

Activated protein C overexpression suppresses the pyroptosis of subarachnoid hemorrhage model cells by regulating the NLRP3 inflammasome pathway

AI YAN, XUYAN PAN, XIANQIANG WEN, XIAOHU NIE and YUNTAO LI

Department of Neurosurgery, Huzhou Central Hospital, Affiliated Hospital of
Huzhou Normal University, Huzhou, Zhejiang 313000, P.R. China

Received July 20, 2021; Accepted September 13, 2021

DOI: 10.3892/etm.2021.10827

Abstract. Subarachnoid hemorrhage (SAH) is a condition with a high associated mortality rate that is caused by hemorrhagic stroke. Activated protein C (APC) serves a neuroprotective role in central nervous system diseases. However, its role in SAH remains unclear. The present study aimed to investigate the role of APC and its regulatory mechanism in SAH. The SAH rat model was constructed through internal carotid artery puncture, while the SAH cell model was established via the application of oxygenated hemoglobin. ELISA was performed to detect the level of cytokines, and flow cytometry was used to determine the population of pyroptotic cells. Reverse transcription-quantitative PCR and western blotting were used to examine the relative mRNA and protein levels of APC. APC was silenced using specific APC short hairpin RNA. Neurological functions of rats were estimated using modified Garcia scoring and the balance beam test, while SAH was estimated using modified Sugawara's scoring. The results demonstrated that the expression of APC was significantly decreased, whereas the expression of NLR family pyrin domain-containing 3 (NLRP3) was increased in the SAH rat model in a time-dependent manner. The application of APC recombinant protein 3K3A-APC could significantly ameliorate SAH and improve neurological functions. In addition, 3K3A-APC could inhibit pyroptosis in a dose-dependent manner in the SAH cell model. Moreover, the NLRP3 inhibitor BAY11-7082 could reverse the upregulation of pyroptosis induced by APC-knockdown. Overall, the present study revealed that APC could ameliorate SAH-induced early brain

injury by suppressing pyroptosis via inhibition of the NLRP3 inflammasome, which could provide a novel strategy for the treatment of SAH.

Introduction

Subarachnoid hemorrhage (SAH) is a type of hemorrhagic stroke defined as hemorrhage in the subarachnoid space, and it is often caused by brain injury or aneurysm rupture (1). SAH is classified into five grades, with grade V being associated with the worst clinical prognosis (2). The average mortality of patients with SAH is 40-50% in a population-based study in the USA (3). Nevertheless, a previous study has suggested that ~46% of patients who survive from SAH often suffer from cognitive impairment (4). Early brain injury (EBI) is termed the brain injury that occurs within 72 h of SAH (5). The abnormal elevation of the intracranial pressure, the reduced cerebral blood perfusion and the development of brain edema are considered to be associated with SAH-induced EBI (6). However, the molecular mechanism underlying SAH-induced EBI remains unknown.

Activated protein C (APC) is an activated form of zymogen that serves an important role in regulating multiple biological processes (7). APC has been first recognized for its anticoagulant activity through the inactivation of coagulation factors and subsequent prevention of thrombosis (8). In addition, APC has also been indicated to exert anti-inflammatory activity (9). In the central nervous system, APC serves a neuroprotective role in various brain diseases, such as ischemic stroke and traumatic brain injury (10,11). APC can be transported through the blood-brain barrier (BBB) via contact with its receptors and the stabilized endothelial cells of the BBB (12). APC is antithrombotic and anti-inflammatory, and has been identified as a neuroprotective factor that is able to exert antiapoptotic effects (13). A previous report has indicated that APC causes biased cytoprotective signaling that reduces ischemia-induced injury by cleaving protease-activated receptor 1 (14). Moreover, APC promotes neurogenesis, and recombinant 3K3A-APC is considered to be a promising agent for ischemic stroke therapy (11). 3K3A-APC is an artificial protein made through the replacement of three lysine residues by three alanine residues, which reduces >90% of the anticoagulant activity

Correspondence to: Professor Ai Yan, Department of Neurosurgery, Huzhou Central Hospital, Affiliated Hospital of Huzhou Normal University, 1558 Sanhuan Bei Road, Huzhou, Zhejiang 313000, P.R. China
E-mail: yanai71@126.com

Key words: subarachnoid hemorrhage, activated protein C, NLR family pyrin domain-containing 3, pyroptosis, 3K3A-activated protein C

but retains the original biological activity of the molecule (15). Furthermore, APC inhibits the activation of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome, which provides a novel framework for the anti-inflammatory activities of APC (16). However, the role of APC and its derivative in SAH-induced EBI requires further elucidation.

