·Basic Research·

# Peroxynitrite-induced expression of inducible nitric oxide synthase and activated apoptosis *via* nuclear factor-kappa B pathway in retinal pigment epithelial cells and antagonism of cholecystokinin octapeptide-8 *in vitro*

*Li-Na Hao [1], Xu-Dong Zhang [2], Min Wang [1], Tao Yang [3], Shou-Zhi He [4]*

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[1]Department of Ophthalmology, People's Hospital of Hebei Province, Shijiazhuang 050051, Hebei Province, China

[2]Pharmaceutical Department of Hebei Province People's Hospital, Shijiazhuang 050051, Hebei Province, China

[3]Internal Department of First Hospital affiliated to the Hebei Medical University, Shijiazhuang 050031, Hebei Province, China

[4]Ophthalmology Department of 301 Hospital, Beijing 100853, China

**Correspondence to:** Li-Na Hao. Department of Ophthalmology, People's Hospital of Hebei Province, Shijiazhuang 050051, Hebei Province, China. jhaolina@yahoo.com.cn

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## Abstract

• AIM: To explore that if peroxynitrite induced the expression of inducible nitric oxide synthase (iNOS)via nuclear factor-kappa B (NF-κ B)pathway in retinal pigment epithelial (RPE) cells and the antagonism of cholecystokinin octapeptide-8 (Melatonin , CCK-8) *in vitro*.

• METHODS: RPE cells were obtained from eyes of C57BL/6 mouse and divided into control, peroxynitrite and CCK-8 groups. Control group was treated with saline, peroxynitrite group was treated with peroxynitrite, and CCK-8 group was treated with CCK-8 after added with peroxynitrite. All changes were observered at 6, 12 and 24 hours after treatment. Gene array analysis, Reverse Transcription Polymerase Chain Reaction (RT-PCR) were used to determine the expression of inducible nitric oxide synthase (iNOS)mRNA in RPE cells. Western blotting was used to test the apoptosis of RPE cells. Immunofluorescent staining was used to determine the NF-κ B pathway signal transduction.

• RESULTS: Compared to the control group, the expression of iNOS mRNA was up-regulated in peroxynitrite group and down-regulated in CCK-8 group with gene array analysis. Apoptosis was increased in peroxynitrite group and decreased in CCK-8 group with western blotting. The NF-κ B pathway signal transduction was more and more stronger in the peroxynitrite group. But in CCK-8 group, little stronger could be observed at 12 hours, then weak at 24 hours with immunofluorescent staining ($P$<0.001).

• CONCLUSION: This study suggested that apoptosis of RPE cells was partly induced by peroxynitrite, which may be the new way of oxidative damage to the RPE cells. The NF-κ B signal transduction may affect and reinforce apoptosis mediated by peroxynitrite. CCK-8 decreased apoptosis of RPE cells induced by peroxynitrite and is a potential agent for therapy of retinopathy. The mechanism of CCK-8 dealing with RPE cells may be related to its direct inhibition of the formation of iNOS to produce peroxynitrite and antagnism of damage of peroxynitrite to the RPE cells.

• KEYWORDS: cholecystokinin octapeptide-8, retinal pigment epithelial cells, oxidative; cell signal

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## INTRODUCTION

The major view of apoptosis in retinal pigment epithelial (RPE) cells is accepted today is that they are formed because of oxidative stress. Oxidtive stress is defined as the generation of reactive oxidant species that exceeds the antioxidant defenses. Traditional reactive species include oxygen hydrogen-peroxide ($H_2O_2$), nitric-oxide (NO) and superoxide-anion ($O_2^-$). Recent studies have focused on peroxynitrite. Which formed from the reaction of $O_2^-$ and

NO, is a cytotoxic species that can oxidize several cellular components such as proteins, lipids, and DNA [1-2]. Due to the lack of endogenous enzymes responsible for peroxynitrite inactivation, developing a specific scavenger is of considerable importance. We have reported that RPE cells oxidation may occur by the Fas/FasL pathway which is antagonized by puerarin [3]. In this study we show that peroxynitrite -induced expression of inducible nitric oxide synthase and activated apoptosis via NF-κ B pathway in RPE cells and that production was attenuated by cholecystokinin octapeptide-8 (CCK-8) in cultured RPE cells.

