The Involvement of PI3K-Mediated and L-VGCC-Gated Transient Ca\textsuperscript{2+} Influx in 17β-Estradiol-Mediated Protection of Retinal Cells from H\textsubscript{2}O\textsubscript{2}-Induced Apoptosis with Ca\textsuperscript{2+} Overload

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Abstract

Intracellular calcium concentration ([Ca\textsuperscript{2+}]) plays an important role in regulating most cellular processes, including apoptosis and survival, but its alterations are different and complicated under diverse conditions. In this study, we focused on the [Ca\textsuperscript{2+}] and its control mechanisms in process of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced apoptosis of primary cultured Sprague-Dawley (SD) rat retinal cells and 17β-estradiol (βE2) anti-apoptosis. Fluo-3AM was used as a Ca\textsuperscript{2+} indicator to detect [Ca\textsuperscript{2+}] through fluorescence-activated cell sorting (FACS), cell viability was assayed using MTT assay, and apoptosis was marked by Hoechst 33342 and annexin V/Propidium Iodide staining. Besides, PI3K activity was detected by Western blotting. Results showed: a) 100 μM H\textsubscript{2}O\textsubscript{2}-induced retinal cell apoptosis occurred at 4 h after H\textsubscript{2}O\textsubscript{2} stress and increased in a time-dependent manner, but [Ca\textsuperscript{2+}] increased earlier at 2 h, sustained to 12 h, and then recovered at 24 h after H\textsubscript{2}O\textsubscript{2} stress; b) 10 μM βE2 treatment for 0.5-24 hrs increased cell viability by transiently increasing [Ca\textsuperscript{2+}], which appeared only at 0.5 h after βE2 application; c) increased [Ca\textsuperscript{2+}] under 100 μM H\textsubscript{2}O\textsubscript{2} treatment for 2 hrs or 10 μM βE2 treatment for 0.5 hrs was, at least partly, due to extracellular Ca\textsuperscript{2+} stores; d) importantly, the transiently increased [Ca\textsuperscript{2+}] induced by 10 μM βE2 treatment for 0.5 hrs was mediated by the phosphatidylinositol-3-kinase (PI3K) and gated by the L-type voltage-gated Ca\textsuperscript{2+} channels (L-VGCC), but the increased [Ca\textsuperscript{2+}] induced by 100 μM H\textsubscript{2}O\textsubscript{2} treatment for 2 hrs was not affected; and e) pretreatment with 10 μM βE2 for 0.5 hrs effectively protected retinal cells from apoptosis induced by 100 μM H\textsubscript{2}O\textsubscript{2}, which was also associated with its transient [Ca\textsuperscript{2+}] increase through L-VGCC and PI3K pathway. These findings will lead to better understanding of the mechanisms of βE2-mediated retinal protection and to exploration of the novel therapeutic strategies for retina degeneration.

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Introduction

Intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) plays a vital role in regulating many fundamental cellular processes, such as gene regulation, cell proliferation, cell survival, and apoptosis [1]. Ca\textsuperscript{2+} homeostasis is tightly regulated and the disturbances in Ca\textsuperscript{2+} homeostasis have been implicated in degenerative diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD) and Huntington’s disease (HD) [2,3]. The increase of [Ca\textsuperscript{2+}], is mediated by two closely related mechanisms: excessive release of Ca\textsuperscript{2+} from endoplasmic reticulum (ER) stores and store-operated Ca\textsuperscript{2+} entry (SOCE), the Ca\textsuperscript{2+} influx process through plasma membrane (PM) channels following the release of Ca\textsuperscript{2+} from the ER stores [4]. Specifically, [Ca\textsuperscript{2+}] alterations are different under diverse conditions. Accumulating evidence suggests that both the excessive elevation of [Ca\textsuperscript{2+}] and the loss of [Ca\textsuperscript{2+}] are crucial for degenerative diseases [5]. Increased [Ca\textsuperscript{2+}] leads to the inappropriate activation of Ca\textsuperscript{2+}-dependent processes, which are normally inactive or operate at low Ca\textsuperscript{2+} levels, thus causing metabolic derangements that ultimately lead to cell death [6]. In contrast, chronic depletion of ER Ca\textsuperscript{2+} influences
ER-dependent processes and also inhibits Ca\(^{2+}\)-dependent
retinal cell injury and apoptosis, while treatment with 10 μM βE2 played a protective role. Both completely different roles were mediated by increasing the [Ca\(^{2+}\)], which occurred at the early stage of apoptosis and at 0.5 h after βE2 treatment. Furthermore, both of the increased [Ca\(^{2+}\)] under completely opposite conditions were partially due to extracellular [Ca\(^{2+}\)]. Importantly, the transient [Ca\(^{2+}\)] increase induced by βE2 was gated by the L-type voltage-gated Ca\(^{2+}\) channels (L-VGCC) and phosphatidylinositol-3-kinase (PI3K) was involved, but it was not involved in the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] increase.

**Materials and Methods**

### 2.1: Animals and Chemicals

SD rats (obtained on postnatal days 0-3, body weights of 5-12 g) were housed in a controlled environment in a specific pathogen-free animal center. The temperature was maintained at 24±2°C, the humidity was 52±10% and fresh air was circulated continuously. All of the procedures used in the experiments were approved by the Institutional Animal Ethics Committee, Medical School of Xi’an Jiaotong University (permission No. 2009-12) and conformed to accepted ethical standards of the Animals in Research and the Association for Research in Vision and Ophthalmology statement for the use of animals in vision and ophthalmic research.

