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Original Research Article

Anthocyanin attenuates oxygen-glucose deprivation/reperfusion-induced apoptosis of PC12 cells via inactivation of ASK1/JNK/p38 signaling pathway

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Abstract

Purpose: *To investigate whether the cytoprotective effect of anthocyanin (Anc) on oxygen-glucose deprivation/reperfusion (OGD/R)-induced cell injury is related to apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK)/p38 signaling pathway.*
Methods: *PC12 cells were pre-treated with various concentrations of Anc (10, 50, and 100 μg/mL) in OGD/R-induced cell injury model. The 3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide (MTT) assay was used to assess cell viability. Cell apoptosis was measured by lactic acid dehydrogenase (LDH) release assay and flow cytometry. Western blot was employed to determine the protein expressions of BCL-2, BAX, caspase-3, p-ASK1 (Thr845), p-JNK, and p-p38.*
Results: *The results indicate that Anc increased the viability of PC12 cells after OGD/R exposure ($p < 0.05$), and also efficiently rescued OGD/R-induced apoptosis ($p < 0.05$). Mechanistic studies showed that these protective roles of Anc are related to the inhibition of ASK1/JNK/p38 signaling pathway.*
Conclusion: *The results indicate Anc protects against OGD/R-induced cell injury by enhancing cell viability and inhibiting cell apoptosis. The underlying mechanism of action is partly via inactivation of ASK1/JNK/p38 signaling pathway. Thus, Anc has promise as a potential natural agent to prevent and treat cerebral ischemia-reperfusion injury.*

Keywords: *Cerebral ischemia-reperfusion injury, OGD/R, Anthocyanin, ASK1/JNK/p38 signaling pathways, Cell apoptosis*

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INTRODUCTION

Brain ischemia-reperfusion (I/R) injury, a cerebrovascular disease with high mortality, is a serious threat to human health [1]. Currently, the treatment for ischemic stroke is fibrinolytic therapy or reoxygenation. However, after reperfusion, various complications may occur, thus aggravate brain tissue injury, which is called reperfusion injury. Ischemia-reperfusion (I/R) injury can induce inflammation, oxidative stress and cytotoxicity through specific programmed cell death mechanisms, and promote neurodegenerative changes [2]. In recent years,

the protective effects of various plant active extracts on cell injury and apoptosis have been attracting wide attention[3]. Therefore, the discovery of natural neuroprotective agents has important clinical and therapeutic significance.

Anthocyanin (Anc), one of the most widespread families of natural pigments, exerts cytoprotective roles in various pathological conditions [4], such as inflammation and cancer [5]. Moreover, Anc has been reported to display neuroprotective and brain health benefits [6]. In addition, Anc reduced D-gal-induced hippocampal and cortical inflammation and prevented memory impairment [7]. Previous studies have illustrated the protective role of Anc on prevent cell damage and hippocampal neuronal apoptosis was through reducing the production of reactive oxygen species (ROS) [8]. Furthermore, there is ample evidence to suggest that Anc improves memory and cognition and displays neuroprotective effects on Alzheimer's disease [9]. However, the mechanism underlying the protective role of Anc remains unclear and needs further investigation.

Anthocyanin has been shown to protect against apoptosis via various signaling pathways. Studies have shown that ASK1/JNK/p38 activation is accompanied by OGD/R-induced injury [10]. Studies have indicated that the activities of p38 and JNK were upregulated in the cerebral ischemic response, and the inhibition of JNK and p38 pathways attenuated ischemic brain injury [11]. Taken together, these investigations indicated that the underlying mechanisms of Anc against OGD/R-induced apoptosis may be related to ASK1/JNK/p38 signaling pathway. Here, the role of Anc was evaluated in OGD/R-induced cell injury.

EXPERIMENTAL

Materials

Anthocyanins were obtained from Extrasynthese (Genay, France). The primary antibodies used were: p-ASK1 (Thr845) (Cell Signaling Technology, USA), p-JNK (Cell Signaling Technology, USA), p-p38 (Cell Signaling Technology, USA), caspase-3 (Cell Signaling Technology, USA), BCL-2 (Santa Cruz, USA), BAX (Santa Cruz, USA) and GAPDH (Santa Cruz, USA). The ASK1 overexpressed plasmid was purchased from Santa Cruz Biotechnology.

