



Modulation of NLRP3 inflammasome-related-inflammation via RIPK1/RIPK3-DRP1 or HIF-1 α signaling by phenothiazine in hypothermic and normothermic neuroprotection after acute ischemic stroke

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ABSTRACT

Background: Inflammation and subsequent mitochondrial dysfunction and cell death worsen outcomes after revascularization in ischemic stroke. Receptor-interacting protein kinase 1 (RIPK1) activated dynamin-related protein 1 (DRP1) in a NLRP3 domain containing 3 (NLRP3) inflammasome-dependent fashion and Hypoxia-Inducible Factor (HIF)-1 α play key roles in the process. This study determined how phenothiazine drugs (chlorpromazine and promethazine (C + P)) with the hypothermic and normothermic modality impacts the RIPK1/RIPK3-DRP1 and HIF-1 α pathways in providing neuroprotection.

Methods: A total of 150 adult male Sprague-Dawley rats were subjected to 2 h middle cerebral artery occlusion (MCAO) followed by 24 h reperfusion. 8 mg/kg of C + P was administered at onset of reperfusion. Infarct volumes, mRNA and protein expressions of HIF-1 α , RIPK1, RIPK3, DRP-1, NLRP3-inflammation and cytochrome c-apoptosis were assessed. Apoptotic cell death, infiltration of neutrophils and macrophages, and mitochondrial function were evaluated. Interaction between RIPK1/RIPK3 and HIF-1 α /NLRP3 were determined. In SH-SY5Y cells subjected to oxygen/glucose deprivation (OGD), the normothermic effect of C + P on inflammation and apoptosis were examined.

Results: C + P significantly reduced infarct volumes, mitochondrial dysfunction (ATP and ROS concentration, citrate synthase and ATPase activity), inflammation and apoptosis with and without induced hypothermia. Overexpression of RIPK1, RIPK3, DRP-1, NLRP3-inflammation and cytochrome c-apoptosis were all significantly reduced by C + P at 33 °C and the RIPK1 inhibitor (Nec1s), suggesting hypothermic effect of C + P via RIPK1/RIPK3-DRP1 pathway. When body temperature was maintained at 37 °C, C + P and HIF-1 α inhibitor (YC-1) reduced HIF-1 α expression, leading to reduction in mitochondrial dysfunction, NLRP3 inflammasome and cytochrome c-apoptosis, as well as the interaction of HIF-1 α and NLRP3. These were also evidenced *in vitro*, indicating a normothermic effect of C + P via HIF-1 α .

Conclusion: Hypothermic and normothermic neuroprotection of C + P involve different pathways. The normothermic effect was mediated by HIF-1 α , while hypothermic effect was via RIPK1/RIPK3-DRP1 signaling. This provides a theoretical basis for future precise exploration of hypothermic and normothermic neuroprotection.

1. Introduction

Acute ischemic stroke (AIS) is one of the most common diseases with high death and disability rates worldwide [1,2]. It accounts for up to 85 % of all types of strokes [3,4]. Inflammatory responses and cell death is

the hallmark of stroke pathology [5,6]. Currently, revascularization is the main treatment for ischemic stroke [7], however patients reached by this therapy often require supplementary neuroprotection strategies [8–10].

Chlorpromazine and promethazine (C + P) are two phenothiazine

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Table 1
Primers used for RT-qPCR analysis.

Primer name	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse
RIPK1	ACCTTAGACGCGTAGGAGCG	CGGAGCTAGGTGCTGAAGTG
RIPK3	GGAGTCAGGGGAATCAAGCC	TGGGTTTGGGAAGGATGCTCG
DRP-1	TGGAAGAGCTCAGTGCTGG	ACTCCATTTTCTTCTCCTGTTGT
HIF-1 α	GGCGAGAACGAGAAGAAAATAGG	AGATGGGAGCTCAGGTTGTG
NLRP3	CTGCATGCCGTATCTGTTG	GCTGAGCAAGCTAAAGGCTTC
ASC	ACAGTACCAGGAGTTCGTG	GGTCTGTCACCAAGTAGGGC
Caspase-1	TGGAGCTTCAGTCAGGTCAT	ATGCGCCACCTTCTTTGTTC
IL-1 β	CAGCTTTCGACAGTGAGGAGA	TTGTGAGATGCTGCTGTGA
IL-18	ACCGCAGTAATACGGAGCAT	TCTGGGATTCTGTTGGCTGTT
cytochrome -c	CTTGGGCTAGAGAGCGGGA	GGTATCTCTCTCCCAAGTGAT
Apaf-1	GTAGACGGCTTCTCCGCTC	GGATGCCCAGGATTTCCGAG
Caspase-9	AGCTGGCCAGTGTAATAC	GCTCCACCTCAGTCAACTC
Caspase-3	GAGCTTGAACGCGAAGAAA	TTGCGAGCTGACATTCCAGT
SHSY5Y-HIF-1 α	CTGAGAGGTTGAGGGACGGA	GGAAGTGCAACTGATGAGC
SHSY5Y-NLRP3	GCTGGCATCTGGGGAAC	GCCCTTCTGGGGAGGATAGT
SHSY5Y-Caspase-1	GAAAAGCCATGGCCGACAAG	GCCCTTTTCGGAATAACGGA
SHSY5Y- cytochrome -c	TTCTAGGCACTGTCGGGGTA	AGCCTGAAGGCATGACGTTT
SHSY5Y-Caspase-3	TGAGGCGGTTGTAGAAGAGTTTCG	TTATTAACGAAAACAGAGCGCC
SHSY5Y- β -actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
β -actin	CAAGAAGGTGGTGAAGCAG	AAAGTGGAAGAATGGGAG

neuroleptic drugs. They conferred neuroprotection in stroke is thought to be due to the induction of a “hibernation”-based state [11,12], however some studies revealed that the neuroprotection of C + P only partially through hypothermia [13]. The neuroprotective mechanisms on hypothermic and normothermic effects by C + P therefore remain to be determined.

The Receptor-interacting protein (RIPK), a serine-threonine kinase family consisting of 7 isoforms RIPK1-7, has been shown to be active the context of brain inflammation and cell death after stroke [14]. RIPK1 combines with RIPK3 through the C-terminal RIP homotypic interaction motif (RHIM) domain to form the RIPK1/RIPK3 complex, which initiates downstream signal transduction and triggers necroptosis [15]. There is a close relationship between RIPK1/RIPK3 activity and inflammation except necroptosis [16]. Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome activation requires the kinase activity of RIPK1 that has relationship with inflammatory cytokine production [17]. RIPK1 induced mitochondrial dysfunction which resulted in cell death in murine fibrosarcoma L929 cells while RIPK1 inhibitor (Nec1s) blocked the release of cytochrome c from mitochondria preventing apoptosis [18]. Moreover, RIPK1 inhibitor reduced caspase-9-mediated apoptosis after rhabdomyolysis in rats [19]. In addition, hypoxia inducible factor 1- α (HIF-1 α) is involved in regulation of biological functions such as inflammation and apoptosis which play a detrimental role in nervous system disorders [20,21]. HIF-1 α exhibited an inflammation amplification effect [22]. HIF-1 α mediates adaptive responses to oxidative stress through nuclear translocation and regulation of gene expression. Mitochondrial alterations are crucial for the adaptive response to oxidative stress [23]. A study has reported that inhibiting the activation of HIF-1 α in acute kidney injury models can reduce the mitochondrial apoptotic pathway and alleviate damage [24]. Our recent study revealed that HIF-1 α regulate NLRP3 inflammasome-mediated apoptosis pathway after ischemic stroke [25].

In the present study, we attempted to delineate the hypothermic or normothermic effect induced by C + P underlying the neuroprotective mechanisms on inflammation and thus apoptosis. We further determined the key roles of RIPK1/3-DRP1 or HIF-1 α and NLRP3, thus cytochrome c-Caspase-3 signaling in the neuroprotective mechanisms.

2. Materials and methods

Subjects. A total of 150 adult male Sprague-Dawley rats (280–300 g, Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were utilized in this study. The Animal Care and Use Committee of the

Capital Medical University approved the methods used, and investigation was carried out in line with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were randomly divided into the nine groups: (1) Sham (n = 8); (2) Stroke with 2 h middle cerebral artery occlusion (MCAO) (n = 22, 8 for Western blot and real-time PCR, 8 for TTC, 6 for immunofluorescence); (3) Stroke and C + P treatment at normothermic condition (37 °C) (n = 22, 8 for Western blot and real-time PCR, 8 for TTC, 6 for immunofluorescence); (4) Stroke and C + P treatment at hypothermic condition (33 °C) (n = 22, 8 for Western blot and real-time PCR, 8 for TTC, 6 for immunofluorescence); (5) Stroke and RIPK1 inhibitor (Nec1s, 6 mg/kg) (n = 16, 8 for Western blot and real-time PCR, 8 for TTC); (6) Stroke and RIPK1 inhibitor (Nec1s, 6 mg/kg) + C + P at 37 °C (n = 16, 8 for Western blot and real-time PCR, 8 for TTC); (7) Stroke and RIPK1 inhibitor (Nec1s, 6 mg/kg) + C + P at 33 °C (n = 16, 8 for Western blot and real-time PCR, 8 for TTC); (8) Stroke and HIF-1 α inhibitor (YC-1, 5 mg/kg) (n = 14, 8 for Western blot and real-time PCR, 6 for immunofluorescence); (9) Stroke and HIF-1 α inhibitor (YC-1, 5 mg/kg) + C + P at 37 °C (n = 14, 8 for Western blot and real-time PCR, 6 for immunofluorescence).

Focal Cerebral Ischemia. The ischemia model used in this study was described previously by us [25]. Rats were anesthetized in a chamber with 1–3% isoflurane and a mixture of 70 % nitrous oxide and 30 % oxygen for a short period of time. Then, their anesthesia was maintained using a facemask with 1 % isoflurane delivered from a calibrated precision vaporizer. Variability between rats was minimized by using poly-L-lysine-coated intraluminal nylon (4.0) sutures to produce consistent infarcts. Blood pCO₂ and pO₂, mean arterial pressure, and rectal temperature were monitored throughout the unilateral, 2-h MCA occlusion process. A circulating heating pad and a heating lamp were used throughout the procedures to maintain rectal temperatures constant between 36.5 and 37.5 °C for temperature control groups by an insulation blanket beneath a warm light during the recovery period of 24–28 h. The animal without temperature control group was placed in a 25 °C environment [26].

Drug Administration. A 1:1 ratio of C + P was dissolved in saline and administered at 8 mg/kg body weight through intraperitoneal (IP) injection at the onset of reperfusion following 2 h MCAO. One-third of the original dose was injected 2 h later to compound the drug’s effects. Nec1s (Selleck, Houston, USA), a RIPK1 inhibitor, was dissolved in 5 % dimethyl sulfoxide (DMSO) and administered at 6 mg/kg body weight through IP injection after 2 h MCAO [27]. YC-1 (Selleck, Houston, USA), a HIF-1 α inhibitor, was dissolved in 1 % dimethyl sulfoxide (DMSO) and administered at 5 mg/kg body weight through IP injection 2 h before MCAO [28].

