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Original Research Article

Carnosic acid attenuates inflammation, oxidative stress and mitochondrial dysfunction in neurons via activation of AMPK/SIRT1 pathway

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Abstract

Purpose: *To determine the effects and mechanism of action of carnosic acid (CA), a natural diterpenoid compound, on the neuroprotective roles of anesthetics.*
Methods: *The effects of carnosic acid (CA) on cell viability and apoptosis were evaluated by MTT assay and flow cytometry, respectively. Its effects on mitochondrial damage was assessed by JC-1 staining, while oxidative stress and inflammatory response were determined by enzyme-linked immunosorbent assay (ELISA) and immunoblot assays, respectively. Immunoblot assays were performed to evaluate the effect of CA on AMPK/SIRT1 pathway.*
Results: *Carnosic acid (CA) increased the survival rate of isoflurane-induced neuronal cells, but inhibited isoflurane-induced mitochondrial damage in neurons ($p < 0.01$). CA also suppressed isoflurane-induced oxidative stress in neurons, and inhibited isoflurane-induced neuronal inflammatory response ($p < 0.01$). Furthermore, CA activated AMPK/SIRT1 pathway, and also attenuated inflammation, oxidative stress and mitochondrial dysfunction in neurons induced by isoflurane ($p < 0.01$).*
Conclusion: *Carnosic acid attenuates inflammation, oxidative stress and mitochondrial dysfunction in neurons via activation of AMPK/SIRT1 pathway. Thus, it has potentials for use in the treatment of neurotoxicity*

Keywords: *Carnosic acid, Isoflurane, Mitochondrial damage, Oxidative stress, AMPK/SIRT1 pathway, Neurotoxicity*

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INTRODUCTION

Inhaled anesthetics are sedatives, but can also cause neurotoxic effects, often manifested in postoperative cognitive decline and neurodegenerative diseases such as Alzheimer's and Parkinson's, as well as neurodevelopmental and behavioral disorders in children [1]. Isoflurane is the most commonly used inhalation anesthetic in developed countries [2,3]. Although isoflurane is favored for its rapid onset of action, it has been associated with increased neurotoxic effects. In cultured cell models, exposure to isoflurane increased the expression of the pro-

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inflammatory cytokine interleukin (IL) -6 and the activation of the nuclear factor -κB (NF-κB) pathway. In addition, the expressions of oxidative stress factors increased in human glioma cells induced by the use of anesthetic isoflurane [4]. To unravel the neurotoxic effects of anesthetics, new drugs still need to be developed.

Carnosic acid (CA), a natural diterpenoid compound, has been identified as a bioactive chemical in many medicinal plant, such as rosemary, sage and saint basil. Studies have shown that CA produces a variety of pharmacological effects [5]. For example, by blocking the collapse of mitochondrial membrane potential (MMP) and the opening of mitochondrial permeability transition pore (mPTP), carnosic acid improves the survival rate of hypoxia/reoxygenation (H/R)-induced cardiomyocytes and weakens mitochondrial dysfunction, thus playing a role in myocardial protection [6]. In spinal cord injury, CA plays a neuroprotective role by activating the Nrf2 pathway to inhibit erastin-induced iron death in PC12 cells [7].

CA alleviates acute ethanol-induced liver injury by activating SIRT1/p66Shc and inhibiting mitochondrial damage [8], and also mitigates high-adipose-induced brain injury through the NF-κB regulation of inflammation and caspase-3-related apoptosis [9]. In terms of mechanism, a large number of studies have shown that CA may activate the AMPK pathway, and the activation of AMPK can reduce the neurotoxicity induced by anesthetics, thus playing a neuroprotective role [10,11]. However, its possible effects and mechanisms on the neuroprotective roles of anesthetics are still unclear.

In this study, the effects and mechanisms of carnosic acid (CA), a natural diterpenoid compound, on the neuroprotective roles of anesthetics were revealed.

EXPERIMENTAL

Cell culture

Primary mouse hippocampal neurons were purchased from Thermo Fisher (Waltham, MA USA), and maintained in Complete Neurobasal™ Medium supplied with GlutaMAX™-I and B-27™ in a serial culture hood containing 5 % CO_2. For isoflurane exposure, cells were placed in the airtight incubator connected to an anesthesia machine that was used to supply isoflurane into the incubator for 24 h. The cells were treated with CA at doses of 2.5, 5, 10 and 20 μM.

