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Calcium Overload Is A Critical Step in Programmed Necrosis of ARPE-19 Cells Induced by High-Concentration H_2O_2

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Objective Oxidative stress plays an important role in retinal pigmental epithelium (RPE) death during aging and the development of age-related macular degeneration. Although early reports indicate that reactive oxygen species (ROS) including H_2O_2 can trigger apoptosis at lower concentrations and necrosis at higher concentrations, the exact molecular mechanism of RPE death is still unclear. The purpose of this study was to investigate the molecular pathways involved in RPE death induced by exogenous ROS, especially at higher concentrations. **Methods** Cultured ARPE-19 cells were treated with H_2O_2 at different concentrations and cell viability was measured with the MTT assay. Cell death was morphologically studied by microscopy using *APOPercentage* assay and PI staining. Furthermore, the impact of oxidative stress on ARPE-19 cells was assessed by HO-1 and PARP-1 Western blotting and by the protection of antioxidant EGCG. Calcium influx was determined using the fura-2 calcium indicator and the role of intracellular calcium overload in ARPE-19 cell death was evaluated following cobalt treatment to block calcium effects. **Results** H_2O_2 reduced the viability of ARPE-19 cells in a concentration-dependent manner, which was presented as a typical s-shaped curve. Cell death caused by high concentrations of H_2O_2 was confirmed to be programmed necrosis. Morphologically, dying ARPE-19 cells were extremely swollen and lost the integrity of their plasma membrane, positively detected with *APOPercentage* assay and PI staining. 24-hour treatment with 500 μmol/L H_2O_2 induced remarkable up-regulation of HO-1 and PARP-1 in ARPE-19 cells. Moreover, antioxidant treatment using EGCG effectively protected cells from H_2O_2-induced injury, increasing cell viability from 14.17%±2.31% to 85.77%±4.58%. After H_2O_2 treatment, intracellular calcium levels were highly elevated with a maximum concentration of 1200nM. Significantly, the calcium channel inhibitor cobalt was able to blunt this calcium influx and blocked the necrotic pathway, rescuing the ARPE-19 cell from H_2O_2-induced death. **Conclusions** At high concentrations, H_2O_2 induces ARPE-19 cell death through a regulated necrotic pathway with calcium overload as a critical step in the cell death program.

Key words: Apoptosis; ARPE-19 cell; Necrosis; Oxidative-stressed injury; Hydrogen peroxide

INTRODUCTION

The RPE monolayer is exposed to high levels of visible light and oxygen under normal circumstances[1], therefore RPE is at a risk for oxidative damage. Although visible light does not directly damage DNA or proteins causing cellular demise, it can lead to the oxidation of key constituents *via* reactions with endogenous photosensitizers, including porphyrins, flavins, and, of specific importance to the RPE, lipofuscin[2-5]. Additional oxidative stress to RPE also results from phagocytosis of photoreceptor outer segment membranes, which are rich in polyunsaturated fatty acids that may undergo peroxidation[6]. Therefore, accumulated oxidative damage in the largely non-mitotic RPE monolayer is likely to cause tissue dysfunction that in turn might contribute to diseases such as age-related macular degeneration (AMD)[7-8].

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates[9]. As to reactive oxygen species, they can be typically defined as molecules or ions formed by the incomplete one-electron reduction of oxygen. ROS includes free radicals such as superoxide, hydroxyl radical, and singlet oxygen, as well as non-radical species such as hydrogen peroxide[10]. Oxygen free radicals are highly reactive and have the capacity to damage cellular components such as proteins, lipids, and nucleic acids[11]. ROS can also activate ion channels within the cell, including Ca^{2+} channels[12]. Mounting evidence shows that calcium channels can respond to pharmacological compounds that reduce or oxidize thiol groups on the channel protein and reactive oxygen species such as hydrogen peroxide and superoxide can mediate changes in calcium channel

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function via alterations of such thiol groups[13]. As a result of calcium channel opening, excessive calcium influx into the cytosol from the extracellular compartment or internal stores can occur after ROS exposure. Although calcium is a well-recognized second messenger necessary for signaling cellular responses, in excessive amounts it can be deleterious to function, causing cell death[14].

