PANoptosis-like cell death in ischemia/reperfusion injury of retinal neurons

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Abstract

PANoptosis is a newly identified type of regulated cell death that consists of pyroptosis, apoptosis, and necroptosis, which simultaneously occur during the pathophysiological process of infectious and inflammatory diseases. Although our previous literature mining study suggested that PANoptosis might occur in neuronal ischemia/reperfusion injury, little experimental research has been reported on the existence of PANoptosis. In this study, we used in vivo and in vitro retinal neuronal models of ischemia/reperfusion injury to investigate whether PANoptosis-like cell death (simultaneous occurrence of pyroptosis, apoptosis, and necroptosis) exists in retinal neuronal ischemia/reperfusion injury. Our results showed that ischemia/reperfusion injury induced changes in morphological features and protein levels that indicate PANoptosis-like cell death in retinal neurons both in vitro and in vivo. Ischemia/reperfusion injury also significantly upregulated caspase-1, caspase-8, and NLRP3 expression, which are important components of the PANoptosome. These results indicate the existence of PANoptosis-like cell death in ischemia/reperfusion injury of retinal neurons and provide preliminary experimental evidence for future study of this new type of regulated cell death.

Key Words: apoptosis; gascdermin-D (GSDMD); ischemia/reperfusion; mixed lineage kinase domain-like protein (MLKL); necroptosis; NOD-like receptor protein 3 (NLRP3); PANoptosis; pyroptosis; receptor-interacting protein kinase 3 (RIPK3); retinal neuron

Introduction

PANoptosis is a phenomenon in which pyroptosis, apoptosis, and necroptosis simultaneously occur during the pathophysiological process of some diseases, and they can be regulated at the same time (Malireddi et al., 2019). A series of studies have reported that PANoptosis is regulated by the PANoptosome complex, which is assembled by some key regulators of pyroptosis, apoptosis, and necroptosis (Malireddi et al., 2019, 2020; Banoth et al., 2020; Christgen et al., 2020; Samir et al., 2020). The protein complex simultaneously regulates pyroptosis, apoptosis, and necroptosis (Christgen et al., 2020; Samir et al., 2020). In addition to infectious diseases, other diseases such as nervous system diseases, involve cell death and immune response, and there are many studies on the regulated cell death forms (RCDs) pyroptosis, apoptosis, and necroptosis (Yuan and Yankner, 2000; Elmore, 2007; Tan et al., 2014; Ofengeim et al., 2015; Kesavardhana and Kanneganti, 2017; Ge et al., 2018; Yuan et al., 2019; Guo et al., 2020; Liao et al., 2021; Yan et al., 2021). These RCDs have been shown to be associated with immune response (Semmler et al., 2005; Takeda et al., 2008; Basuroy et al., 2009; Huang et al., 2018; Liu et al., 2019b; Wang et al., 2019a; Zhou et al., 2019; Chen et al., 2021; McKenzie et al., 2020; Hu et al., 2021; Wu et al., 2021). These studies indicated the possibility that PANoptosis plays a larger role in the nervous system. Thus, we conducted a literature mining study to explore this hypothesis (Yan et al., 2022). In that study, we found that under experimental conditions, the three RCD forms of PANoptosis exist in models of middle cerebral artery occlusion and oxygen-glucose deprivation/recovery (OGD/R). To validate the hypothesis that PANoptosis exists in retinal ischemia/reperfusion injury (I/R) injury, the present study used both in vitro and in vivo I/R models (OGD/R and acute high intraocular pressure (aIOP)) of retinal neurons to investigate the existence of PANoptosis-like cell death under experimental conditions.

Methods

Animals and aIOP model

The animals used in the experiment were male Sprague-Dawley rats (n = 90) with a weight range of 250–300 g and an age of 8 weeks. The experiments were approved by the Animal Ethics Committee of Central South University on March 2, 2021 (approval No. 2021-XMSB-0002). The animals were purchased from the Hunan STJ Laboratory Animal Co., Ltd (Hunan, China) (license No. SCXK (Xiang) 2019-0004), and the experimental protocols and operating procedures were carried out in accordance with the Guidelines for Animal Experiments of Central South University under the Law of Animal Protection and Management of the Chinese Government. Rats were randomly divided into three groups using a random number table: Sham, aIOP, and aIOP + inhibitor (n = 30 rats/group).

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Propidium iodide (PI; MilliporeSigma, Burlington, MA, USA, Cat# P4170) staining inhibitors in the OGD/R model, the final concentration of each inhibitor in dissolved in DMSO at a final concentration of 50 μM. Disulfiram (DSF; Selleck Z-VAD-FMK (Z-VAD; Cat# S7023, Selleck Chemicals, Houston, TX, USA) was Drug treatments for 2 hours. The normal control group was maintained in the normal culture (Chen et al., 2016; Wang et al., 2018, 2020; Huang et al., 2021). The R28 cells oxygen containing air. It was well-tested and applied in our previous studies by our research team, and contained an airtight box, oxygen concentration sensor, Fisher Scientific, Cat# 1966025) with FBS and PS. After the medium was PS) was replaced with glucose-free medium (DMEM, no glucose, Thermo Fisher Scientific). R28 cells were subcultured every 2 or 3 days. The R28 cells NaCl; Thermo Fisher Scientific, Waltham, MA, USA, Cat# SV30010) at 37°C in a 5% CO2 cell culture incubator (Fisher Scientific). R28 cells were subcultured every 2 or 3 days. The R28 cells were used for related experiments after drug treatment.