H\(_2\)O\(_2\) was purchased from Xi’an Pure Chemical Industries (Xi’an, Shaanxi, China). Fetal Bovine Serum (FBS) and phenol red free 1:1 DMEM/F-12 were obtained from Hyclone (Logan, Utah, USA). Poly-lysine, βE2, Hoechst 33342 dye and nifedipine, an L-VGCC blocker, were purchased from Sigma (St. Louis, Missouri, USA). We used 95% ethanol as the solvent to make the βE2 stock solution at a concentration of 1x10\(^{-2}\) M. Fluo-3 AM, an indicator of intracellular Ca\(^{2+}\) levels, was purchased from Biotium (Hayward, Calif., USA). We used Dimethylsulfoxide (DMSO) as the solvent for making 5 mM Fluo-3 AM stock solution and 20% Pluronic F-127 (5900) (offered by Biotium) in DMSO to facilitate AM ester solubilization. Trypsin, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and ethylene glycol tetraacetic acid (EGTA), an extracellular Ca\(^{2+}\) chelator, were purchased from Amresco (Solon, Ohio, USA). LY294002, a PI3K inhibitor, was purchased from Cayman (Ann Arbor, MI, USA). The Annexin V-FITC Apoptosis Assay Kit and bicinechonic acid (BCA) Protein Assay Kit were purchased from Zhuhai Jioncare Bioscience Ltd (Zhuhai, Guangdong, China), and radio immunoprecipitation assay (RIPA) buffer was purchased from Biotec (Biotechnology, Inc. of China). Anti-p-Akt and anti-Akt antibodies were purchased from Cell Signaling (Boston, Massachusetts, USA), and Anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

### 2.2: Primary Retinal Cells Cultures

We cultured primary retinal cells referencing other’s study [28] and making some revision. Neonatal SD rats were sacrificed (10–12 rats were needed for each 24-well or 6-well culture plate) and then the eyeballs were enucleated and immediately placed into a beaker containing D-Hanks solution.
The retinas were removed from the pigment epithelium layer with the aid of a dissecting microscope under sterile conditions and were placed into a glass tube containing 1:1 Ham’s F-12-DMEM medium. The beaker containing the eyeballs and the tube containing the retinas were placed onto ice. The retina fragments were treated with 0.25% trypsin at 37°C for 8 mins and the digestion was terminated by adding three times the volume of 1:1 Ham’s F-12-DMEM containing 10% FBS. The suspension was filtered with a 200-mesh screen and centrifuged at 1000 rpm for 10 mins. After the supernatant was discarded, the cells were suspended, diluted with medium containing 10% FBS to 1x10^6 cells/ml and plated onto 24-well or 6-well plates (Corning Costar) with 1 ml or 3 ml of cell suspension per well. Before culturing, all the plates were coated with poly-lysine (0.1 mg/ml) and maintained in a humid incubator overnight. Next, we washed the plates three times with sterile double distilled water (ddH_2O), once with D-Hanks balanced salt solution, and then with 200 μl of medium, which provided a pre-environment for cell growth. The cells were cultured at 37°C in a 5% CO_2 atmosphere until they were used at 4-6 days in vitro, during which the medium was replaced according to the cell growth and metabolism conditions.

2.3: Drug Treatment

After the cultures were maintained for 4-6 days in vitro, H_2O_2 and/or βE2 were added by bath application. Overall, 1 M H_2O_2 was prepared from 30% H_2O_2 dissolved in sterile cool PBS and was diluted with the medium to 10 mM. Next, the 10 mM H_2O_2 was diluted with the essential medium gradually to 200-25 μM, and 0 μM was regarded as the control. The 0.5-100 μM βE2 was prepared from the 1x10^{-2} M βE2 stock solution with the medium and was added to the cultures. We considered 0 μM as the control. The βE2 stock solution was dissolved in 95% ethanol, and a small amount of ethanol was present in the medium (<1%), but it had no effect on the primary cultured SD rat retinal cells [28]. Except for analyzing the time and dose dependency of H_2O_2 or βE2, we used H_2O_2 at a final concentration of 100 μM for 2 hrs/24 hrs and βE2 at a final concentration of 10 μM for 0.5 hrs to perform the experiments. To discover the source of increased [Ca^{2+}], different concentrations of EGTA were added directly to the medium 1 hr before the application of 100 μM H_2O_2 for 2 hrs or 10 μM βE2 for 0.5 hrs to chelate the extracellular Ca^{2+}. Under the co-application, we pre-treated cells with 10 μM βE2 treatment for 0.5 hrs before the application of 100 μM H_2O_2 for 2 hrs. To conduct the channel experiments and the mechanism study, the cultures were pre-conditioned for 2 hrs by nifedipine or for 0.5 hrs by LY294002 before the other treatments.

2.4 Cell Viability Assay

To determine the cell viability of the primary cultured SD rat retinal cells, we performed an MTT assay. MTT was applied to the cultures at a final concentration of 0.5 mg/ml for 4 hrs at 37°C in 5% CO_2, and the wells with no cells were used as blank controls. The medium was then removed, and DMSO was added to solubilize the colored formazan crystal product. The absorbance was determined at 490 nm on a Measurement Photometric multi-well plate reader (Electron Corporation Multiskan Spectrum, Thermo, Finland) with Skanit RE for Mass 2.2 software after the plates were agitated at 37°C for 10 mins. All absorbance values were subtracted by the blank value, and the untreated cultures were considered as the control group. The mean cell viability for each condition was determined by averaging at least quadruplicate values, the fold change relative to the control was calculated, and the control values were normalized to 1. All experiments were performed using 3-5 separate experiments to confirm reproducibility.

