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Mechanism of RIPK1-RIPK3-MLKL Axis-Mediated Necroptosis in Alzheimer's Disease

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Abstract: To address the unclear mechanism of neuronal loss and the lack of therapeutic targets in Alzheimer's disease (AD), Western blot, real-time PCR, and immunohistochemistry were used to examine brain tissues from 32 AD patients and 20 controls. MTT assay, PI staining, Morris water maze, and Nissl staining were performed to investigate the effects of phosphorylated tau on HT22 cells and the intervention of Nec-1s in APP/PS1 mice. Results showed that mRNA levels of RIPK1, RIPK3, and MLKL in AD brains were increased by 2.86-, 2.43-, and 2.17-fold, respectively, and p-MLKL was positively correlated with CDR scores. 1.0 μmol/L phosphorylated tau reduced cell viability to 52.3%, while Nec-1 increased viability by 56.0%. Nec-1s shortened the escape latency by 28.5% and increased CA1 neurons by 42.6% in model mice. These findings suggest that RIPK1-RIPK3-MLKL axis-mediated necroptosis is involved in AD pathogenesis, and targeting RIPK1 can alleviate pathological damage in AD.

Keywords: Alzheimer's disease; RIPK1-RIPK3-MLKL axis; Necroptosis; Phosphorylated tau protein

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder primarily characterized by cognitive dysfunction and neuronal loss, with its pathogenesis not yet fully understood [1]. Necroptosis, a regulated form of cell death mediated by the RIPK1-RIPK3-MLKL axis, has recently been found to play a significant role in the neuronal loss observed in AD [2]. This study investigates the molecular mechanism of RIPK1-RIPK3-MLKL axis-mediated necroptosis in AD by examining brain tissue from AD patients, as well as cellular and animal models. It also evaluates the interventional effects of targeting RIPK1, aiming to provide new strategies for AD treatment.

2 . Materials and methods

2.1. General information

Brain tissue samples and clinical data were collected from patients with Alzheimer's disease (AD) admitted to the Department of Neurology of our hospital from January 2020 to December 2025. The AD group comprised 32 patients with pathologically confirmed AD, including 18 males and 14 females, aged 65–88 years old, with a mean age of 76.5 ±

6.8 years old. The control group included brain tissue samples from 20 volunteer donors who died of non-neurological diseases during the same period, including 11 males and 9 females, aged 66–85 years old, with a mean age of 75.2 ± 7.1 years old. There were no statistically significant differences in age, sex, or other general information between the two groups ($P > 0.05$), ensuring comparability.

Animal experiments: Male APP/PS1 transgenic AD model mice (n = 30) and age-matched wild-type C57BL/6J mice (n = 30), aged 8 months, weighing 25–30 g, were used. AD model mice were randomly divided into a model group (AD group) and an intervention group (RIPK1 inhibitor group), with 15 mice per group.

Cell experiments: The mouse hippocampal neuronal cell line HT22 was provided by the Institute of Neuroscience of our hospital.

2.2. Diagnostic criteria

AD was diagnosed according to the criteria established by the National Institute on Aging-Alzheimer's Association (NIA-AA):

(1) Core clinical criteria: Meeting the diagnostic criteria for dementia syndrome, with insidious onset, a clear history of cognitive decline, and early and most prominent cognitive deficits being either amnestic or non-amnestic (language, visuospatial, or executive dysfunction);

(2) Exclusion criteria: Excluding other neurological disorders causing cognitive impairment, active cerebrovascular disease, toxic or metabolic abnormalities;

(3) Biomarker evidence: Decreased Aβ42 levels and/or increased total tau or phosphorylated tau levels in cerebrospinal fluid, or positive Aβ deposition on PET imaging.

2.3. Inclusion and exclusion criteria

Inclusion criteria: (1) Meeting the above AD diagnostic criteria; (2) Age ≥ 60 years old; (3) Well-preserved brain tissue samples with qualified RNA and protein quality; (4) Complete clinical data.