Excessive inflammation is hypothesized to be a major cause of SAH-induced EBI (17). The NLRP3 inflammasome is composed of a NOD-like receptor (NLRP3), apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain and an adaptor protein (18). It is activated in response to pathogens and induces the secretion of IL-1 β and IL-18, which can lead to an inflammatory cascade and initiate the programmed cell death pathway known as pyroptosis (19,20). Previous studies have indicated that the activation of the NLRP3 inflammasome is associated with inflammatory injury and neurological disorders in SAH (21,22). Moreover, pyroptosis induced by the NLRP3 inflammasome has been revealed to be associated with neuroinflammatory injury in the case of SAH (23). However, to the best of our knowledge, the role of the NLRP3 inflammasome and pyroptosis in SAH-induced EBI is rarely reported.

A previous study has indicated that APC can prevent ischemia-reperfusion injury through the inhibition of the NLRP3 inflammasome (16). However, the association between APC and the NLRP3 inflammasome in SAH-induced EBI remains unclear. The present study established SAH cell and rat models to explore the biological functions of APC and the NLRP3 inflammasome in SAH-induced EBI, aiming to provide novel strategies for the management of SAH.

Materials and methods

Cell culture and construction of the SAH model. Rat PC12 cells (The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were used in the present study and cultured in RPMI-1640 medium (cat. no. 11875093; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated horse serum (cat. no. 04-124-1A; Shanghai Yaoyun Biological Technology Co., Ltd.) and 5% fetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.). Cells were treated with 10 mM oxygenated hemoglobin (OxyHb) at 37°C to construct the SAH cell model for 24 h, while the control cells were treated with corresponding PBS solution. BAY11-7082 (5 μ mol/l; cat. no. S2913; Selleck Chemicals), an inhibitor of NLRP3 (24), was dissolved in PBS and added to the culture medium at 37°C for 48 h, while PBS was used to culture cells as control. Moreover, 3K3A-APC (FILZB1-03; ZZ Biotech LLC) was used to treat cells at the concentrations of 5, 10 and 20 ng/ml, respectively.

Reverse transcription-quantitative PCR. Total RNA from rat PC12 cells that were treated with 3K3A-APC (FILZB1-03; ZZ Biotech LLC) at the concentrations of 5, 10 and 20 ng/ml were extracted using TRIzol[®] reagent (cat. no. 1596-026; Invitrogen; Thermo Fisher Scientific, Inc.) after co-culturing for 24 h. The cDNA synthesis kit (Thermo Fisher Scientific, Inc.) was used for cDNA synthesis of RNA according to the manufacturer's protocols. The SYBR[®] Green fluorochrome (cat. no. K0223; Thermo Fisher Scientific, Inc.) and the following thermocyc-

ling conditions were used for qPCR: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 45 sec and extension at 72°C for 30 sec, with a final extension at 72°C for 3 min. β -actin was used as the reference gene. Gene expression was calculated using the 2^{- $\Delta\Delta$ C_q} method (25). All primers were listed as follows: APC forward, 5'-CACCCCTTTCAGTTCAAGTAGC-3' and reverse, 5'-AAGACCCAGAATGGCGTTTAG-3'; NLRP3 forward, 5'-ACCTCAACAGACGCTACACCC-3' and reverse, 5'-GCTGCTCCCTGGAACACC-3'; β -actin forward, 5'-CGGTCAGGTCATCACTATC-3' and reverse, 5'-CAGGGCAGTAATCTCCTTC-3'. Each reaction was repeated three times.

Western blotting. The total protein content of cells or brain tissues was obtained using RIPA lysis buffer with protease inhibitor cocktail (Beyotime Institute of Biotechnology). The protein levels were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The protein (20 μ g/lane) was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked using 5% skimmed milk at room temperature for 2 h and incubated overnight at 4°C with the primary antibodies as follows: APC (1:2,000; cat. no. ab40778), NLRP3 (1:1,000; cat. no. ab263899), caspase-1 (1:100; cat. no. ab74279) (all from Abcam), gasdermin D (GSDMD-N; 1:1,000; cat. no. 39754) and β -actin (1:1,000; cat. no. 3700) (both from Cell Signaling Technology, Inc.).