## MATERIALS AND METHODS

**Materials** Peroxynitrite was obtained by reacting ice-cold solutions of sodium nitrite (0.6mol/L) with $H_2O_2$ (0.7mol/L) in acidic medium (0.6mol/L HCl) and rapidly quenching the reaction in NaOH (1.5mol/L), as described previously [4,5]. The reaction mixture solution was frozen at -20℃, and the peroxynitrite concentrated in the upper layer was collected. Its concentration was measured at 302 nm using a molar extinction coefficient of $1670 mol^{-1} \cdot cm^{-1}$. Pathogen-free, aged 2-3 weeks, forty C57BL/6 mice and health, male, Sprague-Dawley (SD, 250g±) rats were used in this study. All animals were treated in accordance with The Association for Research in Vision and Ophthalmology ARVO Resolution "Statement for the use of Animals in Ophthalmic and Vision Research " in USA. RPE were harvested by using a modification of techniques described previously[6]. Freshly enucleated mouse eyes were cleaned of extraocular tissue. Scissors were introduced at the optic nerve into the vitreous cavity and the incision was extended through the sclera, choroid, and retina toward the iris. Three additional radial incisions were made, and these cuts were completed with a number 10 scalpel and the tissue cut into 3-mm squares. The retina was removed carefully and the tissue was incubated with 6.25U/mL dispase (Invitrogen-Gibco) for 8 hours at 4℃. The loosened RPE sheets were separated from the remainder of the ocular tissue and placed on a drop of 25% gelatin with the apical surface of the RPE facing upward. Contamination with choroidal cells was avoided by visualizing the RPE sheets under a dissecting microscope during harvesting. The gelatin film containing the RPE sheet was then incubated in a humidified atmosphere of 5% $CO_2$ and 95% air at 37℃ for 2 minutes to allow the gelatin to melt and encase the RPE sheet. The specimen was kept at 4℃ for 4 minutes to solidify the liquid gelatin, covered with 20 μL of 10% gelatin and 300mol/L sucrose in DPBS at 37℃, returned to 4℃ for 6 minutes, and played in the laboratory overnight in $CO_2$-free medium (CFM; Gibco-Invitrogen) at 4℃. On receipt in the laboratory, the RPE-containing sheet was triturated into small microaggregates in DMEM and diluted to a concentration of approximately 40 000 cells/10μL for injection into the subretinal space. Cytokeratin staining before transplantation indicated that all the cells were of epithelial origin. Then RPE cells were divided into control, peroxynitrite and CCK-8 groups. Control group was treated with saline, peroxynitrite group was treated with peroxynitrite (200 microm) to induce cell death and CCK-8 group was treated with CCK-8 (200 microm) after added with same amount of peroxynitrite. All changes were observered at 6, 12 and 24 hours after treatment.

**Antibodies and reagents** Monoclonal mouse anti-nitrotyrosine (NT, the foot print of peroxynitrite) antibody, NF-κB p50 antibody, goat anti-mousefluoresceint isothio-cyanate (FITC) antibody, CCK-8 were purchased from Sigma Company, USA.

