Pyrroloquinoline Quinone Protects SMA560 Astrocytes Against Thrombin-Induced Cell Death by Improving Mitochondrial Function

Xi Xu¹, Xiaochun Zhang¹, Yanyan Zhao¹, Xiangyan Liang¹, Lijun Zhang¹ and Yu-Feng Zhao¹

Abstract
Thrombin activation after cerebral hemorrhage induces the death of neurons and astrocytes. Pyrroloquinoline quinone (PQQ) shows nutritional functions and cell protection. This study aimed to clarify the protective effects of PQQ on thrombin-induced cell death in astrocytes. Murine SMA560 astrocytoma cells were used in this study. The cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. The changes in reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were observed by CellROX® Deep Red and JC-1, respectively. The expression of apoptotic genes was measured by reverse transcription-quantitative polymerase chain reaction. Thrombin dose- and time-dependently induced SMA560 cell death. PQQ significantly repressed thrombin-induced SMA560 cell death in a dose-dependent manner. Thrombin led to the diminishment of MMP, increased production of ROS, and the upregulated expression of apoptotic genes including c-Jun, TP53, Bim, Puma, and Noxa in SMA560 cells. Meanwhile, PQQ treatment significantly attenuated the effects of thrombin on ROS, MMP, and gene expression in SMA560 cells. In conclusion, PQQ protects SMA560 astrocytes against thrombin-induced cell death by inhibiting oxidative stress and improving mitochondrial function in vitro.

Keywords
pyrroloquinoline quinone, astrocytes, mitochondria, thrombin, apoptosis, oxidative stress, cerebral hemorrhage

Received: September 20th, 2021; Accepted: February 1st, 2022.

Introduction
Cerebral hemorrhage leads to the death of both neurons and astrocytes. Astrocytes play an important role in preserving the viability of neurons through multiple pathways including the release of paracrine molecules, the buffering of ion changes, and the direct connection with neurons.¹ It has been shown that the astrocyte–neuron interaction is critical for the matura-
tion of neurons and synaptic circuits.² Therefore, to investigate the mechanism of astrocytes’ death and their protective methods may be of great significance for the prevention of neuronal loss after cerebral hemorrhage.

It is well known that thrombin is able to induce the death of the cells in the hemorrhagic region when it is activated after cerebral hemorrhage to initiate coagulation.³ Thrombin activation influences the pathogenesis of secondary injury progression of cerebral hemorrhage.³ Thrombin acts through protease-activated receptors (PARs) to elicit intracellular signaling transduction pathways.⁴ It was reported that thrombin could activate c-Jun N-terminal kinase (JNK) and caspase 3 to induce neuronal apoptosis via PAR1 signaling.⁵ Meanwhile, PAR1 is expressed in astrocytes, and thrombin also induces astrocyte apoptosis.⁶,⁸ Repression of thrombin-induced apoptosis of astrocytes can be beneficial to the protection of neural function since astrocytes improve the viability of neurons.

Pyrroloquinoline quinone (PQQ) is an aromatic tricyclic o-quinone and shows a wide range of nutritional functions and improves the viability of many kinds of cells.⁹ PQQ regulates the activities of enzymes that are important for cell metabolism. For example, it binds to lactate dehydrogenase in mammalian cells and augments the conversion of lactate to pyruvate, which may enhance adenosine triphosphate production via the mitochondrial tricarboxylic acid cycle and increase cell viability.¹⁰ PQQ also possesses the ability to influence the

