abcam, ab86255), anti-PSSG (Virogen, Watertown, MA, USA, 101-A-100), anti-SOD2 (signal, HPA001814), anti-catalase (Abcam, ab16731), anti-GAPDH (Santa Cruz, Santa Cruz, CA, USA, sc-32233), anti-B23 antibodies, and horseradish peroxidase-conjugated secondary antibodies (sc2061, sc2060, sc2030; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. Human retinal pigment epithelial (RPE) cell culture

Human fetal RPE cells were purchased at passage one from ScienCell™ Research Laboratories (Carlsbad, CA, USA), and all experiments were performed with cells between passages two to eight. The cells were maintained in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml of penicillin. Cell cultures were maintained at 37 °C in a humid atmosphere incubator with 5% CO_2 and 95% air. The medium was changed every 3-4 days. For H_2O_2-induced apoptotic studies, cells were synchronized by gradual serum deprivation with the following procedure: cells were cultured overnight in DMEM with 2% FBS followed by incubation in serum-free medium for 30 min before exposure to a bolus of 200 μM H_2O_2 for 6 h.

2.3. Cell viability assay

Cell viability was measured by a colorimetric cell viability kit (Promokine, Heidelberg, Germany) with the tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which can be bioreduced to a water-soluble orange formazan dye by dehydrogenases present in the viable cells. The amount of formazan produced is directly proportional to the number of living cells. Cells were seeded at a density of 5000 cells/well (100 μl total volume/well) in a 96-well assay. Cells were incubated with or without RTA 408 (1–100 nM) for 24 h and then treated with 200 μM of H_2O_2 for 6 h. After treatment, 10 μl of WST-8 solution was added to each well of the culture plate and incubated for 2 h in the incubator. The absorption was evaluated at 450 nm using a microplate reader (BioTek, Winooski, VT).

2.4. Hoechst 33342 fluorescent staining

Cells were plated on poly-L-lysine coated glass coverslips for overnight and then incubated with or without RTA 408 (1–100 nM) for 24 h followed by 200 μM H_2O_2 treatment for 6 h. The cells were washed twice with ice cold PBS and then fixed with cold 4% paraformaldehyde for 15 min followed by PBS wash. The fixed cells were then stained with 0.1 μg/mL Hoechst 33342 (Invitrogen, Grand Island, NY) for 10 min and rinsed with PBS. The images were taken using a fluorescence microscope (Olympus, Center Valley, PA).

2.5. Flow cytometry analysis of cell apoptosis

Cells were seeded in 100 mm culture plates and incubated overnight at 37 °C and then incubated with or without RTA 408 (1–100 nM) for 24 h followed by 200 μM H_2O_2 treatment for 6 h. After that, the cells were trypsinized and stained with annexin V and PI using annexin V apoptosis Kit (Invitrogen, Grand island, NY) according to the manufacturer's protocol. The stained cells were then analyzed by flow cytometer (FC500, Beckman Colter, Indianapolis, IN) to differentiate among viable (annexin V^-/PI^-), early apoptotic (annexin V^+/PI^-), late apoptotic (annexin V^+/PI^+) cells, and necrotic cells (annexin V^-/PI^+).