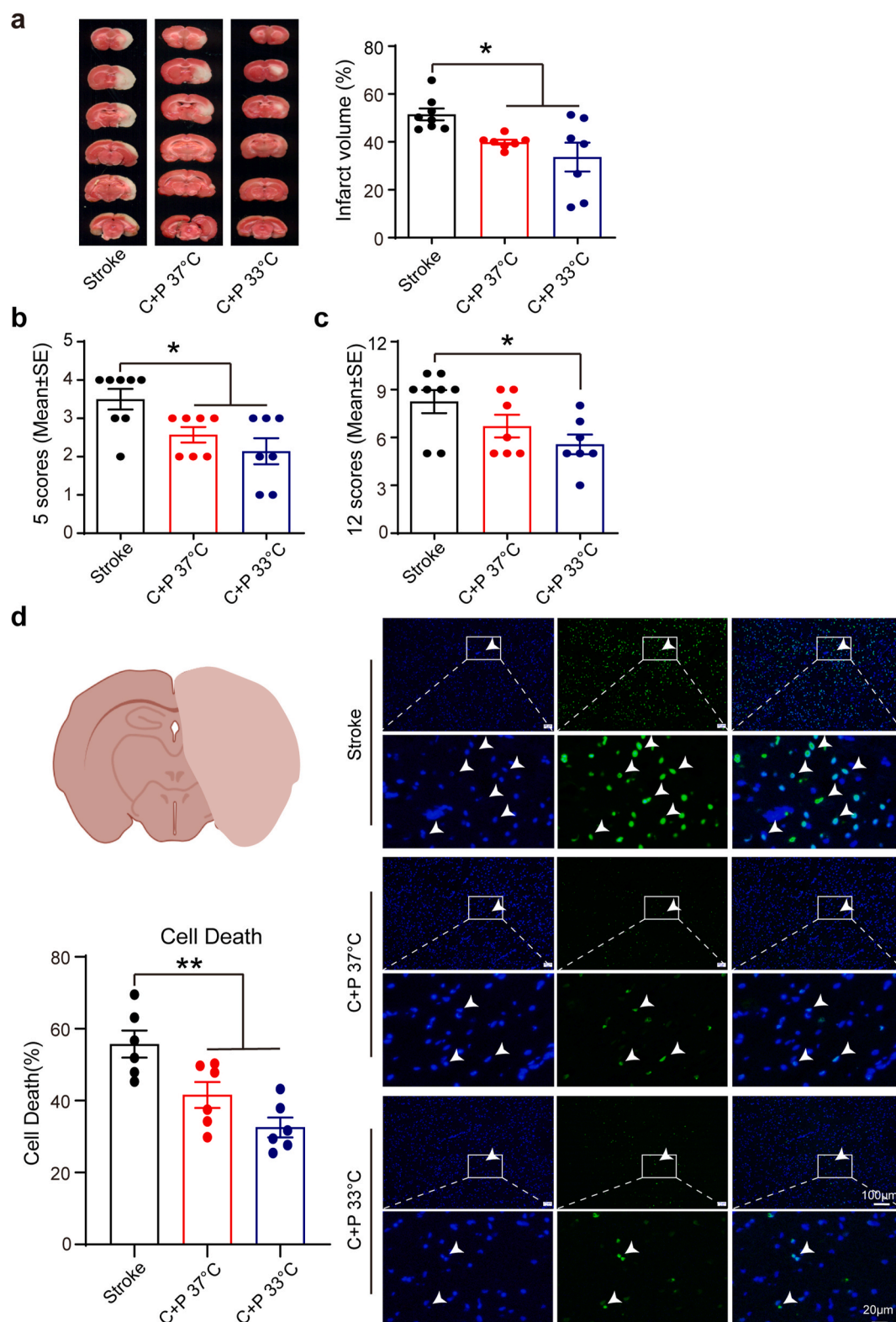


Fig. 1. C + P reduced brain injury and apoptosis post-ischemia reperfusion. (a) The 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining illustrates infarct volumes in MCAO rats with or without C + P treatment at 48 h after reperfusion. (b–c) Neurological deficit after C + P treatment in MCAO rats at 48 h after reperfusion by using 5 score and 12 score system. (d) TUNEL assay of C + P therapy at 24 h after reperfusion. Apoptosis cell death was significantly decreased after C + P treatment. Scale bar = 20 μm. Data are presented as mean ± SEM.

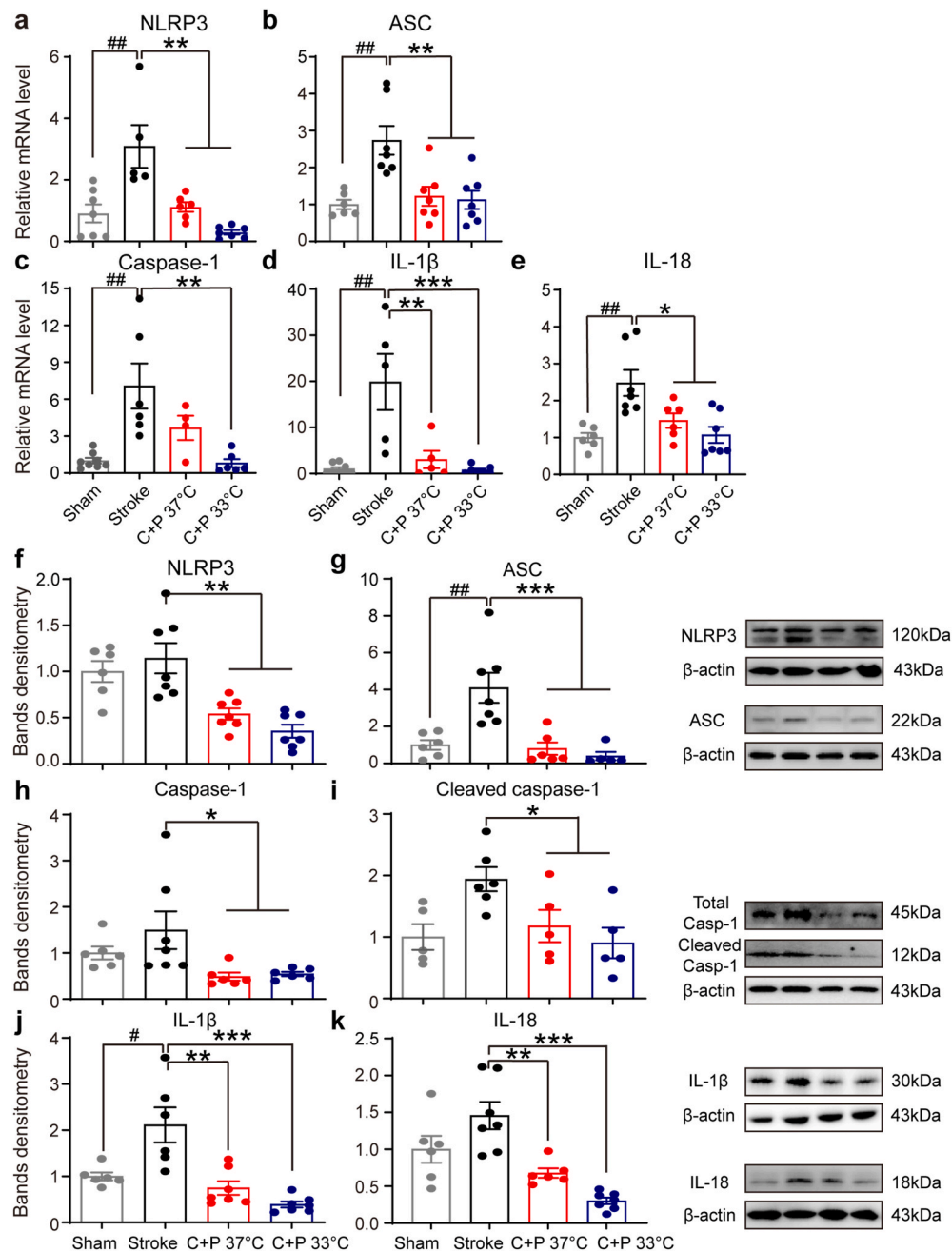


Fig. 2. C + P decreased the activation of NLRP3 inflammasome pathway. NLRP3, ASC, Caspase-1, IL-1 β , and IL-18 mRNA (a–e) and protein (f–k) expression were measured after 24 h reperfusion in brain tissue. mRNA and protein levels of NLRP3, ASC, Caspase-1, IL-1 β and IL-18 were all increased after ischemia and reperfusion and all significantly reversed by C + P at normothermic and hypothermic condition. Data are presented as mean $\pm$ SEM.

Cell Culture, Oxygen Glucose Deprivation/Reoxygenation (OGD/R) and C + P Administration. The cell line of human SHSY5Y was acquired from Cell Resource Center, Peking Union Medical College. We incubated cells in a 25 cm² culture flask with 5 ml of DMEM/F12 and 10 % FBS and cultured nine to eleven passages. We incubated the cells at 5 % CO₂ and 37 °C in a humidified incubator. As previously described [29], we first prepared an anaerobic chamber by flushing it with a 95 % N₂ and 5 % CO₂ (v/v) mixture, maintaining the temperature at 37 °C. The cell culture medium was then replaced with deoxygenated, glucose-free DMEM to initiate a 2-h OGD phase. SHSY5Y cells were then removed from the anaerobic chamber, and the DMEM medium was replaced with the regular maintenance medium. We finally administered C + P treatment at a concentration of 2.5 μ M [30]. The cells were incubated for 24 h in a humidified environment before being harvested.

Infarct Volume Measurement. After 48 h of reperfusion, ischemic rat brains were removed, cut into 2 mm-thick slices (brain matrix), and stained with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA). The infarct volume was calculated using an indirect method to ensure that error attributable to edema was reduced as much as possible [25]. The infarct cross-sectional area in each brain slice was calculated using Image J. The infarct volume (%) of each brain slice [(total volume in the left hemisphere of the same brain - non-infarcted volume in the right hemisphere of the same brain)/(total volume in the left hemisphere of the same brain $\times$ 100 %)]. Lastly, the total infarct volume for each brain was calculated and the sums of the infarct areas of all the brain slices were averaged together.

Neurological Deficits. The neurological deficits were evaluated using the modified scoring systems (5 and 12 scores) proposed by Longa

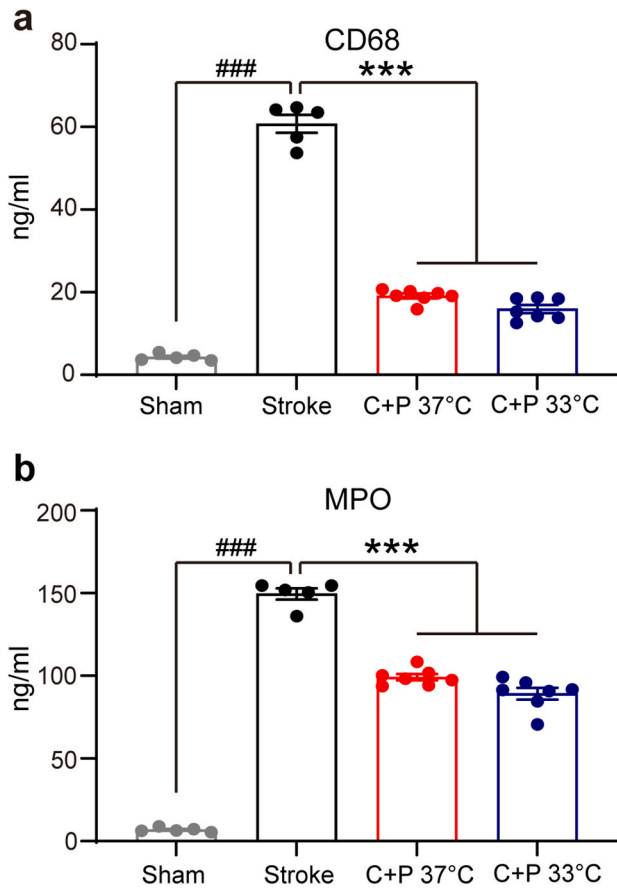


Fig. 3. C + P attenuated the brain inflammation by the levels of CD68 and MPO. Quantitative analysis of (a) CD68 and (b) MPO were measured. CD68 and MPO levels were higher at 24 h of reperfusion. Decreased CD68 and MPO levels were observed in C + P groups regardless of temperature control. Data are presented as mean $\pm$ SEM.

et al. [31] and Belayev et al. [32] before surgery for baseline, after 2 h MCA occlusion (immediately before reperfusion), and after 48 h reperfusion. Higher scores indicate more severe deficits in both scoring systems. Because of the importance of severity and consistency of brain damage in this study, neurological deficits after MCA occlusion were also used to validate brain injury. MCA occlusions were considered unsuccessful, and the rats were excluded from the study if the score was 1 or below (approximately 10 % of animals with MCA occlusion were discarded).

Real-time qRT-PCR. A Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to manufacturer directions. We eliminated DNA contamination with RNase-free DNase I (Invitrogen). Subsequently, RNA (1 μ g) was reverse transcribed into cDNA in a volume of 20 μ l containing 2.5 μ M of random hexamers, 2 μ M dNTP and 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Quantitative PCR was conducted in a 20 μ l reaction mixture containing 0.2 μ l cDNA, 0.5 μ M forward and reverse primers, and 10 μ l Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7500 real-time cycler (Applied Biosystems). The number of transcripts for target genes, including HIF-1 α , RIPK1, RIPK3, DRP-1, NLRP3, ASC, Caspase-1, IL-1 β , IL-18, cytochrome c, Apaf-1, Caspase-9, Caspase-3 were quantified using the comparative $2^{-\Delta\Delta CT}$ method from the Applied Biosystems User Bulletin No.2 (P/N 4303859) [33]. Primer sequences are displayed in Table 1.

Western Blotting. The rat brains were lysed with RIPA buffer, which includes a protease inhibitor cocktail (Sigma). Using the bovine serum

albumin (BSA) protein assay kit (Appligen Technologies Inc., Beijing, China), protein concentration was determined. After separating the proteins (20 μ g/lane) on a 10 % SDS-PAGE gel, they were electro-transferred to PVDF membranes (Millipore, Bedford, MA, USA). They were then blocked on Tris-buffered saline (pH 7.4) consisting of 5 % non-fat milk at room temperature for 1 h. The membranes were incubated with primary antibodies overnight at 4 $^{\circ}$ C, including HIF-1 α (mouse monoclonal IgG, 1:500, ab8633), RIPK1 (mouse monoclonal IgG, 1:500, ab72139), RIPK3 (rabbit polyclonal IgG, 1:1000, ab62344), DRP-1 (rabbit monoclonal IgG, 1:1000, ab184247), NLRP3 (rabbit polyclonal IgG, 1:1000, ab214185), ASC (rabbit polyclonal IgG, 1:1000, ab307560), IL-18 (rabbit polyclonal IgG, 1:1000, ab191860), pro-Caspase-1+p10+p12 (rabbit monoclonal IgG, 1:1000, ab179515), IL-1 β (rabbit polyclonal IgG, 1:500, ab9722), cytochrome c (rabbit polyclonal IgG, 1:1000, ab133504), Apaf-1 (rabbit polyclonal IgG, 1:500, ab234436), Caspase-9 (rabbit polyclonal IgG, 1:1000, ab184786), Caspase-3 (rabbit polyclonal IgG, 1:500, ab184787, Abcam, Cambridge, UK) and p-DRP-1 (rabbit monoclonal IgG, 1:1000, 4867, Cell Signaling, MA, USA). Membranes then underwent two washes with suitable HRP-conjugated secondary antibodies (Zhongshan Biotechnology Co.) at room temperature (RT) for 1 h. After, a chemiluminescence detection kit (Zhongshan Biotechnology Co) was used to visualize the antigen-antibody complexes. The images were analyzed using Image J, version 1.44. Values obtained from the density of all bands per group of target protein were averaged and used for statistical analysis [34].