2.5: Assessment of Apoptosis

After exposure to 100 μM H_2O_2 for 0-24 hrs, apoptosis was assayed by Annexin V/Propidium Iodide (PI) staining and Hoechst 33342 staining. For Annexin V/PI staining, the cells were collected, centrifuged at 1000 rpm for 5 mins, suspended and diluted with 1 x binding buffer (Annexin V-FITC Apoptosis Assay Kit) to 5x10^5 cells/ml. The 500 μl suspension was loaded with 5 μl Annexin V-FITC and 10 μl PI for 15 mins. After incubated in the dark at room temperature, the cells were analyzed within one hour with a flow cytometer (San Jose, California, USA). For Hoechst 33342 staining, 40 μl of suspension was dropped onto the slide, fixed in 4% paraformaldehyde in PBS at room temperature for 20 mins and stained with 2 μg/ml Hoechst 33342 dye in the dark for 10 mins. The samples were then observed under a fluorescence microscope (Nikon, Eclipse Ti, Japan) with fluorescence excitation at 340 nm and emission at 510 nm. The cells with condensed DNA were counted as apoptotic cells, and the average apoptotic cells of each field were calculated. The sample fields with approximately 100 cells were randomly selected, and each sample was evaluated. The cells in 3-5 random fields/cultures were scored, and the counts were based on at least four separate cultures in each treatment condition.

2.6: Intracellular Ca^{2+} Measurement

[Ca^{2+}] detection was performed by FACS analysis [30]. After washing twice with PBS, the adherent cells were digested from plates with 300 μl 0.25% trypsin per well, and the digestion reaction was quenched by the addition of Ca^{2+}-free medium containing 900 μl 10% FBS per well. The suspensions were collected and centrifuged at 1000 rpm for 10 mins. After discarding the supernatant, we suspended the cells with Ca^{2+}-free PBS and incubated it in dark with 2 μM Fluo-3-AM (Molecular Probes, Biotium) at 37°C for 30 mins and at room temperature for 15 mins. The sample without Fluo-3-AM was considered as the blank control, whose fluorescence was represented as F_c. Before detection, we washed the cells twice with PBS to minimize background fluorescence and nonspecific staining. The fluorescence was measured at FL-1 (526 nm) in a flow cytometer (Becton Dickinson, FACSCalibur-E4121, Becton Dickinson Immunocytometry systems driven by 2350 Qume, San Jose, California, USA) with an excitation laser at 488 nm, and at least 10,000 events per sample were acquired. The obtained image data were analyzed with Cell Quest Version 3.3 software and the Geo Mean of fluorescence (F) was used because its standard normal distribution was better compared to the mean fluorescence. All F values were subtracted by F_c to eliminate the background fluorescence and nonspecific
staining. The relative F values of each treated group were expressed as the fold of control, with the F values of the control group normalized to 1. The changes of relative F values of Fluo-3AM represented the [Ca\(^{2+}\)] alteration. To confirm the reproducibility, all experiments were performed at least 3-5 times with separate cultures.

2.7: Western Blot Analysis

The primary cultured retinal cells lysates were made by mixing cold RIPA buffer at a pH of 7.0 (the RIPA buffer consists of 20 mM Tris/HCl, 2 mM ethyleneglycoltetraacetic acid, 25 mM 2-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin) with a 1 mM solution of the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO) and a 10% solution of phosphatase inhibitor mixture P1260 (Applygen Technologies Inc., Beijing, China). The mixture was then homogenized on ice for 5 mins and centrifuged at 12000 g at 4°C for 20 mins. The BCA protein assay reagents (Pierce, Rockford, USA) were used to assess the concentration of the cell lysates. The assays were performed in triplicate, and the cell lysates were subsequently loaded onto a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel, underwent electrophoresis and were subsequently transferred to a nitrocellulose membrane (Millipore, Bedford, MA) that was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS, pH7.4) and incubated with anti-p-Akt and anti-Akt (1:1000, Cell signaling, Boston, USA) at 4°C overnight. After washing the membrane with TBS/T (TBS with 0.1%Tween 20), we applied goat anti-rabbit IgG (1:5000) labeled with horseradish peroxidase (HRP) at room temperature for 4 hrs, and then washed the membrane with TBS. Anti-β-actin antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to verify the protein concentration. The ECL system (Thermo, USA) was used to visualize the protein bands.

2.8: Statistical Analysis

All results were based on 3-5 independent replications with 4-6 samples per condition per experiment. Values shown in this study were expressed as the mean ±SD. Data were analyzed using the T-test for independent samples, or One-way ANOVA and the LSD post hoc test were used for multiple comparisons. P<0.05 was considered statistically significant for all tests.

Results

3.1: H\(_2\)O\(_2\) induced the apoptosis of primary cultured SD rat retinal cells, and the [Ca\(^{2+}\)], increased during the early apoptosis

