Control of peroxynitrite–induced production of inducible nitric oxide synthase isoforms and antagonism of cholecystokinin octapeptide–8 in retinal pigment epithelial cells *in vivo*

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Abstract

• AIM: To explore if peroxynitrite (ONOO⁻) induced iNOS via Fas/ Fas/L pathway in diabetic rats and the effection of cholecystokinin octapeptide–8 (CCK-8) as therapeutic agent for decrease diabetic retinopathy.

• METHODS: Thirty-six rats were taken as control group, seventy two were given (streptozotocin) STZ (45mg/kg) and then divided into ONOO⁻ group and CCK-8 group (peritoneal injection CCK-8). STZ-induced diabetic rats were treated with CCK-8 for 60 days. Western blotting analysis, DNA ladder, RT-PCR, immunohistochemistry and flow cytometry were used for determining the expression of nitrotyrosine (NT, the foot print of ONOO⁻); apoptosis and inducible nitric oxide synthase (iNOS) mRNA as well as Fas/Fasl signal transduction in RPE cells.

• RESULTS: Both RPE cells in ONOO⁻ and CCK-8 group developed apoptosis and expressed NT, iNOS mRNA and Fas/Fasl. But later delayed the all changes in a time-dependent manner compared with control and ONOO⁻ group (*P* < 0.001). iNOS and Fas/Fasl were up-regulated and associated with an increase of expression of ONOO⁻ *in vivo*

• CONCLUSION: The study suggested that apoptosis of RPE was partly induced by ONOO⁻ may be the new way of oxidative damage to the RPE cells. CCK-8 decreased RPE cells apoptosis partly induced by ONOO⁻ and is a potential drug for therapy of diabetic retinopathy. The mechanism of CCK-8 dealing with RPE cells may be related to its direct inhibition of the formation of iNOS to produce ONOO⁻ and antagonism of damage of ONOO⁻ to RPE cells.

• KEYWORDS: CCK-8; retinal pigment epithelial cells; oxidative; cell signal; diabetes

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**INTRODUCTION**

Diabetic retinopathy resists reversal after good glycemic control is reinstituted, and preexisting damage at the time of intervention is considered as the major factor in determining the outcome of the retinopathy. Many of these pathways become activated in response to the production of superoxide anion. Superoxide can interact with nitric oxide under iNOS, forming the potent cytotoxin peroxynitrite (ONOO⁻). ONOO⁻ attacks various biomolecules in the retina eventually leading to its dysfunction via multiple mechanisms. This study focuses on emerging evidence suggesting that ONOO⁻ plays a key role in the pathogenesis of complications of diabetes, which underlie the development and progression of diabetic retinopathy. Several authors have postulated the importance of ONOO⁻ production in the development of diabetic complications[1,2]. But the relations of RPE cells with oxidation and cell signal transduction network work are still far clear. CCK-8 is the subfamily member of melatonin, which is an endogenous neurohormone produced by the pineal gland and retina, has been reported to be a potent antioxidant and free radical scavenger [3,4]. Early inhibition of oxidative stress with use of CCK-8 probably represent safe and effective retin-
protective agents in diabetic retinopathy.

MATERIALS AND METHODS

Materials

Animals

Pathogen-free, male, Sprague-Dawley (SD) rats (5-6 weeks old) were used. All animals were treated in accordance with ARVO Statement for the use of Animals in Ophthalmic and Vision Research in China.

Antibodies and reagents

Monoclonal mouse anti-nitrotyrosine (NT) antibody, Fas/FasL antibody, goat anti-mouse fluorescein isothiocyanate (FITC) antibody, and streptozotocin (STZ), were purchased from Sigma (St. Louis, MO, USA). CCK-8 were purchased from Sigma Company, USA.

Methods

Animals and Isolation of RPE cells

Animals were divided into three groups: control group, ONOO− group and CCK-8 group, each containing 36 animals. SD rats in ONOO− and CCK-8 groups were intraperitoneally (i.p.) injected with STZ (45mg/kg) to establish the animal model. There was one right eye of each rat involved in the experiment. The rats in the control group received the same amount of saline. Three days after starting of experiment, the rats in CCK-8 group received CCK-8 1mg/kg per day. Animals were examined by retinoscope on the days 20, 40 and 60 after STZ injection.

