·Basic Research·

Activation of the ERK 1/2 and STAT3 signaling pathways is required for 661W cell survival following oxidant injury

Shu-Qian Dong, Hui-Zhuo Xu, Xiao-Bo Xia, Sha Wang, Li-Xin Zhang, Shuang-Zhen Liu

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Department of Ophthalmology, Xiangya Hospital of Central South University, Changsha 410006, Hunan Province, China
Correspondence to: Shuang-Zhen Liu. Department of Ophthalmology, Xiangya Hospital of Central South University, Changsha 410006, Hunan Province, China. liushzhxy@163.com

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Abstract

• AIM: To evaluate the influence of hydrogen peroxide (H_2O_2) on mouse photoreceptor-derived 661W cell survival and to determine the effect of PD98059, an inhibitor for MEK1 (the direct upstream activator of ERK1/2), and S3I201, a STAT3-specific inhibitor on 661W cell survival after H_2O_2 exposure.

• METHODS: The mouse photoreceptor-derived 661W cells were cultured. 661W cells were treated for 12 hours with different concentrations (0, 0.25, 0.50, 0.75, 1mmol/L) of H_2O_2 and cell viability was determined by 3- (4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)(MTT) assay. 661W cells were treated with different concentrations H_2O_2 (0, 5, 10, 50, 500, 1000 μ mol/L) for 15 minutes or 1mmol/L H_2O_2 for different time points (0,5,10,15,30 minutes), and p-Tyr705-STAT3, STAT3, Phospho-p44/42 MAPK (Thr202/Tyr204), ERK1/2 were surveyed by immunoblot analysis. After treatment with 50μ mol/L PD98059, or S3I201 for 1 hour, the inhibition efficiency of cell signal pathways was analyzed by immunoblot analysis and the effects of inhibitors on cell viability were determined by MTT.

• RESULTS: After treating with different concentrations of H_2O_2 for 12 hours, the cell viability of 661W cells decreased in concentration-dependent manner ($P<0.05$). Moreover, H_2O_2 induced phosphorylation of ERK1/2 and STAT3 in 661W cells ($P<0.05$). After pretreatment with 50μ mol/L PD98059 or S3I201 for 1 hour, H_2O_2-induced phosphorylation of ERK1/2 or STAT3 was suppressed separately ($P<0.05$). Using PD98059 or S3I201 to inhibit ERK1/2 or STAT3 signal pathway, the cell viability of 661W cells decreased significantly ($P<0.05$).

• CONCLUSION: We demonstrated that the exposure of 661W cells to H_2O_2 increased the activation of ERK1/2 and STAT3 signal pathways. Activation of these pathways is required for 661W cell survival following oxidant injury.

• KEYWORDS: 661W cells; oxidant injury; ERK1/2; STAT3; survival
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INTRODUCTION

Oxidative stress is well documented in the neuronal cell death that is associated with a variety of chronic neurodegenerative disorders, such as age-related macular degeneration (AMD) [1] which is the leading cause of blindness in the elderly and is estimated to affect more than 8 million individuals in the United States alone [2]. In addition, reactive oxygen species (ROS) including H_2O_2 activates an array of signal transduction pathways that serve to coordinate the cellular response and ultimately determine cell fate [3].

The 661W cells line was derived from a mouse retinal tumor and has been characterized as a cone-specific cell line that expresses cone pigments, transducin and arrestin. Clinical and histopathologic studies have revealed that death of photoreceptors [4,5] with rod cells loss preceding that of cone cells, is common to all forms of AMD. Nevertheless, the molecular mechanisms involved in oxidative stress-induced apoptotic 661W cells death are complex and not fully understood. Moreover, numerous studies [6-8] indicate that activation of ERK1/2 and STAT3 plays a central role in cell survival against a variety of stress stimuli, including H_2O_2 in endothelial cell, epithelial cell and so on. However, little is known about the circumstance in neuron cells-661W cells line. In this study, we examined the influence of H_2O_2 on 661W cell survival and the role of

ERK1/2 and STAT3 signal pathways on the cell survival following oxidative stress.