¹Xi’an Medical University, Xi’an, China

Corresponding Author:
Yu-Feng Zhao, Institute of Basic Medical Sciences, Xi’an Medical University, Xi’an, 710021, China.
Email: yufeng.zhao@xiyi.edu.cn
cellular redox state, which plays an important role in regulating cell viability.\textsuperscript{11,12} PQQ scavenges reactive oxygen species (ROS) to attenuate oxidative stress and stimulates mitochondrial biogenesis, and its deficiency influences the normal energy metabolism and mitochondrial function.\textsuperscript{13,14} Since mitochondria and redox state play a central role in cell apoptosis,\textsuperscript{15} PQQ is proposed to be a therapeutic agent to reverse the neural damage that is closely linked to mitochondrial dysfunction after cerebral hemorrhage. It has been shown that PQQ promotes neurite growth of mouse neurons and protects primary cultured rat midbrain neurons against rotenone-induced injury.\textsuperscript{16} On the other hand, the effect of PQQ on the survival of astrocytes after cerebral hemorrhage is not clear. In this study, the beneficial effects of PQQ on the survival of astrocytes under treatment with thrombin, an important mediator of cellular damage after cerebral hemorrhage, were observed and the molecular mechanism was investigated.

Materials and Methods

Materials

PQQ and thrombin were purchased from Sigma-Aldrich (Merck KGaA). JC-1, ROS assay probe CellROX\textsuperscript{®} Deep Red, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and reagent for cell culture were the products of Thermo Fisher. RNA extractive kit, reverse transcription (RT), and quantitative polymerase chain reaction (qPCR) reagents were obtained from Takara Bio USA Inc.

Cell Culture

Murine SMA560 astrogliaoma cells were cultured in an incubator with DMEM containing 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin in 5% \(\text{CO}_2\) at 37 °C. The medium was regularly changed every 3 days. SMA560 cells were subcultured after 80% confluence and planted into either plates or dishes for different measurements.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide Assay

SMA560 cells, grown up to 70% confluence in 96-well plates, were treated with thrombin and the other compounds for the fixed time period. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added into medium (0.5 mg/mL) for 4 h. Then the medium was discarded and 100 \(\mu\)L isopropanol with 0.01 M hydrochloric acid was added to each well. MTT crystals were dissolved and the absorbance values per well at 560 nm wavelength were measured.

ROS Measurement

SMA560 cells were treated with thrombin and the other compounds for a fixed period. Then CellROX\textsuperscript{®} Deep Red (Thermo Fisher Scientific) was added to the medium at a final concentration of 5 \(\mu\)M for 30 min at 37 °C. The cells were washed 3 times with phosphate-buffered saline and visualized under a confocal microscope (Leica SP8). The cellular fluorescence intensity with excitation/emission (Ex/Em) at 640 nm/665 nm was recorded and used for data analysis.

Mitochondrial Membrane Potential Measurement

JC-1 exhibits mitochondrial membrane potential (MMP)-dependent accumulation in mitochondria with the shift of emission wavelength from green to red.\textsuperscript{17} SMA560 cells were stained with JC-1 (5 \(\mu\)g/mL) for 15 min at 37 °C. The cells were washed and the fluorescence was recorded using a confocal microscope (Leica TCS SP8). The red fluorescence (Ex/Em at 560 nm/600 nm) was measured and the intensity of each cell was analyzed by LAS LITE software (Leica Biosystem).

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA of SMA560 cells was extracted and RT using cellular RNA extraction and RT kits (Takara Biotechnology), and the complementary DNA was used for qPCR. The reaction mixture was denatured for 5 min at 95 °C for 30 s and then went to 38 cycles of denaturing at 95 °C for 10 s, annealing, and extending at 60 °C for 15 s. The sequences of primers are listed in Table 1.

