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Inhibition of Calpain on Oxygen Glucose Deprivation-induced RGC-5 Necroptosis*

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Summary: The purpose of this study was to investigate the effect of inhibition of calpain on retinal ganglion cell-5 (RGC-5) necroptosis following oxygen glucose deprivation (OGD). RGC-5 cells were cultured in Dulbecco's-modified essential medium and necroptosis was induced by 8-h OGD. PI staining and flow cytometry were performed to detect RGC-5 necrosis. The calpain expression was detected by Western blotting and immunofluorescence staining. The calpain activity was tested by activity detection kit. Flow cytometry was used to detect the effect of calpain on RGC-5 necroptosis following OGD with or without N-acetyl-leucyl-leucyl-norleucinal (ALLN) pre-treatment. Western blot was used to detect the protein level of truncated apoptosis inducing factor (tAIF) in RGC-5 cells following OGD. The results showed that there was an up-regulation of the calpain expression and activity following OGD. Upon adding ALLN, the calpain activity was inhibited and tAIF was reduced following OGD along with the decreased number of RGC-5 necroptosis. In conclusion, calpain was involved in OGD-induced RGC-5 necroptosis with the increased expression of its downstream molecule tAIF.
Key words: oxygen glucose deprivation; necroptosis; calpain; retinal ganglion cell-5; N-acetyl-leucyl-leucyl-norleucinal; truncated apoptosis inducing factor

Necroptosis is a type of regulated form of cell death which has the necrotic morphological features such as cell swelling, mitochondria dysfunction and cell membrane permeabilization[1, 2]. Necroptosis occurs in many kinds of neuronal damage models such as traumatic brain injury[3], tumor necrosis factor (TNF)-α-induced hippocampal neurons damage[4], motoneurons co-cultured with astrocytes of amyotrophic lateral sclerosis (ALS)[5], retinal ganglion cell (RGC) following high intraocular pressure (HIOP)[6] and primary cultured cortical neurons following oxygen glucose deprivation (OGD)[7], *etc*. Overall, these results suggest that necroptosis might be a form of cell death that widely exists in injured neurons.

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Calpains are calcium-activated neutral proteases, which belong to the family of cytosolic cysteine proteinases. It has been unveiled that calpain is involved in the regulation of the cellular proliferation, differentiation and apoptosis[8–10]. Moreover, some studies showed that calpain may be involved in necrosis as well[11]. Calcium-dependent calpain is activated by increasing calcium concentration in cytoplasm in N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG)-treated cells. The activated calpain cleaves BH3 interacting domain (BID) death agonist to trucked BID, which redistributes from the cytosol to mitochondria where it regulates Bcl-2-associated X (BAX) protein activation[12]. Once activated, Bax would promote the mitochondria injury by combining with mitochondria membrane, which favors the release of truncated apoptosis inducing factor (tAIF) from mitochondria to induce the necroptosis[13, 14]. Cabon *et al*[12, 15] found that AIF in MNNG-treated cells was cleaved into tAIF which released to cytoplasm from mitochondria. tAIF formed a complex with endonuclease Cyc lo philinA (CyPA) and histone family 2A variant (H2AX) towards DNA degradation, finally contributed to necroptosis. Other researches[16] found that the maturation and release of AIF were both related to calpain in the ischemic models of rats. What's more, our previous study also found that tAIF was involved in calpain-mediated early RGC-5 necroptosis induced by high pressure[17]. Collectively, the calpain-tAIF pathway might play an important role in various neuronal necrop-

tosis models.

HIOP is regarded as one of the main risk factors which cause damage of RGC *in vivo*[18–21]. Besides high pressure-induced direct damage, ischemia-hypoxia of RGCs induced by compression of the central retinal artery following HIOP is another important mechanism[22]. Although our previous study suggested that tAIF was involved in calpain-mediated RGC-5 necroptosis induced by high pressure[17], the possible role of calpain-tAIF pathway in RGC-5 necroptosis following ischemia-hypoxia is not yet completely understood. Therefore, in this study, we plan to unveil the effect of calpain-tAIF in RGC-5 necroptosis induced by OGD (the classical model *in vitro* to simulate ischemia-hypoxia). Our study will help gain a better understanding of the mechanism of the early RGC necroptosis in acute ischemic-hypoxic retinal diseases and provide experimental evidence to determine a possible target for inhibiting this process in future alternative medicine.