The death of cells can be classified into apoptotic or necrotic cell death[15]. While apoptosis is known as programmed active cell death, necrosis is viewed as passive cell death. In contrast to apoptosis, necrosis is characterized by the rapid loss of plasma membrane integrity, cell/organelle swelling, mitochondrial dysfunction[16], and the lack of typical apoptotic features such as cell shrinkage and DNA fragmentation[17]. Although necrosis is known to occur under a variety of pathological conditions, little effort has been made to study necrosis due to the belief in its unregulated nature. However, programmed 'occurrence' of necrotic cell death has been documented in some papers, including the newly proposed concept of necroptosis[18-20]. Exogenous hydrogen peroxide (H_2O_2) is commonly used to induce oxidative stress in cells. This study revealed that ARPE-19 cells exposed to strong oxidative stress (caused by high-concentration H_2O_2) exhibited typical features of necrosis, but interestingly this H_2O_2-induced necrosis was further shown to be a regulated process with cellular calcium overload as a critical step.

MATERIALS AND METHODS

Materials

Human retinal pigment epithelial cells (ARPE-19) were purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). Dulbecco's modified Eagle Medium (DMEM), F12 medium, Phosphate-buffered saline (PBS), penicillin/streptomycin (P/S), and fetal bovine serum (FBS) were purchased from Invitrogen Inc (Invitrogen-Life Technology, Rockville, MD). Hydrogen peroxide, MTT [3-(4,5-dimethylthiazol-2-l) -2,5-diphenyltetrazolium bromide], PI (Propidium iodide), trypsin/EDTA, Fura-2, HO-1 and PARP-1 antibody were purchased from Sigma (St. Louis,MO, USA). Proteinase inhibitor was from Roche (Roche Diagnostics, Laval, Quebec, QC, Canada). *APOPercentage*™ assay kit was purchased from Biocolor (Belfast, Northern Ireland). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

ARPE-19 Cell Culture

The human retinal pigment epithelial cell line ARPE-19 was purchased from the American Type Culture Collection (Manassas, VA). ARPE-19 cells within 10 passages from the time of purchase were cultured in a 1:1 mixture of Dulbecco modified Eagle Medium and Ham F12 (DMEM/F12) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 0.1 mg/mL streptomycin (both Invitrogen), at 37 ℃ in 5% CO_2. Cells were passaged twice weekly.

H_2O_2 Treatment

ARPE-19 cells were seeded at 3×10^4 cells/mL onto 96, 24 or 6-well plates and grown for 48 hours to sub-confluence. The cells were treated with 1 to 10 mM hydrogen peroxide in culture media (Sigma, St. Louis, MO) for up to 32 hours as required. For the control group, cells were treated with only culture medium.

MTT Assay

The MTT reduction assay was used as an index of cell viability and was performed as described previously[21]. Briefly, control and H_2O_2- treated ARPE-19 cells were incubated in serum-free medium containing 0.4 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]. During this period of time, mitochondrial and cytosolic dehydrogenases of living cells reduced the yellow tetrazolium salt (MTT) to a purple formazan dye suitable for spectrophotometric detection. After one hour, the MTT solution was aspirated and dimethylsulfoxide (0.3 mL/well) was added. Optical densities of the supernatant were read at 575 nm using a microplate spectrophotometer (Spectra Max 340; Molecular Devices, Sunnyvale, CA). Absorbances were normalized to untreated control cultures representing 100% viability.

APOPercentage™ Assay and PI Staining

The APOPercentage™ assay (Biocolor Ltd., Belfast, Northern Ireland) was performed according to the manufacturer's protocol. When the membrane of apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis under microscopy. For PI staining, cells were cultured in 24-well plate for 24 h, then treated with PI solution (final concentration: 2 μg/mL). After incubating for 10 min at room temperature in the dark, PI-positive cells were scored by using an inverted fluorescence microscope (Leica, Germany).

Measurements of Intracellular Calcium Concentration ($[Ca^{2+}]_i$)

To measure the $[Ca^{2+}]_i$ response of ARPE-19 cells to H_2O_2, a Fura-2 assay was performed as described previously[22-23]. Briefly, cells on coverslips were loaded with 1.25 mol/L Fura-2/AM by immersing the cells in Tyrode's solution (100 mmol/L NaCl, 10 mmol/L KCl, 1.2 mmol/L NaH_2PO_4, 5 mmol/L $MgSO_4$, 20 mmol/L glucose, 10 mmol/L taurine and 10 mmol/L MOPS) containing pluronic F-127 (0.025% W/V) for 30 min at 37 ℃. Then the coverslip was mounted into a chamber on the stage of an inverted fluorescence microscope (Nikon TE2000S, Japan) with a xenon lamp and CCD camera (CoolSNAP DG-4, Photomatrics, USA). Fura-2 emission (510 nm) was obtained by continuous rapid alternating excitation at 340 and 380 nm, and the 340/380 ratios were used to calculate $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ standard calibration curve was obtained using the Fura-2 Calcium Imaging Calibration Kit (Invitrogen). Images were recorded and analyzed using MetaMorph/Fluor software (Molecular Devices Co., USA).