RPE sheets were harvested by a modification of the technique described by Kaplan and Pfeffer [6-8]. Briefly, freshly enucleated porcine eyes were cleaned of extraocular tissue. The suprachoroidal space of the posterior pole was sealed with cyanoacrylate glue, and a small scleral incision was made 3mm posterior to the limbus until the choroidal vessels were exposed. Tenotomy scissors were introduced through this incision into the suprachoroidal space, and the incision was extended circumferentially. Four radial relaxing incisions were made in the sclera, and the sclera was peeled away from the periphery to the optic nerve with care taken to avoid tearing the choroid. The eyecup was then incubated with 25U/mL dispase (Invitrogen-Gibco) for 30 minutes and rinsed with CO2-free medium (CFM; Invitrogen-Gibco) and a circumferential incision was made into the subretinal space along the ora serrata. The loosened RPE sheets were separated from the remainder of the ocular tissue and placed on a slice of 50% gelatin with the apical RPE surface facing upward. Contamination with choroidal cells was avoided by visualizing the RPE sheets under a dissecting microscope during harvest. The gelatin film containing the RPE sheet was then incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C for 5 minutes, to allow the gelatin to melt and encase the RPE sheet. The specimen was kept at 4°C for 5 minutes to solidify the liquid gelatin and then stored in CFM at 4°C.

Western blotting for nitrotyrosine (NT)

The rats were sacrificed and the three eyes were enucleated immediately among three groups at 20, 40 and 60 days respectively. There were total nine eyes in this experiment. Samples were prepared as described and the protein content of the supernatants was determined by the Bradford method.[9] RPE cells were homogenized and solubilized in ice cold PBS containing protease inhibitors, phenylmethylsulfonyl fluoride (1μg/mL), aprotinin (1μg/mL), leupeptin (1μg/mL), pepstatin A (1μg/mL) and EDTA (1mmol/L). The homogenate was centrifuged at 15 000r at 4°C for 10 minutes. 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 semidy electromophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blots were stained at room temperature with a 1:600 dilution of monoclonal mouse anti - NT and C3 antibody over night at 4°C. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution), blots were developed using the enhanced chemiluminescence Western blot analysis detection system (ECL Plus; Amersham Pharmacia Biotech, Arlington Heights, IL).

DNA ladder for apoptosis

The rats were sacrificed and the two eyes were enucleated immediately among three groups at 20, 40 and 60 days respectively. There were total nine eyes in this experiment and DNA ladder technique were prepared as described.[9]

RT-PCR for expression of iNOS mRNA

The rats were sacrificed and the three eyes were enucleated immediately among three groups at 20, 40 and 60 days respectively. There were total nine eyes in this experiment. 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 rat retina in three groups, according to the kit manufacturer's specifications.

The sense and anti-sense oligonucleotide primers for rat iNOS were synthesized by Biological Engineering Corporation. The primer sequences are: iNOS (262bp) sense primer 1: 5'-CGCCCTTCCGACGTTC-3'; sense primer 2: 5'-TCCAGGAGGATCGACGT-3'; β-actin(420bp)sense primer 1: 5'-GAGACCTTCAACACCCAGCC-3'; sense primer 2: 5'-GCCGAGCGATCGGAACCCGCTA-3'. And 4 μg of RNA in a total volume of 20μL (pH8.3) were for
synthesizing the cDNA. RT-PCR was first performed at 24°C for 10 minutes, then at 42°C for 15 minutes. The reaction mixture was heated at 99°C for 5 minutes, and the RT product was mixed with DNA polymerase (AmpliTag Applied Biosystems, USA) and the sense primer in a buffer containing 20mmol Tris-HCl, 50mmol KCl, 2.0mmol MgCl₂ (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°C for 2 minutes and then 45 seconds in each cycle; annealing the primer with DNA at 55°C for 45 seconds; and extending of the primer at 72°C for 10minutes. 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 2% agarose gel.

Western blot results were used to confirm the iNOS change pattern, as described previously in the part of the expression of the NT protein.

**Immunohistochemistry and flow cytometry for Fas/Fasl transduction** There were totally two eyes in immuno-histochemistry at each point of groups (20, 40, 60 days). Which were fixed with 10% buffer formalin, embedded with paraffin and cut into slices of 5 µm. After washed with PBS, the slides were incubated with hydrogen peroxide (peroxidase blocking reagent; Daco, Carpinteria, CA) to block endogenous peroxidase activity, then with 10% goat serum for 30 min at room temperature to block non-specific antigen. After rinsed and washed in PBS the block slides were incubated with streptavidin conjugated with horseradish peroxidase. The color was developed with streptavidin and biotin chromogen (Liquid DAB+Substrate-Chromogen System; Dako).