Exclusion criteria: (1) Comorbid with other neurodegenerative diseases such as Parkinson's disease or frontotemporal dementia; (2) Comorbid with cerebrovascular disease or brain tumors; (3) Recent use of immunosuppressants or cytoprotective agents; (4) Obvious autolysis or contamination of samples.

2.4. Methods

2.4.1. Treatment methods

(1) Animal intervention: The intervention group received daily intraperitoneal injections of the specific RIPK1 inhibitor Nec-1s (1.0 mg/kg) for 8 consecutive weeks; the model group and wild-type group received equal volumes of saline. Behavioral tests and brain tissue sampling were performed after the intervention period.

(2) Cell intervention: HT22 cells were treated with different concentrations (0, 0.1, 0.5, 1.0, 2.0 μmol/L) of phosphorylated tau protein (pTau) for 24 h; in some experiments, cells were pre-incubated with the RIPK1 inhibitor Nec-1 (10 μmol/L) or the RIPK3 inhibitor GSK'872 (1 μmol/L) for 1 h before pTau treatment.

2.4.2. Outcome measures

(1) Human brain tissue samples: Western blot was used to detect the protein expression levels of RIPK1, p-RIPK1, RIPK3, MLKL, and p-MLKL in the hippocampus and cortex; immunohistochemical staining was used to observe the localization and expression distribution of these proteins; real-time PCR was used to detect mRNA levels.

(2) Cell experiments: Cell viability (MTT assay), lactate dehydrogenase (LDH) release rate, and cell death morphology (PI staining) were measured; expression and phosphorylation levels of RIPK1/RIPK3/MLKL were detected; co-immunoprecipitation was used to detect RIPK1-RIPK3 complex formation.

(3) Animal experiments: The Morris water maze test was used to assess learning and memory abilities (escape latency, time spent in the target quadrant, number of platform crossings); Nissl staining was used to observe the number of surviving neurons in the hippocampal CA1 region; immunofluorescence was used to detect the colocalization of p-MLKL with the neuronal marker NeuN; ELISA was used to measure the levels of inflammatory factors TNF-α, IL-1β, and IL-6 in hippocampal tissue.

2.4.3. Criteria for therapeutic efficacy

(1) Molecular level: RIPK1 kinase activity inhibition rate ≥ 50% was defined as effective inhibition; p-MLKL level reduced to below 60% of the model group was considered significant blockade of necroptosis.
(2) Cellular level: Cell viability increased by ≥ 30% compared to the model group, which was considered protective.
(3) Animal level: Escape latency in the water maze shortened by ≥ 20% or time spent in the target quadrant prolonged by ≥ 25% compared to the model group was considered significant cognitive improvement; the number of surviving neurons increased by ≥ 30% compared to the model group was considered significant neuroprotection.

2.4.4. Statistical methods

SPSS 26.0 statistical software was used for data analysis. Measurement data were expressed as mean ± standard deviation (SD). Comparison between the two groups was performed using an independent samples t-test. Comparison among multiple groups was performed using one-way ANOVA, followed by the LSD-t test for pairwise comparisons. Correlation analysis was performed using Pearson correlation. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression changes of RIPK1/RIPK3/MLKL in brain tissue of AD patients

Compared to the control group, RIPK1, RIPK3, and MLKL mRNA levels in the AD group were increased by 2.86, 2.43, and 2.17 times, respectively ($P < 0.001$); the p-RIPK1/RIPK1 and p-MLKL/MLKL ratios were increased by 3.24 and 3.28 times, respectively. RIPK1-positive and p-MLKL-positive neurons were increased by 2.46 and 6.10 times, respectively. p-MLKL levels were positively correlated with CDR scores ($r = 0.672$, $P < 0.01$). See **Table 1**.