Immunofluorescence Labeling. After blocking with 5 % pre-immune goat sera in phosphate-buffered saline (PBS) for 1 h at RT, sections were incubated with primary antibodies, including RIPK1 (mouse monoclonal IgG, 1:200, ab72139), RIPK3 (rabbit polyclonal IgG, 1:200, ab62344), HIF-1 α (mouse monoclonal IgG, 1:100, ab8633), NLRP3 (rabbit polyclonal IgG, 1:200, ab214185, Abcam, Cambridge, UK) overnight at 4 $^{\circ}$ C. After two washes with PBS, the sections were incubated at RT for 1 h with FITC- or TRITC-conjugated secondary antibodies. We used the mouse secondary antibody of FITC (Zhongshan Biotechnology Co, China) for RIPK1 and NLRP3, and the rabbit secondary antibody of TRITC (Zhongshan Biotechnology Co, China) for RIPK3 and HIF-1 α . Antifade mounting medium and DAPI (Zhongshan Biotechnology Co, China) were used to mount the slides. Images were acquired from the infarcted cortex of the brain sections (6 images were randomly chosen per section). RIPK1 $^{+}$ RIPK3 $^{+}$ cells and HIF-1 α $^{+}$ NLRP3 $^{+}$ cells were counted by using the Image J manual cell counting tool. When using the Image J manual cell counting tool, blinded manner was used to each group. The values were averaged and for statistical analysis. The percentage of co-localization cells (%) was calculated as (co-localization cells/total cells) $\times$ 100 % [29].

Enzyme-linked Immunosorbent Assay (ELISA). We dissected the rat brains on ice as quickly as possible to prevent degradation by proteases. For every 5 mg of infarcted tissue, 300 μ l of extraction buffer was added to the tube, which was mixed with an electric homogenizer. It was agitated for 1 h at 4 $^{\circ}$ C, and centrifuged for 20 min at 13,000 rpm. We used ELISA kits to measure MPO (ml003250) and CD68 (ml059367, Shanghai Enzyme-linked Biotechnology Co, China), markers of neutrophils and macrophages respectively. Mitochondrial function markers including ATPase (ml059183), citrate synthase (ml059595), as well as ROS (ml926281) and ATP (ml059601, Shanghai Enzyme-linked Biotechnology Co, China) also were examined.

TUNEL Assay. As outlined in our previous publication [25], the TUNEL assay was used to investigate DNA fragmentation by means of a commercial kit (In situ Cell Death Detection Kit, Fluoresce, Roche, Indianapolis, IN, USA). Slides were placed on ice for 2 min after being permeabilized with 0.1 % (v/v) Triton X-100 containing 0.1 % (w/v) sodium citrate. Samples were then incubated in 50 ml of the TUNEL reaction mixture at 37 $^{\circ}$ C for 1 h. TUNEL staining was visualized using a fluorescence microscope (DM4000, Leica, Germany). We acquired 10 images of different locations from the MCAO-supplied territory (shaded area in Fig. 1D). The TUNEL $^{+}$ cells were then counted with the

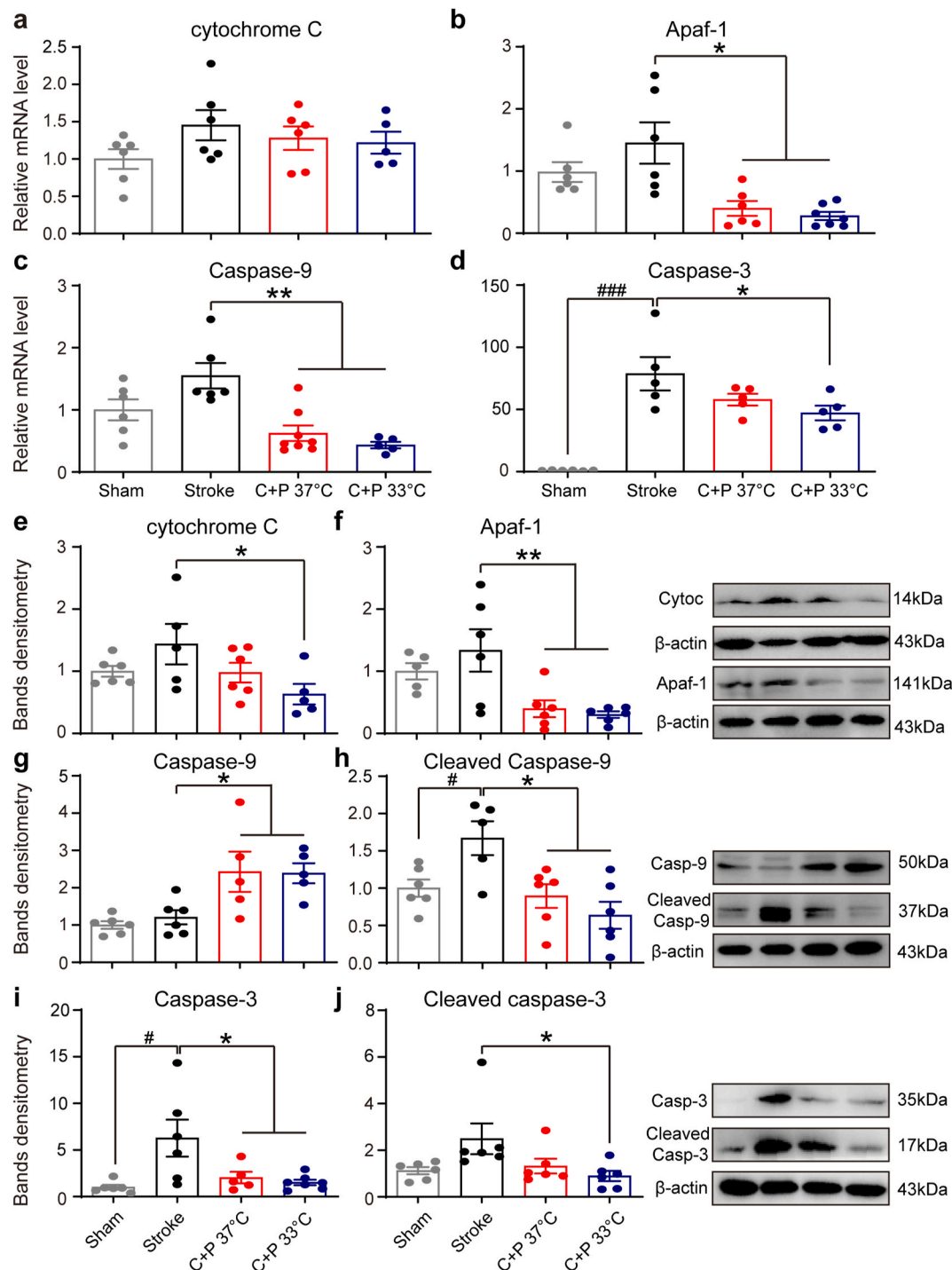


Fig. 4. C + P inhibited cytochrome C-induced apoptosis pathway activation. The mRNA (a–d) and protein (e–j) expression of cytochrome C, Apaf-1, Caspase-9 and Caspase-3 were examined. Levels of cytochrome C, Apaf-1, Caspase-9 and Caspase-3 were increased after 24 h of reperfusion and reversed by C + P groups regardless of temperature control. Data are presented as mean $\pm$ SEM.

manual cell counting tool in Image J. Values from all sections in each group were obtained in a blinded manner and used for subsequent statistical analysis. The percentage of apoptotic cells (%) = (TUNEL⁺ cells/total cells) $\times$ 100 %. The amount of positively staining cells was tallied up to determine apoptotic cell death with the TUNEL index.

Coimmunoprecipitation (Co-IP). Co-IP of HIF-1 α and NLRP3 was performed as described by us [29]. Immunoprecipitation lysis buffer was used to collect brain tissue lysates. The lysates were centrifuged at 12,000 rpm prior to collecting the supernatants after 30 min on the ice. Primary anti-HIF-1 α (PA1-16601, Invitrogen) and IgG antibody (2729,

Cell Signaling Technology) were added to 100 μ l tissue lysate and incubated at 4°C overnight. Antibody immunocomplex solution and lysate were transferred to the protein G magnetic bead-containing tube and incubated with rotation at room temperature for 30 min. Magnetic Separation Rack (7017, Cell Signaling Technology) was used to separate the magnetic beads in accordance with the manufacturing directions and then boiled to denature the protein-bead complex. The immunoprecipitants underwent Western blot analysis.

Statistical Analysis. We conducted our statistical analysis using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA),

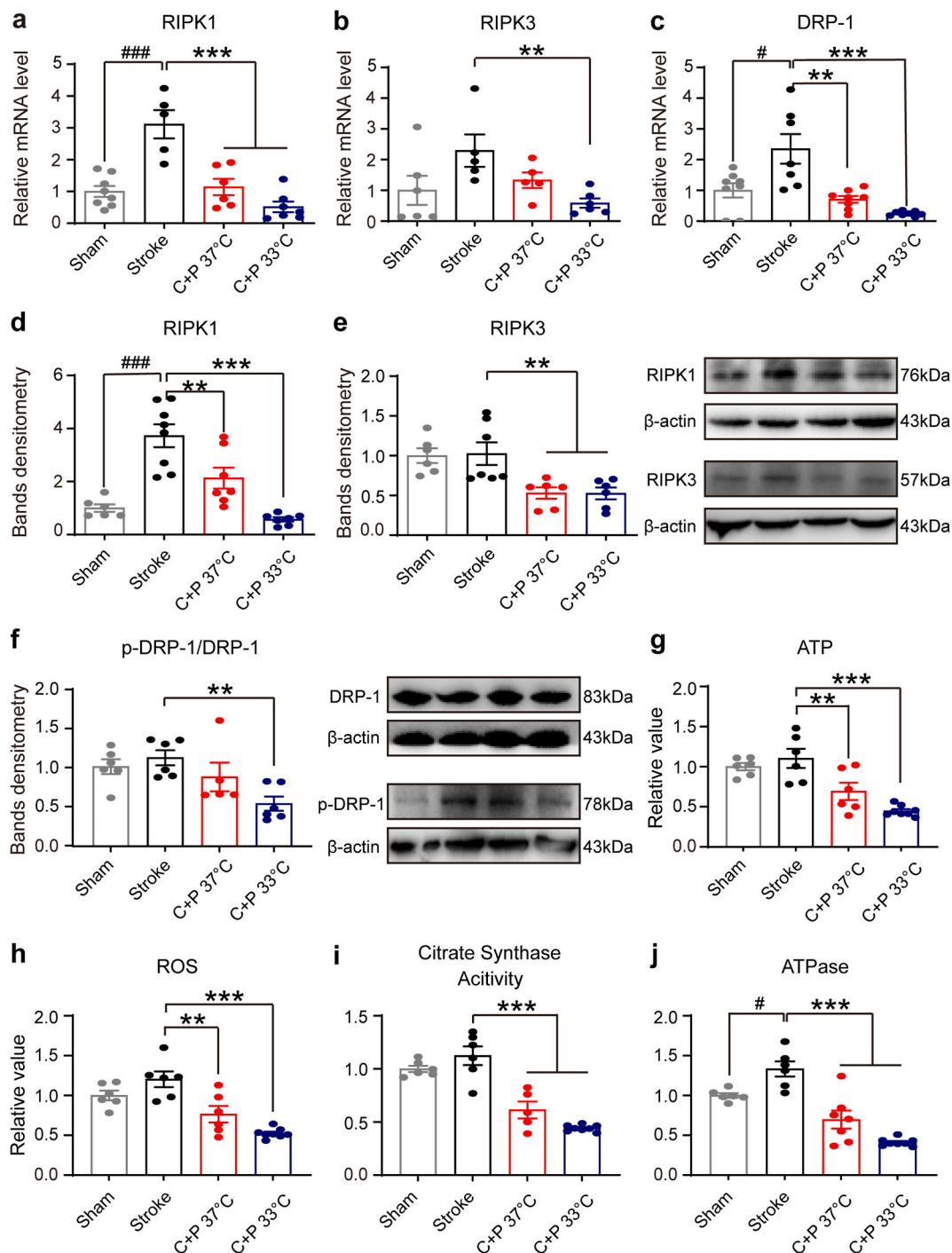


Fig. 5. C + P decreased the level of RIPK1/RIPK3-DRP-1 and mitochondrial function markers. RIPK1/RIPK3-DRP-1 RIP1 mRNA (a–c) and protein levels (d–f) were measured by quantitative real-time PCR and Western blot analysis. Levels of RIPK1 and RIPK3, as well as DRP-1 phosphorylation were decreased by C + P groups regardless of temperature control at 24 h of reperfusion. The activity of ATPase and citrate synthase, as well as the ROS and ATP concentration were measured for mitochondrial function evaluation (g–j). C + P could reduce mitochondrial dysfunction after 24 h reperfusion. Data are presented as mean $\pm$ SEM.

employing a random effects model. Each analysis used data collected per animal. Data was in a normal distribution. Levene's test at $p > 0.05$ demonstrated satisfactory variance. A Kolmogorov-Smirnov test confirmed data normality, as indicated by $p > 0.05$ for all variables. Data meeting both conditions was assessed using parametric tests. We determined differences between groups using one-way ANOVA and t -tests, setting a significance level of $p < 0.05$.