**iNOS mRNA expression assay** RPE cells used in arrays were dissected free of any contaminating tissue and homogenized in trizol reagent. RNA extraction was carried out according to the manufacture's protocol. Concentration and RNA quality were assessed via spectrophotometry and formaldehyde gel electrophoresis. Amplified mRNA was then labeled with Cy3 or Cy5 (Random Primer DNA Labeling Kit, Bao-Boiscience Engineering Corporation, China). Successfully labeled control and experimental targets with 12 housekeepig genes and 12 artificial synthesize 70 mer oligo DNA as positive and negative controls were then combined and prepared for hybridization. Array slides were obtained from Qiagen Corporation, USA. and incubated in prehybridization for 1 hour at 42℃. Targets were dried via vacuum centrifugation then resuspended in 50μL hybridization solution added with 1μL Cot 1 DNA and 1μL poly A oligonucleotide as blocking agents, heated to 95℃ for 5 minutes and then added to the face of one slide. The printed face of the second slide of the pair was then placed face to face with the first, using the same prob. Slide pairs were then placed on a level plastic cover above some 1×SSC moistened tissue in a slide box. The slide box was sealed and placed floating in a water bath and habridized for 24-48 hours at 42 ℃. Following hybridization, slides were washed in wash solution for 20 minutes and repeated for another 20 minutes then dipped in nuclease free water, spray dried. Finally, the backs of the slides were cleaned with $ddH_2O$, wiped with 100% ethanol, then wiped dry and scanned by Scan Array Express Scanner (Packard Bioscience Corporation, USA) Gene Pix Pro 4.0 photo soft ware (Axon Instruments Corporation, USA) was used for clustering analysis. Two folds higher divergence were regarded as divergence expression gene. At same time,

NF-κ B was up-regulated in peroxynitrite group and down-regulated in CCK-8 group. Equal amounts of the total RNA were used to detect the mRNA levels of iNOS by reverse transcription polymerase chain reaction (GeneAmp RNA-PCR kit; Applied Biosystems, USA ). Total RNA was extracted from the RPE cells in three groups, according to the kit manufacturer's specifications. The sense and anti-sense oligonucleotide primers for rat iNOS[7] were synthesized by Biological Engineering Corporation. The primer sequences are: iNOS (262bp) sense primer 1: 5'-CGCCCTTCCGCAG TTCT-3'; sense primer 2:5'-TCCAGGAGGACATGCAG CAC-3'.β-actin (420bp) sense primer 1:5'-GAGAC CTTC AACACCCAGCC-3'; sense primer 2:5'-GCGGGGCATCG GAACCGCTCA-3'. And 4μg of RNA in a total volume of 20μL (pH8.3) were for synthesizing the cDNA. RT-PCR was first performed at 24℃ for 10 minutes, then at 42℃ for 15 minutes. The reaction mixture was heated at 99℃ for 5 minutes, and the RT product was mixed with DNA polymerase (Ampli *Taq*; Applied Biosystems, USA) and the sense primer in a buffer containing 20mmol Tris-HCL, 50mmol KCl, 2.0mmol $MgCl_2$ (PH 8.3), and 50mmol of each dNTP in a 100μL volume. The mixture was then amplified by PCR using 29 cycles. The thermal cycle profile used in this study was as follows: an initial denaturing at 94℃ for 2 minutes and then 45 seconds in each cycle; annealing the primer with DNA at 55℃ for 45 seconds; and extending of the primer at 72 ℃ for 10 minutes. All reactions were normalized for iNOS expression. The negative controls consisted of omission of RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on 20g/L agarose gel.

**RPE cells apoptosis** RPE Cells were prepared as described, which were homogenized and solubilized in ice cold PBS containing protease inhibitors, phenylmethylsulfonyl fluoride (1mg/L), aprotinin (1mg/L), leupeptin (1mg/L), pepstatin A (1mg/L) and EDTA (1mmol/L). The homogenate was centrifuged at 15 000r/min at 4℃ for 10 minutes. The protein content of the supernatants was determined by the Bradford method [8]. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% linear slab gel, under reducing conditions, separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blot was stained at room temperature with a 1:100 dilution of monoclonal mouse anti-NT antibody (1:400) over night at 4℃ . After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:800 dilution), blot was developed using the enhanced chemiluminescence and analyzed with Western blot analysis detection system (ECL Plus; Amersham Pharmacia Biotech, Arlington Heights, IL).