Table 1. Comparison of RIPK1/RIPK3/MLKL expression in brain tissue between AD patients and control group (mean ± SD)

Indicator	Control Group (n = 20)	AD Group (n=32)	t value	*P* value
RIPK1 mRNA	1.00 ± 0.12	2.86 ± 0.35	22.64	< 0.001
RIPK3 mRNA	1.00 ± 0.15	2.43 ± 0.41	18.92	< 0.001
MLKL mRNA	1.00 ± 0.11	2.17 ± 0.38	16.35	< 0.001
p-RIPK1/RIPK1	0.21 ± 0.04	0.68 ± 0.09	21.76	< 0.001
p-MLKL/MLKL	0.18 ± 0.03	0.59 ± 0.08	20.14	< 0.001
RIPK1+ Neurons (cells/mm²)	45.3 ± 8.7	156.8 ± 21.4	22.03	< 0.001
p-MLKL+ Neurons (cells/mm²)	12.6 ± 3.5	89.4 ± 15.2	21.87	< 0.001

3.2. pTau induces neuronal necroptosis via the RIPK1-RIPK3-MLKL axis

After treatment with 1.0 μmol/L pTau for 24 h, cell viability decreased to 52.3% of the control group, and the LDH release rate increased by 3.8 times; the p-MLKL/MLKL ratio increased by 4.73 times, and RIPK1-RIPK3 binding increased by 4.21 times. Nec-1 intervention increased viability by 56.0%, decreased LDH by 56.0%, and decreased p-MLKL/MLKL by 56.3%; GSK'872 intervention increased viability by 50.9%, decreased LDH by 51.8%, and decreased p-MLKL/MLKL by 63.4% (**Table 2**).

Table 2. pTau-Induced Necroptosis in HT22 Cells and the Effect of Inhibitor Intervention (mean ± SD, n = 6)

Group	Cell Viability (%)	LDH Release Rate (U/L)	p-MLKL/MLKL	RIPK1-RIPK3 Binding
Control	100.0 ± 5.3	85.4 ± 9.2	0.15 ± 0.03	1.00 ± 0.11
pTau 0.1 μM	89.6 ± 6.2*	112.3 ± 12.5*	0.28 ± 0.05*	1.56 ± 0.18*
pTau 0.5 μM	71.4 ± 5.8**	198.7 ± 18.6**	0.49 ± 0.06**	2.84 ± 0.26**
pTau 1.0 μM	52.3 ± 4.9**	324.5 ± 25.3**	0.71 ± 0.08**	4.21 ± 0.35**
pTau + Nec-1	81.6 ± 5.7##	142.8 ± 15.4##	0.31 ± 0.04##	1.92 ± 0.21##
pTau + GSK'872	78.9 ± 6.1##	156.3 ± 16.7##	0.26 ± 0.04##	3.98 ± 0.33##

Note: Compared with the control group, *$P < 0.05$, **$P < 0.01$; compared with pTau 1.0 μM group, ##$P < 0.01$.

3.3. Ameliorative effects of inhibiting RIPK1 kinase activity in AD model mice

Nec-1s treatment significantly improved cognitive function in APP/PS1 mice: escape latency was shortened by 28.5% (58.3 → 41.7 s), time spent in the target quadrant was increased by 45.5% (18.9 → 27.5 s), and the number of platform crossings was increased by 65.5% (2.9 → 4.8 times) (all $P < 0.01$). Regarding molecular pathology, p-RIPK1/RIPK1 was reduced by 51.4%, p-MLKL/MLKL was reduced by 55.4%, and TNF-α, IL-1β, and IL-6 were reduced by 44.4%, 46.4%, and 43.5%, respectively. Surviving neurons in the CA1 region increased by 42.6%, and the p-MLKL+NeuN colocalization rate decreased by 60.8% (**Table 3**).