Cell viability

The cells were plated in 96-well plates at a density of 2×10^3 cells/well, treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and then rinsed with PBS twice. Next, the cells were incubated for 4 h before measurement with a microplate reader at 490 nm.

Immunofluorescent staining

The cells were blocked with 4 % paraformaldehyde (PFA) in 5 % BSA in PBST and then incubated with primary PCNA (Santa Cruz Biotechnology). After rinsing with PBS for three times in PBST, secondary antibodies conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) were added. The DAPI was used to counterstain cell nuclei. Images were captured using fluorescent microscope (LSM700, Zeiss model, San Diego, CA).

Cell apoptosis

For the detection of apoptotic cell percentage, Annexin V/PI apoptosis detection was conducted following the manufacturer's protocol (Sigma Aldrich, USA). The cells were digested into single cells and resuspended in reaction buffer containing Annexin V and PI for 5 min at room temperature. Cell apoptosis was analyzed with a flow cytometer (BD Biosciences).

JC-1 staining

To measure the degree of mitochondria damage, the cells were plated into 12-well plates and kept for 24 h for cell adhesion. After rinsing in PBS, the cells were incubated with 2 μM JC-1 for 15 min at 37 °C in the dark. After rinsing in PBS 3 times, the cells were examined by a fluorescent microscope.

Determination of antioxidant activity

The levels of superoxide dismutase (SOD), glutathione, r-glutamyl cysteingl +glycine (GSH), and malondialdehyde (MDA) were assessed using kits from Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China) and the assays performed following the manufacturer's guidelines.

Assay of lactate dehydrogenase (LDH)

Cellular LDH release was monitored with a lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Fisher Scientific). The culture medium was collected and added into a 96-well

flat-bottom plate, and the reaction mixture was then added. After incubation for 30 min in the dark, and the addition of stop solution from the kit, the absorbance in each well was determined using SpectraMax M Series Multi-Mode Microplate Reader at 490 nm.

Enzyme linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6) in the cell lysates were measured using ELISA kit. Samples were added into the wells, and biotin-conjugated primary antibodies were plated into wells before the addition of avidin-conjugated horseradish peroxidase (HRP). Then enzyme substrate was added for color development. The intensity of each well was measured using a microplate reader SpectraMax M Series Multi-Mode Microplate Reader at 350 nm.

Immunoblot assay

Total proteins were extracted using RIPA buffer (Beyotime), and protein concentration was measured through using BCA kit. The proteins were separated using SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked with 5 % BSA in TBST, and subsequently incubated with primary antibodies targeting p-p65 (1:1000, Abcam), p65 (1:1000, Abcam), p-AMPK (1:1000, Abcam), AMPK (1:1000, Abcam), SIRT1 (1:2000, Abcam), and b-actin (1:20000, Abcam) at room temperature for 2 h. Thereafter, the membranes were cultured with secondary antibodies for 2 h at room temperature. The membranes were visualized with ECL kit. The intensity was measured using imageJ 9.0 software.

Statistical analysis

GraphPad 7.0 software was used for statistical analysis. Data are presented as mean ± SEM. Statistical comparison was carried out using Student's t-test, and $p < 0.05$ was considered statistically significant.

RESULTS

CA increased the survival rate of isoflurane-treated neuronal cells

Cell proliferation was determined in response to the increasing dose of CA (0, 2.5, 5, 10, and 20 μM) in neuronal cells. The CA at concentrations of 0, 2.5, 5 and 10, μM scarcely affected cell viability (Figure 1 A). In addition, to confirm the effect of CA in isoflurane-induced neuronal cells, cells were stimulated with 3 % isoflurane. ISO inhibited cell viability, but was reversed by CA treatment (Figure 1 B). CA also alleviated cell apoptosis induced by isoflurane (Figure 1 C and D).