After washing with 0.1 M PBS, the membranes were incubated with the secondary antibodies HRP-labeled goat anti-rabbit IgG (1:1,000, cat. no. A16104; Thermo Fisher Scientific, Inc.) and HRP-labeled goat anti-mouse antibody IgG (1:1,000, cat. no. A0216; Thermo Fisher Scientific, Inc.) at 4°C for 2 h. Enhanced chemiluminescence detection kit (cat. no. WBKLS0100; MilliporeSigma) was used for signal detection. Each reaction was replicated three times.

Knockdown of APC. A total of three short hairpin (sh)RNAs were designed for targeting rat APC (shAPC-1, 5'-GCAUGAACUGCCUCCCAUTT-3'; shAPC-2, 5'-GCAGGAAGCCCAUGAACAAATT-3'; shAPC-3, 5'-GCCACUGACAUAUCUUAUTT-3'). A scrambled siRNA negative control (shNC; 5'-UUCUCCGAACGUGUCACGUTT-3') was used as the control. All the APC shRNAs were inserted into the pLKO.1 vector (Elabo Biotechnology, Co., Ltd.). Rat PC12 cells (5x10⁵ cells/well) were transfected with 100 nM APC shRNAs or the corresponding negative empty using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and cells were cultured for 48 h before analysis.

ELISA. The expression levels of IL-1 β and IL-18 in cells that had been treated with 3K3A-APC or BAY11-7082, as indicated above, were examined using Rat IL-18 ELISA kit (cat. no. E-EL-R0567c) and Rat IL-1 β ELISA kit (cat. no. E-EL-R0012c) (both from Elabscience, Inc.) according to the manufacturer's protocol. Briefly, IL-1 β and IL-18 antibodies were applied at 37°C for 2 h. After rinsing off the washing solution, the secondary antibody was applied. Subsequently, the stop solution was added and the optical density at 450 nm was measured. Each reaction was replicated three times.

Estimation of cell pyroptosis. Activated caspase-1 was estimated to reflect the level of cell pyroptosis using the Caspase-1 p20 Antibody/FITC (cat. no. EL900443-100-FITC; Eterlife, Ltd.) according to the manufacturer's instructions. In brief, PC12 cells were seeded into 6-well plates (5×10^5 cells/well) and allowed to grow until reaching 50% confluence. 3K3A-APC or BAY11-7082-treated cells were incubated with a caspase-1 detection probe for 1 h in the dark at 37°C. After rinsing to remove the unconjugated FLICA reagent, cells were stained with propidium iodide (PI) for 20 min at 37°C. Accuri C6 flow cytometer (v1.0.264; BD Biosciences) using CellQuest Pro software v3.3 (BD Biosciences) was used to identify pyroptotic cells as being identified by double positive for cleaved caspase-1 and PI. Each reaction was replicated three times.

Construction of the SAH rat model. Male Sprague-Dawley rats (weight, 280–320 g; age, 12 weeks; $n=30$) were purchased from Elibo Biotechnology, Co., Ltd. The rats were raised in a 12/12-h dark/light cycle with free access to food and water at $25 \pm 2^\circ\text{C}$ with humidity ($60 \pm 5\%$). The SAH rat model was constructed through internal carotid artery puncture according to a method described previously (26). Rats were initially anesthetized using isoflurane (5%) in an induction chamber and then maintained on isoflurane (2–2.5%) anesthesia with the aid of a nose cone. After anesthesia, the external carotid artery was ligated and sectioned. A nylon thread was inserted through the external carotid artery into the intracranial part of the internal carotid artery. When the branch met resistance, the nylon thread was advanced to puncture the internal carotid artery, which resulted in SAH in rats. Subsequently, 2 mg/kg of 3K3A-APC and corresponding vehicle (PBS) were intraperitoneally injected every 12 h. In the sham-operated group, a similar procedure was performed without perforation. Animals were euthanized by decapitation under deep pentobarbital anesthesia at 6, 12, 24, 48 or 72 h after model constructing (intravenously; 60 mg/kg body weight). Brain tissues were collected for pathological analysis. There were six rats at each time point in different groups. All animal procedures involved in the current study were approved by the Independent Animal Ethics Committee of Huzhou Central Hospital, Affiliated Hospital of Huzhou Normal University (approval no. 16470; Huzhou, China) and was carried out in compliance with the ARRIVE guidelines (27).