MATERIALS AND METHODS

Materials Antibodies to p-Tyr705-STAT3,STAT3, Phospho-p44/42 MAPK (Thr202/Tyr204), ERK1/2, were purchased from Cell Signaling Technology (Danvers, MA, USA). S3I201 and PD98095 were obtained from Calbiochem (San Diego, CA). The in situ cell viability detection kit 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoli- um bromide (MTT) was purchased from ATCC (Manassas, VA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA)

Methods

Cell culture and treatment Mouse photoreceptor-derived 661W cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 1% penicillin/ streptomycin (Sigma, St. Louis, MO, USA). Cells were grown in 5% CO_2 and 95% humidity at 37℃. In all the experiments, 661W cells were starved for 1 hour with serum-free DMEM, before treatment with H_2O_2 to induce apoptosis. When various pharmacological inhibitors were used, the same volume of DMSO was added to control samples.

Inhibition of ERK1/2 and STAT3 signal pathways activation 661W cells were pretreated with the ERK kinase (MEK1) inhibitor PD98059 (50μmol/L) or STAT3- specific inhibitor S3I201 (50μmol/L) for 1 hour prior to treatment with 1mM H_2O_2. Cell survival and activation of ERK1/2 and STAT3 were determined.

Determination of cell viability by MTT assay To test the viability of 661W cells, 10μL MTT was added to the media (final concentration, 0.5mg/mL), and the cells were placed in an incubator overnight. MTT is reduced by metabolically active cells to form a dark blue formazan crystal. The next day, the formazan precipitate was dissolved in detergent reagent, and the optical density was determined with a spectrophotometer at 570nm reader (Molecular Devices, Sunnyvale, CA). The viability of the cells was expressed as percentage of cells surviving. Each experiment was performed at least three times, with 5 replicates for each treatment.

Immunoblot analysis 661W cells were grown to 80% confluence in 6cm plate. After various treatments, cells were washed twice with ice-cold PBS and harvested in the radioimmunoprecipitation assay (RIPA) buffer that contained 100μg/mL phenylmethylsulfonylsuoride (PMSF), 100mmol/L sodium orthovanadate, and 50μL/mL of proteinase inhibitor cocktail. Protein quantification was performed using the Bradford protein assay kit (Pierce Biotechnology, Rockford, IL). Proteins (20μg) were separated by SDS-PAGE and transferred to PVDF membranes. After incubation with 5% skim milk for 1 hour at room temperature, membranes were incubated with primary antibody overnight at 4℃ and then incubated with the appropriate peroxidase-linked secondary antibody for 1 hour at room temperature. Immunoreactivity was visualized by enhanced chemiluminescence using Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and images were captured by a Chemi Genius Image Station (SynGene, Frederick, MD). Band intensities were quantified using the Gene Tools program (SynGene, Frederick, MD).

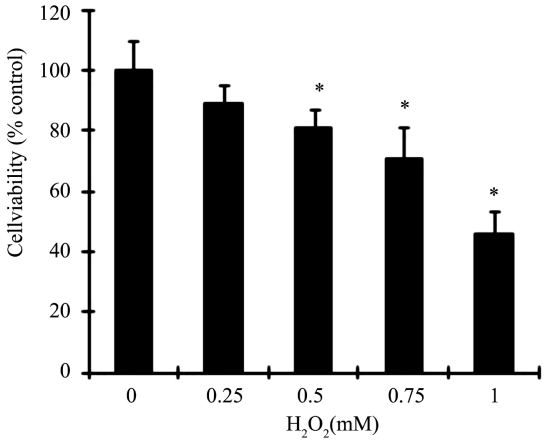

Figure 1 Effect of H_2O_2 on cell viability of 661W cells Freshly grown 661W cells were exposed to different indicated concentrations of H_2O_2 for 12 hours. Cell viability was assessed by MTT assay. Values are means±SD of 3 independent experiments conducted in triplicates and expressed as the percentage of control. * P <0.05 compared with control.

Statistical Analysis Data are presented as means±SD and were subjected to one-way ANOVA. Differences between two groups were determined by student's t test. P <0.05 was considered statistically significant.

RESULTS

Cell viability of 661W cells decreased following oxidant injury H_2O_2 has been reported to influence cell viability of kinds of cells [9-11]. Own to each type of cells has different ability of resistance to H_2O_2, we need to determine the effect of H_2O_2 on 661W cells. 661W cells were exposed to different indicated concentrations of H_2O_2 for 12 hours and cell viability was examined using the MTT assay. Cell viability of 661W cells decreased in concentration-dependent manner. After treating with 0.5mM H_2O_2 for 12 hours, the Cell viability decreased to 81% in 661W cells and further dropped to 70% and 50% in the groups treated with 0.75mmol/L and 1mmol/L H_2O_2, respectively (Figure 1).