Table 3. Effects of Nec-1s on Cognitive Function and Neuropathology in APP/PS1 Mice (mean ± SD, n = 15)

Indicator	Wild-type Control	AD Model Group	AD + Nec-1s Group	F value	*P* value
Escape Latency (s)	28.6 ± 4.2	58.3 ± 6.5**	41.7 ± 5.4##	45.32	< 0.001
Time in Target Quadrant (s)	35.8 ± 4.1	18.9 ± 3.6**	27.5 ± 4.2##	38.76	< 0.001
Platform Crossings	6.8 ± 1.2	2.9 ± 0.8**	4.8 ± 1.1##	32.18	< 0.001
p-RIPK1/RIPK1	0.19 ± 0.04	0.72 ± 0.09**	0.35 ± 0.06##	58.43	< 0.001
p-MLKL/MLKL	0.16 ± 0.03	0.65 ± 0.08**	0.29 ± 0.05##	52.67	< 0.001
TNF-α (pg/mg)	12.4 ± 2.3	38.7 ± 5.2**	21.5 ± 3.6##	46.21	< 0.001
IL-1β (pg/mg)	8.6 ± 1.8	29.5 ± 4.1**	15.8 ± 2.9##	41.85	< 0.001
CA1 Neurons (cells/mm)	185.6 ± 15.3	92.4 ± 11.7**	131.8 ± 13.5##	48.76	< 0.001

Note: Compared with the wild-type group, **P<0.01; compared with the AD model group, ##$P < 0.01$.

4. Discussion

4.1. Necroptosis as a key link between AD pathological features and neuronal loss

This study shows significantly elevated expression of RIPK1, RIPK3, and p-MLKL in the brain tissue of AD patients, positively correlated with the degree of cognitive impairment, indicating abnormal activation of the necroptosis pathway in the AD brain, consistent with previous studies [3].

Analysis of the reasons:

(1) Aβ oligomers can activate microglia via TNF-α signaling, triggering RIPK1/RIPK3/MLKL activation, which is then transmitted to neurons;

(2) This study confirms that phosphorylated tau protein can directly induce neuronal necroptosis, promoting RIPK1-RIPK3 complex formation and MLKL phosphorylation in a concentration-dependent manner. Therefore, necroptosis constitutes a key link between core AD pathologies and neuronal loss.

4.2. Potential of RIPK1 as a therapeutic target in AD and current intervention strategies

RIPK1 is a critical node regulating cell death and inflammation. The use of Nec-1s in this study significantly improved cognitive function in APP/PS1 mice, reduced hippocampal neuronal loss by 42.6%, and decreased inflammatory factor levels, confirming the therapeutic potential of targeting RIPK1.

Analysis of the reasons:

(1) Inhibiting RIPK1 kinase activity directly blocks the execution of necroptosis;

(2) Reducing the release of DAMPs indirectly mitigates neuroinflammation.

Recent research by the Yuan Junying team found [4]:

(1) INPP5D, as a microglial homeostasis regulator, directly binds to RIPK1 via its SH2 domain, inhibiting its excessive activation;

(2) INPP5D deficiency leads to aberrant RIPK1 activation and expression of AD risk genes;

(3) Inhibiting RIPK1 kinase activity alleviates neuroinflammation. The brain-penetrant RIPK1 inhibitor SIR9900 has shown a good safety profile in Phase I clinical trials [5], offering a new strategy for AD treatment.

4.3. Limitations of this study and future research directions

Limitations: (1) Relatively small human sample size and cross-sectional design; (2) Only the APP/PS1 model was used, not encompassing tau pathology models; (3) Long-term safety and optimal intervention timing require further exploration.

Future directions: (1) Develop highly brain-penetrant RIPK1 inhibitors and promote clinical translation; (2) Explore the value of biomarkers like p-MLKL in early AD diagnosis; (3) In-depth study of the microglial INPP5D-RIPK1 axis; (4) Explore the crosstalk between necroptosis and other forms of cell death and comprehensive intervention strategies.

4. Conclusion

This study confirms that RIPK1-RIPK3-MLKL axis-mediated necroptosis plays a crucial role in neuronal loss in Alzheimer's disease, with phosphorylated tau protein acting as a direct activator of this pathway. Targeting RIPK1 significantly improves cognitive function, reduces neuroinflammation, and attenuates neuronal loss in AD model mice. With the revelation of the INPP5D-RIPK1 regulatory axis and the clinical advancement of brain-penetrant RIPK1 inhibitors, targeting necroptosis holds promise as a new therapeutic strategy for AD.

Disclosure statement

The author declares no conflict of interest.

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