Neurological function score. The scoring of neurological functions was performed using modified Garcia scoring and the balance beam test according to the method as indicated in a previous report (28). Modified Garcia scoring included spontaneous activity, the extension of the four limbs, climbing on a metal mesh wall and touch and whisker response of both sides of the trunk. The scoring system was as follows: 0 Points, no symptoms of nerve damage; 1 point, the contralateral forepaws could not be fully extended; 2 points, when walking, the rat turned to the left (paralyzed side), indicating moderate neurological deficits; 3 points, when walking, the body of the rat toppled to the left (paralyzed side), indicating in severe neurological deficits; 4 points, unable to walk spontaneously, loss of consciousness.

Beam walking test. The beam walking test method was performed according to the methods in Manaenko *et al* (29). The score of normal rats in the beam walking test was 4.

SAH score. The scoring of SAH was conducted using modified Sugawara's scoring (30). The rats were decapitated under anesthesia and images of the basal cistern and Willis' circle were captured. The basal cistern was divided into six parts, and the scores for each part were as follows: i) 0 was defined as no SAH; ii) 1 was defined as a small amount of SAH; iii) 2 was defined as medium SAH with several distinguishable blood vessels; and iv) 3 was defined as a large amount of SAH without distinct blood vessels. The final score was the sum of the scores of the six parts, among which 0–7, 8–12 and 13–18 points were defined as mild, moderate and severe hemorrhage, respectively.

Statistical analysis. GraphPad Prism version 7.0 (GraphPad Software, Inc.) was used for data analyses and visualization. Quantitative data are presented as the mean \pm standard deviation, and each experiment was repeated three times. Differences between groups were analyzed using one-way ANOVA followed by Tukey-Kramer multiple comparison test. Moreover, the ordinal data (all scores) were analyzed using Kruskal-Wallis test followed by Dunn's post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

APC and NLRP3 are altered in a time-dependent manner in the SAH rat model. To preliminarily explore the role of APC and NLRP3 in SAH, their expression levels were detected in the SAH rat model at different time points. The results demonstrated that the mRNA expression level of APC was significantly reduced after 48 h ($P < 0.05$), whereas that of NLRP3 was significantly elevated after 12 h compared with the sham group ($P < 0.001$), and these effects occurred in a time-dependent manner (Fig. 1A). Furthermore, the protein level of APC was also revealed to be markedly decreased, whereas NLRP3, caspase-1 and GSDMD-N were increased in the SAH rat model in a time-dependent manner (Fig. 1B). These results indicated that APC and NLRP3 were associated with the development of SAH and that they exhibited reversed expression patterns in SAH.

APC recombinant protein 3K3A-APC ameliorates SAH-induced injury in the rat model. To further explore the function of APC in SAH, a recombinant APC protein known as 3K3A-APC was applied in the SAH rat model. After 24 or 72 h of intervention, 3K3A-APC significantly reduced SAH in rats compared with the vehicle ($P < 0.001$; Fig. 2A and B). In addition, 3K3A-APC significantly improved the neurological function of SAH rats compared with the vehicle ($P < 0.001$), which were significantly impaired compared with the sham group at 24 h ($P < 0.001$; Fig. 2C). In addition, the balance beam score was significantly reduced in the SAH model after the application of 3K3A-APC compared with the vehicle ($P < 0.001$; Fig. 2D). Interestingly, the SAH hemorrhage, neurological function and balance beam score from 24 to 72 h in the model revealed no significant difference after treatment with 3K3A-APC.

