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Original Paper

Apocynin Attenuates Cobalt Chloride-Induced Pheochromocytoma Cell Apoptosis by Inhibiting P38-MAPK/Caspase-3 Pathway

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Key Words
Apocynin • NADPH oxidase • Cobalt chloride • Pheochromocytoma cell • p38-MAPK • Caspase-3 • Apoptosis

Abstract
Background/Aims: Apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, has been identified as a potential neuroprotectant. In this study, we aimed to investigate the protective effect of apocynin against cobalt chloride ($CoCl_2$)-induced pheochromocytoma (PC12) cell apoptosis. ***Methods:*** The PC12 cell culture was pretreated with apocynin and/or SB203580 (p38 mitogen-activated protein kinase [p38-MAPK] inhibitor) at different time points prior to $CoCl_2$ incubation. The cell viability, apoptosis rate, DAN damage, and antioxidant activity were detected using cell counting kit-8 (CCK-8), flow cytometry, enzyme-linked immunosorbent assay (ELISA), and comet assay respectively. The protein and mRNA expressions of p38-MAPK and caspase-3 in the cells were measured by qRT-PCR and Western blotting. ***Results:*** Apocynin inhibited $CoCl_2$-mediated apoptosis, reduced oxidative stress, and down-regulated the expression of p38-MAPK and caspase-3. ***Conclusions:*** Our findings show that apocynin attenuated $CoCl_2$-induced apoptosis by potently restraining p38-MAPK-caspase-3 signaling pathway in PC12 cells, suggesting that apocynin may be a potent prophylactic reagent against $CoCl_2$-mediated PC12 cell apoptosis.

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Introduction

Hypoxia-induced cell injury is one of the pathophysiological mechanisms of cerebrovascular diseases such as cerebral infarction. Neurons are most sensitive to hypoxia, which may contribute to brain dysfunction and a variety of neurological imbalances such as motor dysfunction, learning disabilities, epilepsy, seizure, and dementia [1-4]. Ample evidence has shown that prolonged hypoxia can lead to neuronal necrosis and apoptosis in the brain [5]. In addition, hypoxia is associated with the development of neurodegenerative diseases [6, 7]. It is highly elevated in the brain of patients with Alzheimer's disease and promotes neuron degeneration [8, 9].

Cobalt chloride ($CoCl_2$) is an established chemical inducer of hypoxia-like responses such as erythropoiesis. It was reported that $CoCl_2$ could mimic hypoxic responses observed in cultured cells, including pheochromocytoma (PC12) cells [5]. PC12 cells are widely used as a well-established model for probing various aspects of the cellular biology of neurons [10]. p38 mitogen-activated protein kinases (p38-MAPK) is a mammalian orthologue of the yeast Hog1p MAPK, participating in the signaling cascade controlling cellular responses to cytokines and stress. Abnormal activity of p38 has been implicated in pathological events in several organs and tissues including the neuron, bone, lung, cardiac and skeletal muscle, red blood cells, and fetal tissues [11-13]. The protein product of proto-oncogene RAS can increase the activity of p38, which further induces the excessive activity of transcription factor NF-κB.

In this study, we preconditioned PC12 cells with $CoCl_2$ to generate a chemical hypoxia-induced damage model to explore the role of apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, on the antioxidant stress induced by $CoCl_2$; change of p38-MAPK-caspase-3 in $CoCl_2$-induced damage; and the role of inhibition of p38-MAPK-caspase-3 pathway on the antioxidant and anti-apoptotic effect of apocynin.

Materials and Methods

Cell culture and pretreatment protocols

PC12 cells (Bio-Eng Inst, Nanjing, China) were stored in Dulbecco's modified eagle medium (DMEM) high glucose medium containing 10% fetal bovine serum (FBS) and cultured at 37 °C with 5% CO_2. The treated PC12 cells (they were incubated in the presence or absence of 600 μM $CoCl_2$ for 60 min) were equally randomized into six groups: (1) pure PC12 cells group; (2) apocynin group, wherein apocynin (100 μM) was administered for 30 min; (3) $CoCl_2$ group; (4) apocynin + $CoCl_2$ group, wherein apocynin (100 μM) was administered at 30 min prior to $CoCl_2$; (5) apocynin+SB203580+$CoCl_2$ group, wherein SB203580 (p38-MAPK inhibitor, 20 μM) was administered at 30 min prior to apocynin; and (6) SB203580 group, wherein SB203580 (20 μM) was administered for 30 min.