**NF-κB pathway signal transduction assay** The RPE cells were fixed in 70% ethanol for 24 hours. The suspension were centrifuged 1 000r/min for 4 minutes. Then sediment were mixed with PBS, filtered in screen and centrifuged again, the cells were then suspensioned with PBS. With indirect immuno-flourescent labeling antibody technique, NF-κB antibody p50 (1:400) were added into the suspension and reacted in dark for 30 minutes at room temperature, then added goat anti mouse FITC labeling antibody for another 30 minutes under the same condition. The suspension were examined under fluorescent microscope.

**Statistical Analysis** Data were performed on computer (SPSS 15.0). The results was expressed as mean ±SD. Statistical significance was determined by a one-factor analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons. $P<0.01$ was considered significant.

**RESULTS**

**iNOS mRNA Expression** Yellow color showed no changes, red color showed up-regulation of gene and green color showed down-regulation of gene. As time passed by, control group showed no changes (yellow color). peroxynitrite group showed up-regulation of iNOS mRNA (red color), while CCK-8 group showed down-regulation iNOS mRNA (green color). Taking advantage of gene array technique, we observed that there were no expression of iNOS in control group, but there were continuous up-regulation of iNOS (NOS2) and nNOS (NOS1) genes in peroxynitrite group compared with that in the control and CCK-8 groups. There were minor up-regulation of iNOS gene with 2.1-fold changes and nNOS gene with 1.7-fold changes at 6 hour in CCK-8 group and continued to 5.1-fold up-regulation of iNOS gene and 3.4-fold nNOS gene at 12 hour, but decreased to 2.3-fold of iNOS gene and 2.0-fold nNOS gene at 24 hour. Except the genes iNOS related to nitric-oxide (NO) and production, the other apoptosis-related genes including NF-κB signal transduction way was up-regulated in peroxynitrite group and there was the opposite facts in CCK-8 group (Figure 1). There was no expression of iNOS mRNA in the control group, but there was distinct up-regulation of iNOS mRNA in peroxynitrite group as time passed. Expression of iNOS mRNA in CCK-8 group gradually up-regulated from 6 to 12 hours, then down-regulated at 24 hours (Figure 2). With computer photo-analysis, there were significant differences among the three groups ($P<0.001$, Figure 3).