Statistical Analysis

The data are presented as mean ± SEM. The data were analyzed by one-way analysis of variance and Bonferroni posthoc

| Table 1. The Sequences of Primers Used in the Study |
|---------------------------------|--------------------------------------------------|--------------------------------------------------|
| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| c-Jun | GAACGACCTTTCTCACGTT | CGCCGAGGCTTGGGCGGGA |
| TP53 | GACCGCGCAGCTATCTTA | GCACAAACAGCACCCAA |
| Bim | TAGGTGACGGGGAGGTAGGTTACA | GTCGAGCTGACGGAGTTAAGGC |
| Puma | TCCTACGCTCCTCTCTCAC | CCATTCTCGGCTGCAGCA |
| Noxa | GAAAGGCGGAGTGGAACCCA | CTTTTGCTCTCAATCTCCG |
| Bax | GGTGATGTTGCTATGGTGGATACAG | TGGCTGGGCGTCCGAGTTAAGAG |
| Bel-2 | GTGACCGTACGGTCTACTCTTCA | ACCAGGAGAAAACAA |
| GAPDH | GAGAAGTTCGATGTGTG | ATGCCAGGATAGGTCTTG |

Natural Product Communications
test to calculate the differences of the groups. $P < .05$ was considered as being a statistically significant difference.

**Results**

**Effects of Thrombin and PQQ on the Death of SMA560 Cells**

First, the dose- and time-dependent effects of thrombin on SMA560 cell viability were observed. Thrombin at a concentration below 30 U/mL did not show significant influence on SMA560 cell viability in 48 h incubation, but at concentrations of 300 and 1000 U/mL inhibited SMA560 cell viability after 12 h treatment and showed stronger effects at 48 h ($P < .05$, $n = 12$, Figure 1A). PQQ dose-dependently attenuated thrombin-induced death of SMA560 cells, as indicated by MTT assay. PQQ at the concentration above 10 μmol/L significantly improved the thrombin (300 U/mL) induced decrease in cell viability after 24 h incubation (Figure 1B).

**Effects of Thrombin and PQQ on ROS Levels in SMA560 Cells**

The ROS levels significantly increased in SMA560 cells after thrombin treatment (300 U/mL) for 12 h, as indicated by the intensity of CellROX® Deep Red. PQQ (30 μmol/L) treatment significantly attenuated thrombin-induced increase in ROS in SMA560 cells ($P < .05$, $n = 3$, Figure 2).

**Effects of Thrombin and PQQ on Mitochondrial Function of SMA560 Cells**

Driven by MMP, JC-1 accumulates in mitochondria to emit red fluorescence. Thrombin treatment (300 U/mL) for 12 h resulted in a significant decrease in JC-1 red fluorescence intensity in SMA560 cells, indicating damage to mitochondrial function. PQQ (30 μmol/L) treatment significantly attenuated this thrombin-induced decrease in JC-1 red fluorescence intensity in SMA560 cells (Figure 3).

**Effects of Thrombin and PQQ on Apoptotic Gene Expression in SMA560 Cells**

The expression of apoptotic genes was stimulated by thrombin. Thrombin treatment (300 U/mL) for 12 h induced a significant increase in expression of c-Jun, TP53, Bim, Puma, and Noxa, but the expression of Bax and Bcl-2 was not significantly influenced. PQQ (30 μmol/L) treatment significantly attenuated thrombin-induced increase in the expression ROS in c-Jun, TP53, Bim, Puma, and Noxa in SMA560 cells ($P < .05$, $n = 3$, Figure 4).

**Discussion**

This study found that thrombin dose- and time-dependently decreased SMA560 cell viability and induced cell death. Thrombin led to SMA560 cell apoptosis at a concentration above 100 U/mL at 12 h treatment and showed stronger effects after 24 and 48 h treatment. After 48 h treatment, there were no cytotoxic effects of thrombin at 30 U/mL and below. It was reported that low concentrations of thrombin are neuroprotective and high concentrations stimulate apoptosis of neuronal cells.18,19 Although the neuroprotective effects of thrombin at low concentration did not appear in SMA560 astrocytes, the present results are in accord with the previous reports that showed cytotoxicity on astrocytes of thrombin at high concentration.6