OGD/R injury of R28 cells in vitro

OGD/R injury was induced by simulating the ischemic-reperfusion (I/R) injury in vitro (Hu et al., 2020b; Xie et al., 2020). When R28 cells were subcultured to the 3rd generation and had grown to about 80% density, the OGD/R model was performed on the cells using a protocol based on published literature (Wang et al., 2018b, 2020; Xie et al., 2020). When the R28 cells were cultured in the normal culture state, the initial culture medium (low glucose DMEM with 10% FBS and 1% PS) was replaced with glucose-free medium (DMEM, no glucose, Thermo Fisher Scientific, Cat# 1966025) with FBS and PS. After the medium was changed to glucose-free medium, the OGD treatment began, which maintained a hypoxic condition (< 1%). This device was made by our research team, and contained an airtight box, oxygen concentration sensor, and a hypoxic air supply pipeline (95% nitrogen and 5% CO2) to replace the oxygen containing air. It was well-tested and applied in our previous studies (Chen et al., 2016; Wang et al., 2018, 2020; Huang et al., 2021). The R28 cells subjected to OGD treatment were cultured at 37°C for 2 hours. After OGD injury, the cells were returned to the initial culture condition (low glucose DMEM with 10% FBS and 1% PS at 37°C in a 5% CO2, cell culture incubator) for 2 hours. The normal control group was maintained in the normal culture medium (low glucose DMEM with 10% FBS and 1% PS) in a culture incubator (Thermo Fisher Scientific). The R28 cells were washed with PBS at room temperature, and then the cells were washed with PBS three times for 5 minutes each, and then stained with Hoechst 33342 (see Section “Propidium iodide staining”). After three washes with PBS for 5 minutes each, the cells were observed under a fluorescence microscope (Olympus). Images were taken at six random positions for each sample. The percentage of TUNEL-positive cells was analyzed by ImageJ software.

EtOH-induced macrophage injury

EtOH-induced macrophage injury (EtOH-iMac; Biotium, Fremont, CA, USA, Cat# 40050) staining combined with DSF was used to indicate pyroptosis. DSF inhibits GSDMD expression and interferes with pore formation triggered by GSDMD in the cell membrane to prevent the release of interleukin and other inflammatory factors (Zhang et al., 2021a, b). This special function enables EtOH-iMac to stain necrotic cells that selectively stains dead cells with damaged cell membranes, and is a useful dye for pyroptosis (Pan et al., 2018; Wang et al., 2019b). EtOH-iMac was diluted in 1× PBS and the solution concentration was adjusted to 1 μg/mL. R28 cells were treated with EtOH-iMac solution for 10 minutes at room temperature, followed by three PBS washes for 5 minutes each. Subsequently, they were stained with 4′,6-diamidino-2-phenylindole (DAPI; MilliporeSigma, Cat# 94463) in 0.1 M PBS at a final concentration of 0.5 μg/mL for 10 minutes at room temperature, and then the cells were washed with PBS three times for 5 minutes each.

The cells were cultured with a mixture of glucose and PBS at a ratio of 1:9 and observed under a fluorescence microscope (Olympus, Tokyo, Japan) (Wang et al., 2018, 2020; Huang et al., 2021). Images were taken at six random positions for each sample. The percentage of PI-positive cells was analyzed by ImageJ v1.4.3 software (NIH, Baltimore, MD, USA).

Terminal deoxynucleotidyl transferase-mediated nick end labeling

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) apoptosis detection kit combined with Z-VAD was used to detect apoptosis of R28 cells (green fluorescent, Millipore, Cat# MA0223) and in animal tissue (red fluorescent, Millipore, Cat# MA0224). When R28 and drug treatment, R28 cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes, washed with PBS, and then stained with fluorescein isothiocyanate (FITC)-conjugated proteinase K (20 μg/mL; Millipore, Cat# MA0224-2) at room temperature for 5 minutes. Rat retinal sections were washed with PBS three times for 5 minutes each, then treated with FITC-conjugated proteinase K (20 μg/mL) at 37°C for 20 minutes. After the TdT-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining solution was prepared according to the kit protocol, reaction solution was added to the washed cells and sections, and the cells were treated at 37°C for 2 hours in the dark. Subsequently, the cells and sections were washed with PBS three times for 5 minutes each, and then stained with Hoechst 33342. Images were taken at six random positions for each sample. The percentage of TUNEL-positive cells was analyzed by ImageJ software.