Subsequently, brain tissues were collected from rats followed by ELISA, which revealed that the levels of IL-1 β and IL-18 were elevated in the vehicle SAH rats compared with the sham group; however, 3K3A-APC could significantly reduce

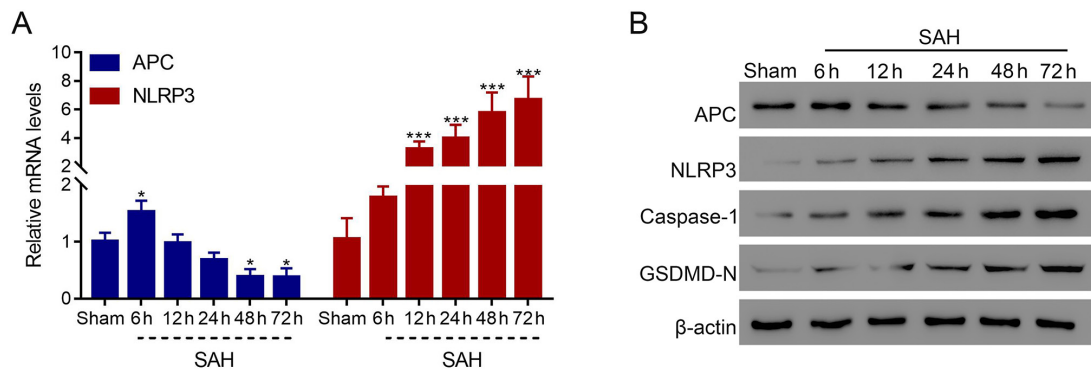


Figure 1. Expression levels of APC and NLRP3 are altered in a time-dependent manner in the SAH model. (A) mRNA level of APC and NLRP3 in the brain of the SAH rat model at 6, 12, 24, 48 and 72 h. (B) Protein levels of APC, NLRP3, caspase-1 and GSDMD-N in the brain of the SAH rat model at 6, 12, 24, 48 and 72 h. *P<0.05, ***P<0.001 vs. sham. APC, activated protein C; NLRP3, NLR family pyrin domain-containing 3; SAH, subarachnoid hemorrhage; GSDMD, gasdermin D.