It is well established that thrombin activates PARs to exhibit a role in the coagulation cascade and regulation of cell viability by stimulating different subtypes of G-proteins and subsequent diverse signaling molecules.20,21 Different signaling pathways have been reported to mediate thrombin-induced cell apoptosis, which include molecules such as JNK, the extracellular signal-regulated kinases 1 and 2, ras homolog family member A, nuclear factor-κB, and signal transducer and activator of transcription.6,7,22 In this study, thrombin stimulated the expression of c-Jun and TP53 in SMA560 cells. The products of c-Jun and TP53 are 2 transcription factors that induce apoptosis after being activated by apoptotic factors.23 Puma and Noxa are
the target genes of p53 and Bim that of gene c-Jun. The expression of Puma, Noxa, and Bim in SMA560 cells was upregulated by thrombin in SMA560 cells. This indicates that thrombin induces SMA560 cell apoptosis by activating the PAR1 and c-Jun/P53 signaling pathways. However, other signaling pathways cannot be excluded since they may also regulate the expression of Puma, Noxa, and Bim. The exact role of other signaling pathways and the interactions among the signaling molecules in thrombin-induced SMA560 cell apoptosis is not well defined and remains to be debated in the future.

Thrombin-induced SMA560 cell apoptosis was blocked by PQQ treatment. PQQ is a nutritionally important growth
factor to improve reproduction ability and stimulate neonatal growth.25 The present study found that PQQ dose-dependently reversed thrombin-induced apoptosis in SMA560 astrocytes. PQQ also protects against apoptosis in other kinds of cells, such as myocardial and neural cells.26 Antioxidation is suggested to be the molecular mechanism of the cytoprotective role of PQQ.29 In the present study, thrombin led to oxidative stress in SMA560 cells, and PQQ completely inhibited thrombin-induced ROS elevation. Antioxidation may be the reason for PQQ to attenuate thrombin-induced SMA560 cell apoptosis. On the other hand, thrombin resulted in MMP loss and mitochondrial dysfunction, which were fully prevented by PQQ treatment. The dysfunction of mitochondria causes excessive ROS production, and this results in further mitochondrial dysfunction. This deterioration of mitochondrial function finally leads to the activation of the apoptotic pathway. PQQ protects mitochondria by attenuating ROS. Meanwhile, PQQ is able to promote mitochondria biogenesis by stimulating the expression of peroxisome proliferator-activated receptor-γ coactivator-1α.9,14,30,31 Thus, a mutual improvement between ROS reduction and mitochondrial biogenesis under PQQ stimulation protects SMA560 astrocytes against thrombin-stimulated cell apoptosis. The detailed mechanism of PQQ to elicit the mutual improvement between ROS and mitochondria remains to be clarified in future studies.

The favorable prognosis after cerebral hemorrhage depends on the survival of neurons. A previous study has shown that PQQ protects neurons against cerebral hemorrhage-induced apoptosis.32 The present study demonstrated that PQQ also protects astrocytes against cell death that is induced by thrombin, an important mediator of cerebral hemorrhage-induced cell apoptosis. It has been known that astrocytes interact with neurons through multiple pathways to preserve neural function.33 Thus, it is suggested that PQQ benefits neural function not only by direct action on neurons, but also by protection of astrocytes after cerebral hemorrhage. The present study further establishes the foundation for PQQ usage in the early stage of cerebral hemorrhage when thrombin is activated.

In conclusion, PQQ protects SMA560 astrocytes against thrombin-induced cell death by inhibiting oxidative stress and improving mitochondrial function in vitro.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: This work was supported by the Natural Science Basic Research Program of Shaanxi (Program No. 2019JZ-38).

Ethical Approval
Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent
Not applicable, because this article does not contain any studies with human or animal subjects.

Trial Registration
Not applicable, because this article does not contain any clinical trials.