Most cell culture models of oxidative stress employ H\(_2\)O\(_2\) as the pro-oxidant to induce oxidative stress because it is capable of altering the intracellular redox state of a cell and causing oxidative damage by its conversion to the highly reactive hydroxyl radical OH \([28,31,32]\). Furthermore, 100 µM H\(_2\)O\(_2\) treatment for 24 hrs induced retinal cell apoptosis \([28]\). To ascertain the role of [Ca\(^{2+}\)] in our study model and to dynamically observe the [Ca\(^{2+}\)], alteration during apoptosis under a modest treatment condition, we performed the following experiments. First, cell viability and the [Ca\(^{2+}\)] were assayed simultaneously at 2 h after treatment with different concentrations of H\(_2\)O\(_2\). As shown in Figure 1, 25-200 µM H\(_2\)O\(_2\) decreased cell viability (Figure 1A) but increased [Ca\(^{2+}\)] in a dose-dependent manner (Figure 1B, C), which was significant at 100-200 µM. This finding indicated that 2 hrs after the application, 100-200 µM H\(_2\)O\(_2\) reduced cell viability and caused Ca\(^{2+}\) overload. Next and importantly, we used 100 µM as the H\(_2\)O\(_2\) concentration to dynamically and continuously observe apoptosis by Hoechst 33342 staining and [Ca\(^{2+}\)] alteration during apoptosis, and cell viability was also assayed. The results showed that apoptosis was significant at 4 h, the significance increased over time (Figure 1G, H); however, the [Ca\(^{2+}\)] increased remarkably at 2 h and 4 h, and this increase remained until 12 h but then gradually recovered to the control level at 24 h (Figure 1E, F). Cell viability was reduced in a time-dependent manner from 0 to 24 hrs (Figure 1D). Compared with control group, the 100 µM H\(_2\)O\(_2\) treatment for 2 hrs caused a dramatic increase in [Ca\(^{2+}\)] \((P<0.001)\) and a slight decrease in cell viability; however, the 100 µM H\(_2\)O\(_2\) treatment for 24 hrs caused a remarkable decrease in cell viability \((P<0.001)\), but no significant alteration was discovered in [Ca\(^{2+}\)] (Figure 1D, E), suggesting that the [Ca\(^{2+}\)] increase occurs at the early stage of H\(_2\)O\(_2\) induced apoptosis when cell injury is minimal.

3.2: βE2 increased cell viability and protected primary cultured SD rat retinal cells from H\(_2\)O\(_2\) injury, and the transient [Ca\(^{2+}\)] increase was found to be involved in protection

Pretreatment with 10 µM βE2 for 0.5 hrs effectively protected retinal cells from 100 µM H\(_2\)O\(_2\)-induced apoptosis \([28]\). To confirm whether or not [Ca\(^{2+}\)] was involved in βE2-mediated protection in our model, we first observed the effects of different concentrations of βE2 treatment for 0.5 hrs and 10 µM βE2 treatment for different periods on cell viability and [Ca\(^{2+}\)], respectively. The results showed that a range of 0.5-100 µM βE2 treatment for 0.5 hrs significantly increased [Ca\(^{2+}\)] in a dose-dependent manner (Figure 2B, C), and 5-50 µM βE2 significantly increased cell viability (Figure 2A). However, at lower (0.5 and 1 µM) or higher (100 µM) concentrations of βE2, the treatment only increased [Ca\(^{2+}\)] but had no effect on cell viability, which may be due to the concentration selectivity or because lower concentrations (0.5 and 1 µM) of βE2 are insufficient to increase cell viability and higher concentrations (100 µM) of βE2 are toxic for retinal cells. Interestingly, cell viability was significantly increased at 0.5-24 h after the application of 10 µM βE2 (Figure 2D), but the [Ca\(^{2+}\)] increased significantly and rapidly only at 0.5 h after 10 µM βE2 treatment, fluctuated near the control level at 1-18 h, and then restored to the control level at 24 h (Figure 2E, F). Furthermore, under 10 µM βE2 pretreatment for 0.5 hrs and then 100 µM H\(_2\)O\(_2\) treatment for 2 hrs, 10 µM βE2 pretreatment for 0.5 hrs significantly restored the decreased cell viability but significantly sharpened the increased [Ca\(^{2+}\)] induced by 100 µM H\(_2\)O\(_2\) for 2 hrs (Figure 2G, H), suggesting that βE2 increased cell viability and protected primary cultured SD rat
retinal cells from H$_2$O$_2$ injury that is associated with immediate and transient [Ca$^{2+}$] increases.

3.3: Both increased [Ca$^{2+}$] induced by 100 μM H$_2$O$_2$ treatment for 2 hrs and 10 μM βE2 treatment for 0.5 hrs were caused by extracellular Ca$^{2+}$ influx

Ca$^{2+}$ homeostasis is strictly controlled by channels, pumps and exchangers functioning as gates for Ca$^{2+}$ entry and release. A cell becomes activated because of an external signal, which results in up to a 100-fold increase in the [Ca$^{2+}$] caused by the uptake of extracellular Ca$^{2+}$ and/or the release of intracellular Ca$^{2+}$ stores. To confirm whether the increased [Ca$^{2+}$] in our model treated with 100 μM H$_2$O$_2$ for 2 hrs or 10 μM βE2 for 0.5 hrs is due to the extracellular Ca$^{2+}$ influx, we preliminarily detected the [Ca$^{2+}$] before and after adding EGTA, a chelator of extracellular Ca$^{2+}$, in the presence and absence of H$_2$O$_2$ or βE2, respectively. Simultaneously, cell viability was assayed. As shown in Figure 3, 0.2-5 mM EGTA treatment for 24 hrs decreased cell viability (Figure 3A), treatment with 1-5 mM EGTA for 1 hr had no effect on the [Ca$^{2+}$] (Figure 3B, C). However, the effect of EGTA on the [Ca$^{2+}$] was different in the presence of H$_2$O$_2$ or βE2. Based on previous experiments, we selected to pretreat the cells with 0.1-5 mM EGTA for 1 hr to chelate the extracellular Ca$^{2+}$ before H$_2$O$_2$ or βE2 treatment.
The results showed that 1-5 mM EGTA significantly aggravated the decrease in cell viability (Figure 3D), but 0.5-5 mM EGTA significantly attenuated the increase in [Ca\(^{2+}\)] caused by the 100 μM H\(_2\)O\(_2\)-induced injury for 2 hrs (Figure 3E, F). This aggravating or attenuating effect was dose-dependent. Furthermore, 1-5 mM EGTA dose-dependently attenuated the increased cell viability and the increased [Ca\(^{2+}\)] caused by 10 μM βE2 treatment for 0.5 hrs (Figure 3G, H, I). The attenuating impact of EGTA on the increased [Ca\(^{2+}\)] induced by H\(_2\)O\(_2\) or βE2 implicated that [Ca\(^{2+}\)] increases under the two conditions were, at least, caused by extracellular sources. In this experiment, we monitored the pH before and after EGTA application and found that the low dose of EGTA did not alter the pH value of the medium, eliminating the effect of a change in pH as the cause of the increase in [Ca\(^{2+}\)].