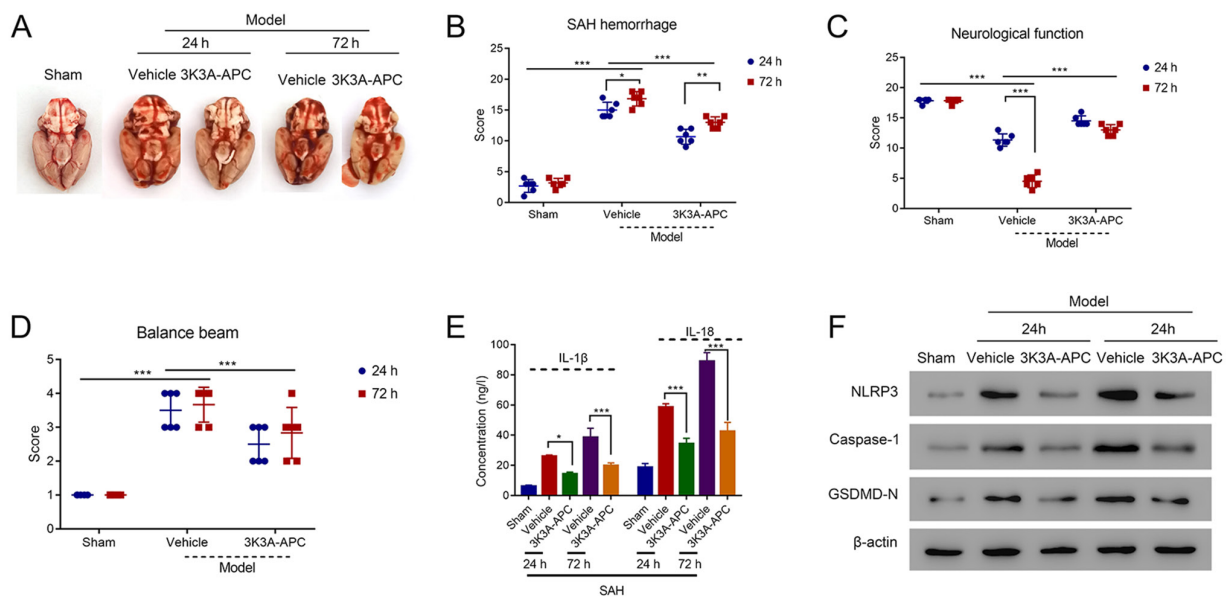


Figure 2. 3K3A-APC ameliorates SAH-induced injury in the rat model. (A) Morphology of brain tissues from the SAH rat model treated with 3K3A-APC for 24 and 72 h. (B) SAH, (C) neurological function and (D) balance beam test scores of the SAH rat model treated with 3K3A-APC for 24 and 72 h. (E) Expression levels of IL-1β and IL-18 in brain tissues from the SAH rat model treated with 3K3A-APC for 24 and 72 h. (F) Protein expression levels of NLRP3, caspase-1 and GSDMD-N in brain tissues from the SAH rat model treated with 3K3A-APC for 24 and 72 h. *P<0.05, **P<0.01, ***P<0.001. APC, activated protein C; NLRP3, NLR family pyrin domain-containing 3; SAH, subarachnoid hemorrhage; GSDMD, gasdermin D.

their concentrations (all $P<0.05$; Fig. 2E). Western blotting revealed that the expression levels of NLRP3, caspase-1 and GSDMD-N were reduced after the intervention of 3K3A-APC compared with that in vehicle group both at 24 and 48 h (Fig. 2F). Overall, these results suggested that recombinant APC protein 3K3A-APC could ameliorate the hemorrhage and improve neurological functions in SAH.

APC recombinant protein 3K3A-APC inhibits pyroptosis in a dose-dependent manner in the SAH cell model. The association between 3K3A-APC and pyroptosis in the SAH cell model was investigated. The construction of the SAH cell model was performed via application of 10 mM OxyHb, where the application of 5, 10 and 20 ng/ml 3K3A-APC significantly reduced the proportion of pyroptotic cells that were PI- and caspase-1-positive in a dose-dependent manner compared with cells treated with 0 ng/ml 3K3A-APC (all $P<0.05$; Fig. 3A).

In addition, the levels of IL-1β and IL-18 were significantly reduced after the application of 5, 10 and 20 ng/ml 3K3A-APC in a dose-dependent manner compared with cells treated with 0 ng/ml (all $P<0.01$; Fig. 3B). Moreover, the protein levels of NLRP3, caspase-1 and GSDMD-N were suppressed in a dose-dependent manner in cells treated with 3K3A-APC (Fig. 3C). These results indicated that 3K3A-APC could inhibit pyroptosis in the SAH cell model in a dose-dependent manner.

NLRP3 inhibitors reverse the activity of 3K3A-APC in the SAH cell model. To further verify the association between 3K3A-APC and pyroptosis in SAH, a specific inhibitor of NLRP3, BAY11-7082, was applied to explore its effect on 3K3A-APC in PC12 cells. A total of three specific shRNAs targeting APC were designed to inhibit the expression of APC at the mRNA and protein levels ($P<0.001$; Fig. S1). The inhibition of APC with shAPC-2 significantly increased