EtOH-induced cell death

EtOH-induced cell death (EtOH-Id) was induced by EtOH-iMac (Biotium, Fremont, CA, USA, Cat# 40050) staining combined with DSF was used to indicate pyroptosis. DSF inhibits GSDMD expression and interferes with pore formation triggered by GSDMD in the cell membrane to prevent the release of interleukin and other inflammatory factors (Zhang et al., 2021a, b). This special function enables EtOH-iMac to stain necrotic cells that selectively stains dead cells with damaged cell membranes, and is a useful dye for pyroptosis (Pan et al., 2018; Wang et al., 2019b). EtOH-Id was dissolved in 1× PBS and the solution concentration was adjusted to 1 μg/mL. R28 cells were treated with EtOH-Id solution for 10 minutes at room temperature, followed by three PBS washes for 5 minutes each. Subsequently, they were stained with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 20 minutes, followed by three PBS washes for 5 minutes each. Then, 5 μL of EtOH-Id (100 μg/mL) was injected into the intravitreal space of the eye 30 minutes before the animals were killed. Cells and sections stained with EtOH-Id were observed using a fluorescence microscope (Olympus). Images were taken at random positions for each sample. The percentage of EthD-III-positive cells was analyzed by ImageJ software.

Western blot assay

R28 cells were lysed in ice-cold RIPA buffer (CWBio, Beijing, China) with 1% octyl-β-D-glucopyranoside (OG) and 1% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes (Pall, New York, USA, Cat# PD8485). The membranes were incubated at room temperature for 2 hours in 5% nonfat dry milk, and then incubated with primary antibodies overnight at 4°C. The reactions were followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000; CWBio). The bands were visualized using an enhanced chemiluminescence (ECL) kit (Pierce). The primary antibodies used were: anti-BAX (1:1000, Proteintech, Rosemont, IL, USA, Cat# 50599-2-lg, RRID: AB_2061561); anti-BCL-2 (1:2000, Proteintech, Cat# 26593-1-AP, RRID: 358 | NEURAL REGENERATION RESEARCH | Vol 18 | No 2 | February 2023 | NEURAL REGENERATION RESEARCH | www.nrronline.org
Enzyme-linked immunosorbent assay

The expression levels of IL-1β and IL-18 in R28 cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) kits (rat IL-1β, F2923-A; rat IL-18, F3070-A; KX-Bio Co., Shanghai, China). Following OGD/R treatment, cell culture supernatants were collected and centrifuged at 3000 r/min for 20 minutes. Then IL-1β and IL-18 concentrations were determined according to the manufacturer’s instructions.

Immunofluorescence staining

Frozen sections of rat retinal tissue were placed at room temperature for 30 minutes, washed three times with PBS for 5 minutes each, sealed with 1× PBS containing 5% bovine serum albumin and 0.3% Triton X-100 for 2 hours, and incubated with primary antibodies overnight at 4 °C. Primary antibodies included an affinity-purified donkey anti-rabbit IgG (1:5000, Jackson Immuno Research, West Grove, PA, USA, Cat# 711-035-152, RRID: AB_1001528) and donkey anti-mouse IgG (H+L, 1:5000, Jackson Immuno Research, Cat# 715-035-150, RRID: AB_2340770). The sections were developed with diaminobenzidine (DAB) reagents, and the band intensities were quantitated with Image J software. The level of performance was normalized to the level of β-actin. All cell experiments were performed at least three times independently.

Statistical analysis

Data were summarized as the mean ± standard deviation of independent replicates. The significance of differences between two groups was determined by two-tailed Student’s t-test using GraphPad Prism 8 software. All animal experiments were performed at least five times independently. DSF: Disulfiram; EthD-III: Ethidium iodide (PI staining for R28 cells). R28 cells were treated with OGD/R and Nec-1. Scale bar: 100 μm. OGD treatment lasted for 2 hours. After OGD treatment, the cells were returned to the initial culture condition for 2 hours. The normal control group was maintained in the normal culture condition for the same length of time. In the aHIOP model (bottom panel), inhibitors (or DMSO for control) were injected into the vitreous cavity 30 minutes before HDIOP/sham surgery. To induce ocular hypertension, normal saline was injected into the anterior chamber to form artificial intraocular pressure (IOP). IOP was slowly increased to 110 mmHg and maintained for 60 minutes, then gradually returned to normal level and maintained for 48 hours before retinal tissue collection. In the sham group, a sterilized needle was inserted into the anterior chamber without elevating the IOP at the same time point. aHIOP: Acute high intraocular pressure; DMSO: dimethyl sulfoxide; OGD/R: oxygen-glucose deprivation/recovery.

Results

OGD/R induces morphological changes of PANoptosis-like cell death in R28 cells

We first investigated whether OGD/R simultaneously induces the morphological changes of PANoptosis-like cell death in R28 cells (Figure 1). The OGD/R treatment and staining timeline were the same in each group. Cell staining results indicated that the OGD/R treatment significantly induced apoptosis (TUNEL staining; Figure 2A and D), pyroptosis (EthD-III staining; Figure 2B and D), and necroptosis (PI staining; Figure 2C and D). The RCD morphology of cells treated with OGD/R after pretreatment with inhibitors of apoptosis (Z-VAD; Figure 2A and D), pyroptosis (DSF; Figure 2B and D), and necroptosis (Nec-1; Figure 2C and D) was significantly reversed. These results indicated that PANoptosis-like cell death occurred in R28 cells following OGD/R injury, which is similar to those observed in PANoptosis in other models (Kurilko and Kang, 2006; S. Iwata et al., 2021).