3. Results

C + P Reduced Brain Damage and Apoptosis under Normothermic and Hypothermic Conditions. At 48 h after reperfusion (Fig. 1a), infarct volume was decreased by C + P with body temperature at both 37 °C and 33 °C ($p < 0.05$). Neurologic deficits, analyzed by 5- or 12-point scoring systems after 48 h reperfusion (Fig. 1b and c), was also decreased by C + P ($p < 0.05$). Furthermore, apoptotic cell death, determined by TUNEL (Fig. 1d), was diminished by C + P ($p < 0.01$).

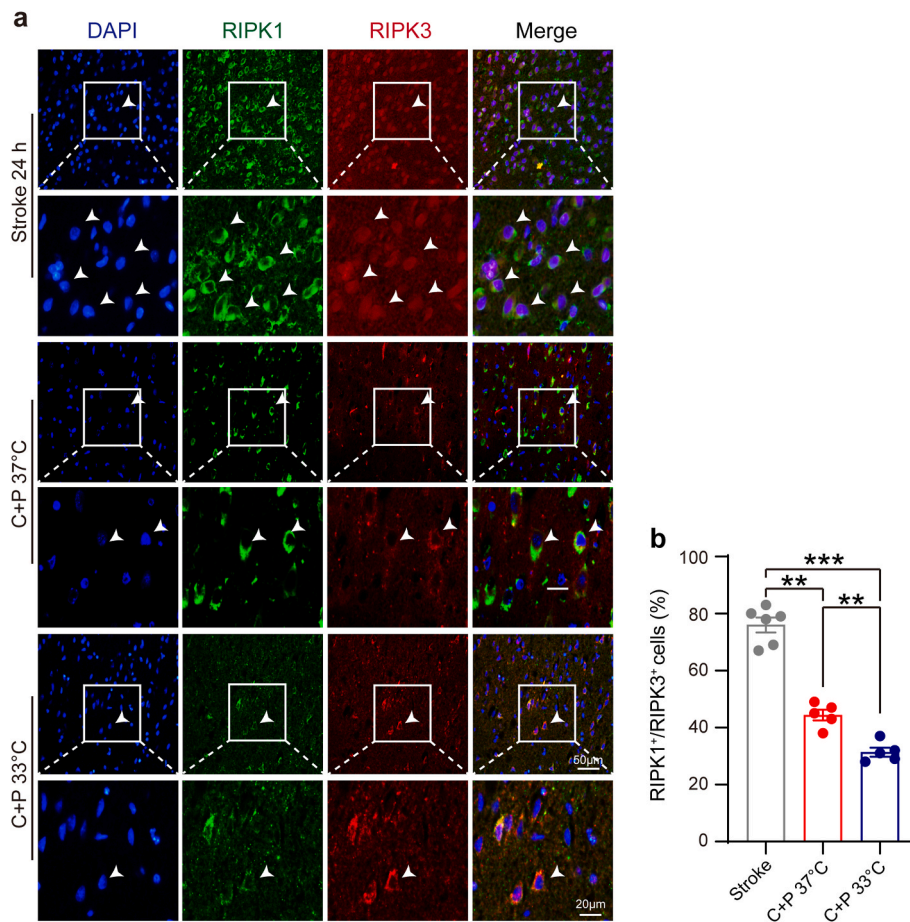


Fig. 6. Co-localization of RIPK1 and RIPK3. (a–b) RIPK1-positive cells (green) co-localized with RIPK3-positive cells (red) at 24 h after reperfusion. After C + P treatment whether temperature controlled, the number of brain RIPK1⁺RIPK3⁺ cells was reduced. Scale bar = 50 μm. Data are presented as mean ± SEM.

Normothermic and Hypothermic Effects of C + P on Suppressed Inflammatory Response, NLRP3 Inflammasome Activation and Immune Cells Infiltration. NLRP3 inflammasome activation after 24 h of reperfusion was determined by Real-time qRT-PCR and Western blot. mRNA levels of NLRP3, ASC, Caspase-1, IL-1β and IL-18 were all increased after ischemia and reperfusion ($p < 0.01$) and reversed by C + P at 37 °C or 33 °C ($p < 0.05$) (Fig. 2a–e). In addition, C + P significantly reduced the protein expression of NLRP3 inflammasome complex, including NLRP3, ASC, Caspase-1, IL-1β and IL-18 at 24 h of reperfusion (Fig. 2f–k). ELISA revealed that C + P significantly reduced levels of MPO (a neutrophil marker) and CD68 (a macrophage marker) (Fig. 3a–b) ($p < 0.05$, $p < 0.01$, $p < 0.001$).

C + P at Normothermic and Hypothermic Condition Attenuated Apoptotic Cell Death via Cytochrome c/Apaf-1-caspase-3. Expression of cytochrome c/Apaf-1-caspase-3 after 24 h of reperfusion was determined by real-time qRT-PCR and Western blot. mRNA levels of Apaf-1, Caspase-9 and Caspase-3 were all significantly reversed by C + P (Fig. 4a–d) ($p < 0.05$, $p < 0.01$, $p < 0.001$). Similarly, C + P inhibited the protein expression of cytochrome c, Apaf-1, Caspase-9 and -3 (Fig. 4e–j) ($p < 0.05$, $p < 0.01$).

C + P at Both Normothermic or Hypothermic Condition Decreased Expression of RIPK1/RIPK3-DRP-1, Mitochondrial Dysfunction and Co-Localization of RIPK1 and RIPK3. Expression of RIPK1, RIPK3 and DRP-1 after 24 h of reperfusion was determined by Real-time qRT-PCR and Western blot. C + P treatment significantly decreased mRNA and protein expression of RIPK1 and RIPK3 as compared to stroke group. The phosphorylation level of DRP-1 was reduced (Fig. 5a–f) ($p < 0.05$, $p < 0.01$, $p < 0.001$). We further examined the mitochondrial function markers by ELISA. Levels of ATP, ROS,

citrate synthase and ATPase were all significantly decreased by C + P treatment (Fig. 5g–j) ($p < 0.01$, $p < 0.001$). Additionally, RIPK1 was present in RIPK3⁺ cells, determined by immunofluorescence, at 24 h after reperfusion. After C + P treatment at both 37 °C and 33 °C, the number of RIPK1⁺/RIPK3⁺ cells were decreased with a significantly reduction being found under hypothermic condition (Fig. 6a–b) ($p < 0.01$, $p < 0.001$).

Hypothermic Effect of C + P on RIPK1. At 48 h after reperfusion, infarct volume and neurologic deficits were significantly decreased by Nec1s, the inhibitor of RIPK1 (Fig. 7a–b) ($p < 0.01$). Nec1s also significantly reduced mRNA (Fig. 8a–e) and protein (Fig. 9a–f) levels of RIPK1, RIPK3, NLRP3, cytochrome c and Caspase-3 ($p < 0.05$, $p < 0.01$, $p < 0.001$). This finding suggests that RIPK1 play an important role in the suppression of inflammation and apoptosis after cerebral ischemia. To explore the mechanism of hypothermic and normothermic neuroprotection of C + P on inflammation and apoptosis after ischemic stroke, we examined the brain damage, mitochondrial function, and expression of RIPK1, RIPK3, NLRP3, cytochrome c and Caspase-3 with Nec1s after 24 h of reperfusion. The administration of Nec1s in addition to C + P at hypothermic condition did not further reduce infarct volume, neurological deficits and mitochondrial dysfunction (Fig. 7c–h) and the levels of RIPK1, RIPK3, NLRP3, cytochrome c and Caspase-3 (Fig. 8f–j and Fig. 9g–m). These findings suggest that the hypothermic neuroprotection of C + P relies on the RIPK1 pathway. Furthermore, the effect of mitochondrial function, anti-inflammation, and apoptosis induced by C + P at normothermic condition was significantly enhanced by the addition of RIPK1 inhibitor Nec1s ($p < 0.05$, $p < 0.01$, $p < 0.001$), suggesting the normothermic effect of C + P was due to the molecules other than RIPK1.

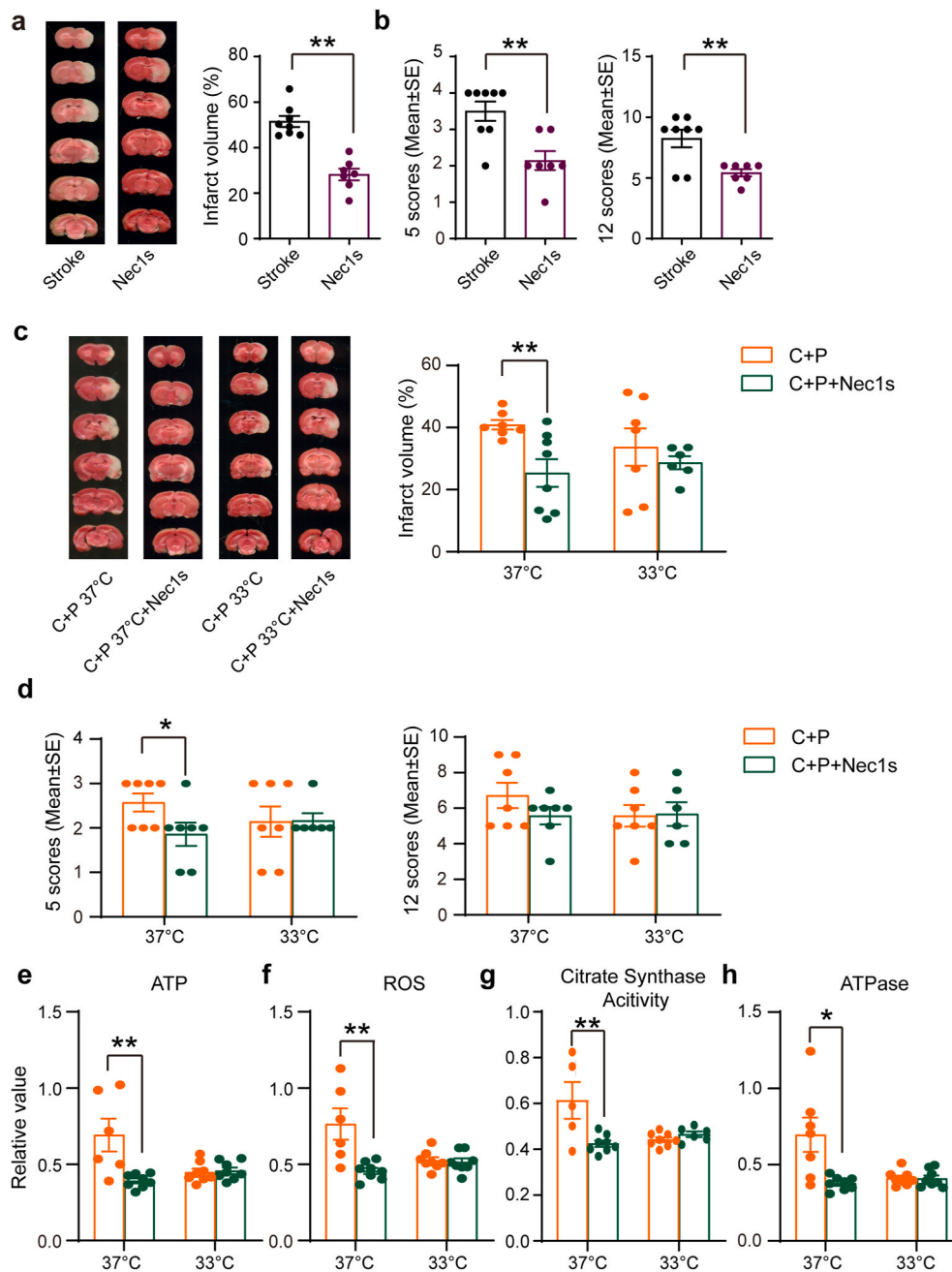


Fig. 7. C + P at hypothermic condition reduced brain damage and mitochondrial dysfunction via RIPK1. TTC staining illustrates infarct volumes and Neurological deficit in MCAO rats for Nec1s (a–b) and Nec1s plus C + P regardless of temperature control (c–d) at 48 h after reperfusion. (e–h) The activity of ATPase and citrate synthase, as well as ROS and ATP concentration were measured. The additional administration of Nec1s in C + P at hypothermic condition did not further reduce infarct volume, neurological deficits and mitochondrial dysfunction. Furthermore, the neuroprotective effect induced by C + P at normothermic condition was significantly enhanced by additional Nec1s. Data are presented as mean ± SEM.