3.4: L-VGCC mediated the [Ca\(^{2+}\)] increase induced by 10 μM βE2 treatment for 0.5 hrs but did not mediate the [Ca\(^{2+}\)] increase induced by 100 μM H\(_2\)O\(_2\) for 2 hrs

It has been suggested that estrogen potentiates L-VGCC in other cells [20–22]; however, it remained unknown whether L-VGCC gated the extracellular Ca\(^{2+}\) influx caused by 10 μM βE2 treatment for 0.5 hrs or 100 μM H\(_2\)O\(_2\) treatment for 2 hrs in our model. To this end, we conducted several experiments using the L-VGCC blocker nifedipine. First, we measured the effect of nifedipine on the cell viability and found that treatment for 24 hrs with 10 μM and 20 μM nifedipine showed no effect on the cell viability, but 30 μM nifedipine significantly decreased the cell viability (Figure 4A). Second, we measured the [Ca\(^{2+}\)] at different time points after 20 μM nifedipine treatment and found that the [Ca\(^{2+}\)] increased at 0.5-1 h after 20 μM nifedipine application but later recovered (Figure 4B). When specifically blocking L-VGCC, the reactively impermanent increase in [Ca\(^{2+}\)] occurred at 0.5-1 h after 20 μM nifedipine application because of the Ca\(^{2+}\) homeostasis. Afterwards, the [Ca\(^{2+}\)] recovered to the resting level, and nifedipine began to develop its stable and innate effect. Third, we detected the blocking effect of nifedipine on increased [Ca\(^{2+}\)] under two conditions and found that 20 μM nifedipine pretreatment for 2 hrs significantly attenuated the increased [Ca\(^{2+}\)] induced by 10 μM βE2 treatment for 0.5 hrs (Figure 4C) but did not attenuate the increased [Ca\(^{2+}\)] induced by 100 μM H\(_2\)O\(_2\) treatment for 2 hrs (Figure 4D). L-VGCC gated the transient [Ca\(^{2+}\)] increase induced by βE2 but did not gate the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] increase. Fourth, we analyzed the impact of nifedipine on βE2-mediated retinal protection and discovered that 20 μM nifedipine pretreatment for 2 hrs significantly attenuated βE2 protection against H\(_2\)O\(_2\) injury (P=0.029, Figure 4E) and also significantly attenuated the increased [Ca\(^{2+}\)] induced by βE2 and H\(_2\)O\(_2\) co-treatment (P=0.018, Figure 4F). Therefore, βE2 protection on primary cultured SD rat retinal cells was associated with transient Ca\(^{2+}\) influx gated by L-VGCC.

3.5: βE2 pretreatment protected primary cultured SD rat retinal cells from H\(_2\)O\(_2\)-induced apoptosis by activating the PI3K pathway and then transiently up-regulating the [Ca\(^{2+}\)]

βE2 plays a protective role in the retina via the PI3K/Akt pathway [28]. Our results showed that βE2 protected primary cultured SD rat retinal cells from H\(_2\)O\(_2\) injury, which was associated with a transient [Ca\(^{2+}\)] increase (Figure 2). Therefore, we hypothesized that βE2 plays a protective role in our study model by activating the PI3K pathway and then transiently increasing [Ca\(^{2+}\)]. To test this hypothesis, we performed the following experiments using the PI3K inhibitor LY294002. First, we confirmed that 10 μM βE2 treatment for 0.5 hrs up-regulated the p-Akt level via Western blotting (Figure
Second, we measured the effects of LY294002 on the cell viability and the \([\text{Ca}^{2+}]_i\) of the retinal cells and found that 1-50 μM LY294002 treatment for 24 hrs dose-dependently decreased the cell viability (Figure 5C), but treatment for 0.5 hrs had no effect on the resting \([\text{Ca}^{2+}]_i\) (Figure 5D). Third, we detected the inhibitory effects of LY294002 on the alteration of \([\text{Ca}^{2+}]_i\) and cell viability due to 10 μM βE2 treatment for 0.5 hrs or 100 μM \(\text{H}_2\text{O}_2\) treatment for 2 hrs. Results showed that pretreatment for 0.5 hrs with 10 μM or 20 μM LY294002 significantly attenuated the increased cell viability and \([\text{Ca}^{2+}]_i\) due to βE2 (Figure 5E, F). However, 10 μM LY294002 did not reverse the cell viability decrease induced by \(\text{H}_2\text{O}_2\) but instead...
promoted the decrease in cell viability (Figure 5G). In addition, both 10 μM and 20 μM LY294002 had no effect on the [Ca\(^{2+}\)] increase induced by H\(_2\)O\(_2\) (Figure 5H). PI3K was involved in the βE2-induced increase of [Ca\(^{2+}\)] and cell viability but was not involved in the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] increase and cell viability decrease. Fourth, we verified that PI3K-mediated βE2 protection against H\(_2\)O\(_2\) injury was associated with transiently up-regulating [Ca\(^{2+}\)]. As shown in Figures 5i and j, 20-50 μM LY294002 dose-dependently attenuated the βE2-mediated protective effect against H\(_2\)O\(_2\) injury and dose-dependently restored the increased [Ca\(^{2+}\)] induced by co-treatment with 10 μM βE2 for 0.5 hrs and 100 μM H\(_2\)O\(_2\) for 2 hrs.