**RPE cells Apoptosis** With Western blotting analysis, a faint expression of NT could be seen in the control group. A

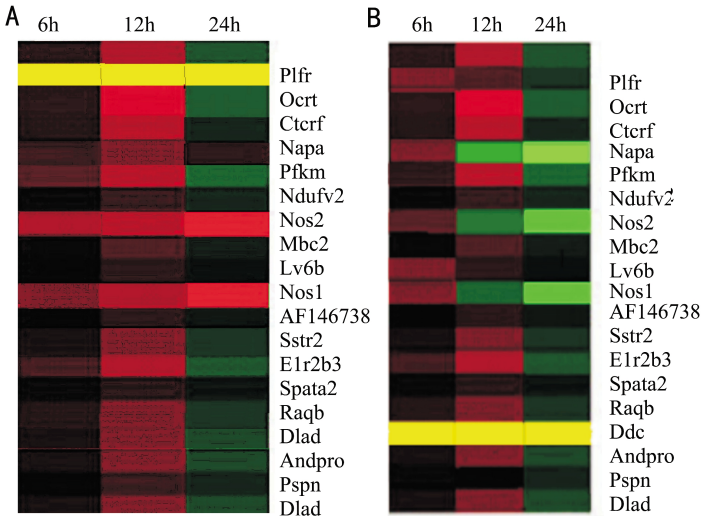


**Figure 1 Gene array expression** A:Peroxynitrite; B:CCK-8

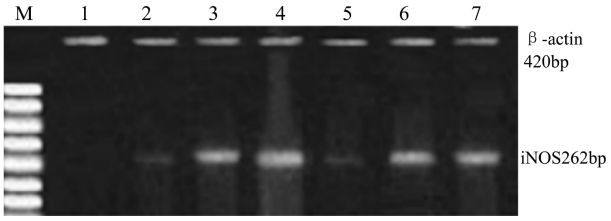


**Figure 2 RT-PCR detection of iNOS mRNA expression in RPE cells with CCK-8** M: DNA marker; 1-7: Control and β-actin; second row; 2-4: Peroxynitrite at 6,12,24 hours; 5-7: CCK-8 at 6,12,24 hours

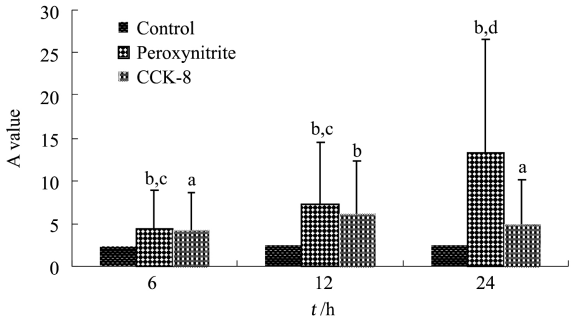


**Figure 3 Comparison of RT-PCR detection of iNOS mRNA expression in RPE cells with CCK-8** $^{a}P<0.05$, $^{b}P<0.01$ *vs* Control; $^{c}P<0.05$, $^{d}P<0.01$ *vs* CCK-8

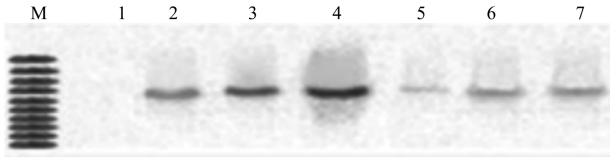


**Figure 4 Detection of NT protein expression in RPE cells with Western blotting** M: Maker; 1: Control; 2-4: Peroxynitrite at 6,12,24 hours; 5-7:CCK-8 at 6,12,24 hours

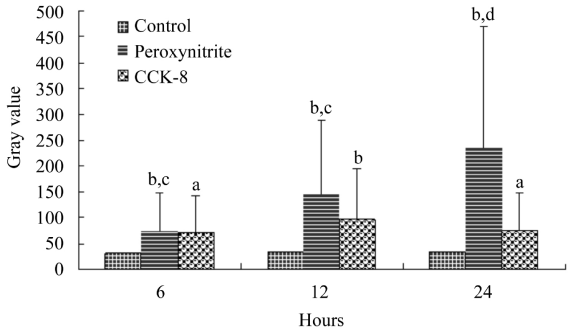


**Figure 5 Computer photo analysis for detection of NT protein in RPE cells with Western blotting** $^{a}P<0.05$, $^{b}P<0.01$ *vs* Control; $^{c}P<0.05$, $^{d}P<0.01$ *vs* CCK-8

gradual to strong expression of NT was observed at different stages of the experiment in peroxynitrite group. But expression of NT in CCK-8 group changed gradually from faint to strong during the period of 6 to 12 hours, then turn to weak at 24 hour (Figure 4). Computer photo analysis indicated that there were significant differences among three groups ($P<0.001$, Figure 5). The trial was repeated at least three times.

**NF-κB pathway signal transduction** The NF-κB negative antigen was visible as a faint-green color in the nucleus and cytoplasm and the NF-κB positive antigen appeared as an orange-yellow color under the fluorescent microscope. Therefore a faint-green color was visible in the nucleus and cytoplasm of the control group. The color changed from green-yellow to orange-yellow color during the period of 6, 12 and 24 hours in the peroxynitrite group. In comparison, the color changed from faint-green to yellow color during the period of 6 to 12 hours of experiment, then turned to green-yellow at 24 hours in the CCK-8 group (Figure 6).