Protein Extraction and Western Blot Assay

Protein was isolated from confluent ARPE-19 cells growing on 6-well plates by washing in PBS at 4 ℃ and then adding lysis buffer (50 mmol/L Tris-HCl [pH 8], 150 mmol/L NaCl, 0.02% N_3Na, 100 μg/mL phenylmethylsulfonyl fluoride, 1% NP-40, 50 mmol/L NaF, 2 mmol/L EDTA, and proteinase inhibitor [Roche Diagnostics, Laval, Quebec, QC, Canada]). Cell lysates were collected as previously described[24]. Reducing SDS-PAGE and Western blotting was performed as previously described[25]. Membranes were probed using anti-HO-1 or anti-PARP-1 antibodies (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:1 000 with anti–rabbit horseradish peroxidase-conjugated antibody as secondary reagent (1:2 000). Detection was performed using the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, IL). To ensure equal protein loading, nitrocellulose membranes were stripped (REblot; Chemicon International, Temecula, CA) and reprobed with actin antibody at a dilution of 1:5 000 (Cedarlan e Laboratories, Hornby, ON, Canada).

Statistical Analysis

All data are given as the mean ± SEM of at least three experiments. For multigroup comparisons, ANOVA followed by Bonferroni test was performed. $P<0.05$ was considered statistically significant.

RESULTS

Effect of Hydrogen Peroxide on the Cell Viability

Hydrogen peroxide reduced viability of ARPE-19 cells in a concentration-dependent manner, which was presented as a typical S-shape curve. H_2O_2 at low concentrations (<300 μmol/L) gradually reduced cell viability, but at higher concentrations (300-500 μmol/L), a remarkable reduction of cell viability was detected. Above 500 μmol/L H_2O_2, cellular viability had only a smooth decline since most of the cells were dead and mitochondria had lost their cellular metabolic ability (Fig. 1).

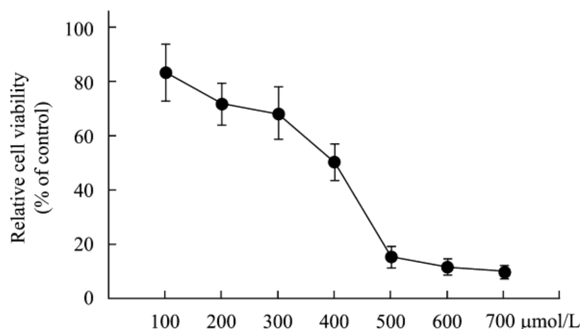


FIG. 1. The viability of ARPE-19 cells is reduced following treatment with hydrogen peroxide at various concentrations for 24 h, determined using the MTT assay. Results are expressed as a percentage of the control, and are the means ± SEM (n=48, three individual cultures).

Cell Death Pattern Induced by Hydrogen Peroxide

The death of ARPE-19 cells caused by high concentrations of H_2O_2 exhibited typical morphological features of necrosis. As shown in Fig. 2B, dying ARPE-19 cells were extremely swollen, and numerous cells had lost plasma membrane integrity, as indicated by *APOPercentage* dye staining. In contrast, apoptotic cells displayed shrinkage rather than swollen cell bodies (Fig. 2C). Necrosis was further confirmed by use of PI staining, which specifically labeled condensed nuclei and was a marker for plasma membrane integrity. As shown in Fig. 2Bb, necrotic cells exhibited positive PI staining of the nuclei with intensive red fluorescence.

Intracellular Oxidative Stress

Treatment with high concentrations of H_2O_2 caused severe oxidative stress in ARPE cells. The cellular redox status was assessed by HO-1 and PARP-1 Western blot. As shown in Fig. 3A, after treatment with 500 μmol/L of H_2O_2 for 24 h, HO-1, an intracellular marker in response to oxidative stress,

was significantly up-regulated. PARP-1, another nuclear enzyme involved in DNA repair after oxidative damage, was strongly up-regulated, indicating that nuclear DNA was massively damaged by the exogenous ROS (Fig. 3B). Furthermore, antioxidantive treatment with EGCG effectively protected ARPE cells from H_2O_2 injury, increasing cell viability from 14.17% ±2.31% to 85.77% ±4.58% (Fig. 3C).