Cell culture

The rat pheochromocytoma-derived cell line PC12 was purchased from ATCC and cultivated in DMEM (Invitrogen, USA) containing 10 % fetal bovine serum (FBS; Gibco, USA), 1 % penicillin/streptomycin (P/S; Sigma, USA) at 37 °C.

OGD/R model

PC12 cells were cultured for 24 h and rinsed 3 times with PBS. Dulbecco's modified Eagle medium (DMEM) without serum and low glucose was added to the cells, which were then placed in a hypoxia chamber with 94 % N_2, 5 % CO_2, and 1 % O_2 at 37 °C for 3 h as OGD treatment. After OGD treatment, the cells were quickly reoxygenated by changing to normal medium containing FBS and glucose under normoxic conditions containing 5 % CO_2 for another 24 h at 37 °C. Next, different concentrations of Anc (10, 50, or 100 μg/ml) were added into the cells for 2 h, and then were treated with OGD for another 3 h, then followed by 24 h of reoxygenation.

MTT assay

PC12 cells (5×10^3 cells/well) were pretreated with Anc (10, 50, or 100 μg/mL) for 2 h. After OGD/R, cells were treated with MTT (5 mg/ml) and placed at 37°C for another 4 h. Then removed the medium and added dimethyl sulfoxide (DMSO), followed by shaking gently for 30 min. The absorbance at 490 nm was evaluated using a microplate reader (Bio-Rad, USA).

Lactic acid dehydrogenase (LDH) release assay

The apoptosis was partly detected by evaluating LDH release. The culture supernatants were collected immediately after exposure to OGD/R, and LDH cytotoxicity assay detection Kit (Beyotime, China) to evaluate the content of LDH release. The absorbance at 450 nm was obtained by the microplate reader (Takara Biotechnology, China), and calculated as follows: cell mortality = 100 × (Re – Rs) / (Rm - Rs). (Re = experimental release, Rs = spontaneous release, Rm = maximum release.

Flow cytometric analysis

The apoptosis was partly detected by the FITC Annexin V Apoptosis Detection Kit I (KeyGen Biotech, Jiangsu, China). The early apoptosis cells were stained with positive Annexin V-FITC staining and negative propidium iodide (PI). Cells with positive PI and Annexin V-FITC staining were at the end of apoptosis. Annexin V-FITC and PI-negative cells were survived without

apoptosis.

Western blot analysis

Lysed the cells in RIPA (Beyotime, Shanghai, China), and then centrifugated at 1.2 × 10^4 rpm for 15 min, the BCA assay kit (Beyotime) was used to qualify the supernatant protein concentration. 30 μg samples were subjected to 10 % SDS-PAGE, and then transferred to nitrocellulose membranes. 5 % non-fat milk was used to block the NC membranes and then incubated with the corresponding primary antibodies at 4 °C overnight. The next day, appropriate secondary antibodies were used to incubate the NC membranes at room temperature for 2 h. The density of proteins was detected by an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Trizol (Invitrogen) was used to isolate total mRNA while reverse transcription kit (Tiangen Biotech, China) was used for reverse-transcription of the total RNA. Quantitative RT-PCR was conducted using the SYBR Green PCR Master Mix (Qiagen, Shanghai, China). The qPCR primer sequences used are as shown in Table 1.

Table 1: Primer sequences used in PCR studies

ASK	Forward: AGACCCTGACAAG AGAGCCT	Reverse: TCTCCGTGCAACC ACATACC
GAP DH	Forward: ACTCTACCCACGG CAAGTTC	Reverse: TGGGTTTCCCGTT GATGACC

Statistical analysis

At least triplicate independent experiments were performed and the data presented as mean ± standard deviation (SD). Group differences were statistically analyzed using one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

Anc increased cell viability after OGD/R exposure

Neuron cell injury caused by OGD/R, which is used to mimic brain I/R injury *in vitro* [22]. In order to identify the effect of Anc on cell viability after OGD/R treatment, the OGD/R-stimulated cell injury model was first established and then pretreated with Anc (10 μg/ml, 50, or 100 μg/mL). As shown in Figure 1 A, OGD/R exposure significantly decreased cell viability. After treatment with Anc, cell viability was increased in a dose-dependent manner (10–100 μg/mL) (Figure 1 A). Next, the amount of LDH release from PC12 cells was measured after treatment with Anc (10, 50 or 100 μg/mL) following OGD/R-stimulated PC12 cell injury. The LDH content was significantly increased after OGD/R exposure. Lactate acid dehydrogenase (LDH) was decreased after treatment with Anc in a dose-dependent manner (Figure 1 B). These results suggest that Anc increased cell viability after OGD/R-induced injury.