Normothermic Effect of C + P on HIF-1 α . To further explore the normothermic effect of C + P, we examined the regulation of mitochondrial function, inflammation and apoptosis by HIF-1 α . Levels of mitochondrial function markers were significantly decreased by C + P at 37 °C or YC-1 (HIF-1 α inhibitor) (Fig. 10a–d) ($p < 0.05$, $p < 0.01$, $p < 0.001$). Level of HIF-1 α was increased after ischemia and reperfusion ($p < 0.01$, $p < 0.001$), and significantly reversed by either C + P at 37 °C or YC-1, as well as by YC-1 plus C + P at 37 °C (Fig. 10e and 11a) ($p < 0.01$). Notably, the combination of YC-1 and C + P did not further reduce HIF-1 α expression, suggesting that C + P at normothermic condition induced neuroprotection by inhibiting HIF-1 α activation. We further examine the expression of key molecules in the NLRP3-

inflammation and cytochrome c-apoptosis pathway (Fig. 10f–i and Fig. 11b–g) with C + P at 37 °C, YC-1 plus C + P at 37 °C, as well as YC-1 group, the all treatments significantly reduced the expression of NLRP3, caspase-1 and caspase-3 after stroke ($p < 0.05$, $p < 0.01$). Again, YC-1 and C + P at 37 °C did not provide further neuroprotection as compared to C + P at 37 °C, suggesting normothermic neuroprotection by C + P on inflammation and apoptosis depend on the HIF-1 α pathway.

Interaction of HIF-1 α and NLRP3. To analyze the relationship of HIF-1 α and NLRP3, the co-localization of HIF-1 α and NLRP3 were measured by immunofluorescence in the brain. HIF-1 α was present in NLRP3⁺ cells after stroke. C+P at 37 °C, YC-1 plus C + P at 37 °C, and YC-1 all significantly reduced the number of HIF-1 α ⁺/NLRP3⁺ cells

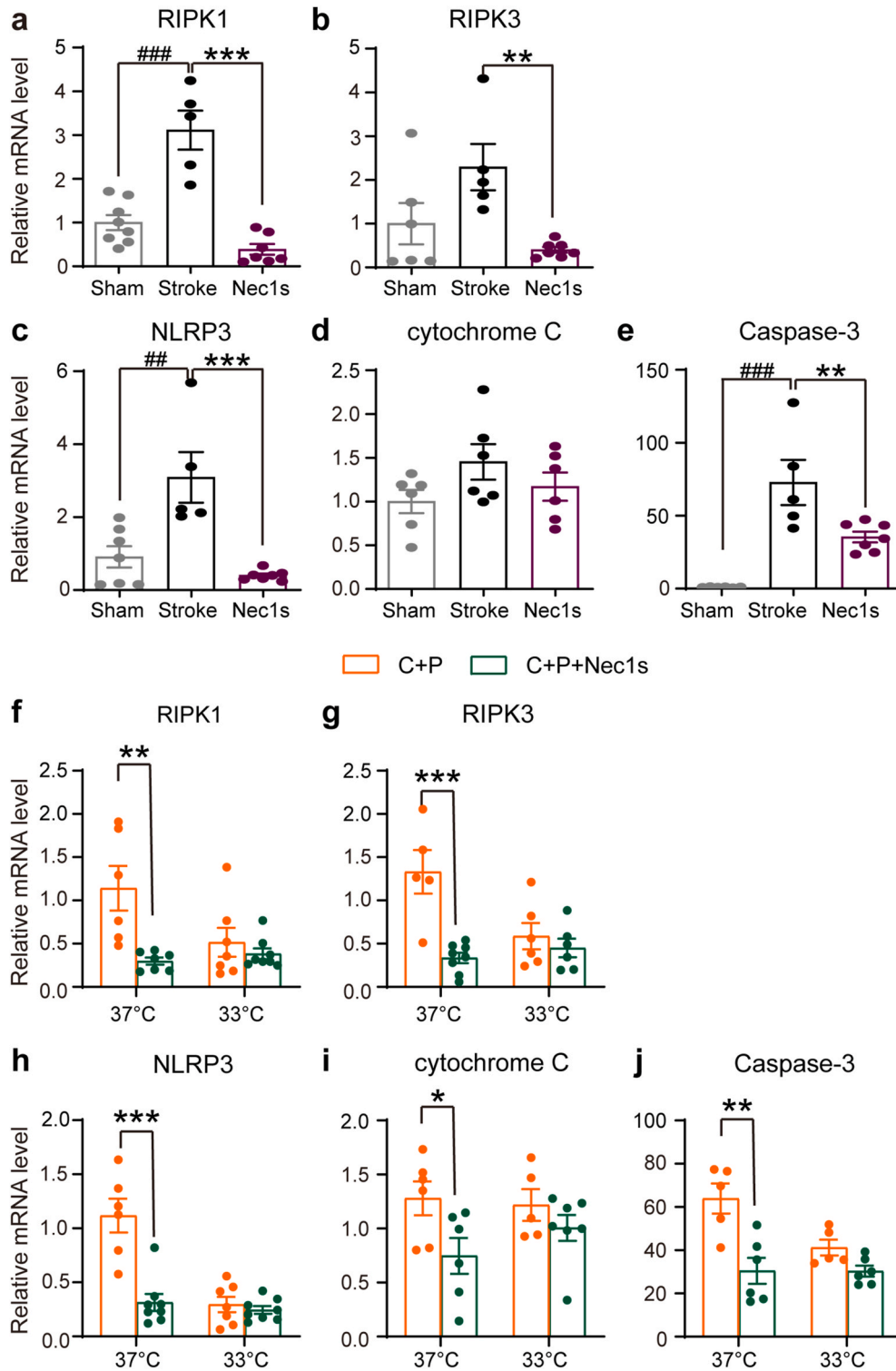


Fig. 8. C + P with hypothermia decreased the mRNA expression of NLRP3-inflammation and cytochrome C-apoptosis pathway via RIPK1. RIPK1, RIPK3, NLRP3, cytochrome C and Caspase-3 mRNA levels examined by quantitative real-time PCR. (a-e)mRNA expression of RIPK1, RIPK3, NLRP3 and Caspase-3 were significantly decreased by Nec1s at 24 h after reperfusion.(f-j)The additional administration of Nec1s in C + P at hypothermic condition did not further reduce the mRNA levels of the aforementioned molecules. Furthermore, the effect of anti-inflammation and apoptosis induced by C + P at normothermic condition was significantly enhanced by additional Nec1s. Data are presented as mean $\pm$ SEM.

(Fig. 12a). By immunoprecipitating with HIF-1 α or IgG antibody followed by antibody treatment against HIF-1 α and NLRP3 with Western blot (Fig. 12b), we measured the interaction of HIF-1 α and NLRP3 in the tissue lysates of ischemic penumbra. Compared to the IgG control, significant increases in NLRP3 were detected in the immunoprecipitation

complex with HIF-1 α antibody. An interaction between HIF-1 α and NLRP3 was therefore observed. We further found that the interaction of HIF-1 α and NLRP3 was increased at 24 h of reperfusion. With the treatments of C + P at 37 °C or YC-1, the interaction has been show ($p < 0.001$). However, YC-1 did not further significantly enhance the

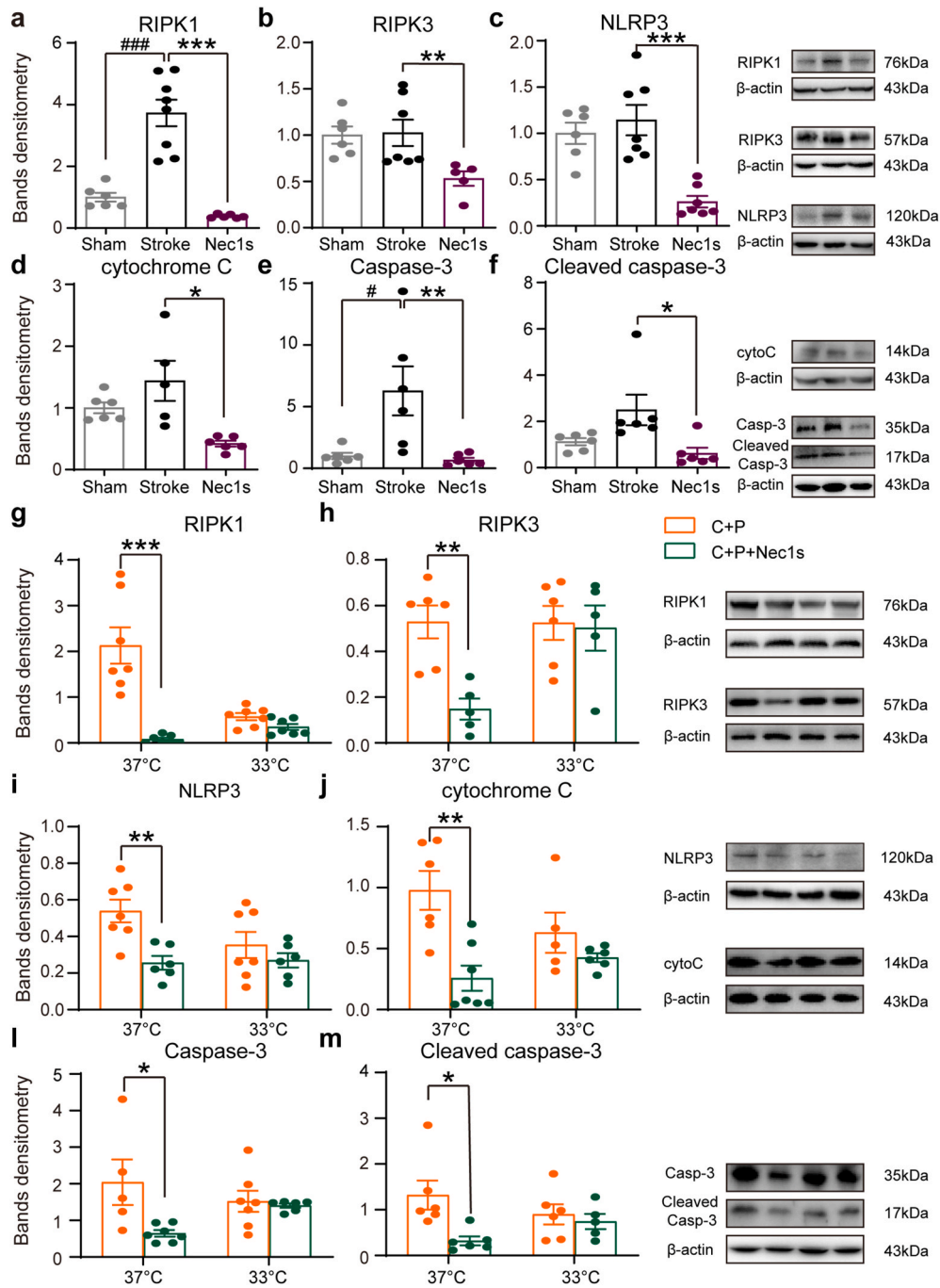


Fig. 9. Protein levels of NLRP3-inflammation and cytochrome C-apoptosis pathway after C + P with hypothermia or Nec1s. RIPK1, RIPK3, NLRP3, cytochrome C, Caspase-3 and Cleaved caspase-3 protein levels examined by Western blot analysis. (a–f) The protein expression of RIPK1, RIPK3, NLRP3, cytochrome C, and Cleaved caspase-3 were significantly decreased by Nec1s at 24 h after reperfusion. (g–m) The additional administration of Nec1s in C + P at hypothermic condition did not further reduce the protein levels of the aforementioned molecules. Furthermore, the effect of anti-inflammation and apoptosis induced by C + P at normothermic condition was significantly enhanced by additional Nec1s. Data are presented as mean $\pm$ SEM.

decreased interaction of HIF-1 α and NLRP3 by C + P at 37 °C, suggesting that C + P at normothermic condition induced neuroprotection through the inhibition of HIF-1 α and NLRP3 interplay.

In Vitro Normothermic Effect of C + P on Inflammation and Apoptosis via HIF-1 α . In SHSY5Y cells with OGD/R, Real time-PCR and Western blot were performed to investigate HIF-1 α , NLRP3, caspase-1, cytochrome c, and caspase-3 expression after C + P at 37 °C (Fig. 13a–l). Compared to the control group, the expression of HIF-1 α , NLRP3, caspase-1 and caspase-3 were all increased following OGD/R at 24 h, and C + P reversed these increases ($p < 0.05$, $p < 0.01$).