There were total another two eyes in flow cytometry. The eye balls were dissected via posterior incision under a dissecting microscope and the retina with RPE cells were taken out. Which were fixed with 70% ethanol for 24 hours. Then fluorescence intensity of Fas/Fasl and amount of positive Fas/Fasl cells in RPE were examined. Cells were washed three times in PBS and re-suspended in PBS at 2x10⁷/mL. After Staining with primary and secondary antibodies, cells were analyzed by FACSscan. The optical system was 2w laser producer with an output of 300mW and exciting wavelength at 488 nm, projected wavelength at 605nm. The data was input into an HP-300 Consort 30 model computer and processed with related software. The DNA content distributing square pattern and dual parameter dimension pattern was obtained in this way. The cell number was calculated with cell circle analyzing sequence. Chicken blood erythrocyte was taken as a standard sample to adjust the instrument coefficient (CV) <5.0%.

**Statistical Analysis** Statistical analyses of the data were performed on computer (SPSS 16.0). The results were expressed as means ±SD. Statistical significance was determined by a one-factor analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons. P<0.05 was considered significant.

**RESULTS**

**Animal Model** Comparing the control and CCK-8 groups, there were typical diabetic symptoms in ONOO⁻ group. There were also remarkable differences in glucose concentration and body weight among the three groups during 20, 40 and 60 experiment days.

**Western Blotting for NT** With Western blot analysis, a faint expression of NT could be seen in the control group. A gradual to strong expression of NT was observed at different stages of the experiment in ONOO⁻ group. But expression of NT in CCK-8 group changed gradually from faint to strong during the period of 20 to 40 days, then turn to weak at 60 days (Figure 1). Computer photo analysis indicated that there were significant differences among three groups (P<0.001, Figure 2).

**DNA Ladder for Apoptosis** There was no appearance of DNA ladder band in the control group, but there was distinct typical DNA ladder band in the ONOO⁻ group as time passed. Expression of DNA ladder band in the CCK-8 group appeared gradually strong during the period of 20 to 40 days of the experiment, then weak at 60 days (Figure 3).

**RT–PCR for Expression of iNOS mRNA** There was no expression of iNOS mRNA in the control group, but there was distinct up-regulation of iNOS mRNA in the ONOO⁻ group as time passed by. Expression of iNOS mRNA in the CCK-8 group appeared gradually with up-regulation of iNOS mRNA during the period of 20 to 40 days of the experiment, then down-regulation at 60 days (Figure 4). With computer photo-analysis, there were significant differences among the three groups (P<0.001, Figure 5). Western blot results to confirm the iNOS change pattern. The results verified the iNOS change pattern(Figure 6, 7).

**Immunohistochemistry and flow cytometry for Fas/Fasl transduction** Immuno-histochemistry staining revealed that the specific expression of Fas/Fasl was yellow, brown-yellow or brown staining in the cell nucleus and cytoplasm.
ONOO\(^-\) induced iNOS \(\rightarrow\) Fas/ Fas/L pathway in diabetic rats

Figure 1 Detection of NT protein expression in RPE cells of diabetic rat with Western blotting  Lane 1: Control group; Lane 2-4: ONOO\(^-\) group at 20, 40, 60d respectively; Lane 5-7: CCK-8 group at 20, 40, 60d respectively

Figure 2 Statistics for gray value of NT among three groups

\(a P<0.05, b P<0.01 \) vs control group; \(c P<0.05 \) vs CCK-8 group

Figure 3 DNA ladder for apoptosis of RPE cells in diabetic rat  Lane M: marker; Lane 1: Control group; Lane 2-4: ONOO\(^-\) group at 20, 40, 60d respectively; Lane 5-7: CCK-8 group at 20, 40, 60d respectively

Figure 4 RT-PCR for expression of iNOS mRNA in RPE cells  Lane M: marker; Lane 1: Control group. Lane 2-4: ONOO\(^-\) group at 20, 40, 60d respectively; Lane 5-7: CCK-8 group at 20, 40, 60d respectively

Figure 5 Statistics for OD value of iNOS among three groups

\(a P<0.05, b P<0.01 \) vs control group; \(c P<0.05 \) vs puerarin group

Figure 6 Detection of iNOS protein expression in RPE cells of diabetic rat with Western blotting  Lane 1: Control group; Lane 2-4: ONOO\(^-\) group at 20, 40, 60d respectively; Lane 5-7: CCK-8 group at 20, 40, 60d respectively

Figure 7 Statistics for gray value of iNOS protein among three groups

\(a P<0.05, b P<0.01 \) vs control group; \(c P<0.05 \) vs CCK-8 group

of RPE. In the control group, a very faint yellow color could be observed. At different times in the ONOO\(^-\) group, staining ranged from yellow to brown-yellow, then to brown in the cell nucleus and cytoplasm. Gradually decreasing staining expression could be observed in 20 to 60 days of the ONOO\(^-\) and CCK-8 groups (Figure 8).