## DISCUSSION

Oxidative stress is involved in the pathogenesis of many fundus diseases such as age-related macular degeneration (AMD). Administration of conventional antioxidants has been shown to slow the progression of AMD and vision loss. CCK-8, an endogenous neurohormone produced by the

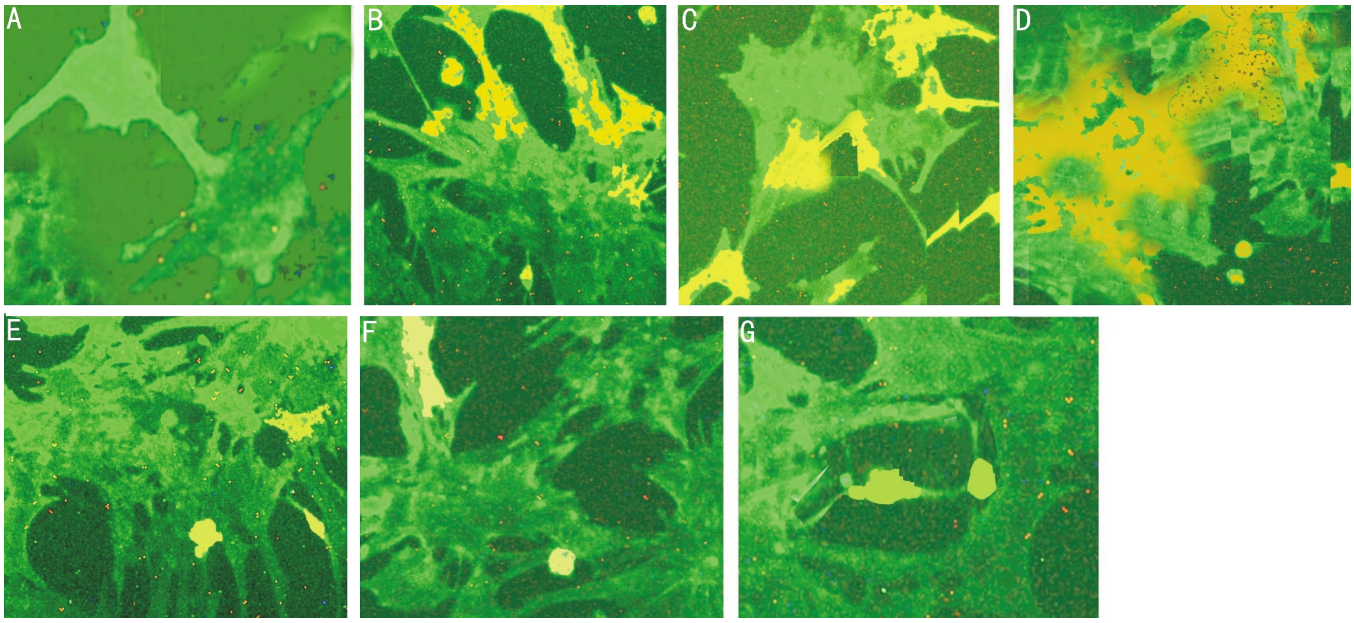


**Figure 6 NF-κB antigen in RPE cells was examined by immune fluorescent staining** A: Control group; B,C,D: 6,12,24 hours in peroxynitrite group; E,F,G: 6,12,24 hours in CCK-8 group, respectively

pineal gland and retina, has been reported to be a potent antioxidant and free radical scavenger [9-12]. In this study we tested whether CCK-8 can protect RPE cells against peroxynitrite -induced cell apoptosis.