1 MATERIALS AND METHODS

1.1 Cell Culture

The RGC-5 cell line was provided by Department of Ophthalmology, Second Hospital of Jilin University in China[23]. RGC-5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Company, USA) supplemented with 3 mL 10% fetal bovine serum (HyClone Laboratories, Inc., USA) and 1% streptomycin (HyClone Laboratories, Inc., USA). The cells were grown at 37°C under atmosphere of 5% CO_2. The RGC-5 cells used in the experiment was within 2–3 passages post-thawed to minimize the variability in the assays based on our observations. The density of RGC-5 cells was around 80% in 6 mL culture media in 50 mL flask before OGD.

1.2 OGD Model and ALLN Administration

When the density of RGC-5 cells was about 80%, cells were washed twice with 6 mL glucose-free DMEM (Life Technologies Company, USA) after twice of wash with 2 mL PBS and 1 mL 0.25% parenzyme (HyClone Laboratories, Inc., USA) digestion, then incubated in an anaerobic chamber with 95% N_2 and 5% CO_2 to induce 8 h OGD[24, 25], while control group (CTL) was not given any treatment. After 8 h, OGD was terminated by removing the cell culture flasks from chamber and replacing the glucose-free DMEM with regular culture medium, and then cells were incubated at 37°C under atmosphere of 5% CO_2 to recover for each time point (6, 12, 24 and 36 h). ALLN (Merck, Germany, an inhibitor of calpain and widely used to reduce calpain-mediated cell death[26, 27]) was dissolved in dimethyl sulfoxide (DMSO) for storage in 10 mmol/L. The RGC-5 cells were exposed to ALLN at the working concentration of 10 μmol/L for 24 h before ODG treatment.

1.3 Immunofluorescence Staining

After cell climbing and OGD treatment, the coverslips with cells were washed in 0.01 mol/L PBS 3 times, fixed in 2 mL 4% PF for 20 min and then reacted with calpain antibody (Abcam, ab39170, UK, 1:500) overnight at 4°C before incubation with Immnol Fluorence staining secondary antibodies (Abcam Company, UK, 1:200) for 2 h, washed in PBS, counterstained with DAPI (4',6-diamidino-2-phenylin dole, Vector, USA), captured and observed under the fluorescence microscope (Nikon, Eclipse 80i, Japan).

1.4 Western Blotting

At each recovery time point following OGD, proteins in cells were separated in a protein extraction buffer containing a cocktail of protease inhibitors. Cell lysates (1.5 mL) collected in a centrifuge tube were incubated on ice for 30 min and then centrifuged at 10 000 *g* for 20 min at 4°C. The supernatants were collected, and the protein concentration was measured by bicinnchoninic acid assay (Pierce, USA). The remains of proteins were boiled for 5 min in 4× denaturing buffer (Fermentas, USA) and electrophoresed until objective bands were separated appropriately. Then the polypeptides were electrotransferred to PVDF membrane which was blocked with 5% nonfat milk (Bio-Rad, USA) for 1 h and then incubated with calpain (Abcam, ab39170, UK, 1:500), tAIF (Santa Cruz, SC-13116, USA, 1:200), β-tubulin (Abcam, ab6046, UK, 1:1000) and GAPDH (Beyotime, AT019, China, 1:1000) overnight at 4°C, and then in HRP-conjugated secondary antibodies (Bio-Rad, USA, 1:20 000) for 1 h. Immunoblotting products were visualized with an ECL Plus™ Western Blotting Detection kit according to manufacturer's instruction (GE Healthcare Life Sci., USA). Images were captured in a Molecular Dynamics Phosphor imager (Nucleo Tech Inc., USA). Western blot bands were measured with Image J (NIH, USA) to analyze the integrated density value (IDV).

1.5 Calpain Activity Assay

Calpain activity was examined by cleavage of the substrate Ac-LLY-AFC (Abcam, UK). At each recovery time point following OGD, cells were digested by sonication on ice in a digestion buffer [150 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS] containing a cocktail of protease inhibitors (Sigma, USA). Cell lysates were centrifuged at 10 000 *g* for 20 min at 4°C. The supernatants were collected, and the protein concentration was determined by Coomassie brilliant blue assay (Pierce, USA). The fluorescence density was measured after 1 h incubation at 37°C in the reaction buffer without light, recorded by a Tecan Infinite M1000 Multifunctional microplate reader (setting excitation wavelength/emission wavelength=400:505 nm advanced, Tecan, Austria). The values of calpain activity were expressed as relative fluorescent unit (RFU) per mg protein of each sample.