Activation of ERK1/2 and STAT3 signal pathways by H_2O_2 on 661w cells To investigate whether ERK 1/2 and STAT3 signal pathways are activated by ROS in 661W cells, the cells were treated with 1mmol/L H_2O_2 for different

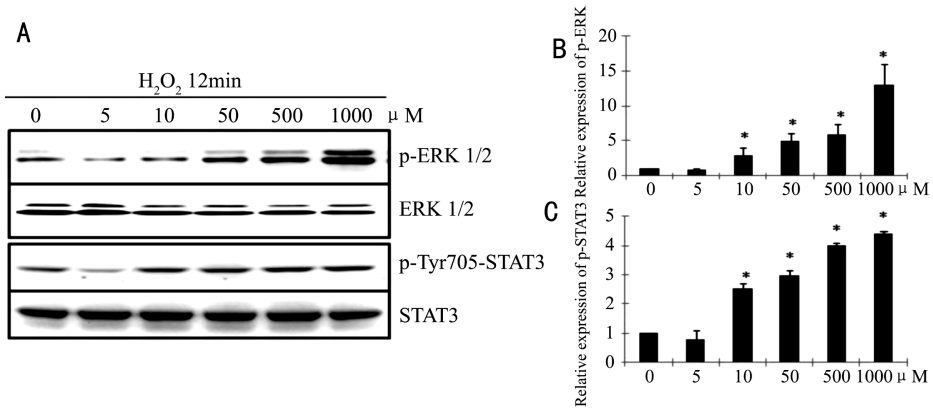

Figure 2 H_2O_2 –induced activation of ERK1/2 and STAT3. 661W cells were exposed to different indicated concentrations of H_2O_2 for 15 minutes Cell lysates were prepared and subject to immunoblot analysis with antibodies for Phospho-p44/42 MAPK (Thr202/Tyr204), ERK1/2,p-Tyr705-STAT3,STAT3. Representative immunoblots from 3 experiments are shown (A). Quantiative analysis of the expression of p-ERK1/2 and p-Tyr705-STAT3 was conducted. Values are means±SD of 3 independent experiments conducted in triplicates and expressed as the percentage of ERK1/2 and STAT3, respectively (B, C). * P<0.05 compared with control.

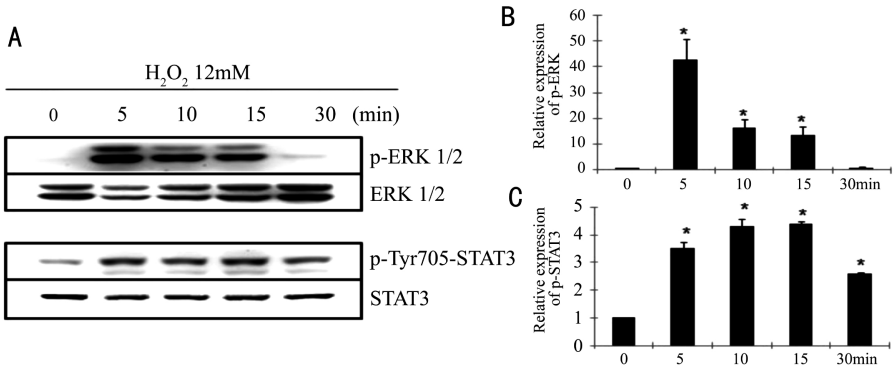

Figure 3 H_2O_2 –induced activation of ERK1/2 and STAT3 signal pathways 661W cells were exposed to 1mM H_2O_2 for indicated time. Cell lysates were prepared and subject to immunoblot analysis with antibodies for Phospho-p44/42 MAPK (Thr202/Tyr204), ERK1/2, p-Tyr705-STAT3, STAT3 (A). Quantiative analysis of the expression of p-ERK1/2 and p-Thy705- STAT3 was conducted. Values are means±SD of 3 independent experiments conducted in triplicates and expressed as the percentage of ERK1/2 and STAT3, respectively (B, C). * P<0.05 compared with control.

indicated time or with different indicated concentrations of H_2O_2 for 15 minutes. Activation of these pathways was measured by immunoblot analysis using antibodies that recognize phosphorylated ERK1/2 and STAT3, respectively. Total ERK1/2 and STAT3 were measured by immunoblot analysis with antibodies that recognize total ERK1/2 and STAT3.