Based on the results of cell viability and apoptosis assay in Figure 1D and H, 100 μM H\(_2\)O\(_2\) treatment for 2 hrs led to a significant decrease in cell viability but did not induce apoptosis. Therefore, we tested the role of βE2 in anti-apoptosis induced by 100 μM H\(_2\)O\(_2\) for 24 hrs and the inhibitory effect of LY294002. In this experiment, we assayed the cell viability by the MTT assay and apoptosis by Annexin V/Propidium iodide staining, and meanwhile, [Ca\(^{2+}\)] measurements and Western blotting were performed. The results showed that 10 μM βE2 pretreatment for 0.5 hrs effectively protected the retinal cells from injury and apoptosis induced by 100 μM H\(_2\)O\(_2\)-mediated stressing for 24 hrs. Moreover, application of 10 μM LY294002 for 0.5 hrs before βE2 treatment significantly inhibited the βE2-mediated protective effect against H\(_2\)O\(_2\)-induced cell viability decrease and apoptosis (Figure 6A-C). Nevertheless, the [Ca\(^{2+}\)] showed no alteration in all treated groups compared to the control group (Figure 6D), which further implicated that the [Ca\(^{2+}\)] increase in the [Ca\(^{2+}\)] overload induced by H\(_2\)O\(_2\) occurred during the early stage of apoptosis but did not occur at the later stages of apoptosis. Western blot results also showed that 10 μM βE2 pretreatment for 0.5 hrs markedly activated the PI3K/Akt pathway, which was significantly inhibited by 10 μM LY294002 (Figure 6E, F).

Briefly, the PI3K pathway mediated the βE2-induced [Ca\(^{2+}\)] increase but did not mediate the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] increase. Pretreatment with 10 μM βE2 for 0.5 hrs protected primary cultured SD rat retinal cells from injury and apoptosis induced by H\(_2\)O\(_2\) by activating the PI3K pathway, and then transiently up-regulated the [Ca\(^{2+}\)], which was detectable at 2 h but not at 24 h after H\(_2\)O\(_2\)-induced stress.

**Discussion and Conclusion**

[Ca\(^{2+}\)] plays an important role in regulating most cellular processes and it is regulated by complex mechanisms. While brief elevations in [Ca\(^{2+}\)] are required to control membrane excitability and to modulate essential processes, chronic elevations in [Ca\(^{2+}\)] trigger toxic signaling cascades that lead to cell death [6,33–35]. Nevertheless, the selection of Ca\(^{2+}\) indicator and method of [Ca\(^{2+}\)] measurement are very important as well. They will affect the result of [Ca\(^{2+}\)] measurement. Fluo-3 AM ester is a membrane-permeating form of fluo-3. It can passively diffuse across cell membranes and can be loaded into most of cells. Fluo-3 AM itself does not respond to Ca\(^{2+}\). However, once inside the cells, it is hydrolyzed to fluo-3 and can bind to Ca\(^{2+}\). Fluo-3 is one of the most suitable fluorescent Ca\(^{2+}\) indicators for flow cytometry. It is a good probe because of its high sensitivity, but a few limited cells can be loaded directly with Ca\(^{2+}\) indicators [36]. Consequently, it is feasible and reasonable that we detected the [Ca\(^{2+}\)] by FACS using Fluo-3 AM. The fluorescence of Fluo-3 AM precisely represents the actual [Ca\(^{2+}\)].

Recent evidence indicates that [Ca\(^{2+}\)] is abnormal in many degenerative disorders in CNS. A number of studies suggest that alterations in [Ca\(^{2+}\)] may result in cell apoptosis [37], which supports the relevance of [Ca\(^{2+}\)], in the mechanisms leading to apoptosis. Several studies show that exposure to H\(_2\)O\(_2\) induces the apoptosis of cultured neurons, which is mediated by increasing the [Ca\(^{2+}\)]. Several channels have been proposed to be involved in the H\(_2\)O\(_2\)-mediated [Ca\(^{2+}\)] increase, including the N-methyl-D-aspartate (NMDA) receptor, the α-amino-3-hydroxy-5-methyl-4-isoxa-zole propionic acid (AMPA) receptor and VGCC [38–40]. The Transient Receptor Potential (TRP) protein superfamily is a group of voltage-independent Ca\(^{2+}\)-permeable cation channels expressed in mammalian cells and consists of six subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML [41,42]. Recent evidence suggests that Ca\(^{2+}\) influx through TRP channels is an important mechanism through which oxidative stress mediates cell death and TRPC, and TRPM subfamily members are also activated by oxidative stress [42]. In our present study, we found that Ca\(^{2+}\) plays a substantial role in H\(_2\)O\(_2\)-induced apoptosis, and the [Ca\(^{2+}\)] increase occurs at the early stage of apoptosis but not during the later stages of this process. Moreover, the increased [Ca\(^{2+}\)] induced by H\(_2\)O\(_2\) is partially caused by extracellular stores.