1.6 Flow Cytometry

The recovery time point was selected (calpain activity up-regulated significantly) to detect necroptosis cells by flow cytometry[28]. Briefly, RGC-5 cells that attached to the flasks were trypsinized, followed by a gentle wash with PBS. Then cells were resuspended in 200 μL of 1× binding buffer, 5 μL of 20 μg/mL Annexin V and 10 μL of 50 mg/mL PI were added to the suspension, and cells were incubated at RT for 15 min without light. After the cells were washed and analyzed by FACS Calibur (Becton, Dickinson Company, USA). The percentages of cells in each quadrant were analyzed using ModFit software (Verity Software House Topsham, USA). Statistical analyses of flow cytometry were conducted by calculating the number of PI positive cells.

1.7 Statistical Analysis

Figure panels were processed by using Adobe Photoshop7.0. The data of calpain activity assay and flow cytometry were both analyzed by using SPSS 19.0 (SPSS, USA). One-way analysis of variance (one-way ANOVA) was performed to test differences in average value between groups. All results were presented as $x \pm s$. A value of $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Immunofluorescence Staining of Calpain Distribution Following OGD

Immunofluorescence staining (fig. 1) showed that calpain was expressed in CTL at different survival time points. No discrepancy in calpain distribution was observed under microscope. There was no difference in fluorescence intensity of calpain under microscope 6, 12 and 24 h after re-oxygenation following OGD. However, the fluorescence intensity of calpain in OGD-36 h group was more distinct and heavier than that in CTL.

2.2 Up-regulation of Calpain Expression 36 h after OGD

The Western blot results indicated that calpain was exhibited mainly as a single 75 kD band. The bands were remarkably thicker and larger in OGD-36 h group than those in CTL (fig. 2A). Ratio of calpain/β-Tubulin in OGD-36 h group was different from that in CTL (fig. 2B). The results indicate that calpain is up-regulated in RGC-5 cells 36 h following OGD.