The results showed that phosphorylated ERK1/2 and STAT3 were activated in response to H_2O_2. A concentration-response experiment indicates that phosphorylated ERK1/2 activation was first detected at 50μmol/L H_2O_2 and increased with H_2O_2 concentrations up to 1mmol/L. The expression of phosphorylated ERK1/2 was faint when the concentrations of H_2O_2 were under 50μmol/L. Moreover, the phosphorylated STAT3 activation by H_2O_2 showed a similar concentration-dependent manner, which was detected at 10μmol/L H_2O_2 and reached maximum at 1mmol/L H_2O_2. In addition, the total expression levels of ERK1/2 and STAT3 did not change(Figure 2).

The results of a time-response experiment were show that within 5 minutes after exposure to H_2O_2, there was a rapid activation of phosphorylated ERK1/2, reaching the peak level as much as 40 times control(Figure 3).

However, the expression of phosphorylated ERK1/2 decreased from 5 to 30 minutes. For comparison, phosphorylated STAT3 peaked at 5 minutes after H_2O_2 treatment, as much as 5 times control. However, the expression of phosphorylated STAT3 decreased from 15 minutes. In addition, the total ERK1/2 and STAT3 expression has unchanged.

Activation of ERK1/2 and STAT3 is required for 661W cell survival following oxidant injury To elucidate the role of ERK1/2 and STAT3 signal pathways in 661W cell survival following oxidant injury, we pretreated 661W cells with PD98059 and S3I201, and then exposed them to 1mmol/L H_2O_2. Activation of ERK1/2 and STAT3 was detected by immunoblot analysis and the cell viability was measured by the MTT assay. As shown in Figure 4, these two inhibitors abolished phosphorylation of ERK1/2 and STAT3, respectively. In addition，treatment with either

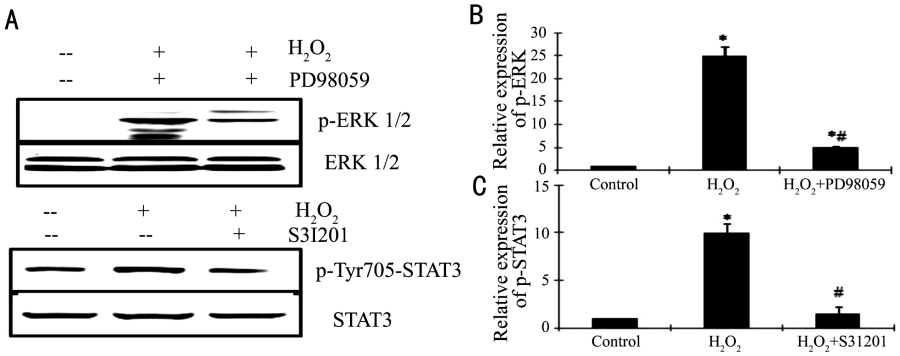

Figure 4 PD98059 and S3I201 reduced H_2O_2 -induced ERK1/2 and STAT3 activation, respectively After pretreating with PD98059 and S3I201 for 1 hour and then exposing them to 1mM H_2O_2 for 15 minutes, 661W cell lysates were prepared and subjected to immunoblot analysis with antibodies for Phospho- p44/42 MAPK (Thr202/Tyr204), ERK1/2, p-Tyr705-STAT3, STAT3 (A). Quantiative analysis of the expression of p-ERK1/2 and p-Thy705-STAT3 was conducted. Values are means±SD of 3 independent experiments conducted in triplicates and expressed as the percentage of ERK1/2 and STAT3, respectively (B,C). * P< 0.05 compared with control. # P< 0.05 compared with the group treated with H_2O_2 only.

PD98059 or S3I201 enhanced H_2O_2-induced apoptotic cell death. Cell viability was reduced to 50% in 661W cells treated with 1mM H_2O_2 for 12 hours and further decreased to 30% and 31% in the presence of 50μmol/L PD98059 or 50μmol/L S3I201, respectively (Figure 5). Taken together, these data indicate that activation of ERK1/2 and STAT3 pathways is required for cell survival in H_2O_2-treated 661W cells.