4. Discussion

In this investigation, we have explored the multifaceted neuroprotective effects of C + P in acute ischemic stroke. Our findings provide evidence that the mechanisms underlying C + P-induced neuroprotection involve distinct signaling pathways under hypothermic and normothermic conditions (Fig. 14).

In hypothermic conditions, C + P exerts its effects largely through the attenuation of the RIPK1/RIPK3-DRP1 signaling axis. This pathway is instrumental in the induction of mitochondrial dysfunction, the

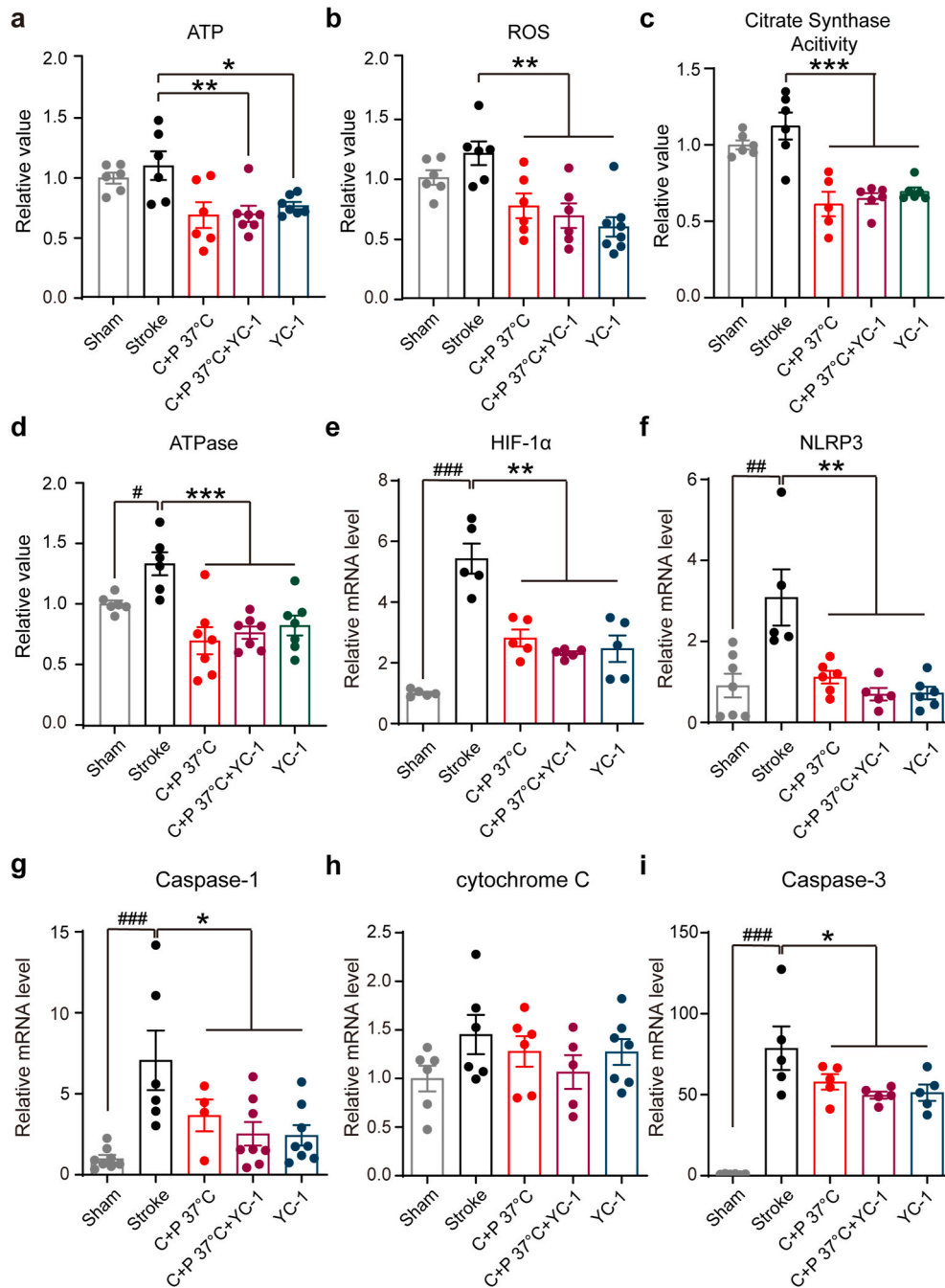


Fig. 10. Normothermic Effect by C + P inhibited mitochondrial dysfunction and the mRNA expression of NLRP3 inflammasome and cytochrome C-apoptosis pathway modulated by HIF-1 α . (a-d)The activity of ATPase and citrate synthase, as well as the ROS and ATP concentration were measured by ELISA. (e-i)Additional, HIF-1 α , NLRP3, Caspase-1, cytochrome C and Caspase-3 mRNA levels examined by quantitative real-time PCR after C + P at 37 °C with or without HIF-1 α inhibitor (YC-1) in MCAO rats at 24 h after reperfusion. Levels of mitochondrial function markers, HIF-1 α , NLRP3, Caspase-1 and Caspase-3 were both significantly reduced in C + P at normothermic condition or YC-1 groups. Notably, YC-1 plus C + P at 37 °C did not provide further neuroprotection as compared to C + P at 37 °C. Data are presented as mean $\pm$ SEM.

activation of the NLRP3 inflammasome, and the promotion of the apoptotic cascade, both of which contribute to the exacerbation of ischemic brain damage. By inhibiting components of this pathway, C + P reduces mitochondrial dysfunction, the expression of inflammatory mediators, and mitigates cellular apoptosis, thereby preserving neural tissue integrity and function.

Under normothermic conditions, HIF-1 α mediates the neuroprotective effects of C + P. HIF-1 α is a transcription factor that responds to hypoxic conditions and is implicated in the modulation of inflammatory processes within the ischemic issue. The normothermic

neuroprotection offered by C + P involves the downregulation of HIF-1 α , leading to a concomitant decrease in mitochondrial function markers, NLRP3 inflammasome activity, and a reduction in apoptotic cell death. This is also corroborated by *in vitro* evidence, in which the expression of HIF-1 α , NLRP3, caspase-1 and caspase-3 were all increased following OGD/R, and reversed by C + P. This data further supports the pivotal role of HIF-1 α in the normothermic neuroprotective effects of C + P.

In this study, we dissect the role of RIPK1 and RIPK3 as key regulators of cell death modalities, including apoptosis and necroptosis.

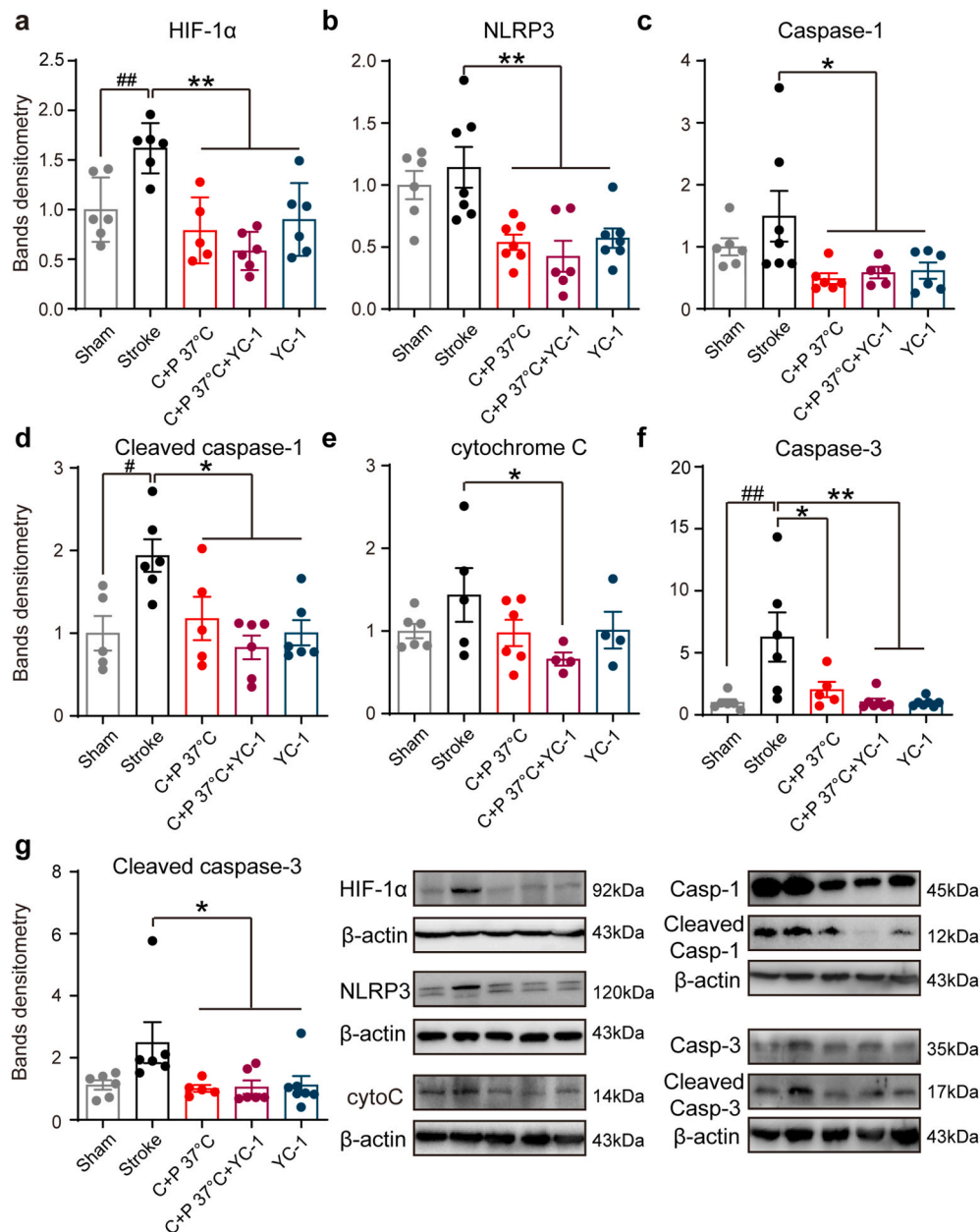


Fig. 11. Normothermic Effect of C + P on the protein expression of NLRP3 inflammasome and cytochrome C-apoptosis pathway with or without YC-1. (a) HIF-1 α , (b) NLRP3, (c) Caspase-1, (d) Cleaved caspase-1, (e) cytochrome C, (f) Caspase-3 and (g) Cleaved caspase-3 protein levels examined by Western blot analysis after C + P temperature control with or without YC-1 in MCAO rats at 24 h after reperfusion, in which β -actin was used for our loading controls. C + P at 37 $^{\circ}$ C, YC-1 plus C + P at 37 $^{\circ}$ C, as well as YC-1 group, significantly reduce the expression of NLRP3, caspase-1 and caspase-3 after stroke. Data are presented as mean $\pm$ SEM.

Contemporary research has expanded our understanding of the RIPK1/RIPK3 complex, highlighting its role in driving inflammatory responses that are distinct from the necroptotic pathway [23,35]. This complex activates DRP1 through phosphorylation at Ser616 in a RIPK3-dependent manner, a process that precipitates mitochondrial damage and ROS production [36]. The ROS act as stimulants for the activation of the NLRP3 inflammasome, a pivotal event in the inflammatory cascade following ischemic injury [37,38]. Our current findings support with emerging literature, which suggests the involvement of the RIPK1/RIPK3 complex in mitochondrial fission through DRP1, leading to activation of the NLRP3 inflammasome. Experiments demonstrate that RIPK3 deletion diminishes NLRP3 inflammasome activation, thereby attenuating inflammation within the hippocampus in models of traumatic brain injury (TBI) [39]. Similarly, vesicular stomatitis virus (VSV) infections that assemble the RIPK1-RIPK3 complex can trigger

DRP1 activation and its translocation to the mitochondria, leading to mitochondrial distress and subsequent activation of the NLRP3 inflammasome [40].

In the pathological context of ischemic stroke, the activation of the NLRP3 inflammasome is a key factor in the development of brain edema, neuronal injury, and subsequent neural dysfunction [41]. The inflammasome facilitates the maturation of inflammatory cytokines such as IL-1 β through the activation of pro-caspase-1. Our findings support the hypothesis that suppressing the NLRP3 inflammasome can mitigate brain damage by modulating the inflammatory response [25]. In addition, we have demonstrated that C + P therapy diminishes ROS levels post-ischemia, contributing to a reduction in oxidative stress and cellular damage [29].