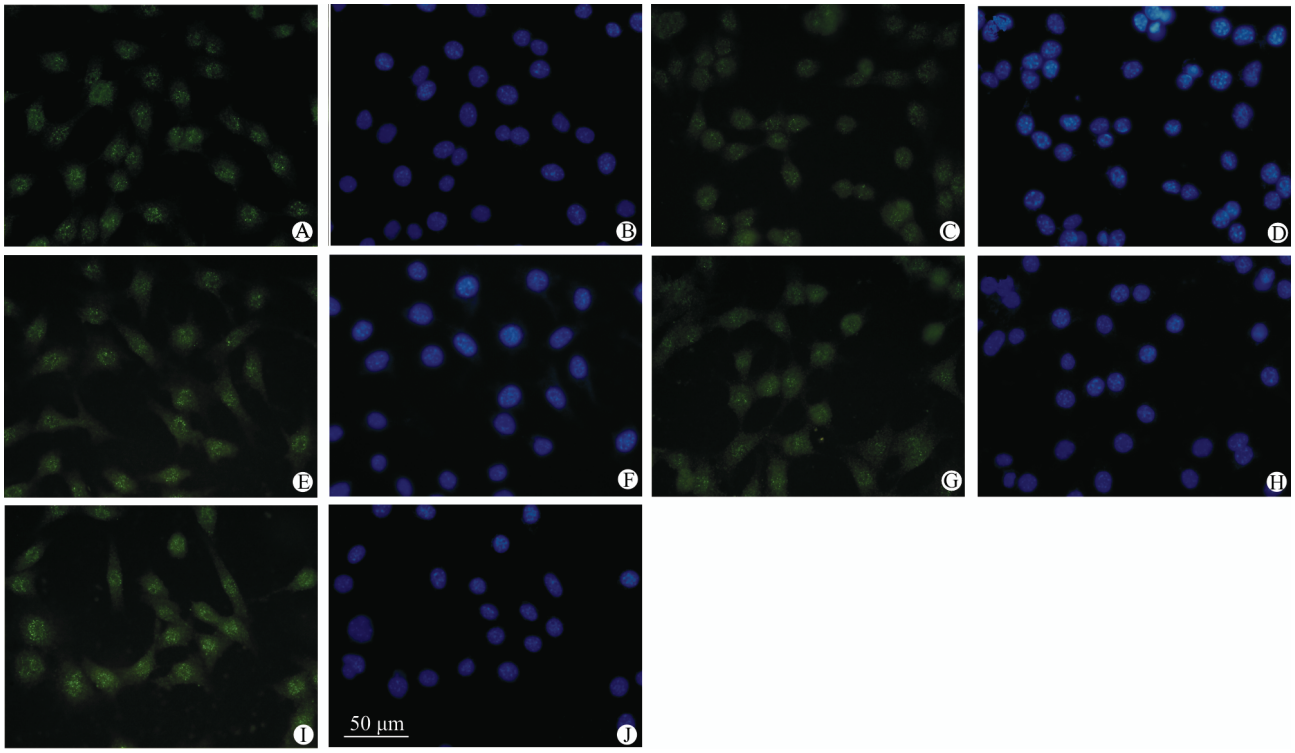

Fig. 1 Immunohistochemical staining of calpain in RGC-5 cells following OGD at different survival time points

A, C, E, G and I: immunohistochemical staining of calpain; B, D, F, H and J: DAPI staining in corresponding cells; A and B: CTL; C and D: OGD-6 h group; E and F: OGD-12 h group; G and H: OGD-24 h group; I and J: OGD-36 h group. Scale bar=50 μm applied to all panels

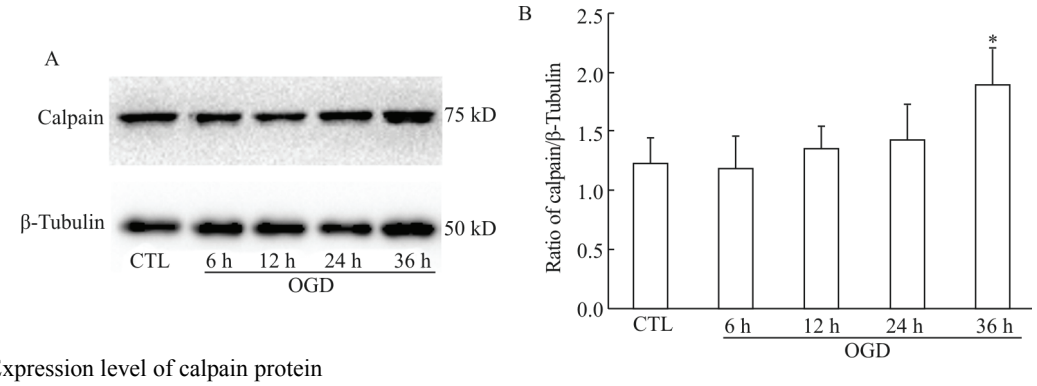

Fig. 2 Expression level of calpain protein

A: Western blot of calpain in RGC-5 at different survival time points following OGD; B: statistical results of IDV by Western blotting

*$P<0.05$ *vs.* CTL

2.3 Increased Calpain Activity 6 h Following OGD

The calpain activity assay suggested that calpain activity remarkably increased in all injury groups except the ODG-36 h group compared with CTL (fig. 3). The activity reached the peak in the OGD-6 h group (P<0.05), and decreased gradually until 36 h. There was significant difference between the OGD-6 h group and 12 or 24 h groups (P<0.05). We conclude that calpain activity is up-regulated in RGC-5 cells following OGD and reaches the highest in 6 h re-oxygenation.