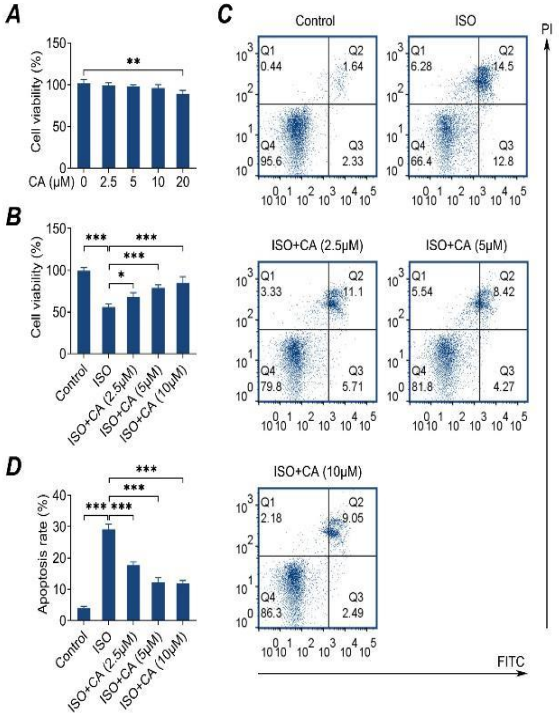

Figure 1: CA increased the survival rate of isoflurane-induced neuronal cells. (A). Cell proliferation of neurons in response to increasing dose of CA; (B): Cell proliferation of neurons in response to ISO and increasing the dose of CA. (C, D): Cell apoptosis in response to ISO. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

CA inhibited isoflurane-induced mitochondrial damage in neurons

The aggregate dye, JC-1, increased JC-1 monomers, and reduced JC-1 aggregates were found in ISO treated cells, but CA treatment reversed these alterations, indicating increased membrane potential (Figure 2).

CA lowered oxidative stress in isoflurane-induced neurons

The level of MDA was enhanced and SOD and GSH were reduced in ISO - stimulated cells (Figure 3 A), and treatment using CA significantly improved these parameters (Figure 3 B). Moreover, ISO increased the level of cellular LDH, and CA reversed the induction of LDH in neurons (Figure 3 B). The data indicate that CA reduced oxidative stress in isoflurane-treated neurons.

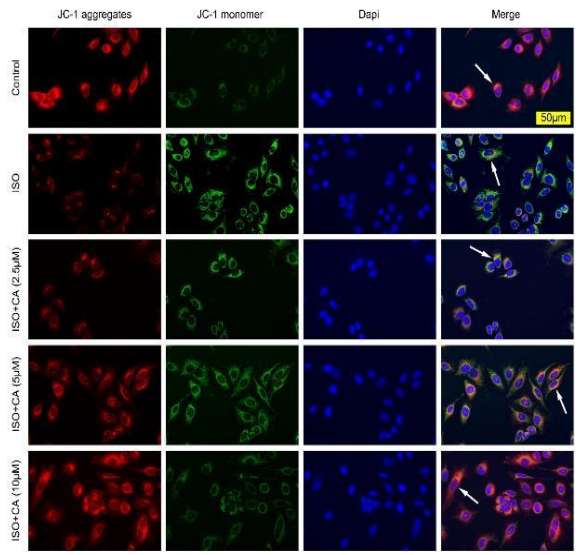

Figure 2: CA inhibited isoflurane - induced mitochondrial damage in neurons. JC-1 staining in cells in response to ISO and increasing dose of CA. *P < 0.05, **p < 0.01, ***p < 0.001