Cell viability measurement

PC12 cells in exponential phase were transferred into 96-well plates at a concentration of 1×10^4 per well. The cell viability in different treatment groups was measured using cell counting kit-8 (CCK-8) (Nanjing, China) according to the manufacturer's instructions. The absorbance at 450 nm was measured with an enzyme-labeled meter (Multi-skanAscent, Finland). The percentage of relative cell viability was calculated by comparing the value of treated cells to that of untreated cells, and the result was expressed as $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times100\%$.

Apoptosis evaluation by Flow Cytometry

The evaluation of nuclear staining for apoptosis was performed based on previous methods [10]. The apoptosis rate in different groups was analyzed by Annexin V-FITC/PI double stain assay using Cytomics FACSVerse (BD, USA) according to the manufacturer's instructions (Zoman, Beijing, China).

Comet assay

The single cell gel electrophoresis assay (SCGE, also known as comet assay) is an uncomplicated and sensitive technique for detection of DNA damage (the length of DNA) at the level of individual eukaryotic cells [14, 15]. The length of DNA tail was examined using Comet Assay kits (Sigma-Aldrich, Shanghai) according to the manufacturer's protocols, and observed and analyzed under the fluorescence microscopy and Comet Assay software Pect (CASP, 1.2.3beta2).

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

Total RNA was isolated from the treated PC12 cells samples using TRIzol reagent (Invitrogen, USA). cDNA was synthesized by reverse transcription following the manufacturer's protocols (MBI Fermentas, Lithuania). qRT-PCR was performed with a standard SYBR-green PCR kit (Toyobo, Japan), and gene-specific PCR amplification was performed using the ABI 7300 (Applied Biosystems, Germany). The primers sequences were shown in Table 1. Relative mRNA expression levels of p38MAPK and caspase-3 were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the expression of GAPDH [16, 17].

Western Blot

Western blot analysis was performed as previously described [18, 19]. Briefly, total protein in the treated cells was measured using the BCA protein assay kit, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBST for 1.5 h, incubated with primary anti-p38MAPK antibody at 4 °C overnight and subsequently with an alkaline phosphatase-conjugated secondary antibody, respectively. Blots were stained with an anti-β-actin antibody, and the level of protein was normalized with respect to β-actin band density.

Enzyme-linked immunosorbent assay

The treated PC12 cells were transferred to 96-well plates at a density of 3×10^5 per well. The cells were dealt with trypsin digestion and ultrasonic breaking, and then centrifugated at 12, 000 rpm at 4 °C for 15 min to harvest the liquid supernatant. Afterward, the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were measured by the enzyme-linked immunosorbent assay (ELISA) employing a commercial ELISA kit (Roche, Germany) according to the manufacturer's instructions.

Statistical analysis

All data were expressed as the mean ± standard deviation (Mean ± SD). Comparisons between groups were performed by analyses of variance (ANOVA) test and followed by LSD post hoc comparison test. A P value of less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

Apocynin-pretreatment enhanced cell viability in PC12 cells

The cell viability of $CoCl_2$-preconditioned PC12 cells was lower than that in untreated-, apocynin-, and SB203580-pretreated PC12 cells (all $P<0.05$) (Fig. 1). Administration of apocynin displayed the protective effect of SB203580 on $CoCl_2$-induced PC12 cell injury.

Preconditioning with apocynin inhibited PC12 cell apoptosis

The cell apoptosis rate in $CoCl_2$-preconditioned PC12 cells was higher than

Table 1. The primer used for qRT-PCR

Primer name	Primer sequence
p38mapk-F	5'-GCTTTGACGCAGGTGCTAAG-3'
p38mapk-R	5'-TGTCCCCATAACCGGAGTAGG-3'
caspase3-F	5'-ATGGAGAACAATAAAACCT-3'
caspase3-R	5'-CTAGTGATAAAAGTAGAGTTC-3'
gapdh-F	5'-GGTGAAGGTCGGAGTCAACG-3'
gapdh-R	5'-CAAAGTTGTCATGGATGACC-3'

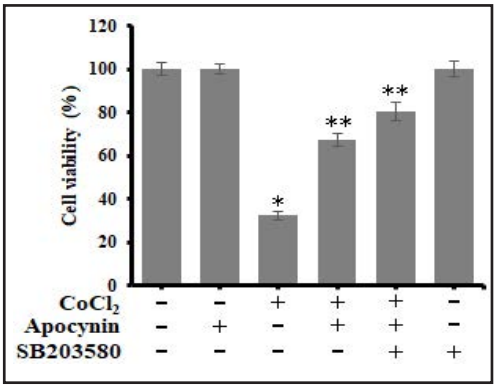

Fig. 1. Cell viability in treated PC12 cells. Note: *P<0.01, **P<0.05 vs. non-$CoCl_2$-treated groups.

that in untreated, apocynin-, and SB203580-preconditioned PC12 cells (all $P<0.05$). The cell apoptosis rate was decreased in $CoCl_2$-preconditioned PC12 cells after administration of apocynin and apocynin+SB203580 (all $P<0.05$) (Fig. 2). The results suggest that apocynin may inhibit the $CoCl_2$-induced PC12 cell apoptosis as SB203580 do.

