Neuroprotective effects of vitexin against isoflurane-induced neurotoxicity by targeting the TRPV1 and NR2B signaling pathways

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Abstract. Vitexin is a bioactive compound extracted from hawthorn leaves, which reduces blood pressure and has anti-inflammatory and potential anticancer effects. However, the mechanisms underlying the protective effects of vitexin against isoflurane-induced neurotoxicity remain elusive. Therefore, the aim of the present study was to investigate these mechanisms further. Sprague Dawley rats received 1.4% isoflurane in a 100% oxygen environment for 2 h. Human PC12 pheochromocytoma neurosecretory cells were exposed to 2% isoflurane for 12 h before they were treated with 1, 10 or 100 µM vitexin for a further 24 h. Vitexin inhibited the isoflurane-induced cell cytotoxicity and weakened isoflurane-induced neuroinflammation and oxidative stress pathways in PC12 cells. In addition, treatment with vitexin suppressed isoflurane-induced caspase-3 activation and increased β-secretase 1 levels in PC12 cells. Furthermore, vitexin treatment decreased the levels of isoflurane-induced cytosolic calcium and reactive oxygen species, and down-regulated the expression of transient receptor potential cation channel subfamily V member 1 (TRPV1) and glutamate ionotropic receptor NMDA type subunit 2B (NR2B) protein expression in isoflurane-treated PC12 cells. These results suggest that vitexin mediates its protective effects against isoflurane-induced neurotoxicity by targeting the TRPV1 and NR2B signaling pathways.

Introduction

Since the discovery of inhalation anesthetics and their clinical application, researchers have gained an improved understanding of ‘inhalation anesthetics and inhalation anesthesia’ (1). The advantages and disadvantages of inhalation anesthetics are continuously being elucidated, and inhalation anesthetics with obvious deficiencies subsequently fall into disuse (2). Recently, inhalation anesthesia has become the primary method for general anesthesia (3). Inhalation anesthetics are metabolized and decompose in organisms (4). The majority of inhalation anesthetics can discharge through the lungs in their primary forms. Therefore, they are safe and effective, and have a higher controllability (5). However, researchers are continuously trying to identify safer inhalation anesthetics (6). Isoflurane is a type of halocarbon inhalation anesthetic first identified in the 1970s, which has a high efficiency and controllability, and serves an important role in maintaining the effects of general anesthesia (7,8).

Recent studies investigating the mechanisms of neuron damage and protection in the central nervous system have made significant progress (8,9). These studies have provided information about how calcium channels, the cell membrane potential and various transmitters influence neuron damage and protection (10). Thus, the principles of general inhalation anesthesia may be further elucidated, including the clinical anesthesia phenomenon, and an increased understanding of the unwanted effects of these may help to avoid their side effects.

The state of consciousness is dependent on the electrophysiological characteristics of the central nervous system (11). The mechanisms of general anesthesia by isoflurane involve the hyperpolarization of nerve cells (12). However, a previous study demonstrated that isoflurane can induce virulence in different nerve cells (12). By contrast, the pre-processing of isoflurane by nerve cells has also been shown to exhibit neuroprotective effects, however, the mechanisms involved in this process are currently unclear (13). Ca²⁺ serves an essential role as a signaling molecule in nerve cells. An imbalance can result in excess Ca²⁺, which may be caused by neurocyte injury (14).

The transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor is universally expressed across the central nervous system, including the hippocampus, cerebral cortex and thalamus (15). During isoflurane-induced neurotoxicity, cellular edema resulting from disrupted energy metabolism may activate the TRPV1 receptor by altering the tension of the cytomembrane (16). In addition, lipid dysbolism of the cytomembrane occurs when the brain is ischemic, which increases the levels of free arachidonic acid (17). Oxygen and glucose deprivation, due to pharacoan-mediated TRPV1...
receptor inhibition, leads to neuronal loss in region 1 of the hippocampus proper due to cellular edema, which has protective functions (18).

Glutamate excitotoxicity is considered to be the primary mechanism underlying neuron injury induced by cerebral ischemia (19). When isoflurane-induced neurotoxicity occurs, a large quantity of excitatory neurotransmitters are released from presynaptic membranes due to disruption of metabolic cellular energy and the depolarization of cytomembranes (20). This leads to the elimination of glutamic acid, which accumulates between synaptic clefts. Glutamic acid binds to and activates postsynaptic membranes, resulting in the opening of N-methyl-D-aspartate (NMDA) ion channels, and an excess influx of Ca\(^{2+}\) (21).

The rosaceous hawthorn plant is used as a digestion aid in traditional Chinese medicine (22). Modern pharmacological methods have discovered that flavonoid compounds extracted from Chinese hawthorn leaves can regulate the lipid profile of blood, reduce blood pressure, enhance the outflow volume of the extracorporeal coronary artery, resist oxidation and protect the ischemic myocardium (23). Vitexin is an active compound extracted from hawthorn leaves, which has protective functions during myocardial ischemia (24). A previous study demonstrated that Chinese hawthorn leaves may possess cardio tonic, antianginal, antiarrhythmic and antioxidative properties, and mitigate the effects of acute myocardial ischemia (25). Therefore, the aim of the present study was to investigate whether vitexin may also protect against isoflurane-induced neurotoxicity.