As for the mechanisms involved in βE2 retinal protection in our model, we speculated that βE2 resisted H\(_2\)O\(_2\) stress by weakening the increased [Ca\(^{2+}\)], due to H\(_2\)O\(_2\). Inconsistent with our hypothesis, we found that 10 μM βE2 played a protective role by immediately sharpening but not restoring the increased [Ca\(^{2+}\)] induced by H\(_2\)O\(_2\). Furthermore, up to 2-5 mM doses of EGTA significantly attenuated the sharpening effect of βE2, indicating that this effect may be caused by a large Ca\(^{2+}\) transient influx. Many studies have proposed that L-VGCC plays an important role in the protective process in CNS, including retina [20–22,43]. In addition, several studies have indicated that the release of Ca\(^{2+}\) from the ER through the inositol 1, 4, 5-trisphosphate receptors (IP3Rs) is essential for cell survival and neuroprotection [44–46]. The members of the TRPM and TRPC subfamilies also play important roles in cell survival [47–50]. βE2 has been shown to be involved in the regulation of Ca\(^{2+}\) influx via the TRPV5 channels [51], and preconditioned cells with a relatively low level of Ca\(^{2+}\) before an excitotoxic insult experienced neuroprotection in retinal ganglion cells [52]. Therefore, we hypothesized that βE2 increased the [Ca\(^{2+}\)] through one or more relevant Ca\(^{2+}\) channels and signaling pathways. Excitedly, we discovered that the retinal protective role of βE2 through potentiating Ca\(^{2+}\) influx is controlled by L-VGCC and mediated by PI3K pathway.

Perplexedly, the results in our present study showed that both H\(_2\)O\(_2\) injury and βE2 protection are mediated by increasing the [Ca\(^{2+}\)] sourced from extracellular Ca\(^{2+}\) influx. These findings can be explained by the following ideas. First, Ca\(^{2+}\) exerts a
**Figure 5.** The effect of the PI3K inhibitor LY294002 (LY) on the cell viability and the \([\text{Ca}^{2+}]\), of primary cultured SD rat retinal cells in \(\text{H}_2\text{O}_2\) injury and \(\beta\text{E2}\) protection. A: Western blot results of the activation of the PI3K/Akt pathway after \(\beta\text{E2}\) treatment for 0.5 hrs; B: Quantitative data of A; C and D: The effects of LY treatments for 24 hrs and 0.5 hrs on the cell viability and the resting \([\text{Ca}^{2+}]\); E and F: The inhibitory effect of LY pretreatment for 0.5 hrs on the increased cell viability and \([\text{Ca}^{2+}]\) induced by 10 \(\mu\text{M}\ \beta\text{E2}\) treatment for 0.5 hrs (10 \(\mu\text{M}\ \text{LY}\) in E, 10 \(\mu\text{M}\) and 20 \(\mu\text{M}\ \text{LY}\) in F); G and H: The effect of LY pretreatment for 0.5 hrs on the decreased cell viability and increased \([\text{Ca}^{2+}]\) induced by 100 \(\mu\text{M}\ \text{H}_2\text{O}_2\) treatment for 2 hrs (10 \(\mu\text{M}\ \text{LY}\) in G, 10 \(\mu\text{M}\) and 20 \(\mu\text{M}\ \text{LY}\) in H); I and J: The dose-dependent attenuating impact of 20-50 \(\mu\text{M}\ \text{LY}\) pretreatment for 0.5 hrs on the \(\beta\text{E2}\) retinal protective role against \(\text{H}_2\text{O}_2\) injury, which is associated with the dose-dependent attenuation of the increased \([\text{Ca}^{2+}]\). (Protocol of drug application: LY for 0.5 hrs, \(\beta\text{E2}\) for 0.5 hrs and \(\text{H}_2\text{O}_2\) for 2 hrs). Values shown are the Mean ±SD. *represents \(P<0.05\), **represents \(P<0.01\) and ***represents \(P<0.001\) compared with the control group; # represents \(P<0.05\), ## represents \(P<0.01\) and ### represents \(P<0.001\) compared with the \(\beta\text{E2}\) (E, F) or \(\text{H}_2\text{O}_2\) (G, I, J) application groups; $ represents \(P<0.05\), $$ represents \(P<0.01\) and $$$ represents \(P<0.001\) compared with the \(\beta\text{E2}\) and \(\text{H}_2\text{O}_2\) co-application group by one-way ANOVA statistical analysis. (B: \(n\) indicates 3 independent replicates; C, E, G, I: \(n\) indicates 3 independent replicates with 4 samples per condition per experiment; D, F, H, J: \(n\) indicates 3 independent replicates with 5 samples per condition per experiment.).

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10 µM βE2 pretreatment for 0.5 hrs protected primary cultured SD rat retinal cells from apoptosis induced by 100 µM H$_2$O$_2$ treatment for 24 hrs. The PI3K/Akt pathway mediated this process, but the alteration in [Ca$^{2+}$] was undetectable. A: The Annexin V/Propidium Iodide staining apoptosis assay; B: Quantitative data of A; C and D: Cell viability and [Ca$^{2+}$]; quantitative data; 10 µM βE2 pretreatment for 0.5 hrs significantly restored the decrease in cell viability and apoptosis, which was significantly inhibited by 10 µM LY (B, C), but the [Ca$^{2+}$], was not significantly altered in all treated groups (D); E: Western blot results, 10 µM βE2 pretreatment for 0.5 hrs promoted p-Akt level, which was inhibited by 10 µM LY pretreatment for 0.5 hrs before βE2 and H$_2$O$_2$ co-treatment. F: Quantitative data of E. Values shown are the Mean ±SD. *represents P<0.05, **represents P<0.01 and ***represents P<0.001 compared with the control group by the T-test or one-way ANOVA statistical analysis; ### represents P<0.001 compared with the H$_2$O$_2$ application group by one-way ANOVA statistical analysis; $$$ represents P<0.001 compared with the βE2 and H$_2$O$_2$ co-application group by one-way ANOVA statistical analysis. (B, C, D: n indicates 3 independent replicates with 4 samples per condition per experiment; F: n indicates 3 independent replicates.). doi: 10.1371/journal.pone.0077218.g006