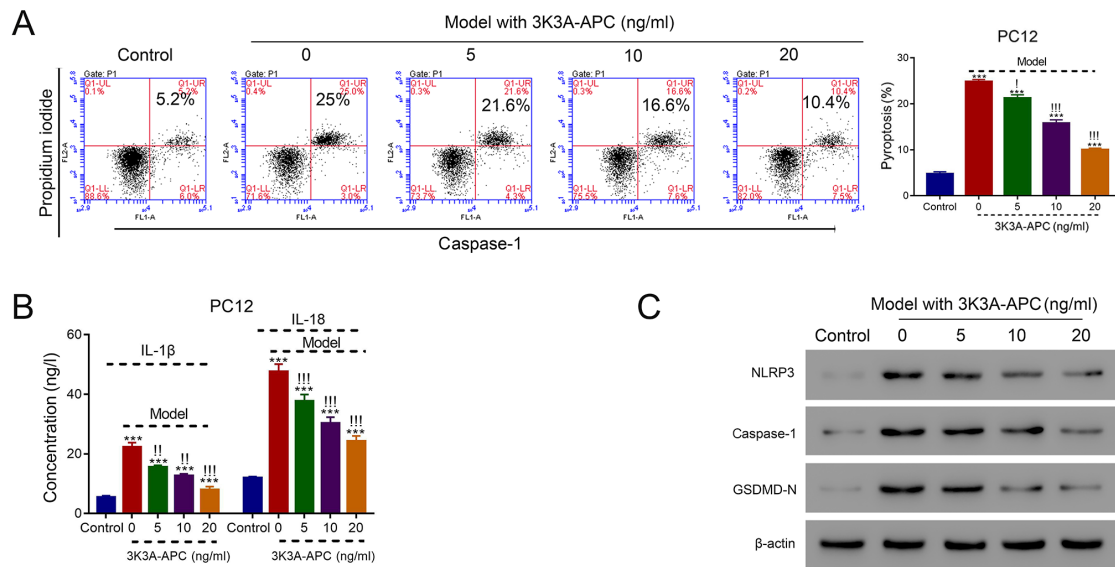


Figure 3. 3K3A-APC inhibits pyroptosis in the SAH cell model in a dose-dependent manner. (A) Flow cytometry revealed the population of pyroptotic cells after treatment with different concentrations of 3K3A-APC. (B) Levels of IL-1 β and IL-18 in the SAH cell model treated with different concentrations of 3K3A-APC. (C) Protein expression levels of NLRP3, caspase-1 and GSDMD-N in the SAH cell model treated with different concentrations of 3K3A-APC. ***P<0.001 vs. control; *P<0.05, **P<0.01, ***P<0.001 vs. 0 ng/ml. APC, activated protein C; SAH, subarachnoid hemorrhage; NLRP3, NLR family pyrin domain-containing 3; GSDMD, gasdermin D.

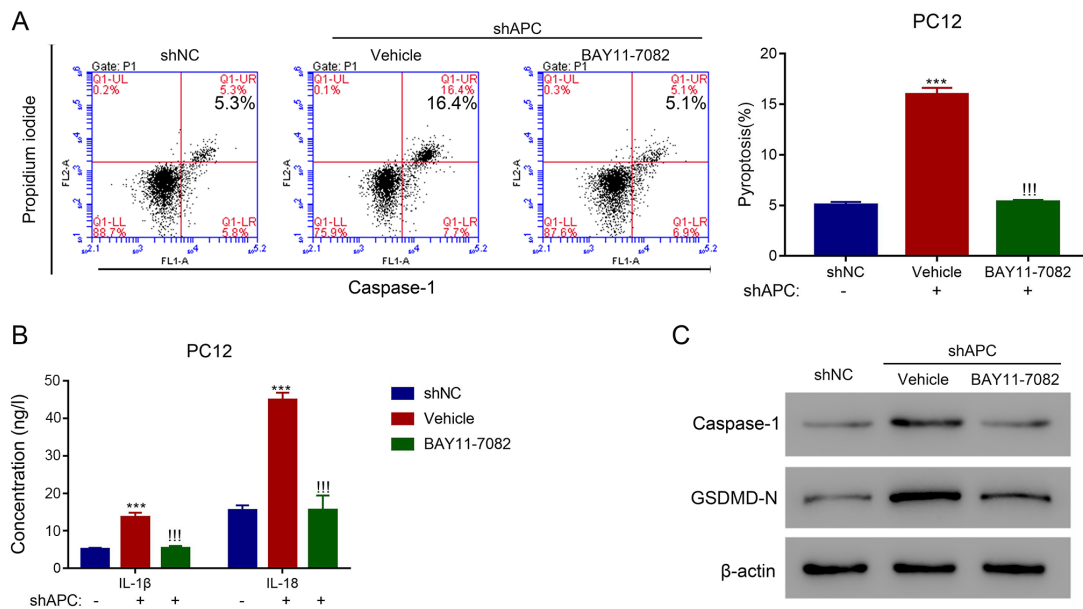


Figure 4. BAY11-7082 rescues the activities of APC-silenced rat PC12 cells. (A) Flow cytometry revealed the population of pyroptotic PC12 cells treated with BAY11-7082 and shAPC. (B) Levels of IL-1 β and IL-18 in cells treated with shAPC and BAY11-7082. (C) Protein levels of caspase-1 and GSDMD-N in cells treated with shAPC and BAY11-7082. ***P<0.001 vs. shNC; ***P<0.001 vs. shAPC + vehicle. APC, activated protein C; sh, short hairpin; GSDMD, gasdermin D; NC, negative control.