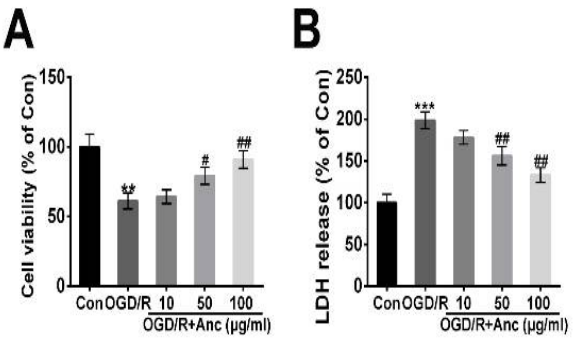

Figure 1: The effect of Anc on PC12 cell viability after OGD/R exposure. (A) Cell viability of PC12 in different groups was determined by MTT assay. (B) Lactate acid dehydrogenase (LDH) release of different groups was detected by a commercial assay.12 independent experiments were repeated and presented as mean ± SD; $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with OGD/R group

Anc decreased cell apoptosis following OGD/R exposure

To further explore the effect of Anc on OGD/R-induced cell apoptosis, the apoptosis of PC12 cells was detected following treatment with different concentrations of Anc. The flow cytometry results illustrated that the apoptotic ratio was significantly increased after OGD/R treatment. However, Anc treatment significantly decreased apoptosis in a dose-dependent manner (Figure 2 A). Furthermore, the levels of apoptosis-associated proteins were also examined. In the OGD/R group, the protein expression of BCL-2 was significantly reduced, while the protein expressions of cleaved caspase-3 and BAX were significantly increased as compared with the control group (Figure 2 B). The levels of several pro-apoptotic proteins were reduced following treatment with different concentrations of Anc, suggesting that Anc

showed a protective role in OGD/R-stimulated cell apoptosis.

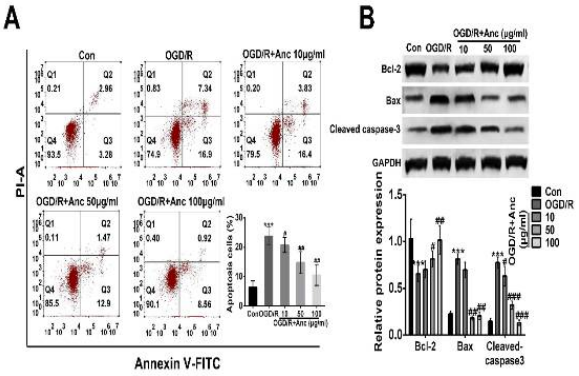

Figure 2: Anc decreased cell apoptosis after OGD/R exposure. PC12 cells were pretreated with Anc (10, 50, or 100 μg/mL). **(A)** PC12 cells were double-stained with PI and Annexin V-FITC and were identified by the flow cytometry. The vertical axis and horizontal axis represented the positive area of PI and positive area of FITC, respectively. After flow cytometry identification, the cells were divided into four parts: Q1: Annexin V-FITC-PI+, indicated mechanical error; Q2: Annexin V-FITC+PI+, indicated late apoptosis or necrosis cells; Q3: Annexin V-FITC-PI-, indicated living cells; Q4: Annexin V-FITC+PI-, indicated early apoptosis cells. **(B)** Levels of apoptosis-related proteins by Western blot analysis. Three independent experiments were performed; *$p < 0.05$ compared with control group, $^{\#}p < 0.05$ compared with OGD/R group

Anc inhibited ASK1/JNK/p38 activation

To examine the role of ASK1/JNK/p38 signaling pathway in OGD/R-induced apoptosis, the phosphorylated protein levels of the ASK1/JNK/p38 pathway from OGD/R-treated cells and normal cells were analyzed. As shown in Figure 3, in PC12 cells, OGD/R exposure upregulated the levels of p-p38, p-JNK and p-ASK1 (Thr845), indicating the activation of ASK1/JNK/p38 signaling pathway. However, following treatment with different concentrations of Anc, the levels of p-ASK1 (Thr845), p-p38 and p-JNK were decreased in a dose-dependent manner, indicating that Anc could inhibit ASK1/JNK/p38 activation.