ORCID iD
Yu-Feng Zhao https://orcid.org/0000-0002-5296-3568

References
1. Khakh BS, Sofroniew MV. Diversity of astrocyte functions and phenotypes in neural circuits. Nat Neurosci. 2015;18(7):942-952. DOI: 10.1038/nn.4043.
2. Durkee CA, Araque A. Diversity and specificity of astrocyte-neuron communication. Neuroscience. 2019;396:73-78. DOI: 10.1016/j.neuroscience.2018.11.010.
3. Posma JJN, Posthuma JJ, Spronk HMH. Coagulation and non-coagulation effects of thrombin. J Thromb Haemost. 2016;14(10):1908-1916. DOI: 10.1111/jth.13441.
4. Shlobin NA, Har-Even M, Itsekson-Hayosh Z, Haroof S, Pick CG. Role of thrombin in central nervous system injury and disease. Biomolecules. 2021;11(4):562. DOI: 10.3390/biom11040562.
5. Han X, Nieman MT, Kerlin BA. Protease-activated receptors: an illustrated review. Res Pract Thromb Haemost. 2021;5(1):17-26. DOI: 10.1002/rth2.12454.
6. Donovan FM, Pike CJ, Cotman CW, Cunningham DD. Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities. J Neurosci. 1997;17(14):5316-5326. DOI: 10.1523/jneurosci.17-14-05316.1997.
7. Zain J, Huang YQ, Feng X, Nierodzik ML, Li JJ, Karpatkin S. Concentration-dependent dual effect of thrombin on impaired growth/apoptosis or mitogenesis in tumor cells. Blood. 2000;95(10):3133-3138.

In conclusion, PQQ protects SMA560 astrocytes against thrombin-induced cell death by inhibiting oxidative stress and improving mitochondrial function in vitro.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: This work was supported by the Natural Science Basic Research Program of Shaanxi (Program No. 2019JZ-38).

Ethical Approval
Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent
Not applicable, because this article does not contain any studies with human or animal subjects.

Trial Registration
Not applicable, because this article does not contain any clinical trials.

ORCID iD
Yu-Feng Zhao https://orcid.org/0000-0002-5296-3568

References
1. Khakh BS, Sofroniew MV. Diversity of astrocyte functions and phenotypes in neural circuits. Nat Neurosci. 2015;18(7):942-952. DOI: 10.1038/nn.4043.
2. Durkee CA, Araque A. Diversity and specificity of astrocyte-neuron communication. Neuroscience. 2019;396:73-78. DOI: 10.1016/j.neuroscience.2018.11.010.
3. Posma JJN, Posthuma JJ, Spronk HMH. Coagulation and non-coagulation effects of thrombin. J Thromb Haemost. 2016;14(10):1908-1916. DOI: 10.1111/jth.13441.
4. Shlobin NA, Har-Even M, Itsekson-Hayosh Z, Haroof S, Pick CG. Role of thrombin in central nervous system injury and disease. Biomolecules. 2021;11(4):562. DOI: 10.3390/biom11040562.
5. Han X, Nieman MT, Kerlin BA. Protease-activated receptors: an illustrated review. Res Pract Thromb Haemost. 2021;5(1):17-26. DOI: 10.1002/rth2.12454.
6. Donovan FM, Pike CJ, Cotman CW, Cunningham DD. Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities. J Neurosci. 1997;17(14):5316-5326. DOI: 10.1523/jneurosci.17-14-05316.1997.
7. Zain J, Huang YQ, Feng X, Nierodzik ML, Li JJ, Karpatkin S. Concentration-dependent dual effect of thrombin on impaired growth/apoptosis or mitogenesis in tumor cells. Blood. 2000;95(10):3133-3138.
Huang C, Fan Z, Han D, Johnston LJ, Ma X, Wang F. Recent progress in studies on the health benefits of pyrroloquinoline quinone. *Biomed Biochim Acta*. 2016;80(1):13-22. DOI: 10.1080/091168451.2015.1062715.

Akagawa M, Nakano M, Ikemoto K. Recent progress in studies on the health benefits of pyrroloquinoline quinone. *Biomed Biochim Acta*. 2016;80(1):13-22. DOI: 10.1080/091168451.2015.1062715.