Pretreatment with apocynin attenuated DNA damage in PC12 cells

Qualitative and quantitative analyses are shown in Fig. 3. The percentage change of tail length (distance from DNA head to the end of DNA tail, represents the severity of DNA damage) and tail DNA percentage in $CoCl_2$-preconditioned PC12 cells was higher than those in untreated-, apocynin-, and SB203580-preconditioned PC12 cells (all $P<0.01$). The percentage change of tail length and tail DNA percentage were decreased in $CoCl_2$-preconditioned PC12 cells after administration of apocynin and apocynin+SB203580 decreased (all $P<0.05$). These results further show that apocynin can inhibit cell damage and has a cytoprotective effect on $CoCl_2$-induced PC12 cell injury.

The mRNA and protein expressions of p38MAPK and caspase-3 in the PC12 cells

The expressions of p38MAPK and caspase-3 in $CoCl_2$-insulted PC12 cells were higher than those in untreated-, apocynin-, and SB203580-preconditioned PC12 cells (all $P<0.05$). The expressions of them were decreased in $CoCl_2$-preconditioned PC12 cells after the administration of apocynin and apocynin+SB203580 (all $P<0.05$) (Fig. 4). These results suggest that the p38MAPK-caspase-3 signal pathway might be involved in the cytoprotection of apocynin.

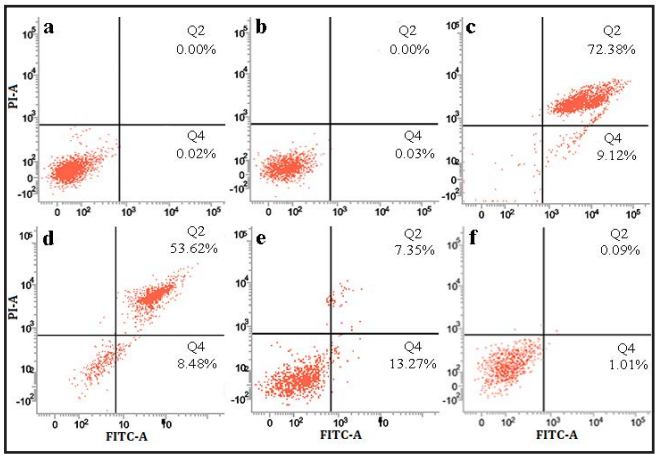

Fig. 2. Cell apoptosis in treated PC12 cells. (a) pure PC12 cells; (b) apocynin treatment; (c) $CoCl_2$ treatment; (d) apocynin+$CoCl_2$ treatment; (e) apocynin+SB203580+$CoCl_2$ treatment; (f) SB203580 treatment.

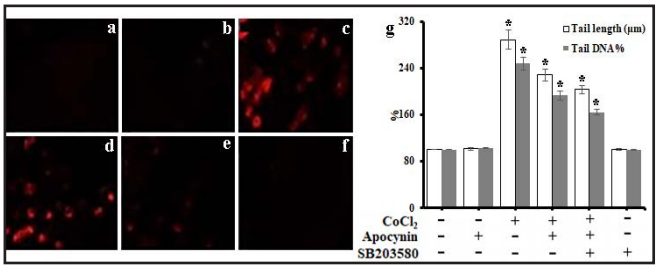

Fig. 3. DNA damage in treated PC12 cells. (a) pure PC12 cells; (b) apocynin treatment; (c) $CoCl_2$ treatment; (d) apocynin+$CoCl_2$ treatment; (e) apocynin+SB203580+$CoCl_2$ treatment; (f) SB203580 treatment; (g) quantitative analyses of DNA damage. Note: *P<0.01 vs. non-$CoCl_2$-treated group.