Our current study presents evidence that C + P treatment leads to a decrease in DRP-1 phosphorylation, ATP, ROS concentration, citrate

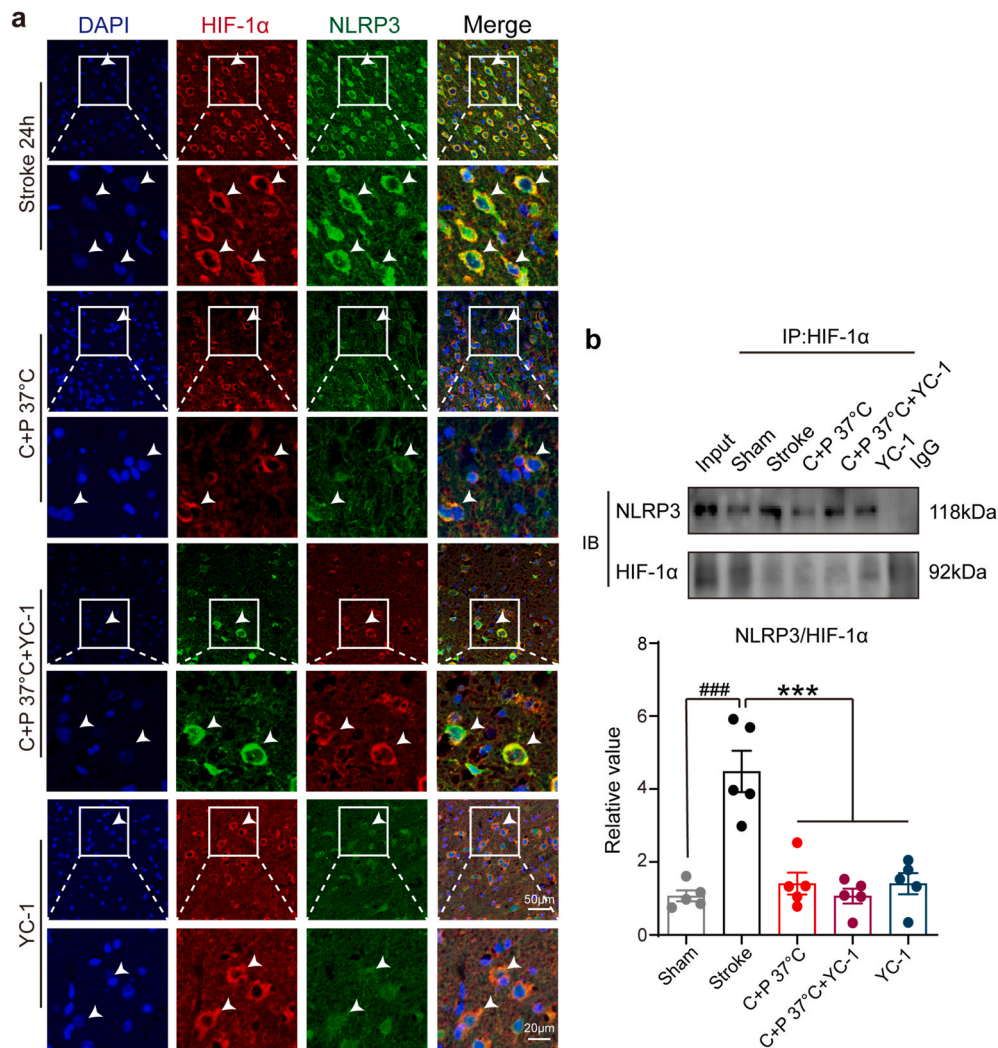


Fig. 12. Interaction of HIF-1α and NLRP3. Interaction of HIF-1α and NLRP3 were measured by immunofluorescence (a) and co-immunoprecipitation (b). NLRP3-positive cells (green) co-localized with HIF-1α-positive cells (red) at 24 h after reperfusion. The number of brain HIF-1α⁺NLRP3⁺ cells and the interaction of HIF-1α and NLRP3 were reduced by C + P at 37 °C with or without YC-1. Data are presented as mean ± SEM.

synthase, ATPase activity as well as markers of inflammation and apoptosis, including RIPK1, RIPK3, NLRP3, and caspase activation. These observations are consistent with the effects noted in the RIPK1 inhibitor group, suggesting that the neuroprotective mechanism of C + P operates largely through the RIPK1/RIPK3-DRP1 pathway. RIPK1/RIPK3/DRP-1-induced mitochondrial dysfunction is reported as a precursor to cell damage and death [42]. The critical role of mitochondria in apoptotic cell death has been well documented, with mitochondrial dynamics and imbalances triggering the intrinsic apoptosis pathway [43,44]. DRP-1 is the primary mediator of mitochondrial fission, a dynamic process that is intimately involved in the response to oxygen/glucose deprivation and subsequent reoxygenation [45]. This study observed the release of cytochrome c from fragmented mitochondria during cellular stress, emphasizing its role in the apoptotic cascade [46]. Cytochrome c serves not only as a trigger for ROS generation but is also as a catalyst for apoptosome formation, thereby initiating the activation of caspases and the cell death pathway [47]. Inhibition of mitochondrial cytochrome c release has been previously noted to confer protection against apoptosis in the context of cardiac injury [48]. That is in concordance with our results that C + P therapy, alongside Nec1s, significantly reduces levels of mitochondrial function markers as well as both the mRNA and protein expressions of cytochrome c, Apaf-1, Caspase-9, and Caspase-3. Collectively, these findings underscore the

capacity of C + P therapy to inhibit both inflammatory responses and apoptotic cell death by mitochondrial dysfunction, primarily via the RIPK1/RIPK3-DRP1 pathway.

Within the realm of phenothiazine derivatives, C + P have established their utility as neuroleptics, with chlorpromazine being recognized for its low potency antipsychotic properties and significant anticholinergic and antiadrenergic actions [49]. Similarly, promethazine is known to block the mitochondrial permeability transition pore, a mechanism that may underlie its protective effects against ischemic neuronal injury [50]. In the present study, we extend these findings by demonstrating that the neuroprotective efficacy of C + P transcends hypothermia. Nec1s, a RIPK1 inhibitor, has been shown to expand the neuroprotective impact of C + P at normothermic temperatures, particularly against inflammatory and apoptotic outcomes post-stroke. However, its addition does not further reduce brain injury as compared to C + P alone at hypothermic temperatures. This observation hints at the existence of additional neuroprotective targets of C + P that operate in normothermic conditions.

The dual assault of hypoxia and inflammation is a hallmark of the pathophysiological progression of stroke, within which the transcription factor HIF-1α emerges as a pivotal player. The role of HIF-1α in adapting cellular responses to hypoxic conditions is well-documented, and recent evidence positions it as a crucial regulator of inflammatory and cell

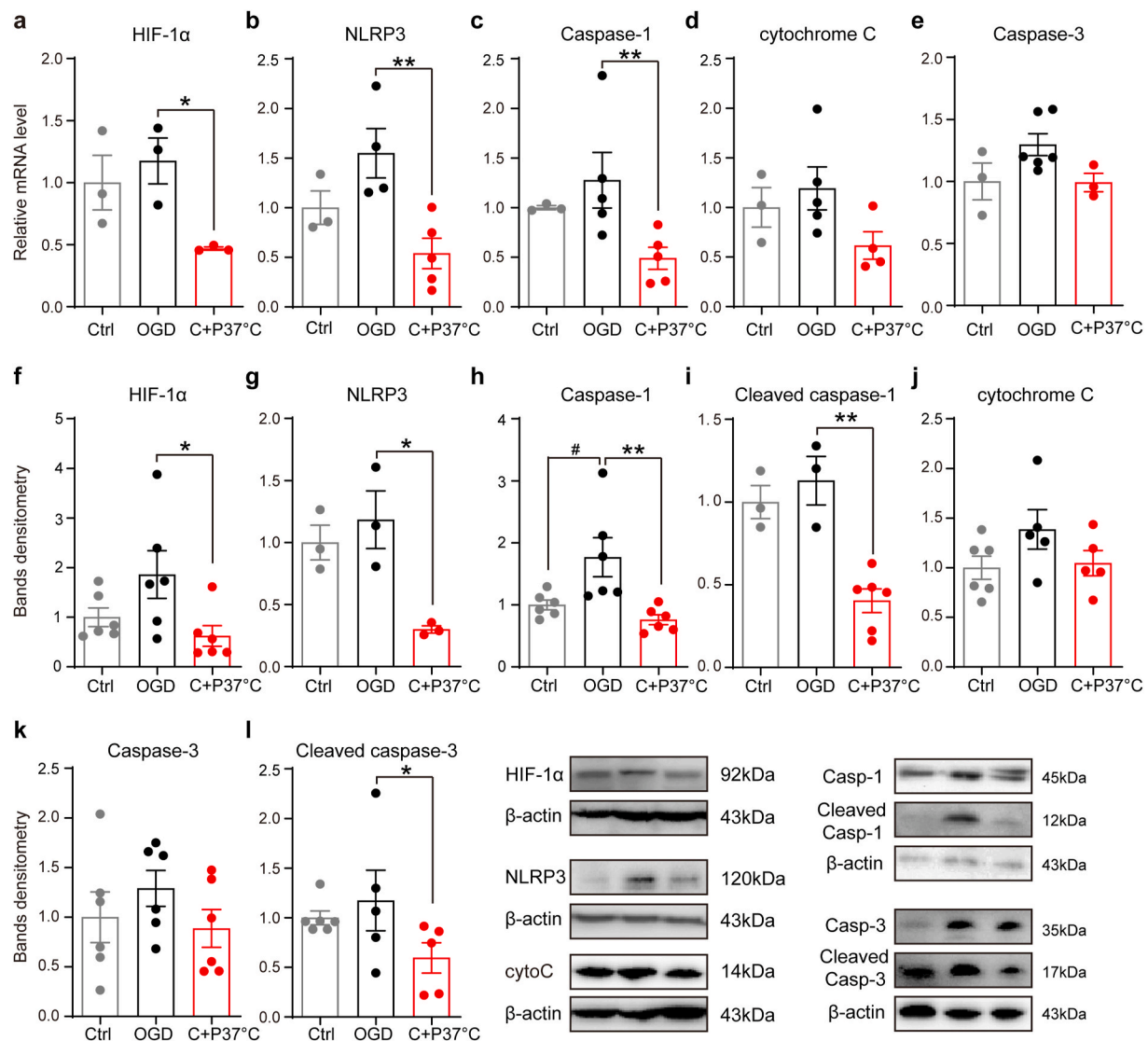


Fig. 13. Normothermic Effect of C + P on inflammatory response and apoptosis induced by HIF-1α in vitro. Establishment of OGD model by culturing SHSY5Y cells, treated with C + P at 37 °C, and detection of HIF-1α, NLRP3, Caspase-1, cytochrome C and Caspase-3 mRNA (a–e) and protein (f–l) expression using quantitative real-time PCR and Western blot. The expression of HIF-1α, inflammation and apoptosis proteins were significantly decreased in C + P at 37 °C group. Data are presented as mean ± SEM.

death processes in various diseases [20]. Inhibition of HIF-1α can protect mitochondria from damage by reducing excessive production of ROS [51]. This is further highlighted by the fact that HIF-1α inhibition can mitigate inflammatory conditions such as synovitis and fibrosis in osteoarthritis models [52,53]. Consistently, our study has observed that C + P administration at 37 °C diminishes HIF-1α expression and mitochondria dysfunction, which corresponds to inflammatory responses and cell death, as verified in SHSY5Y cellular models. This suggests that HIF-1α may represent a strategic target for the neuroprotective effects of C + P, independent of thermal regulation.

In conclusion, the dual modality of C + P therapy emerges as a compelling neuroprotective strategy in the outcome of ischemic stroke. The evidence presented in this study, along with prior research, robustly establishes the efficacy of C + P in mitigating neuronal damage through mechanisms distinctly influenced by the thermal state. At the core of the hypothermic neuroprotection, C + P targets the RIPK1/RIPK3-DRP1 pathway, attenuating mitochondrial dysfunction and the subsequent inflammatory cascade that exacerbates cerebral injury. Conversely, under normothermic conditions, C + P exerts its protective effects by modulating the HIF-1α pathway, offering a shield against the

inflammation and apoptosis perpetuated by hypoxic challenges inherent to stroke pathology. In addition, our findings highlight the nuanced role of temperature modulation in stroke therapy and suggest that the therapeutic application of C + P can be optimized by considering the thermal context. With hypothermia-mediated pathways offering one avenue of protection and normothermia engaging alternative mechanisms, the versatility of C + P is evident, providing a broader therapeutic window and adaptability to patient-specific conditions.

Future studies should aim to explore the possibility of combination therapies that could simultaneously or sequentially modulate these pathways to enhance neuroprotection. The potential modulatory effects of C + P on other cell types within the neurovascular unit, such as astrocytes and endothelial cells may play significant roles in the stroke outcome by C + P. The promising outcomes of this study provide a base for such future explorations, with the ultimate aim of developing precisely targeted, patient-tailored stroke therapies.

Consent for publication

Not applicable.