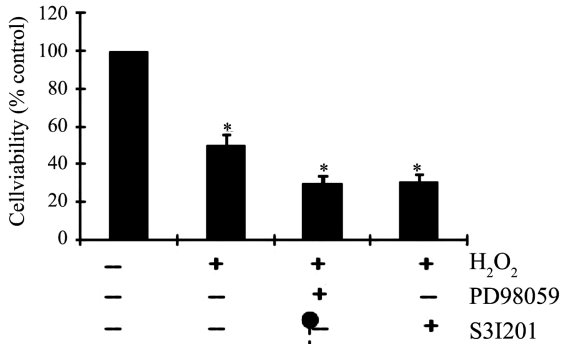

Figure 5 Inhibition of ERK1/2 or STAT3 decreases the viability of 661W cells 661W cells were incubated with PD98059 (50μmol/L) or S3I201 (50μmol/L) for 1 hour and then exposed to 1mmol/ L H_2O_2 for 12 hours. Cell viability was determined by MTT assay. Values are means±SD of 3 independent experiments conducted in triplicates and expressed as the percentage of control. * P <0.05 compared with control.

DISCUSSION

ROS include several oxygen-nitrogen-derived free radicals and oxidants, H_2O_2, superoxide, hypochlorite, nitric oxide, hydroxyl radical, nitrogen dioxide, peroxynitrite and other amino acid- and lipid-derived radicals. Oxidative processes have been proposed to play a causative or contributing role in certain types of eye disorders, such as AMD which is the most important causes of visual impairment in the elderly.

The aetiology of AMD is multifactorial with well established risk factors, such as low plasma concentration of antioxidants. The cone cells in macula area are particularly prone to direct light exposure, in fact playing the role of mediating light transduction into neuronal impulses. Moreover, their cell membranes have the highest polyunsaturated fatty acid content of any known tissue. In addition, retinal oxygen turnover is very high and its cellular mitochondria are abundant. As a result, macula, cone cells particularly, are highly susceptible to oxidative stress. The ability of cells to survive a variety of stresses including oxidant stress often depends on the activation of survival signaling pathways. However, there is little known about the molecular mechanisms involved in the association between oxidative stress and cone cells. In this study, we surveyed the 661W cells line which is cone-specific cell line that has the properties of cone photoreceptors. We demonstrated that the exposure of 661W cells to H_2O_2 reduced the cell viability and increased the activation of ERK1/2 and STAT3 signal pathways. Moreover, either ERK1/2 or STAT3 signal pathway inhibitor aggravated H_2O_2-induced cell death.

By varying the concentrations of H_2O_2, we were able to study the effect of H_2O_2 on the cell viability of 661W cells. Virtually every known organism has evolved specific mechanisms to protect itself from oxidative damage. An important part of the cellular defense to oxidative stress is the specific induction of cell signal pathways in response to specific oxidative stressors.

Increasing publications showed that inhibition of ERK1/2 signal pathway significantly increased cell death after H_2O_2 treatment in various cell types, including epithelial and neuronal cells as well as chondrocytes [12-14] .Our data also suggest that treatment with H_2O_2 induced phosphorylaton of ERK1/2 signal pathway in 661W cells. Using ERK kinase (MEK1) inhibitor PD98059 to prohibit the effect of ERK1/2 signal pathway enhanced H_2O_2 -induced apoptotic cell death. The results showed activation of ERK1/2 is required for

661W cell survival following oxidant injury.

Kim U.S. *et al.* reported that exposure to H_2O_2 induces phosphorylaton of STAT3 signal pathway in lens epithelial cells, a finding that agrees well with our results [15]. We have shown in 661W cells that the activation of STAT3 in response to H_2O_2 occurs within minutes and is independent of new protein synthesis. We used STAT3 specific inhibitor S3I201 to inhibit STAT3 signal pathway, and the MTT result also showed that treatment with STAT3 inhibitor enhanced H_2O_2-induced cell death.

In summary, the exposure of 661W cells to H_2O_2 reduced the cell viability in concentration- dependent manner. In addition, treatment with H_2O_2 increased the activation of ERK1/2 and STAT3 signal pathway. Furthermore, PD98059 and S3I201 can inhibit ERK1/2 and STAT3 signal pathway, respectively. The inhibitors aggravated H_2O_2-induced cell death, suggesting that activation of ERK1/2 and STAT3 signal pathways was required for 661W cell survival following oxidant injury.

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