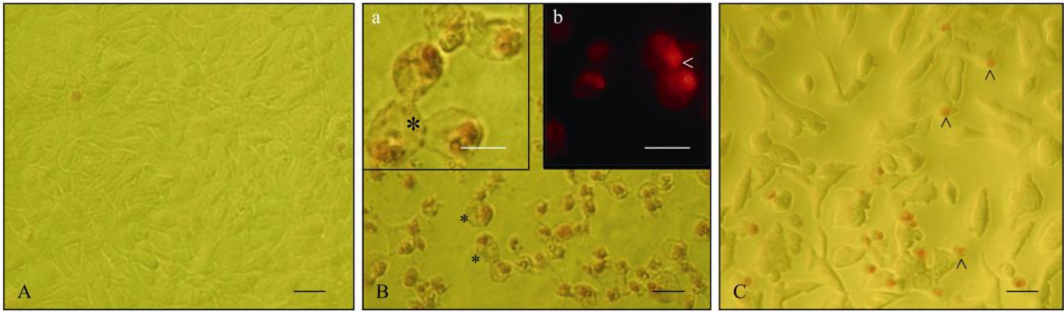
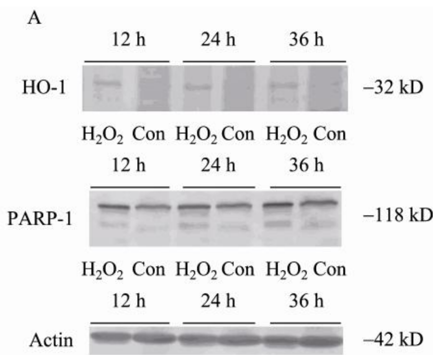


FIG. 2. Hydrogen peroxide induces necrosis in ARPE-19 cells. The *APOPercentage* assay was used to analyze cell death. A. Normal Control: ARPE-19 cells cultured in normal media for 24 h did not display staining. Scale bars=30 μm. B. Necrotic cells: ARPE-19 cells treated with 500 μmol/L H_2O_2 for 24 h were stained positive for *APOPercentage* dye (red) with swollen body and condensed nuclei as typical features of necrosis, denoted with asterisks (*). B(a) high magnification. B(b) PI staining was used to further confirm necrotic cell death, denoted with an arrow (<). Scale bars=30 μm. C. Apoptotic cells: RGC-5 cells cultured in glucose-free media for 48 h were positively stained in red but cells showed shrink bodies which is an important feature of apoptosis (^). Scale bars=30 μm.



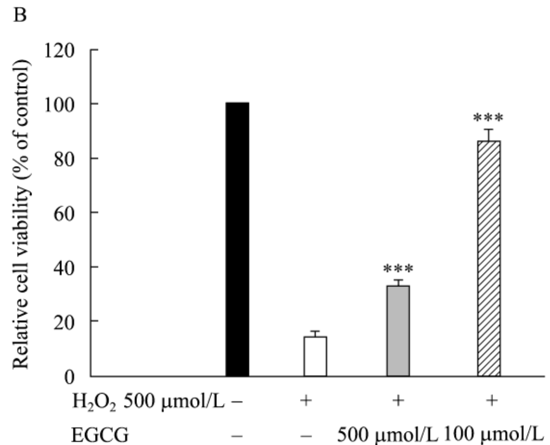


FIG. 3. Hydrogen peroxide leads to an increase in oxidative stress in ARPE-19 cells. A: ARPE-19 cells were cultured in normal media as control or treated with 500 μmol/L H_2O_2 for 24 h, and HO-1 and PARP-1 expression level were then examined by Western blotting. The levels of HO-1 and PARP-1 protein were significantly unregulated after 24 h treatment with 500 μmol/L H_2O_2. Con: control; H_2O_2: treated with 500 μmol/L H_2O_2. B: ARPE-19 cells were pre-cultured in normal media with EGCG (50 or 100 μmol/L) and then treated with 500 μmol/L H_2O_2 for 24 h. Cellular viability was determined by MTT assay. Pre-treatment with 50 or 100 μmol/L EGCG significantly increased cell viability against oxidative-stressed injury. The results are expressed as the percentage of control cells, and are mean values ± SEM (n=48, three separate cultures). (***P<0.001, one-way ANOVA and Bonferroni test).