OGD/R induces expression changes of key proteins in PANoptosis-like cell death in R28 cells

The above results indicated that PANoptosis-like cell death occurred at the morphological level, thus it was important to next determine the expression changes of hallmark proteins for apoptosis, pyroptosis, and necrosis in R28 cells following OGD/R injury. As shown in Figure 3A, western blot assay showed that OGD/R treatment caused a significant increase of cleaved caspase-3 compared with the control and caspase inhibitor (Z-VAD) groups. The results also showed that compared with the control and Z-VAD groups, OGD/R treatment significantly increased the expression of pro-apoptotic protein BAX and decreased the expression of anti-apoptotic protein BCL-2. Western blot showed that OGD/R treatment increased expression of NLRP3 and cleaved caspase-1 (CASP1 p20) (Figure 3B), key proteins in the pyroptosis pathway. Expression of cleaved GSDMD and GSDMD-N (p30), a driver of pyroptosis, were also upregulated by the OGD/R treatment. Elevated expression of pro-inflammatory cytokines IL-1β (p17) and IL-18 (p22) were also observed in the OGD/R group. These expression changes were reversed...
by the specific GSDMD inhibitor DSF. Moreover, the ELISA assay indicated that expressions of mature IL-1β and IL-18 were significantly increased in the R28 cell culture supernatant after OGD/R treatment, and these increases were reversed by DSF inhibitor (Figure 3D). The above results indicated caspase-1/NLRP3/GSDMD-mediated pyroptosis in R28 cells following OGD/R treatment.

Increases in the protein levels of phosphorylated RIPK3 and phosphorylated MLKL are hallmarks of necroptosis (Vandenbergbe et al., 2010; Pasparakis and Vandenbergbe, 2015). As depicted in Figure 3C, the OGD/R treatment remarkably upregulated the expressions of phosphorylated RIPK3 and phosphorylated MLKL, and inhibitors of necroptosis prevented this increase. Taken together, these results indicated that OGD/R treatment induced pyroptosis, apoptosis and necroptosis in R28 cells at the same time point, and further suggest the existence of PANoptosis-like cell death in R28 cells.

Combination of three RCD inhibitors significantly protects R28 cells following OGD/R

The results above indicated that OGD/R treatment induced PANoptosis-like cell death in R28 cells at the same time point. Next, we investigated whether the cell loss following OGD/R treatment is mainly dependent on this kind of combined cell death. Thus, pretreatment with different combinations of inhibitors for apoptosis, pyroptosis, and necroptosis was used to assess the protective effects on cell loss against OGD/R treatment. As shown in Figure 4A and B, Z-VAD combined with either DSF or Nec-1 had a better protective effect than that of Z-VAD alone, as measured by TUNEL-positive cell death, following OGD/R treatment. There was no significant difference between the triple combination and the double combinations, and the TUNEL-positive cell death was largely reversed by the combined pretreatments. The combination of DSF with Z-VAD, or with both Z-VAD and Nec-1, had a better protective effect on EthD-III-positive cell death than DSF alone (Figure 4A and B). The triple combination of DSF, Z-VAD and Nec-1 had the largest protective effect on EthD-III-positive cell death compared with each of the double combinations. PI staining also indicated that the combination of Nec-1 with Z-VAD, or with both Z-VAD and DSF, had a larger protective effect than Nec-1 alone (Figure 4A and B). Moreover, the triple combination of Nec-1, Z-VAD and DSF had a larger protective effect on PI-positive cell death than the double combination of Nec-1 and DSF. Taken together, these results suggested that OGD/R-induced R28 cell death is mainly driven by PANoptosis-like cell death.

An aHIOP model induces PANoptosis-like cell death in vivo

The above experiments indicated that OGD/R injury can induce PANoptosis-like cell death in vitro. Next, we used a rat aHIOP model to investigate whether R/I injury can induce PANoptosis-like cell death in vivo (Figure 1). As shown in Figure 7A, aHIOP treatment induced apoptotic cell death of retinal neurons (indicated by TUNEL staining) in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). The increased TUNEL staining was significantly reduced by Z-VAD pretreatment. Increased caspase-3 expression was detected by immunofluorescence staining in retinal neurons, which further indicated the occurrence of apoptosis in retinal neurons following aHIOP treatment (Figure 8A). Furthermore, the co-immunofluorescence staining of caspase-3 and NeuN indicated that caspase-3 was activated in the retinal ganglion cells (RGCs) of aHIOP-treated retina.