In the experiment of flow cytometry, there were rare Fas/FasL positive cells in the control group. As time passed, positive cells continued to be increased in ONOO\(^-\) group. On the days 20, there were mildly positive cells in CCK-8 group, but apparently increased on the days 40. While on the days 60, positive cells were decreased. There were distinct differences among the three groups (\(P<0.05\), Table 1).

DISCUSSION

Diabetic retinopathy, which complicate even well-controlled cases of diabetes. This study investigated the role of ONOO\(^-\) accumulation in the RPE of diabetic rat retina in the failure of retinopathy to reverse after reestablishment of RPE, and to determine the effect of this reversal on the activity of the CCK-8 responsible for scavenging ONOO\(^-\) and other related products.

The traditional oxygen-free radical stress mechanism pays more attention to the role of hydrogen-peroxide (\(\text{H}_2\text{O}_2\)), nitric-oxide (NO) and superoxide-anion (\(\text{O}_2^-\)), while the new theory includes ONOO\(^-\), a product from rapid reaction of NO and \(\text{O}_2^-\), which may be an important mediator of cytotoxicity in oxidation \([11-12]\). It is also highly reactive and interacts with cellular constituents inflicting damage on cells \([13-15]\). Our
previous study supports the new theory[16-17]. In this study, we also observed the ONOO-mediated protein nitration product- NT, was located in RPE cells of diabetic rats and decreased under the intervention of CCK-8. We found that NT greatly increased in RPE cells of diabetic rats, which indicated that high glucose could induce the production of NT. Due to high blood glucose, there was no enzyme-glucose reaction took place in blood plasma and tissue protein. Therefore the structure and role of protein changed and hemoglobin-associated-crystals-glucose (HbAIC) was developed. Increase of HbAIC enhanced the combination of hemoglobin and oxygen and decreased their separation, leading to the lack of oxygen in plasma and tissue but increase of free radical, which is one of main causes of chronic diabetic complications. On one hand, purerarin alleviated the oxidation of RPE cells and other tissues induced by ONOO in diabetic rat, which would decrease the production of HbAIC and free radical. On the other hand, purerarin could inhibit the expression of iNOS, therefore decreased the formation of ONOO. It is likely that iNOS may contribute to oxidation stress by helping develop more powerful oxidative agents such as ONOO[18]. Some authors[19-20] examined the influence of iNOS isoforms and ONOO generation after experimental stroke.

Cell apoptosis is the result of cascade gene expression. Up to date, more genes contribute to production and regulation of cell apoptosis. It is believed that genes in the inner layer of the cell directly regulate the production and development of the apoptosis, while related elements in the outer layer of the cell affect the expression of the genes through signal transduction way [21-23]. Interaction of the death receptor and death ligand is one of the main ways to induce apoptosis, of which, the Fas/FasL system is considered as the major signal transduction pathway to mediate apoptosis[24-28]. Loss of RPE cells via apoptosis plays a prominent role in several retinal degenerative diseases, such as age-related macular degeneration. That means the occurrence and development of many eye diseases are related to the regulation imbalance of RPE cell's apoptosis. Strategies for preservation of vision that would interrupt the apoptotic signal require understanding the molecular events associated with apoptosis. This study investigated the susceptibility of RPE to Fas/FasL-dependent apoptotic pathways when challenged with different stimuli, including oxidants ONOO, anti-Fas/FasL antibody, activated iNOS and antagonism of CCK-8. We found, intensity of NT, DNA ladder band, iNOS mRNA and positive Fas/FasL cells continued to be increased in ONOO group. In CCK-8 group, it was increased from 20 to 40 days, but decreased from 40 to 60 days which may indicate a protective role of CCK-8 on RPE cells. Purerarin could inhibit the expression of iNOS, therefore decreased the formation of ONOO. To summarize, apoptosis of RPE cells was partly induced by ONOO may be the new way of oxidative damage to the RPE cells. CCK-8 decreased RPE cells apoptosis partly induced by ONOO and is a potential drug for therapy of diabetic retinopathy. Fas/FasL cell signal transduction route and many other apoptotic factors may affect and strengthen the apoptosis process mediated by ONOO. The mechanism of CCK-8 dealing with RPE cells may be related to its direct
inhibition of apoptosis of RPE cells and antagonism of damage of ONOO− to RPE cells.

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