the proportion of pyroptotic cells in the vehicle compared with the shNC group (P<0.001); however, the application of BAY11-7082 could significantly reverse this effect (P<0.001; Fig. 4A). In addition, the expression levels of IL-1 β and IL-18 were significantly elevated with shAPC in the vehicle compared with the shNC group (P<0.001); however, they were significantly suppressed after treatment with BAY11-7082 (P<0.001; Fig. 4B). Moreover, the expression levels of caspase-1 and GSDMD-N were increased with shAPC in the vehicle group compared with the shNC group, which were then decreased

after BAY11-7082 treatment (Fig. 4C). These results suggested that inhibition of APC could promote pyroptosis in the SAH cell model through activating the NLRP3 inflammasome.

Discussion

Long-term neurocognitive impairment significantly disrupts the quality of life of patients with SAH (31). A previous report has demonstrated that microglial pyroptosis is involved in the development of postcardiac arrest brain injury (32). The results

of the present study indicated that APC was significantly reduced, while NLRP3 was significantly elevated in the SAH rat model in a time-dependent manner, and the inhibition of these effects provided a protective role in SAH-induced EBI. Moreover, 3K3A-APC was identified as a promising strategy for SAH treatment.

As an engineered product of APC, 3K3A-APC is synthesized using three alanine residues to replace three lysine residues, which inhibits its coagulative activity and maintains its biological activities (15). It has been demonstrated that 3K3A-APC exerts a promising neuroprotective activity in the treatment of ischemic stroke (15,33). In a phase II clinical trial, patients with acute stroke treated with 3K3A-APC exhibited a decreased incidence of hemorrhage (34). Moreover, 3K3A-APC can prevent the deposition of amyloid- β and diminish neuro-inflammatory responses in mice (35). Therefore, 3K3A-APC is a promising therapy for brain disorders. In the current study, 3K3A-APC significantly ameliorated the hemorrhage and improved neurological functions in the SAH rat model. Moreover, the present study indicated that 3K3A-APC may ameliorate SAH-induced injury and exhibit a protective role in SAH through the inhibition of pyroptosis.

The NLRP3 inflammasome serves an important role in SAH-induced EBI. Hu *et al* (36) reported that the application of a specific G-protein-coupled bile acid receptor 1 agonist, INT-777, can significantly reduce neuroinflammation via the inhibition of the NLRP3 inflammasome. Moreover, the specific blockade of NLRP3 can alleviate SAH-induced EBI by suppressing inflammation (37). In the present study, the expression of NLRP3 was significantly elevated in the SAH rat model. The application of an NLRP3 inhibitor, BAY11-7082, could suppress pyroptosis and reverse the function of APC inhibition in the SAH cell model. These results indicated that NLRP3 was involved in the neuroprotective activity of APC in SAH. The current study demonstrated that the application of 3K3A-APC and the inhibition of NLRP3 could significantly reduce the levels of IL-1 β and IL-18 as well as the proportion of pyroptotic cells in SAH rat and cell models. Therefore, the present study indicated that the neuroprotective role of APC was mediated by the suppression of pyroptosis via the inhibition of the NLRP3 inflammasome. The rationale of the current study is presented as a graphical abstract in Fig. S2.

In the present study, the effects of APC and 3K3A-APC were not examined in a clinical context, and this limitation discounted the clinical significance of the results. In the future, it will be necessary to further examine the role of APC in a clinical context in subsequent analyses. To summarize, the present study indicated that APC could ameliorate SAH-induced EBI by suppressing pyroptosis and inhibiting NLRP3 inflammasome, which could provide a novel strategy for the treatment of SAH.

Acknowledgements

Not applicable.

Funding

The present research was financially supported by the Medical Science and Technology Project of Zhejiang Province (grant

no. 2020KY302) and Huzhou General Science and Social Development Project Foundation (grant no. 2019GY39).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AY designed the project and revised the manuscript. XP performed the experiments and wrote the draft. XW analyzed the data and edited diagrams. XN and YL performed the analysis and interpretation of data and confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures of the current study were approved by the Independent Animal Ethics Committee of Huzhou Central Hospital, Affiliated Hospital of Huzhou Normal University (approval no. 16470; Huzhou, China) and were carried out in compliance with the ARRIVE guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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