biphasic effect on cellular growth, and a modest increase in [Ca$^{2+}$], promotes cell proliferation, whereas relatively high [Ca$^{2+}$] leads to increased mitochondrial Ca$^{2+}$ and accounts for the release of pro-apoptotic factors resulting in cell death [8,9]. Second, a short increase in [Ca$^{2+}$] is tolerated and may be needed to modulate biological functions, but the sustained increase in [Ca$^{2+}$] leads to various degrees of cell damage until cell death. Third, under the two treatment conditions, the increased [Ca$^{2+}$] may be due to different channels, and Ca$^{2+}$ influx through different routes may perform different biological functions [53]. For example, equally high Ca$^{2+}$ loads are toxic when entering via the NMDA channels but not when entering via the VGCC [54]. Our present results showed that 2-12 hrs of a sustained [Ca$^{2+}$] induced by H$_2$O$_2$ is harmful, but a transient [Ca$^{2+}$] increase induced by βE2 for only 0.5 hrs is protective. Furthermore, the favorable [Ca$^{2+}$] increase due to βE2 was gated by L-VGCC and was mediated by the PI3K pathway, but the harmful [Ca$^{2+}$] increase caused by H$_2$O$_2$ was not gated by L-VGCC or mediated by the PI3K pathway.

The majority of the results in this study are easily interpreted; nevertheless, several results are difficult to understand. For example, EGTA attenuated the increase of [Ca$^{2+}$] induced by the 100 µM H$_2$O$_2$-induced injury (Figure 3E and F) but did not attenuate and inversely aggravated the decrease in cell viability (Figure 3D), which is most likely because extracellular Ca$^{2+}$ is necessary for cell growth and chelating the extracellular Ca$^{2+}$ leads to a decrease in cell viability. In our present study, we chelated the extracellular Ca$^{2+}$, but we did not chelate the increased intracellular Ca$^{2+}$, and we did not specifically block the channels controlling the extracellular Ca$^{2+}$ influx due to the H$_2$O$_2$ injury. Further specific chelating and blocking experiments are being performed. Surprisingly, 20 µM nifedipine treatment for 0.5-1 hr increased the [Ca$^{2+}$] significantly (Figure 4B); however, it was the reactively impermanent [Ca$^{2+}$] increase. In this phenomenon, [Ca$^{2+}$] may have reactively increased through other channels when L-VGCC was specifically blocked due to Ca$^{2+}$ homeostasis at resting condition. After 0.5-1 hr of an impermanent [Ca$^{2+}$] increase, the nifedipine developed its innate effect. However, this finding is novel and needs to be further investigated.

In summary, 100 µM H$_2$O$_2$-induced stress led to primary cultured SD rat retinal cell injury and apoptosis; however, 10 µM βE2 played a protective role on retinal cells. Both completely different roles were mediated by increasing the [Ca$^{2+}$], which occurred at the early stage of 100 µM H$_2$O$_2$-induced apoptosis and 10 µM βE2 treatment for 0.5 hrs. Furthermore, the increase in [Ca$^{2+}$] under completely opposite conditions were partially due to extracellular Ca$^{2+}$ stores. Meaningfully, the transient [Ca$^{2+}$] increase induced by βE2 was gated by L-VGCC, and the PI3K pathway was found to be involved but was not found to be involved in the H$_2$O$_2$-induced [Ca$^{2+}$] increase. This finding may be due to different sources of Ca$^{2+}$ through different channels activating pro-apoptotic or pro-survival pathways, thus performing the injury or the protective roles. Our present findings are very important for understanding the mechanism of retina degeneration and the search for preventative treatment targets. The detailed mechanisms and downstream signaling pathways of Ca$^{2+}$ are
mostly unknown; therefore, it is important to direct future efforts towards the mechanisms and pathways of βE2-mediated anti-apoptosis through regulating \([Ca^{2+}]\), and the downstream signals of \([Ca^{2+}].\)

The data from our present study were based on a primary mixed cell culture of retinal cell population. The in vitro model is widely used for studying the pathogenesis of diseases. The primary retinal cell culture began in the late 1950’s. Today, it is routinely used for studies and remains the most widely-used form of retinal cell culture [55]. Moreover, primary mixed culture of retinal cell population includes various retinal cells and may better represent the in vivo condition than a cell line. Besides, \(H_2O_2\) triggers apoptosis and becomes a well-established in vitro model for studying the pathology of oxidative stress in degenerative disorders of CNS such as AMD, which is relative to the producing of reactive oxygen species (ROS) [11, 13]. Therefore, the model of \(H_2O_2\)-induced apoptosis of primary cultured retinal cells represents the pathogenesis of multiple retinal degenerative diseases. Certainly, in our future studies, we will do some research using in vivo model to obtain results that are more closely applicable to in vivo conditions.

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Author Contributions

Conceived and designed the experiments: Yan Feng, Xiaorui Yu. Performed the experiments: Yan Feng, Baoying Wang, Fangying Du, Hongbo Li, Shaolan Wang. Analyzed the data: Yan Feng, Xiaorui Yu, Baoying Wang. Wrote the manuscript: Yan Feng, Xiaorui Yu. Other: Prepared reagents and materials: Baoying Wang, Fangying Du, Hongbo Li, Chunhui Zhu. Had extensive scientific discussion for this study: Yan Feng, Xiaorui Yu, Baoying Wang, Chenghu Hu.

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