Overload of Intracellular Calcium

Calcium influx is a critical molecular step in the H_2O_2-induced necrosis of ARPE-19 cells. As shown in Fig. 4A,B, treatment with 500 μmol/L of H_2O_2 caused a massive increase of $[Ca^{2+}]i$ (about 10-fold) within 100 sec, with maximal $[Ca^{2+}]i$ levels reaching nearly 1 200 nmol/L. As a calcium channel blocker, cobalt significantly blunted the entry of extracellular calcium, reducing maximal $[Ca^{2+}]i$ levels to 560 nmol/L. Furthermore and most interestingly, treatment with 100 μmol/L cobalt prior to H_2O_2 application was able to significantly attenuate ROS-induced cell death, increasing cellular viability from 16.81% ±3.25% to 88.47% ±6.15% of control treated cells as determined with the MTT assay (Fig. 4B).

DISCUSSION

Treatment with hydrogen peroxide decreases the

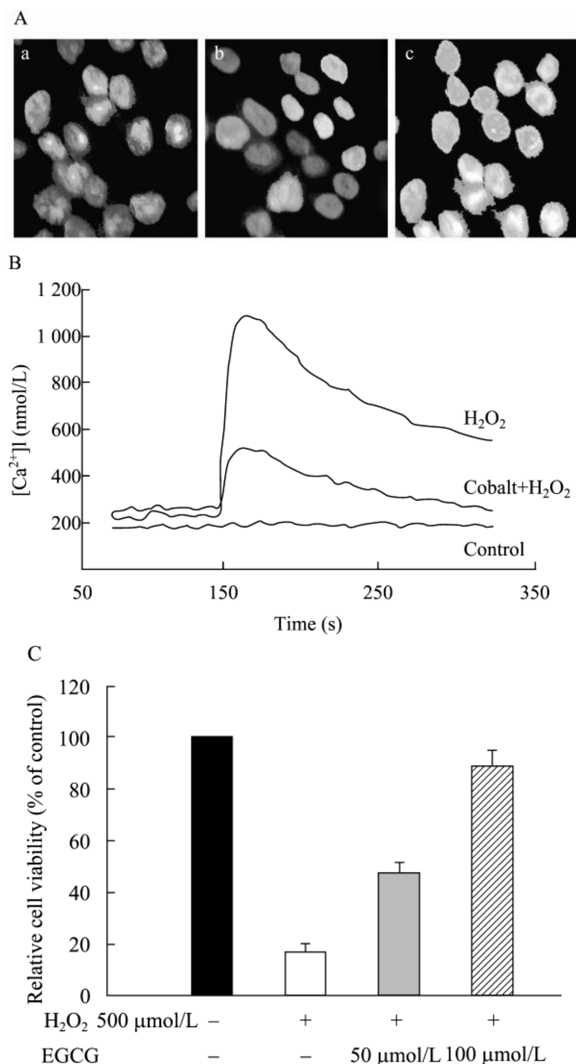


FIG. 4. Hydrogen peroxide causes massive calcium influx in ARPE-19 cells. A: The calcium influx was indicated by fura-2 with an inverted fluorescence microscope. a: control; b: treated with 500 μmol/L H_2O_2; c: pretreated with cobalt 100 μmol/L and then treated with 500 μmol/L H_2O_2. B: Expanded overlay of typical responses. ARPE-19 cells were cultured in normal media with or without 100 μmol/L cobalt and then treated with 500 μmol/L H_2O_2. The intracellular free calcium concentration was measured using the fura-2/Am methodology. $[Ca^{2+}]$i was significantly increased by 500 μmol/L H_2O_2. This increase was blocked by addition of 100 μmol/L cobalt resulting in calcium channel inhibition. Scale bar: 30-second each pulse. C: ARPE-19 cells were pre-cultured in normal media with cobalt (50 or 100 μmol/L) and then treated with 500 μmol/L H_2O_2 for 24 h. Cell viability was determined by MTT assay. Pre-treatment with 50 or 100 μmol/L cobalt significantly increased cell viability following oxidative insult. The results are expressed as the percentage of control cells, and are mean values ± SEM (n=48, three separate cultures). (P<0.001, one-way ANOVA and Bonferroni test).