The existence of pyroptosis in retinal neurons was indicated by EthD-III-positive staining in GCL and INL of aHIOP-treated retina (Figure 7B). EthD-III-positive staining in the retina was significantly reduced by pretreatment of specific pyroptosis inhibitor DSF. Moreover, as shown in Figure 8B, aHIOP treatment significantly increased GSDMD expression (indicated by immunofluorescence staining) in the GCL and INL, which was consistent with the EthD-III staining. Also, the co-immunofluorescence staining of GSDMD and NeuN indicated the activation of GSDMD in RGCs. Thus, these results indicated that aHIOP treatment induced retinal cell death by PANoptosis in vivo.

Similarly, aHIOP treatment increased PI staining, suggesting that aHIOP treatment induced necroptotic cell death, in the GCL, INL, and ONL of the retina, and Nec-1 pretreatment significantly reduced the PI-positive staining (Figure 7C). Furthermore, after aHIOP treatment, immunofluorescence staining showed increased expression of MLKL, a key protein of necroptosis execution, indicating that aHIOP treatment induced retinal cell death by necroptosis (Figure 8C). The co-immunofluorescence staining of MLKL and NeuN indicated the activation of MLKL in RGCs of aHIOP-treated retina. Taken together, the results demonstrated that PANoptosis-like cell death occurred in vivo in retinal neurons following aHIOP injury.
**Figure 5** | Combination of RCD inhibitors decreases the OGD/R-induced EthD-III-positive cells.
(A) EthD-III staining for R28 cells treated with combinations of RCD inhibitors following OGD/R treatment. Red: EthD-III-positive cells. Scale bars: 50 μm. (B) Percentage of EthD-III-positive cells (mean ± SD, n = 3). **P < 0.05, ***P < 0.001, vs. OGD/R + Z-VAD + DSF + Nec-1; #P < 0.05, ##P < 0.01, vs. OGD/R + DSF (Student’s t-test). DSF: Disulfiram; EthD-III: Ethidium Homodimer III; Nec-1: necrostatin-1; OGD/R: oxygen-glucose deprivation/recovery; RCD: regulated cell death; Z-VAD: Z-VAD-FMK.

**Figure 6** | Combination of RCD inhibitors decreases the OGD/R-induced PI-positive cells.
(A) PI staining for R28 cells treated with OGD/R and Z-VAD, DSF, and Nec-1. Red: PI-positive cells. Scale bars: 50 μm. (B) Percentage of PI-positive cells (mean ± SD, n = 3). **P < 0.05, ***P < 0.001, vs. OGD/R + Z-VAD + DSF + Nec-1; #P < 0.05, ##P < 0.01, vs. OGD/R + DSF (Student’s t-test). DSF: Disulfiram; EthD-III: Ethidium Homodimer III; Nec-1: necrostatin-1; OGD/R: oxygen-glucose deprivation/recovery; PI: propidium iodide; RCD: regulated cell death; Z-VAD: Z-VAD-FMK.

**Figure 7** | aHIOP induces pyroptosis, apoptosis, and necroptosis in vivo in rat retina.
(A) TUNEL staining for rat retinal sections. The rats were treated with aHIOP and Z-VAD. (B) EthD-III staining for rat retinal sections. The rats were treated with aHIOP and Nec-1. Scale bars: 50 μm. Percentage of TUNEL-, EthD-III-, and PI-positive cells shown to the right of images (mean ± SD, n = 5 rats per group). ***P < 0.001, vs. Sham; #P < 0.05, vs. aHIOP (Student’s t-test). aHIOP: Acute high intraocular pressure; CASP3: caspase-3; Co-F: coinmunofluorescence; GSDMD: gasdermin-D; MLKL: mixed lineage kinase domain-like; NeuN: neuronal nuclei; OGD/R: oxygen-glucose deprivation/recovery; PI: propidium iodide; RCD: regulated cell death; Z-VAD: Z-VAD-FMK.

**Figure 8** | aHIOP induces high expression of pyroptosis, apoptosis, and necroptosis-related proteins in rat retina.
Immunofluorescence staining of CASP3 (A), GSDMD (B) and MLKL (C) of rat retinal tissue sections. NeuN staining was used to indicate ganglion cells in the retina. Scale bars: 50 μm. Quantification of pyroptosis, apoptosis, and necroptosis-related protein expressions shown on the right of images (mean ± SD, n = 5 rats per group). Relative fold intensity of immunofluorescence staining was normalized relative to the intensity of the sham group. **P < 0.01, ***P < 0.001, vs. Sham; #P < 0.05, ##P < 0.01, vs. OGD/R (Student’s t-test). aHIOP: Acute high intraocular pressure; CASP: caspase; Co-F: coinmunofluorescence; DSF: disulfiram; GCL: ganglion cell layer; GSDMD: gasdermin-D; INL: inner nuclear layer; MLKL: mixed lineage kinase domain-like; ONL: outer nuclear layer; Nec-1: necrostatin-1.
Discussion