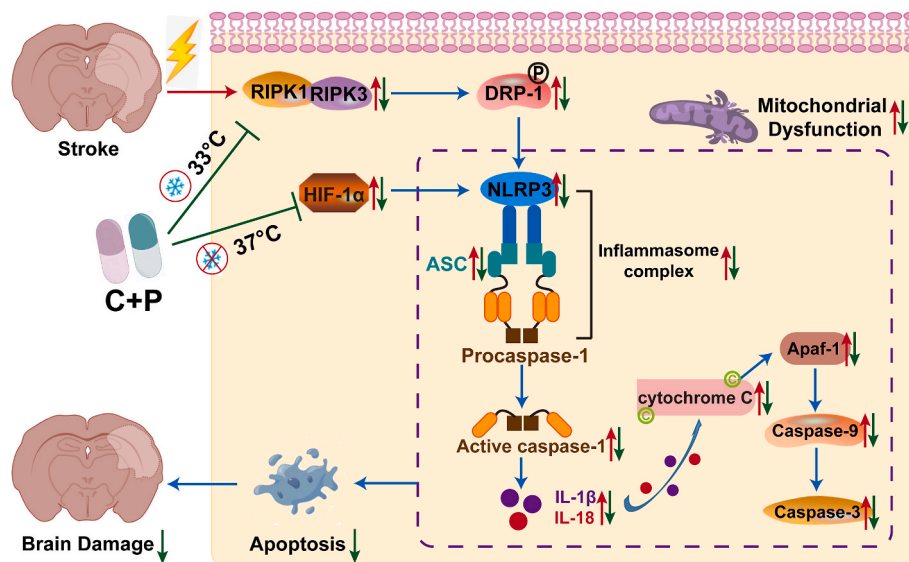


Fig. 14. Neuroprotective Pathways of Chlorpromazine and Promethazine (C + P) in Ischemic Stroke. This schematic diagram delineates the molecular interactions following a stroke, focusing on how C + P modulates neuroprotection through two distinct temperature-dependent mechanisms. Under hypothermic conditions (33 °C), C + P coordinates with a RIPK1 inhibition to reduce mitochondrial dysfunction and inflammatory responses, reducing apoptotic activity, as evidenced by decreased cytochrome c release and caspase activation. This is mediated by the RIPK1/RIPK3-DRP1 pathway. Alternatively, at normothermic levels (37 °C), C + P attenuates HIF-1α expression and its downstream effects, leading to decreased NLRP3 inflammasome assembly and mitochondrial dysfunction, ultimately attenuating cell death pathways. This suggests two separate neuroprotective routes: one hypothermic involving RIPK1/RIPK3-DRP1 signaling, and the other normothermic via HIF-1α modulation. These insights elaborate on the potential of C + P in preserving mitochondrial integrity and preventing apoptosis, offering a theoretical framework for targeted neuroprotective strategies in acute ischemic stroke.

CRediT authorship contribution statement

Qian Jiang: Writing – original draft, Funding acquisition, Data curation. **Yuchuan Ding:** Writing – review & editing, Project administration. **Fengwu Li:** Methodology, Data curation. **Aminah I. Fayyaz:** Writing – review & editing. **Honglian Duan:** Visualization, Methodology. **Xiaokun Geng:** Writing – review & editing, Project administration, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

- [1] S.K. Feske, Ischemic stroke, *Am. J. Med.* 134 (12) (2021) 1457–1464.
- [2] F. Eren, S.E. Yilmaz, Neuroprotective approach in acute ischemic stroke: a systematic review of clinical and experimental studies, *Brain Circ* 8 (4) (2022) 172–179.
- [3] S.E. Lakhan, et al., Corrigendum: matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke, *Front. Neurol.* 9 (2018) 202.
- [4] N. Uzuner, G.T. Uzuner, Risk factors for multiple recurrent ischemic strokes, *Brain Circ* 9 (1) (2023) 21–24.
- [5] Y. Xu, Y. Wang, X. Ji, Immune and inflammatory mechanism of remote ischemic conditioning: a narrative review, *Brain Circ* 9 (2) (2023) 77–87.
- [6] D. He, et al., The inflammatory puzzle: piecing together the links between neuroinflammation and amyotrophic lateral sclerosis, *Aging Dis* 15 (1) (2024) 96–114.
- [7] M.S. Sun, et al., Free radical damage in ischemia-reperfusion injury: an obstacle in acute ischemic stroke after revascularization therapy, *Oxid. Med. Cell. Longev.* 2018 (2018) 3804979.
- [8] J. Zheng, et al., HIF-1α in myocardial ischemia-reperfusion injury, *Mol. Med. Rep.* 23 (5) (2021) 352.
- [9] Z.J. Fu, et al., HIF-1α-BNIP3-mediated mitophagy in tubular cells protects against renal ischemia/reperfusion injury, *Redox Biol.* 36 (2020) 101671.
- [10] Y. Gao, et al., A narrative review of retinal vascular parameters and the applications (Part II): diagnosis in stroke, *Brain Circ* 9 (3) (2023) 129–134.
- [11] L. Guan, et al., Artificial hibernation by phenothiazines: a potential neuroprotective therapy against cerebral inflammation in stroke, *Curr. Neurovasc. Res.* 16 (3) (2019) 232–240.
- [12] F. Li, et al., Perspectives on benefit of early and pre reperfusion hypothermia by pharmacological approach in stroke, *Brain Circ* 8 (2) (2022) 69–75.
- [13] S. Guo, et al., Phenothiazine inhibits neuroinflammation and inflammasome activation independent of hypothermia after ischemic stroke, *Mol. Neurobiol.* 58 (12) (2021) 6136–6152.
- [14] Y. Liu, et al., RIP1/RIP3-regulated necroptosis as a target for multifaceted disease therapy, *Int. J. Mol. Med.* 44 (3) (2019) 771–786.
- [15] H. Hu, et al., RIP3-mediated necroptosis is regulated by inter-filament assembly of RIP homotypic interaction motif, *Cell Death Differ.* 28 (1) (2021) 251–266.
- [16] M. Speir, K.E. Lawlor, RIP-roaring inflammation: RIPK1 and RIPK3 driven NLRP3 inflammasome activation and autoinflammatory disease, *Semin. Cell Dev. Biol.* 109 (2021) 114–124.
- [17] B.H. Duong, et al., A20 restricts ubiquitination of pro-interleukin-1β protein complexes and suppresses NLRP3 inflammasome activity, *Immunity* 42 (1) (2015) 55–67.
- [18] Y.C. Ye, et al., RIP1-mediated mitochondrial dysfunction and ROS production contributed to tumor necrosis factor alpha-induced L929 cell necroptosis and autophagy, *Int. Immunopharm.* 14 (4) (2012) 674–682.
- [19] E. Homsí, et al., TNF-α-mediated cardiorenal injury after rhabdomyolysis in rats, *Am. J. Physiol. Ren. Physiol.* 308 (11) (2015) F1259–F1267.
- [20] D. Yuan, et al., HIF-1α aggravated traumatic brain injury by NLRP3 inflammasome-mediated pyroptosis and activation of microglia, *J. Chem. Neuroanat.* 116 (2021) 101994.
- [21] S. Shen, et al., Positive feedback regulation of circular RNA Hsa_circ_0000566 and HIF-1α promotes osteosarcoma progression and glycolysis metabolism, *Aging Dis* 14 (2) (2023) 529–547.
- [22] C. Fan, et al., Modulation of hypoxia-inducible factor-1α/cyclo-oxygenase-2 pathway associated with attenuation of intestinal mucosa inflammatory damage by Acanthopanax senticosus polysaccharides in lipopolysaccharide-challenged piglets, *Br. J. Nutr.* 122 (6) (2019) 666–675.
- [23] H.S. Li, et al., HIF-1α protects against oxidative stress by directly targeting mitochondria, *Redox Biol.* 25 (2019) 101109.

- [24] P. Zhang, et al., CIRP attenuates acute kidney injury after hypothermic cardiovascular surgery by inhibiting PHD3/HIF-1 α -mediated ROS-TGF- β 1/p38 MAPK activation and mitochondrial apoptotic pathways, *Mol. Med.* 29 (1) (2023) 61.
- [25] Q. Jiang, et al., Hypoxia inducible factor-1 α (HIF-1 α) mediates NLRP3 inflammasome-dependent-pyrototic and apoptotic cell death following ischemic stroke, *Neuroscience* 448 (2020) 126–139.
- [26] X. Geng, et al., Neuroprotection by chlorpromazine and promethazine in severe transient and permanent ischemic stroke, *Mol. Neurobiol.* 54 (10) (2017) 8140–8150.
- [27] N. Takahashi, et al., Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models, *Cell Death Dis.* 3 (2012) e437.
- [28] W.L. Yeh, et al., Inhibition of hypoxia-induced increase of blood-brain barrier permeability by YC-1 through the antagonism of HIF-1 α accumulation and VEGF expression, *Mol. Pharmacol.* 72 (2) (2007) 440–449.
- [29] S. Guo, et al., Chlorpromazine and promethazine (C+P) reduce brain injury after ischemic stroke through the PKC- δ /NOX/MnSOD pathway, *Mediat. Inflamm.* 2022 (2022) 6886752.
- [30] S. Lv, et al., Phenothiazines reduced autophagy in ischemic stroke through endoplasmic reticulum (ER) stress-associated PERK-eIF2 α pathway, *Exp. Neurol.* 369 (2023) 114524.
- [31] E.Z. Longa, et al., Reversible middle cerebral artery occlusion without craniectomy in rats, *Stroke* 20 (1) (1989) 84–91.
- [32] L. Belayev, et al., Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model, *Stroke* 27 (9) (1996) 1616–1622 ; discussion 1623.
- [33] X. Geng, et al., Phosphoenolpyruvate carboxykinase (PCK) in the brain gluconeogenic pathway contributes to oxidative and lactic injury after stroke, *Mol. Neurobiol.* 58 (5) (2021) 2309–2321.
- [34] L. Guan, et al., Neuroprotective effects of pharmacological hypothermia on hyperglycolysis and gluconeogenesis in rats after ischemic stroke, *Biomolecules* 12 (6) (2022).
- [35] K.E. Lawlor, et al., RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL, *Nat. Commun.* 6 (2015) 6282.
- [36] S. Liu, et al., RIPK3 signaling and its role in the pathogenesis of cancers, *Cell. Mol. Life Sci.* 78 (23) (2021) 7199–7217.
- [37] Z. Wang, et al., The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways, *Cell* 148 (1–2) (2012) 228–243.
- [38] Y. Ding, et al., Perspectives on effect of spleen in ischemic stroke, *Brain Circ* 8 (3) (2022) 117–120.
- [39] Z.M. Liu, et al., RIP3 deficiency protects against traumatic brain injury (TBI) through suppressing oxidative stress, inflammation and apoptosis: dependent on AMPK pathway, *Biochem. Biophys. Res. Commun.* 499 (2) (2018) 112–119.
- [40] X. Wang, et al., RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway, *Nat. Immunol.* 15 (12) (2014) 1126–1133.
- [41] Y.S. Feng, et al., Inhibition of NLRP3 inflammasome: a prospective target for the treatment of ischemic stroke, *Front. Cell. Neurosci.* 14 (2020) 155.
- [42] K. Sinha, et al., Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis, *Arch. Toxicol.* 87 (7) (2013) 1157–1180.
- [43] P.A. Li, X. Hou, S. Hao, Mitochondrial biogenesis in neurodegeneration, *J. Neurosci. Res.* 95 (10) (2017) 2025–2029.
- [44] Y. Wang, et al., Skeletal muscle mitochondrial dysfunction in chronic obstructive pulmonary disease: underlying mechanisms and physical therapy perspectives, *Aging Dis* 14 (1) (2023) 33–45.
- [45] E. Smirnova, et al., Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells, *Mol. Biol. Cell* 12 (8) (2001) 2245–2256.
- [46] A.R. Anzell, et al., Mitochondrial quality control and disease: insights into ischemia-reperfusion injury, *Mol. Neurobiol.* 55 (3) (2018) 2547–2564.
- [47] H.A. Kalpage, et al., Tissue-specific regulation of cytochrome c by post-translational modifications: respiration, the mitochondrial membrane potential, ROS, and apoptosis, *Faseb. J.* 33 (2) (2019) 1540–1553.
- [48] Y. Che, et al., IL-22 ameliorated cardiomyocyte apoptosis in cardiac ischemia/reperfusion injury by blocking mitochondrial membrane potential decrease, inhibiting ROS and cytochrome C, *Biochim. Biophys. Acta, Mol. Basis Dis.* 1867 (9) (2021) 166171.
- [49] C.J. Cote, et al., Adverse sedation events in pediatrics: analysis of medications used for sedation, *Pediatrics* 106 (4) (2000) 633–644.
- [50] O. Adolph, et al., Promethazine inhibits NMDA-induced currents - new pharmacological aspects of an old drug, *Neuropharmacology* 63 (2) (2012) 280–291.
- [51] H. Teng, et al., Design and synthesis of 3,4-seco-lupane triterpene derivatives to resist myocardial ischemia-reperfusion injury by inhibiting oxidative stress-mediated mitochondrial dysfunction via the PI3K/AKT/HIF-1 α axis, *Biomed. Pharmacother.* 167 (2023) 115452.
- [52] J. Cosin-Roger, et al., Hypoxia ameliorates intestinal inflammation through NLRP3/mTOR downregulation and autophagy activation, *Nat. Commun.* 8 (1) (2017) 98.
- [53] L. Zhang, et al., Agnuside alleviates synovitis and fibrosis in knee osteoarthritis through the inhibition of HIF-1 α and NLRP3 inflammasome, *Mediat. Inflamm.* 2021 (2021) 5534614.