viability of ARPE-19 cells in a concentration-dependent manner. Findings from a previous study demonstrated that hydrogen peroxide at relatively low concentrations caused apoptosis in ARPE-19 cells in the form of DNA fragmentation and caspase-3 activation. However, high-concentrations of H_2O_2 mainly induced necrotic cell death, characterized by the presence of swollen organelles and loss of plasma membrane integrity[26]. Results from our study are consistent with these previous findings. We observed that ARPE-19 cells were highly resistant to injuries caused by low-concentration of H_2O_2. However, prolonged treatment increased death in ARPE-19 cells, with a typical apoptotic morphology. In addition, we found that H_2O_2 at concentrations over 400 μmol/L was able to cause massive cell death associated with typical features of necrosis, including a swollen cell body, ruptured membrane and condensed nuclei. Interestingly, although the APO Percentage assay was originally used to specifically identify apoptotic cells[27], it was found in our study that swollen plasma membranes and condensed nuclei in necrotic cells could also be clearly stained with APO Percentage dye, thus helping to morphologically differentiate cell death under the microscope.

More importantly, it was further shown by our study that necrosis in ARPE-19 cells caused by high-concentrations of hydrogen peroxide was a regulated process. First, the elevation of cellular ROS could be an initiating step to trigger necrotic cell death, since exogenous H_2O_2 could enter the cells and cause severe oxidative stress. In support of this assumption, we found that H_2O_2-induced injuries were attenuated by the pretreatment with anti-oxidant. The role of ROS in necrotic cell death has been identified in some previous studies[28-32] and oxidative radical stress itself is known to further amplify the production of ROS within mitochondria in a vicious cycle[33]. ROS production may therefore directly oxidize cellular proteins including calcium channels, thus initiating cell death processes through affecting various signaling cascades[34]. Second, the expression and function of cellular enzymes are greatly influenced by high-concentration of H_2O_2. After ARPE-19 cells were treated with 500 μmol/L H_2O_2 for 24 h, we detected that HO-1 and PARP-1 were significantly up-regulated. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradation[35]. Excess free heme is released from heme-proteins under oxidative stress and may contribute to oxidative stress due to its ability to catalyze the formation of reactive oxygen species (ROS)[36]. In various model systems, HO-1 induction confers protection from further oxidative injuries

while its down-regulation accelerates cellular injuries[37]. Furthermore, DNA strand breakages triggered by oxidative stress activate the nuclear enzyme poly (ADP-ribose) polymerase (PARP). However, PARP activation may dramatically lower the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rate of glycolysis, electron transport, and subsequently ATP formation. This process can result in cell dysfunction and cell death[38]. Therefore, these alternations indicate that some cellular proteins or enzymes may be involved in the process of necrosis in ARPE-19 cells. Third, calcium overload is a critical molecular step in H_2O_2-induced necrosis. We detected that levels of intracellular calcium were drastically elevated in ARPE cells after the treatment with H_2O_2 and that the calcium channel inhibitor, cobalt, was capable of effectively blocking this signaling pathway, rescuing ARPE-19 cells from death. Hara Y previously reported a widely expressed Ca^{2+}-permeable cation channel, LTRPC2, which was activated by high concentrations of H_2O_2 in HEK cells. RNAi which mediated down-regulation of LTRPC2 significantly suppressed Ca^{2+} influx and H_2O_2-induced cell death. Thus, LTRPC2 probably represents an important intrinsic mechanism that mediates Ca^{2+} overload in response to the disturbance of a redox state in cell death[39].

Recently, the traditional view of necrosis as passive, accidental cell death has been challenged by ever increasing number of studies which reveal that necrosis can be a programmed process like apoptosis. Meanwhile, a new term necroptosis has been aroused wide attention. In some cells, the death domain receptor-associated adaptor, RIP1, is proved to be the key upstream kinase involved in the activation of necroptosis[40]. Necrostatin-1, a selective allosteric inhibitor of RIP1 kinase, is able to reduce necrotic morphological changes and cause a sharp decrease in necrotic death[41]. In addition, Degterev *et al.* demonstrated that death receptor-mediated apoptosis could be diverted to necroptosis when apoptosis signaling was blocked, suggesting that necroptosis might function as a backup mechanism to insure the elimination of damaged cells under certain conditions even when apoptosis was inhibited[20].

Although it was shown in this study that H_2O_2-induced necrosis in ARPE-19 cells could be a regulated process which was blocked by the calcium channel blocker and antioxidant, the question whether it belongs to necroptosis still need further evidence. To the best of our knowledge, this is the frontier report on programmed necrosis in ARPE-19 cell death pathwayswhich may offer a new therapeutic target and an extended window for novel cell protections.

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