Cheng Q, Chen J, Guo H, et al. Pyrroloquinoline quinone promotes mitochondrial biogenesis in rotenone-induced Parkinson’s disease model via AMPK activation. *Cell Death Dis*. 2017;8(5):e3240. DOI: 10.1038/cddis.2017.35.

Zhang Q, Chen S, Yu S, et al. Neuroprotective effects of pyrroloquinoline quinone (PQQ) against rotenone injury in primary cultured midbrain neurons and in a rat model of Parkinson’s disease. *J Neurochem*. 2015;134(6):1091-1103. DOI: 10.1111/jnc.13128.

Steinberg F, Sites TE, Anderson P, et al. Pyrroloquinoline quinone improves growth and reproductive performance in mice fed chemically defined diets. *Exp Biol Med (Maywood)*. 2003;228(2):160-166. DOI: 10.1177/15353702020322800205.

Xu F, Yu H, Liu J, Cheng L. Pyrroloquinoline quinone inhibits oxygen/glucose deprivation-induced apoptosis by activating the PI3K/AKT pathway in cardiomyocytes. *Mol Cell Biochem*. 2014;386(1-2):107-115. DOI: 10.1007/s10571-013-2384-5.

Zhang P, Ye Y, Qian Y, et al. The effect of pyrroloquinoline quinone on apoptosis and autophagy in traumatic brain injury. *CNS Neurol Disord Drug Targets*. 2017;16(6):724-736. DOI: 10.2174/187152316666170124164306.

Yamada Y, Nishii K, Kuwata K, et al. Effects of pyrroloquinoline quinone and imidazole pyrroloquinoline on biological activities and neural functions. *Helicyon*. 2020;16(6):e03240. DOI: 10.1016/j.helijon.2020.e03240.

Wen H, He Y, Zhang K, et al. Mini-review: functions and action mechanisms of PQQ in osteoporosis and neuro injury. *Curr Stem Cell Res Ther*. 2020;15(1):32-36. DOI: 10.2174/15748881846618120165539.

Huang YQ, Li JJ, Karpatsik S. Thrombin inhibits tumor cell growth in association with up-regulation of p21(waf1/cip1) and caspases via a p53-independent, STAT-1-dependent pathway. *J Biol Chem*. 2000;275(9):6462-6468. DOI: 10.1074/jbc.275.9.6462.

Wong HK, Fricker M, Wyttenbach A, et al. Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenate. *Mol Cell Biol*. 2005;25(19):8732-8747. DOI: 10.1128/mcb.25.19.8732-8747.2005.

Akhter R, Sanphui P, Das H, Saha P, Biswas SC. The regulation of p53 up-regulated modulator of apoptosis by JNK/c-Jun pathway in β-amyloid-induced neuron death. *J Neurochem*. 2015;134(6):1091-1103. DOI: 10.1111/jnc.13128.

Chandrahala A, Ramachandran R. Molecular mechanisms regulating proteinase-activated receptors (PARs). *FEBS J*. 2021;288(8):2697-2726. DOI: 10.1111/febs.15829.

Chowanadisai W, Bauerly KA, Tchaparian E, Wong A, Cortopassi GA, Rucker RB. Pyrroloquinoline quinone stimulates mitochondrial biogenesis through AMP response element-binding protein phosphorylation and increased PGC-1 alpha expression. *J Biol Chem*. 2010;285(1):142-152. DOI: 10.1074/jbc.M109.031030.

Lo H, Shen J, Song X, et al. Protective effect of pyrroloquinoline quinone (PQQ) in rat model of intracerebral hemorrhage. *Cell Mol Neurobiol*. 2015;35(7):921-930. DOI: 10.1007/s10571-015-0187-5.

Halassa MM, Haydon PG. Integrated brain circuits: astrocyte networks modulate neuronal activity and behavior. *Ann Rev Physiol*. 2010;72:335-355. DOI: 10.1146/annurev-physiol-021909-135843.