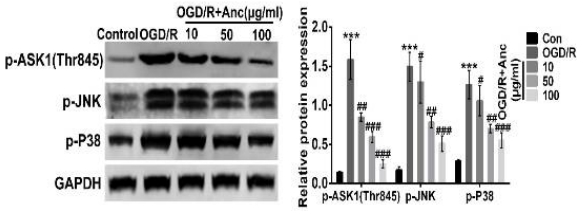

Figure 3: Anc inhibited ASK1/JNK/p38 activation. Western blot was used to analyze the protein levels of ASK1/JNK/p38. The data were shown at least in three independent experiments; *$p < 0.05$, compared with control group, $^{\#}p < 0.05$ compared with OGD/R group

Anc decreased OGD/R-induced PC12 cell apoptosis via ASK1/JNK/p38 inactivation

Studies have shown that crosstalk between the ASK1/JNK/p38 and brain ischemia and reperfusion. Here, we investigated whether Anc reduced OGD/R-induced apoptosis via ASK1/JNK/p38 signaling. As shown in Figures 4 A and B, ASK1-overexpressed plasmid successfully increased the mRNA and protein levels of ASK1. Though anthocyanin decreased the protein levels of the phosphorylation of ASK1, JNK and p38 induced by OGD/R, ASK1 overexpression reversed these results, indicating that Anc inactivated the ASK1/JNK/p38 pathway through ASK1 (Figure 4 C). In addition, Anc treatment decreased the percentage of apoptotic cells after exposure to OGD/R, However, ASK1 overexpression significantly increased the apoptotic cells after exposure to OGD/R. **(Figure 4D)**. In summary, these results indicated that Anc decreased OGD/R-induced cell apoptosis by inhibiting the ASK1/JNK/p38 activation.

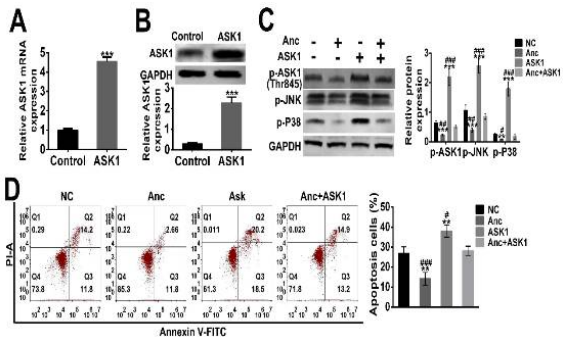

Figure 4: Anc decreased OGD/R-induced PC12 cell apoptosis via ASK1/JNK/p38 inactivation. (A) and (B) Efficiency of ASK1 plasmid transduction. The overexpression plasmid encoding ASK1 were transfected into PC12 cells**. (A)** Real-time PCR. (B) Western blotting. ASK1, transfection with ASK1 overexpressed plasmid; *$p < 0.01$ vs Control. **(C)** and **(D)** Overexpression of ASK1 promotes PC12 cell apoptosis following OGD/R exposure. **(C)** Western blot analyses for the detection of p-ASK1, p-JNK, and p-p38. **(D)** Flow cytometric analysis was used to detect apoptosis; *$p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the Anc group

DISCUSSION

Cerebral I/R injury triggered by hypoxia and glucose deficiency commonly causes permanent disability and is a serious threat to human health [1] [12]. This study used a classic oxygen-glucose deprivation/reperfusion model to detect

the effects of Anc on OGD/R-induced injury and apoptosis [13].

Anthocyanins are cytoprotective agents against various stress conditions [4]. At present, researches have indicated that Anc has antioxidant, anti-inflammatory, anti-cancer and other pharmacological effects [5]. Besides, Anc has been reported to have neuroprotective and brain health benefits [6]. For example, Anc could protect against oxidative stress induced by ethanol and neuronal apoptosis [15] and has protective effects against D-gal-stimulated neuroinflammation and neurodegenerative changes [7]. Anc was also reported to protect against cell damage by reducing the production of ROS and inhibiting hippocampal neuronal apoptosis [8]. The results of the present study show that Anc increased viability and decreased apoptosis of OGD/R-treated PC12 cells, suggesting that Anc can potentially be used as a new natural medicine to prevent and treat brain I/R injury.