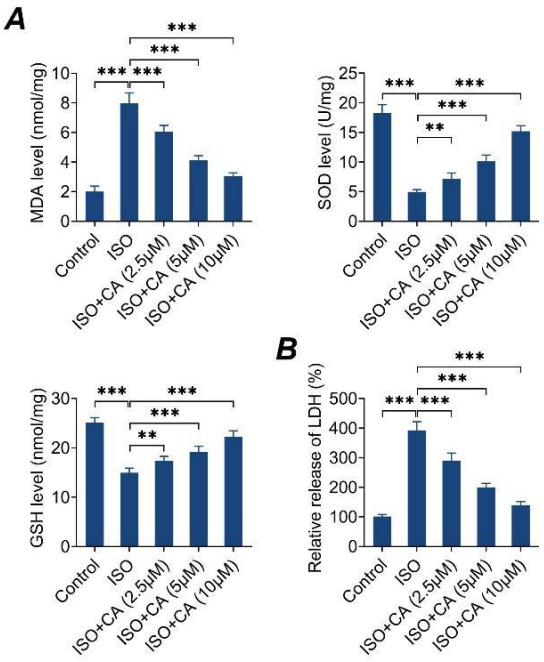

Figure 3: CA lowers oxidative stress in isoflurane-induced neurons. (A) The level of MDA, SOD, and GSH in neurons treated with ISO and increasing dose of CA; (B) Level of LDH in neurons treated with ISO and increasing dose of CA. *P < 0.05, **p < 0.01, ***p < 0.001

CA alleviates ISO-induced cell inflammation

To examine the inflammatory response in ISO treated cells, the levels of IL-18, IL-1β, and TNF-α were monitored in cells. ISO significantly induced the levels IL-18, IL-1β and TNF-α (Figure 4 A), abut CA reversed the increased levels (Figure 4 A). Administration of CA suppressed p-p65 level in ISO-treated neurons (Figure 4 B).

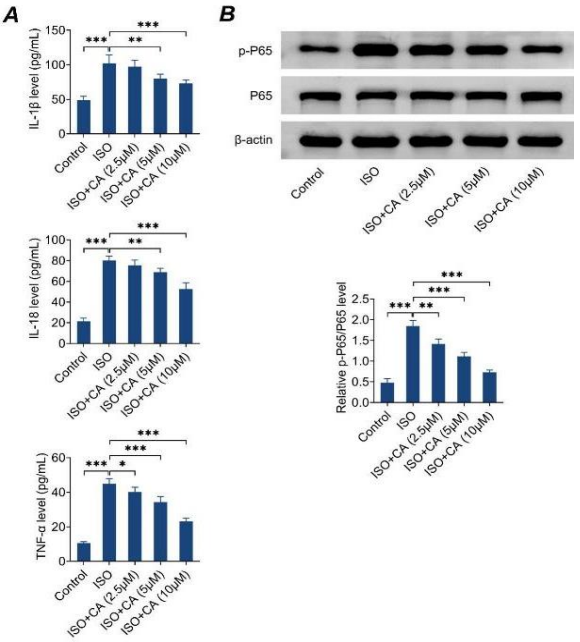

Figure 4: CA alleviates ISO-induced cell inflammation. (A) The level of IL-1b, IL-18, and TNF-a in neurons treated with ISO and increasing dose of CA. *P < 0.05, **p < 0.01, ***p < 0.001

CA reduces isoflurane-induced neuronal inflammation and oxidative stress by activating AMPK/SIRT1 pathway

To unravel the mechanism of action, AMPK/SIRT1 pathway in ISO treated cells was investigated. The level of p-AMPK and SIRT1 was inhibited in ISO treated cells (Figure 5). I CA suppressed the elevation of AMPK and SIRT1 in ISO treated neurons (Figure 5). These results implied that CA reduced isoflurane-induced neuronal inflammation and oxidative stress by activating AMPK/SIRT1 pathway.

DISCUSSION

In the past, it was clinically thought that the effects of anesthetics on the central nervous system were reversible to a certain extent. The central nervous system was completely restored to its original state without any adverse reactions [12]. However, studies have shown that general anesthetics exert level of neurotoxic effect on the brain, especially in the developing brain, which can cause neuronal withering and death, affect the growth of synapses, and ultimately affected the learning and cognitive ability in adulthood [13].