Recent studies have indicated extensive crosstalk between RCD pathways, and PANoptosis is a novel form of programmed cell death in which pyroptosis, apoptosis, and necroptosis simultaneously occur in infectious disease, pathogen-induced immune response or inflammation (Malireddi et al., 2019). Furthermore, it has been shown that PANoptosis is regulated by the Panoptosome complex, which is composed of caspase-1, caspase-8, NLRC3, and other components (Christgen et al., 2020; Samir et al., 2020; Briard et al., 2021). Recent studies have indicated that the Panoptosome can be regulated by z-DNA binding protein 1 and TGF-β-activated kinase 1 to regulate the outcome of pyroptosis, apoptosis, and necroptosis (Christgen et al., 2020; Samir et al., 2020). Findings from these studies suggested that identification and characterization of PANoptosis were vital to inform the development of targeted inhibitors for controlling the occurrence of inflammatory cell death and therapeutic modulation of inflammation and the immune response. However, the existence of PANoptosis in noninfectious injuries and the nervous system remains unknown. Previous studies have established a process to investigate PANoptosis in disease: the first level is to confirm the existence of PANoptosis-like cell death (Simultaneous occurrence of pyroptosis, apoptosis, and necroptosis). The second level is to study the existence of the Panoptosome that regulates the three RCDs simultaneously. And the third level is to study the role of the Panoptosome. The main purpose of this study was to investigate whether PANoptosis-like cell death exists in nervous system I/R injury, and characterize the manifestation of PANoptosis-like cell death.

To investigate whether PANoptosis-like cell death plays a role in retinal I/R injury, R28 cells were selected for in vitro experimental study, and an OGD/R model was used to simulate I/R injury. Additionally, we selected Sprague Dawley rats for in vivo experiments, and used an aHIOP model to simulate in vivo I/R injury of retinal neurons. R28 cells are immortal retinal progenitor cells that are widely used to study retinal diseases and injuries (Herbort et al., 2013), and have an endogenous cell retention reaction (Mathew et al., 2019, 2021). OGD/R and aHIOP models simulate I/R injury of retinal neurons (Osborne et al., 1995; Rosenbaum et al., 1998, 2001; Dvoriantchikova et al., 2010, 2014). They are widely used and generally accepted models at present (Rosenbaum et al., 1998, 2001; Dvoriantchikova et al., 2010).

Our experimental data showed that under the same model conditions and the same treatment time, pyroptosis, apoptosis, and necroptosis occurred simultaneously after retinal I/R injury induced by OGD/R in R28 cells and aHIOP in rat retina. These data support the first level of the definition of PANoptosis that PANoptosis-like cell death exists in retinal nerve I/R injury. Previous studies on PANoptosis suggest that immune response and inflammatory response are closely related to the occurrence of PANoptosis (Kuriakose and Kanneganti, 2019; Banoth et al., 2020; Malireddi et al., 2020; Place et al., 2021). Similarly, studies on RCD of retinal neurons and other neurons have reported that the occurrence of pyroptosis is closely related to inflammatory factors (Homme et al., 2018; Zheng et al., 2019; Chen et al., 2020). The present study showed that the protective effects of a combination of two potential crosstalk between those RCDs. Moreover, our results showed that the protective effects of a combination of two key molecules that regulate PANoptosis.

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References

Banoth B, Tuladhar S, Karki R, Sharma BR, Briard B, Kesavardhana S, Burton A, Kanneganti TD (2020) ZBP1 promotes fungi-induced inflammasome activation and pyroptosis, apoptosis, and necroptosis (PANoptosis). J Biol Chem 295:18276-18283.

Basuroy S, Bhattacharya S, Leffler CW, Parfenova H (2009) Nox4 NADPH oxidase mediates oxidative stress and apoptosis caused by TNF-α in cerebral vascular endothelial cells. Am J Physiol Cell Physiol 296:C422-432.

Briard B, Malireddi RKS, Kanneganti TD (2021) Role of inflammasomes/pyroptosis and PANoptosis during fungal infection. PLoS Pathog 17:e1009358.

Chen H, Deng Y, Gan X, Li Y, Huang W, Lu L, Wei L, Lu L, Jow B, Hong Y, Cao Y, Liu Y, Chi W (2020) NLPR12 collaborates with NLRC3 and NLRC4 to promote pyroptosis inducing ganglion cell death of acute glaucoma. Mol Neurodegener 15:26.

Chen S, Yan J, Deng HK, Long LL, Hu YI, Wang M, Shang I, Chen D, Huang JF, Xiong K (2016) Inhibition of calpain on oxygen glucose deprivation-induced RGC-5 necroptosis. J Neurotrauma 33:301-311.

Chen Y, Li Y, Guo L, Hong J, Zhao M, Hu X, Xu C, Chang C, Liu W, Xiong K (2021) Bibliometric analysis of the PANoptosis and inflammasome in brain. Front Pharmacol 12:625052.

Christgen S, Zheng M, Kesavardhana S, Karki R, Malireddi RKS, Banoth B, Place DE, Briard B, Sharma BR, Tuladhar S, Samir P, Burton A, Kanneganti TD (2020) Identification of the Panoptosome: A molecular platform triggering pyroptosis, apoptosis, and necroptosis (PANoptosis). Front Cell Infect Microbiol 10:2307.