Using RT-PCR, Gene array technique, we verified that inducible nitric-oxide syntheses (iNOS) might contribute to oxidative stress by helping to develop to produce more powerful oxidants such as peroxynitrite. iNOS is the major enzyme involved in inhibiting the production of NO, which is a signaling molecule with multi-regulation functions. Under pathological conditions, up-regulation of iNOS mRNA in RPE is over production of NO, accompanied by activation of the oxidant enzyme as well as increasing the $O_2^-$. Extra NO and $O_2^-$ produce extra peroxynitrite which acts as a strong oxidant. It is likely that iNOS may contribute to oxidation stress by helping develop more powerful oxidative agents such as peroxynitrite. With gene array analysis, we found that iNOS divergence genes appeared in six micro-array slides (NOS2, 10400U12611). They were up-regulated in peroxynitrite group and down-regulated in CCK-8 group. These results verified our previous work [13-15]. Meanwhile, nNOS (NOS1, 10573U67309) showed the same results as iNOS. nNOS was expressed in normal physiological conditions and mainly located in the brain and its activation depends on $Ca^{2+}$ and calmodine (CaM). Many studies have shown that iNOS affects and regulates apoptosis [16-17]. Therefore, the relationship between iNOS and nNOS still needs to be studied and clarified.

With western blotting analysis, we observed the peroxynitrite-mediated protein nitration product, nitrotyrosine (NT), was located in RPE and decreased under the intervention of CCK-8. Apoptoic RPE cells comtinued to be increased in peroxynitrite group compared to control and CCK-8 group. In CCK-8 group, apoptotic cells were increased from 6 to 12 hours, but decreased from 12 to 24 hours. which may indicate a protective role of CCK-8 on RPE. These results are consistent with our previous work [13-15]. The traditional oxygen-free radical stress mechanism pays more attention to the role of hydrogen-peroxide ($H_2O_2$), NO and superoxide-anion ($O_2^-$), while the new theory includes peroxynitrite, a product from rapid reaction of NO and $O_2^-$, which may be an important mediator of cytotoxicity in oxidation[18-19].

Similarly, using immuno-flurescent staining technique, we verified that NF-κB was expressed during the apoptosis of RPE cells. Which was also observered in apoptosis-related genes and signal transduction in this study. NF-κB was translocalized into the nuclei of RPE cells and activated NF-κB and iNOS were highly expressed which paralleled the expression of iNOS. These results suggest that the increased expression of NF-κB and iNOS caused by peroxynitrite is involved in RPE apoptosis.

NF-κB was up-regulated in peroxynitrite group. We observered that NF-κB induced apoptosis in RPE but showed strong and weak up-regulation in peroxynitrite and CCK-8 groups. CCK-8 decreased the apoptosis effect of NF-κB with its anti-oxidative ability. Lacking of CCK-8 anti-oxidative ability, the strong apoptosis effect of NF-κB was shown in CCK-8 group. The activation of NF-κB could also induce the iNOS transcription [20]. That is probably the part reason why peroxynitrite was expressed in both two groups.

CCK-8 has long been implicated in body energetics, first as a gasrointestinal hormone assisting fat utilization and later as a neuropeptide acting either peripherally or centrally in the regulation of body mass [21]. Our study found that the damaging effect of peroxynitrite and iNOS could be antagonized by CCK-8 as assessed with other methods was significantly decreased in RPE cells pretreated with CCK-8 as compared to those without CCK-8 treatment. Therefore, CCK-8 delayed the development of apoptosis in RPE and may be an useful therapeutic agent against RPE apoptosis. This pathogenesis may be related to its anti-oxidant role, anti-iNOS-NO system, therefore lessened RPE oxidation. We found during the period of 6-12 hours, the effect of CCK-8 was not apparently, while after 12 hours, its effect was dramatic. The reason for this maybe related to the concentration of CCK-8 which had reached a critical level. These results suggest that CCK-8 may play a role in protecting RPE cells from oxidative stress.

In conclusion, our findings demonstrate that experimentally induced apoptosis in RPE is mediated by many apoptotic factors. Like other agents, CCK-8 regulates the expression of anti-apoptotic genes, which inhibits the production of iNOS and peroxynitrite, resulting in increase of RPE cells survival.

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