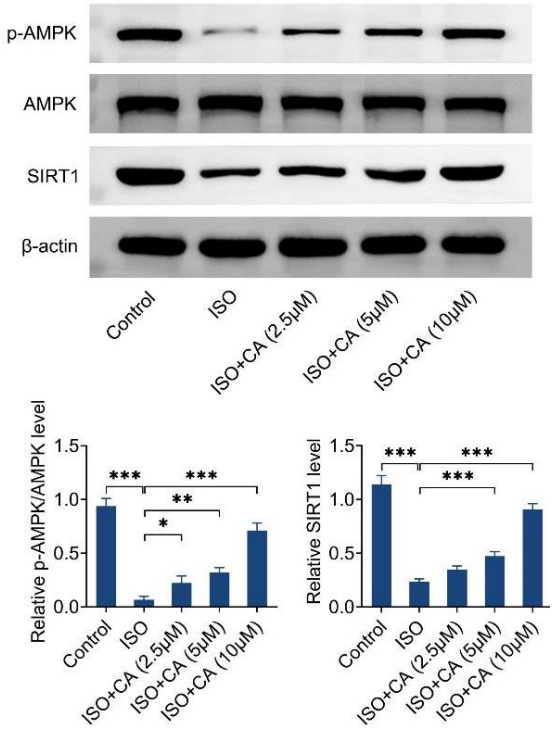

Figure 5: CA reduces isoflurane induced neuronal inflammation and oxidative stress by activating AMPK/SIRT1 pathway. *P < 0.05, **p < 0.01, ***p < 0.001

The neurotoxic effect of narcotic drugs is often manifested in the clinic [14]. The toxicity of anesthetics in the central nervous system is due to the sudden increase in the concentration of anesthetics in the blood, which can cause a series of toxic symptoms [1]. It is therefore important to find new methods or drugs to overcome the neurotoxicity of anesthetics. In this study, carnosic acid attenuated inflammation, oxidative stress and mitochondrial dysfunction in isoflurane-treated neurons. Therefore, CA may serve as a potential drug for the treatment of neurotoxic effects caused by anesthetics.

Isoflurane is the most frequently used inhalation anesthetic. Although isoflurane is favored for its rapid onset of action and minimal effects on respiratory disturbances, it has been associated with increased neurotoxic effects [15]. Isoflurane was used to establish a neuron model to simulate neurotoxicity *in vitro*. Through MTT, FCM, and JC-1 staining, CA could increase isoflurane-induced neuronal cell survival and inhibit mitochondrial damage. Performing a series of *in vitro* assays, CA suppressed isoflurane-treated oxidative stress and inflammatory response in neurons. Therefore, these results indicate that CA could serve as a promising drug for neurotoxic effects caused by anesthetics.

Several mechanisms of neuronal toxicity are induced by anesthetics, such as neuronal apoptosis [16]. A large number of studies have shown that anesthesia may induce the activation of pro-apoptotic proteins in the hippocampal region of rats, which may lead to mitochondrial membrane rupture and cystease activation, leading to apoptosis[17]. In addition, studies have shown that anesthetics may induce oxidative stress and inflammation in the brain, resulting in neuronal toxicity. Therefore, inhibition of neuronal apoptosis may protect from anesthetic-induced brain injury. In this study, CA suppressed neuronal toxicity caused by isoflurane.

CA exhibits a variety of pharmacological effects, including blocking of the collapse of mitochondrial membrane potential (MMP) and opening of mitochondrial permeability transition pore (mPTP). Carnosic acid may improve the survival rate of H/R-induced cardiomyocytes and weakens mitochondrial dysfunction, thereby promoting myocardial protection [18]. Similarly, CA increased isoflurane-induced neuronal cell survival, and alleviated acute ethanol-induced liver injury by activating SIRT1/p66Shc and inhibiting mitochondrial damage [19]. CA also inhibits mitochondrial damage. A previous study showed that CA activated AMPK pathway, and this activation alleviates anesthetics-induced neurotoxicity, therefore playing a neuroprotective role. The data also confirmed that CA alleviated anesthetics-induced neurotoxicity via this pathway, suggesting that CA may be used for the treatment of neurotoxicity caused by anesthetics.

CONCLUSION

CA attenuates inflammation, oxidative stress, as well as mitochondrial dysfunction in neurons treated by isoflurane via AMPK/SIRT1 pathway. Therefore, CA is a potential neuroprotective agent for the treatment of neurotoxicity caused by anesthetics.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ou Liao and Keqi Xie designed the study and carried them out: Ou Liao, Keqi Xie, Xianjie Zhang, Wencai Jiang, Wen Li and An Xie supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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