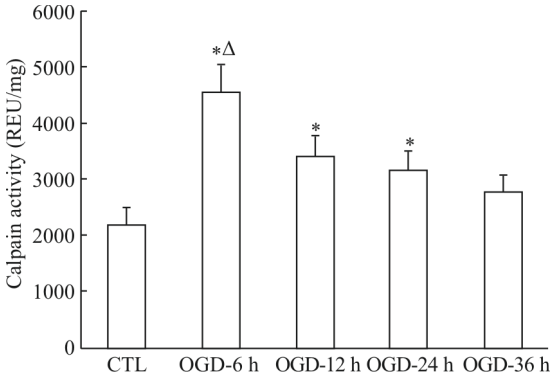

Fig. 3 The statistical analysis of calpain activity assay
*P<0.05 *vs.* CTL, $^{\Delta}P$<0.05 *vs.* OGD-12 h and OGD-24 h groups

2.4 ALLN Inhibiting Calpain Activity Following OGD

According to the results above, we selected the 6 h after re-oxygenation as the key time point to explore the potential role of calpain in the RGC-5 necroptosis induced by OGD. The assay results showed that there was significant difference between the OGD group and OGD-ALLN group (P<0.05, fig. 4), which suggested that ALLN may inhibit the up-regulation of calpain activity effectively in RGC-5 cells following OGD.

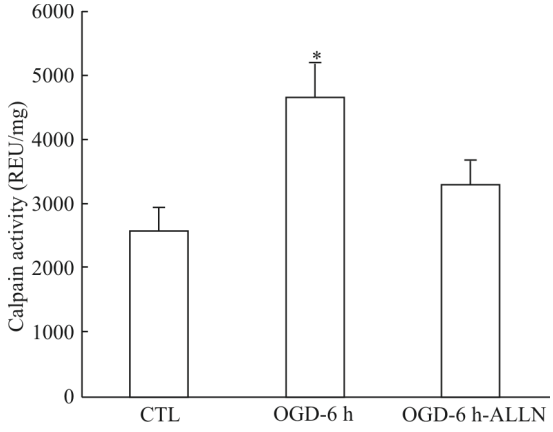

Fig. 4 The statistical analysis of calpain activity assay under OGD and ALLN usage
*P<0.05 *vs.* CTL and OGD-6 h-ALLN group

2.5 Effect of ALLN on Calpain Expression

As the Western blot results reported, there were no significant differences in calpain positive bands among CTL, OGD group and OGD-ALLN group (fig. 5A). In the meantime, it was demonstrated that there were no significant differences in IDV between the OGD group and OGD-ALLN group (fig. 5B). Collectively, these results suggested that ALLN mainly inhibits the activity of calpain instead of the expression of calpain.

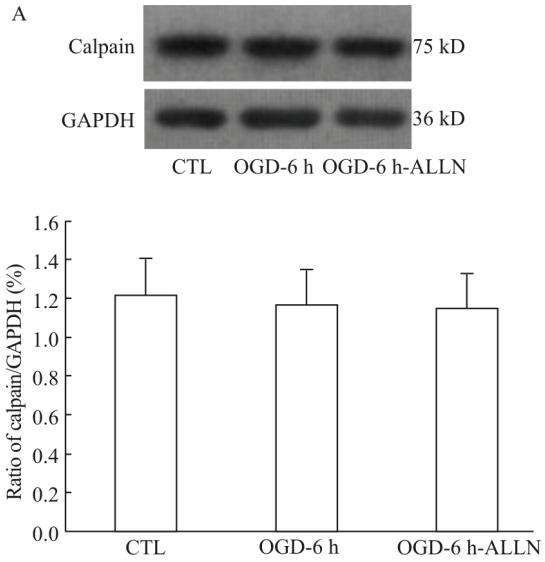

Fig. 5 Calpain protein expression level detection under OGD and ALLN usage
A: Western blot result of caplain; B: statistical results of IDV of Western blot

2.6 Decreased Rate of RGC-5 Necrosis by Inhibiting Calpain Activity

We analyzed cellular necroptosis by using flow cytometry with PI/Annexin V double staining to detect whether inhibition of calpain activity could decrease the rate of necrosis in RGC-5 cells following OGD. The results showed that the ratio of necrosis cells was about 15% (fig. 6B), the percentage decreased to nearly 5% upon adding ALLN (fig. 6C) in the injury group in 6 h. It was demonstrated that the ratio of RGC-5 necrosis decreased when treated with ALLN under OGD condition. Meanwhile, statistical analysis indicated that there were significant changes in PI-positive cells upon adding ALLN compared to CTL and OGD group (fig. 6D). These results indicate that RGC-5 necrosis in the early stage may be related to the up-regulated calpain activity.