Cuenca N, Fernández-Sánchez L, Campello I, Maneu V, De la Villa P, Lax P, Pinilla I (2014) Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. Prog Retin Eye Res 43:17-75.

Degterev A, Hitomi J, Germerscheid M, Ch’en IL, Korkina O, Teng X, Abbott D, Cundy GY, Yuan C, Wagner G, Hedrick SM, Gerber SA, Luskovsky A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313-321.

Dupertez J, Takahashi N, Van Hauwermeiren F, Vandenriessche B, Goossens V, Vanden Bergh T, Declercq W, Libert C, Cauxeels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Nat Immunol 12:98-108.

Dvoriantchikova G, Barakat DJ, Hernandez E, Shestopalov VI, Ivanov D (2010) Liposome-delivered AAT effectively protects the retina against ischemia-reperfusion injury. Mol Vis 16:2882-2890.

Dvoriantchikova G, Degterev A, Ivanov D (2014) Retinal ganglion cell (RGC) programmed necrosis contributes to ischemia-reperfusion-induced retinal damage. Exp Eye Res 123:1-7.

Elmore S (2007) Apoptosis: A review of programmed cell death. Toxicol Pathol 35:495-516.

Ge XT, Li WZ, Huang S, Yin ZY, Xu X, Chen FL, Kong XD, Wang HC, Zhang JN, Lei P (2018) The pathological role of NLRs and AIM2 inflammasome-mediated pyroptosis in damaged blood-brain barrier after traumatic brain injury. Brain Res 1697:10-20.

Guo LM, Wang Z, Li SP, Wang M, Yan WT, Liu FX, Wang CD, Zhang XD, Chen Y, Duan J, Xiong K (2020) RIPA/XMGL-mediated neuronal necroptosis induced by methamphetamine at 39°C. Nat Regen Reg 15:855-874.

He CL, Liu R, Zhang J, He J, Zhang L, Meng W, Zhao K, Zhang Y, Yuan C (2021) Modulation of inflammatory responses in retina by AMPK activation. J Biol Chem 296:18268-18281.

He CL, Liu R, Zhang J, He J, Zhang L, Meng W, Zhao K, Zhang Y, Yuan C (2021) Modulation of inflammatory responses in retina by AMPK activation. J Biol Chem 296:18268-18281.

Hu XM, Li ZX, Lin RH, Shan JQ, Yu QW, Wang RX, Liao LS, Yan WT, Wang Z, Shang L, Huang Y, Zhang Q, Xiong K (2021) Guidelines for regulated cell death assays: a systematic summary, a categorical comparison, a prospective. Front Cell Dev Biol 9:634690.

Hu YD, Tang CL, Jiang JZ, Lu HY, Wu YB, Qin XD, Shi S, Zhao B, Zhu XN, Xia ZY (2020b) Neuroprotective effects of deoxymetadione preconditioning on oxygen-glucose deprivation-reoxygenation injury in PC12 cells via regulation of Ca(2+)-STIM1/Orai1 signaling. Curr Med Sci 40:699-707.
Huang JF, Wang L, Zhai MQ, Wang H, Quan CY, Liu T, Chen X (2013) Differential neuronal expression of receptor interacting protein 3 in rat retina: involvement in ischemic stress response. BMC Neurosci 14:16.

Huang Y, Wang S, Huang F, Zhang Q, Qin B, Liu L, Wang M, Han Y, Han W, Chen D, Liu F, Jiang B, Ji D, Xia X, Huang J, Xiong K (2021) c-FIP regulates pyroptosis in retinal neurons following oxygen-glucose deprivation/recovery via a GSDMD-mediated pathway. Ann Anat 235:151-167.

Huang Z, Zhou T, Sun X, Zheng Y, Cheng B, Li M, Liu X, He C (2018) Necroptosis in microglia contributes to neuroinflammation and retinal degeneration through TLIR4 activation. Cell Death Differ 25:180-189.

Kaczmarek A, Vandenabeele P, Krysko DV (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 38:209-223.

Karlik R, Sharma BA, Tuladhar S, Williams EP, Zalduondo L, Samar P, Zeng M, Sundaram B, Banerji B, Malireddi RK, Riccardi C, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1:1458-1461.

Liao L, Su L, Yan WT, Wang SC, Lu S, Yan K, Huang J, Xiong K (2021) Synergism of TLR-α and TLR-β triggers inflammatory cell death, tissue damage, and mortality in SARS-CoV-2 infection and cytokine shock syndromes. Cell 184:149-168.e17.

Kesavardhana S, Kanneganti TD (2017) Mechanisms governing inflammasome activation, apoptosis, and pyroptosis induction. Nat Immunol 20:291-210.

Kuriakose T, Kanneganti TD (2019) Pyroptosis in antiviral immunity. Curr Top Microbiol Immunol doi:10.1007/82_2019_189.

Liao LS, Su L, Yan WT, Wang SC, Guo LM, Yang YD, Huang K, Xu XM, Zhang Q, Yan J, Xiong K (2021) The role of HSP90α in methamphetamine/hyperthermia-induced necroptosis in rat striatal neurons. Front Pharmacol 12:716394.