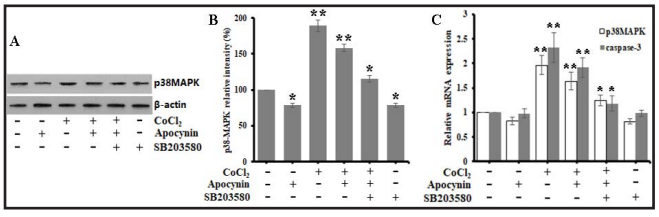

Fig. 4. Relative protein and mRNA expressions of p38-MAPK and caspase-3 in treated PC12 cells. (A) p38MAPK expression; (B) p38MAPK protein relative intensity; (C) relative mRNA expression of p38MAPK and caspase-3. Note: *P<0.05, **P<0.01 vs. pure PC12 cells group.

Antioxidant activity analysis

As shown in Fig. 5, the level of MDA in $CoCl_2$-preconditioned PC12 cells was higher than that in both untreated PC12 cells and apocynin-preconditioned PC12 cells (both $P<0.01$). It was decreased after the administration of apocynin ($P<0.01$). Correspondingly, pretreatment with apocynin remarkedly elevated SOD activity which was weakened by $CoCl_2$. Taken together, these data indicate apocynin could inhibit $CoCl_2$-induced PC12 cell oxidative stress.

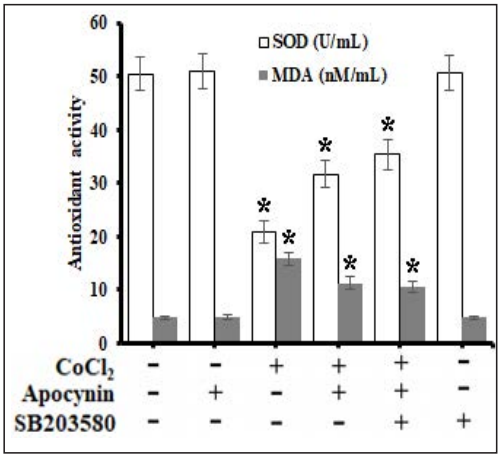

Fig. 5. The antioxidant activity in the treated PC12 cells. Note: *P<0.01 vs. non-$CoCl_2$-treated group.

Discussion

In the present study, we affirmed that apocynin protected against the $CoCl_2$-induced oxidative stress injury by inhibiting p38-MAPK-caspase-3 pathway in PC12 cells. Cerebral ischemia is known to induce a series of neurodegenerative diseases such as dementia, which incurs high mortality risks and imposes a serious threat to human health [20, 21]. Many studies demonstrated that apocynin could attenuate cerebral infarction and subsequent neurological impairments following transient focal ischemia in rats and mice [22-25], suggesting that the administration of apocynin may prove to be a potential therapeutic strategy for the treatment to brain ischemic diseases. In the present study, it was found that apocynin attenuated $CoCl_2$-induced PC12 cell apoptosis, which is contradictory to recent controversies about the potential use of apocynin in clinical situations.

p38-MAPK is one of the activations of mitogen-activated protein kinase (MAPK) families and acts as intracellular signal transduction pathway [13, 26]. p38-MAPK is implicated in the oxidative stress stimulus-induced Reactive oxygen species (ROS) production [27, 28]. In our study, we found that $CoCl_2$ significantly upregulated p38-MAPK expression, and that SB203580, a p38-MAPK inhibitor, inhibited the $CoCl_2$-induced p38-MAPK expression and oxidative stress. Apocynin displayed the same cytoprotective effect as SB203580 did. Our research provides significant evidence that the effect of the p38-MAPK-caspase-3 pathway in chemical hypoxia-induced oxidative stress and damages.

Previous studies showed that administration of apocynin in a middle cerebral artery occlusion (MCAO) model produced significant abatement in the infarct volume and reduced the degree of inflammatory disease [29-32]. They concluded that apocynin possessed a neuroprotective effect. In our study, we observed that apocynin reduced MDA production and increased SOD activity in impaired PC12 cells. In addition, we also found the role of apocynin in $CoCl_2$-induced activation of the p38-MAPK-caspase-3 pathway, suggesting that the inhibition of p38-MAPK-caspase-3 pathway activation may be part of the mechanism underlying the role of apocynin in antioxidative stress and antiapoptosis.

Taken together, our data reveal a key role in apocynin-pretreatment on the endogenous p38-MAPK-caspase-3 pathway in the regulation of oxidative stress induced after $CoCl_2$-insulted PC12 cell injury. Apocynin could reduce $CoCl_2$-induced PC12 cell injury, partly by inhibiting the p38-MAPK-caspase-3 pathway. The modulation of endogenous p38-MAPK-caspase-3 expression and activity and oxidative stress production could be a therapeutic target via apocynin administration.

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Disclosure Statement

All the authors declared no competing interests.

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