2.7 AIF Cleavage Product Decreased after Calpain Inhibition

After calpain was inhibited by ALLN, the Western blot results indicated (fig. 7A) that positive bands of tAIF became remarkably thicker and larger in OGD-6 h group than those in CTL group and OGD-ALLN group. Percentage of IDV of tAIF in OGD group was significantly higher than that in CTL group and OGD-ALLN group (fig. 7B). Moreover, percentage of IDV of tAIF in OGD-ALLN group was significantly lower than that in OGD group (fig. 7B).

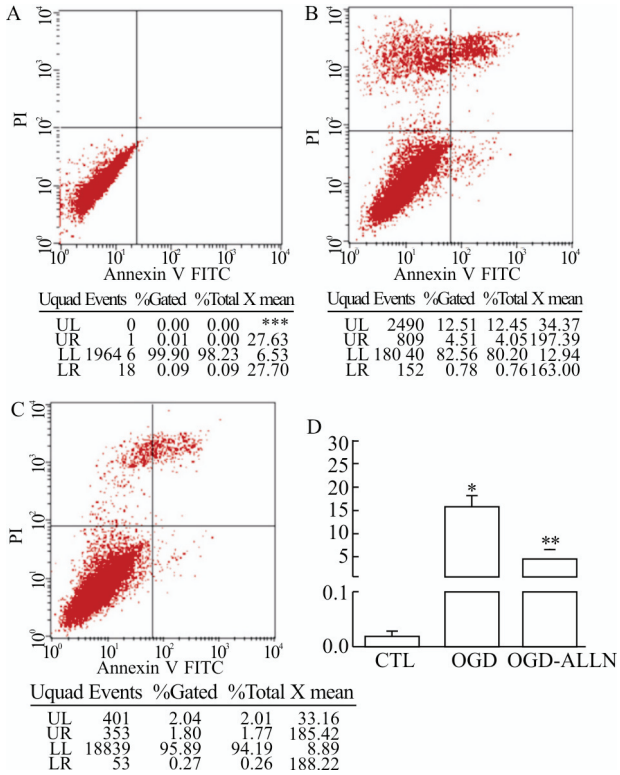

Fig. 6 Quantitation of necrotic cells following OGD and ALLN treatment

A: CTL; B: OGD group; C: OGD-ALLN group; D: statistical results of flow cytometry

$^{*}P<0.05$ *vs* CTL and OGD-ALLN group; $^{**}P<0.01$ *vs* CTL

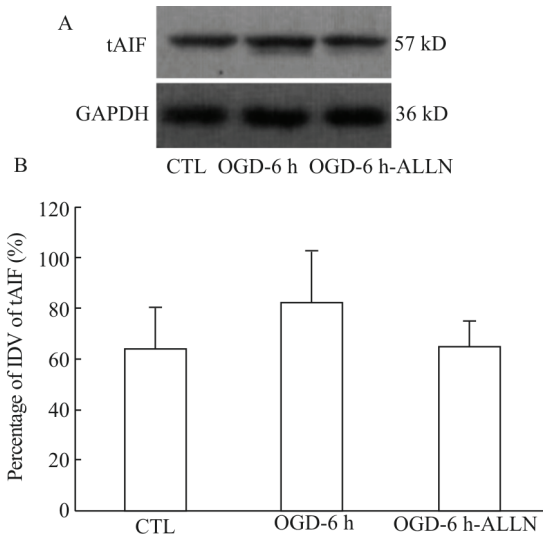

Fig. 7 tAIF protein expression level detection under OGD and ALLN usage

A: Western blots of tAIF; B: statistical analysis of IDV of Western blot

$^{*}P<0.05$ *vs* CTL and OGD-6 h-ALLN group

3 DISCUSSION

A growing number of studies had confirmed that calpain plays an important role in lots of neuronal disorders. The mechanism of apoptosis mediated by calpain had attracted extensive attention in previous researches[29, 30]. It showed that calpain could activate the upstream signal of caspase or cleave some cytoskeleton proteins towards apoptosis[31, 32]. However, calpain could participate in not only the regulatory progress of neuronal apoptosis but that of necrosis. Necrotic death of neuron was caused by activated calpain *via* the deposition of β-amyloid in the brain of Alzheimer's patients[30]. Otherwise, referring to our previous studies and experiment results in this paper, we speculate that calpain also may be one of the regulatory molecules taking part in RGC-5 necrosis under acute injury.