Lin Y, Yu M, Fan T (2020) Insights into mechanisms of panpronefrin-induced apoptosis and necroptosis in human corneal stromal cells. Toxicol Lett 320:9-18.

Liu W, Xia F, Ha Y, Zou L, Yu L, Folorunso O, Pashaee-Marandi A, Lin PY, Tilton RG, Pierce AP, Li H, Zhang W (2019a) Neuropeptidergic effects of HSFI in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 60:955-977.

Liu Y, Liu T, Lei T, Zhang D, Su S, Girani L, Qi D, Ling C, Tong R, Wang Y (2019b) RIPK1/RIP3-regulated necroptosis as a target for multifaceted disease therapy (Review). Int J Mol Med 44:771-786.

Malireddi RK, Kesavardhana S, Kanneganti TD (2019) ZBP1 and TAK1: master regulators of necroptosis in human corneal stromal cells. Toxicol Lett 320:9-18.

Vandenabeele P, Galluzzo I, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700-714.

Tan MS, Tan L, Jiang T, Xu XC, Wang HF, Hia JF, Cai YJ (2014) Amyloid-beta induces NLRP1-dependent neuronal pyroptosis in models of Alzheimer’s disease. Cell Death Dis 5:12.

Toda N, Nakano-Ida T (2007) Nitric oxide: outer blood flow, glaucoma, and diabetic retinopathy. Prog Retin Eye Res 26:205-238.

Van Noorden CJF (2001) The history of Z-VAL-FMK, a tool for understanding the significance of caspase inhibition. Acta Histochem 103:241-251.

Vandenabeele P, Galluzzo I, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700-714.

Wang M, Wang H, Wang S, Liu Q, Lu S, Huang Y, Gou L, Liu F, Shang L, Huang J, Ji D, Xia X, Jiang B, Chen D, Xiong K (2020) RIPK3 mediates necroptosis by regulating phosphorylation of RIP3 in rat retinal ganglion cells. J Cell Sci 137:23-49.

Wang S, Huang J, Yan T, Zhou H, Wang M, Liu L, Wang Z, Chen D, Ji D, Xia X, Liu F, Huang J, Xiong K (2019a) Caspase2 but not caspase1 mediates by calpastatin following glutamate-induced regulated necrosis in rat retinal neurons. Ann Anat 221:57-67.

Wang X, Pan J, Liu H, Zhang M, Liu D, Li L, Tian J, Liu M, Jin T, An F (2019b) A27M gene silencing attenuates diabetic cardiomyopathy in type 2 diabetic rat model. Life Sci 221:249-258.

Wang Z, Guo LW, Yang Y, Zhou HK, Wang SC, Chen D, Huang JF, Xiong K (2018) Inhibition of HSP90α protects cultured neurons from oxygen-glucose deprivation induced necroptosis by decreasing RIP3 expression. J Cell Physiol 233:4864-4884.

Wu X, Hu X, Zhang Q, Li F, Xiong K (2021) Regulatory role of Chinese herbal medicine in regulated neuronal death. CNS Neurol Drug Targets 20:228-248.

Xie P, Ren ZK, Lu J, Hu YM, Guo LM, Zhang Q, Xiong K (2020) Berberine ameliorates oxygen-glucose deprivation/reperfusion-induced apoptosis by inhibiting endoplasmic reticulum stress and autophagy in PC12 cells. Curr Med Sci 40:1470-1475.

Yan WT, Yang YD, Xu XM, Ning W, Yao LS, Lu S, Zhao WJ, Zhang Q, Xiong K (2022) Do pyroptosis, apoptosis, and necroptosis (PANoptosis) exist in cerebral ischemia? Evidence from cell and rodent studies. Neural Regen Res 17:1761-1768.

Yan WT, Lu S, Yang YD, Ning WY, Cai Y, Hu XM, Zhang Q, Xiong K (2021) Research trends, hot spots and prospects for necroptosis in the field of neuroscience. Neural Regen Res 16:1628-1637.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Yuan J, Amin P, Ofengeim D (2019) Necroptosis and RIPK1-mediated neuroinflammation in CNS diseases. Nat Rev Neurosci 20:19-33.

Zhang J, Dai Y, Yang Y, Yu J (2012a) Calcitriol alleviates hypersomatic stress-induced cerebral endothelial cell damage by inhibing the pro-APC–GASPC–Caspase–1/GSDMD pyroptosis pathway in dry eye disease. J Immunol 184:2955-2966.

Zhang Y, Zhang R, Han X (2021b) Disulfiram inhibits inflammation and fibrosis in a rat unilateral urethral obstruction model by inhibiting gaskdemerin D cleavage and pyroptosis. Inflamm Res 70:543-552.

Zheng Q, Zou H, Gao L, Chen K, Liu W, Su J, Chu Y, Yuan R, Ye J (2019) The levels and significance of inflammatory mediators in acute and chronic heart failure. Curr Med Sci 39:767-773.

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