It is well-known that cell apoptosis is accompanied by brain I/R injury [16]. In this work, three methods were used to determine the function of Anc in OGD/R-stimulated PC12 cell apoptosis, including evaluating LDH release, flow cytometry analysis and the expression levels of apoptosis-associated proteins. Anc decreased LDH release, and cell apoptosis was reduced significantly by flow cytometry analysis. Apoptosis-associated proteins include two important groups of proteins: BCL-2 family members and caspases[17]. BCL-2 prevents cytochrome C release into the cytosol and is an anti-apoptotic protein, activating caspases [18]. Bcl-2-associated X, a pro-apoptotic protein, has been reported to induce neuronal apoptosis [19].

Caspase-3 is the ultimate executor of apoptosis [20] and brain I/R injury could induce apoptosis by releasing apoptotic factors [21]. Indeed, this study showed that the expression levels of BAX and cleaved caspase-3 were significantly increased while the expression level of BCL-2 was reduced in the OGD/R group. Anc pretreatment effectively reversed these effects, implying that Anc protected against OGD/R-stimulated damage by inhibiting apoptosis. Therefore, Anc may be considered a promising natural agent to prevent OGD/R-induced apoptosis.

The present work also explored the underlying mechanisms on the effect of Anc on OGD/R-stimulated apoptosis of PC12 cell. Signaling pathways such as MAPK pathway that related to cellular processes including cell cycle and apoptosis, might provide potential therapeutic targets [5]. Previous evidence indicated that MAPKs are involved in I/R. Besides, p38 and JNK, members of MAPKs, are reported to regulate apoptotic signals.

JNK and p38 phosphorylation levels have been reported upregulated in the cerebral ischemic response, and the inhibition of JNK and p38 phosphorylation could attenuate ischemic brain injury [11]. In addition, studies also showed that p-p38 and p-JNK levels were upregulated after OGD/R exposure[11], while AKT signaling via the phosphorylation of ASK1 inhibited JNK- and p38-mediated apoptosis [22]. This work showed that the expression levels of p-p38, p-JNK and p-ASK1 increased after OGD/R treatment but Anc significantly reversed this phenomenon, suggesting that Anc inactivated the phosphorylation of ASK1, p38 and JNK proteins.

Since Anc decreased OGD/R-mediated apoptosis and also inhibited the ASK1/JNK/p38 pathway activation, the results suggested that Anc decreased OGD/R-induced apoptosis via inhibiting ASK1/JNK/p38 activity. JNK, p38 and ASK1 are key regulators of cell death [23], and JNK and p38 could be the downstream of ASK1 [24].

Studies have shown the new mechanisms by which ASK1 mediates various types of extracellular and intracellular signals [25]. Thus, it raised the possibility that the neuroprotective effect of Anc might be mediated by inactivation of OGD/R-induced ASK1/JNK/p38 pathway. Therefore, ASK1-overexpressed plasmid was transfected into PC12 cells and was proved to promote OGD/R-induced cell death, however, treatment with both Anc and ASK1-overexpressed plasmid reversed this phenomenon, suggesting that the neuroprotective effects of Anc against OGD/R-induced cell injury and apoptosis were mediated via the ASK1/JNK/p38 inactivation.

The results of this work demonstrated that Anc is a potential natural agent which prevents OGD/R-induced PC12 cell apoptosis. To extend the findings for clinical application, future work is still needed to further investigate the physiological role of Anc in the pathological processes of OGD/R *in vivo*.

CONCLUSION

The findings of the present study show that Anc protects against OGD/R-stimulated PC-12 cell injury and apoptosis. The protective effect of Anc is mediated by inactivation of ASK1/JNK/p38

signaling pathway. Therefore, Anc is a potential drug for the management of brain I/R injury.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors listed in this manuscript. All liabilities related to the content of this article will be borne by the authors. Hong Zhu and Lan Xiao designed the experiments and revised the manuscript. Dan Ren and Ting Zhang formed the experiments, Ruomeng Li, Bin Guan and Jing Zhang wrote the manuscript.

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