Presently, scientists are much more concerned about the damage mediated through receptor interacting protein 3 (RIP3) pathways which was mainly described as the molecular mechanism of necroptosis[33–38]. Li *et al*[39] identified RIP3 as a molecular switch between TNF-induced apoptosis and necrosis in NIH 3T3 cells. Liu *et al*[4] reported that RIP3-mediated necroptosis was activated in the mouse hippocampus after intracerebroventricular injection of TNF-α. However, our previous studies showed that inhibition of RIP3-mediated necroptosis pathway rescued not all necrotic RGC-5 following OGD[40], which meant other molecules might be involved in RGC-5 necrptosis under this condition. Besides Ubiquitin carboxyl-terminal hydrolase L-1 and heat shock protein 90, *etc*[41–43], some scholars found that calpain might be one of the important regulatory molecules participating in early necroptosis of kidney cells, HeLa cells and vascular endothelial cells, *etc*[11, 44, 45]. Nevertheless, the biological effect of calpain on mediating the early neuronal necroptosis especially in visual nervous system needed further exploration. Our Western blot results showed that the expression of calpain increased in OGD-36 h group, which was confirmed by immunofluorescence staining, suggesting that the expression of calpain is up-regulated generally over time. What aroused our more interests was calpain activity was up-regulated more quickly than expression in RGC-5 cells following OGD. The remarkable increase of calpain activity could be observed in 6 h survival group, which suggested that the change of calpain activity has precedence over the change of calpain quantity under OGD injury. Related studies have confirmed that the change of calpain activity depended on the change of Ca^{2+} concentration. For example, Averna pointed out that activation of calpain occurs as an early event in correlation with an increase in Ca^{2+} concentration in rat brain upon treatment with a high salt diet for a prolonged period of time[46]. Researches on various of diseases showed that the quick change of Ca^{2+} concentration appeared on early stages of diseases. For example, cerebral ischemia led to the diversification of mechanism of Na^{+}/Ca^{2+} exchange and damage of cell membrane towards Ca^{2+} influx[47]. When epileptic attacked, the excitatory neurotransmitter released made the ion channels open towards Ca^{2+} influx[48]. The changes of cell membrane permeability caused by inflammatory response induced intracellular Ca^{2+} overload[49]. Subsequently the intracellular Ca^{2+} overload activated calpain. Therefore, we predict that the up-regulatory calpain activity in earlier injury might be correlated with the increase of Ca^{2+} influx in our own

experiment.

According to the calpain activity remarkably increased in OGD-6 h survival group, we explored whether calpain could regulate RGC-5 necroptosis by inhibiting its activity. Necrosis was reduced to a certain extent when exposed to ALLN (the calpain inhibitor targeting the activity rather than the expression[50]), which meant necrosis had been modulated artificially under this condition. Collectively, we speculate that calpain might play an important role in early RGC-5 necroptosis following OGD. Our result is regarded as a confirmation proof to attest the regulatory effect of calpain on RGC necroptosis, which is new understanding apart from our previous acute pressure results[17].

Based on the possible regulatory effect of calpain on RGC-5 necroptosis, we explored the feasible downstream mechanism initially. Cabon[12] found that AIF was cleaved into tAIF by calpain, which contributed to necroptosis due to DNA degradation. As investigated previously, AIF played a vital role in necroptosis induced by calpain[51]. In our experiment, calpain activity was inhibited by ALLN while the expression of tAIF did not significantly increase in RGC-5 cells under OGD. It further demonstrated that calpain in RGC-5 cells was mediated by the downstream molecule of tAIF to modulate necroptosis pathway. Overall, the necroptosis regulated by calpain mediated by tAIF may be one of patterns of RGCs death induced by OGD. Finally, there is no doubt that our study provides more evidence for the molecular mechanism of early RGC necroptosis (non RIP3 pathway) under retinal ischemia-hypoxia injury. Also it is of great value for the target investigation of early necroptosis in RGC.

In conclusion, our study suggests that OGD-induced RGC-5 necroptosis is partly mediated by calpain, which may be via tAIF-modulation pathway.

Conflict of Interest Statement

The authors declare that there are no competing interests and financial relationship with the commercial identities mentioned in this paper.

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