Materials and methods

Rat details. A total of 30 male Sprague Dawley rats (250-300 g) were obtained from the Experimental Animal Center of the Central Hospital of Cangzhou (Hebei, China). The rats were maintained in 12 h dark/light cycles at 23±2°C with 55±5% humidity, and provided with food and water ad libitum. The animal procedures used in this study were approved by the Standing Committee on Animals at The Central Hospital of Cangzhou.

I Hofluran and vitexin treatment of rats and visualization of neuron cells. A total of 30 Sprague Dawley rats were separated at random into the following five equally-sized treatment groups: i) Control; ii) isoflurane-treated; and iii) 1 mg/kg, iv) 3 mg/kg; and v) 10 mg/kg vitexin-treated groups, respectively. The isoflurane and vitexin-treated groups were exposed to 1.4% isoflurane (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a 100% oxygen environment for 2 h. Following isoflurane treatment, the vitexin-treated group additionally received 1, 3 and 10 mg/kg vitexin (Sigma-Aldrich; Merck Millipore) for 30 min. The rats were euthanized using decollation under anesthesia. Samples of rat brain tissue slices were fixed in 10% formalin buffer overnight and then dehydrated using 90% ethanol for 1 h and 100% ethanol for 2 h. They were subsequently cleared with xylene for 2 h and then embedded in paraffin at 60°C.

Cell lines. Human PC12 pheochromocytoma neurosecretory cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 9% heat-inactivated fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Thermo Fisher Scientific, Inc.), and were maintained in an incubator at 37°C in 5% CO\(_2\) and with 95% humidity.

Cell treatment and viability analysis. PC12 cells were seeded at a density of 1x10\(^4\) cells/well in 96-well plates before they were exposed to 2% isoflurane for 12 h and then cultured with 1, 10 and 100 µM vitexin for 24 h. PC12 cells were subsequently incubated at 37°C for 4 h. Dimethyl sulfoxide solution (98%; 150 µl; Sangon Biotech Co., Ltd., Shanghai, China) was then added to each well. The optical density (OD) was read at 570 nm using the Universal Microplate Reader (Elx800; BioTek Instruments, Inc., Winooki, VT, USA).

Enzyme-linked immunosorbent assay (ELISA). PC12 cells were seeded at a density of 1x10\(^4\) cells/well in 96-well plates before they were exposed to 2% isoflurane for 12 h and then treated with 1, 10 and 100 µM vitexin for 24 h. PC12 cells were immediately collected and centrifuged at 4,000 x g for 10 min. ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to measure serum tumor necrosis factor-α (TNF-α; cat. no. R019), interleukin-6 (IL-6; cat. no. R016), glutathione synthetase (GSH; cat. no. A005) and superoxide dismutase (SOD; cat. no. A001-1) concentrations.

Western blot analysis. PC12 cells were seeded at a density of 2x10\(^4\) cells/well in 6-well plates before they were exposed to 2% isoflurane for 12 h and then treated with 1, 10 and 100 µM vitexin for 24 h. PC12 cells were subsequently harvested in RIPA Lysis Buffer with protease inhibitors (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and total protein was extracted by centrifuging at 12,000 x g for 10 min at 4°C, and according to the manufacturer’s instructions. Protein samples (50 µg) were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk diluted in tris-phosphate buffer containing 0.05% Tween 20 for 1 h, and incubated overnight at 4°C with the following primary antibodies: Polyclonal caspase-3 (cat. no. sc-56052; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.); b-secretase 1 (BACE; cat. no. sc-365948; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.); transient receptor potential cation channel subfamily V member 1 (TRPV1; cat. no. PAB27817; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.); glutamate ionotropic receptor NMDA type subunit 2B (NR2B; cat. no. 14544; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA); and GAPDH (cat. no. sc-365062; dilution, 1:10,000; Santa Cruz Biotechnology, Inc.). The membranes were incubated with the anti-mouse or anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody, (cat. nos. SN133 and SN134, respectively; dilution, 1:5,000; Sunshine Biotechnology Co., Ltd., Nanjing, China),
and bands were visualized using an enhanced chemiluminescence method.

**Reactive oxygen species (ROS) measurement.** PC12 cells were seeded at a density of 1x10^4 cells/well in 96-well plates before they were exposed to 2% isoflurane for 12 h and treated with 1, 10 and 100 µM vitexin for 24 h. PC12 cells were cultured with 2',7'-dichlorofluorescein diacetate for 6 h, then incubated with cell lysis buffer (OxiSelect ROS assay kit; Cell Biolabs, Inc., San Diego, CA, USA) for 5 min at room temperature. The OD was read at 480/530 nm using the aforementioned microplate reader (Bio-Tek instruments, Inc.).

**Analysis of cytosolic calcium levels.** The levels of cytosolic calcium were determined as described previously (26). PC12 cells were treated with isoflurane and vitexin using the aforementioned procedures, before they were treated with Pura-2 (Invitrogen; Thermo Fisher Scientific, Inc.) and perfused with Tyrode's buffer. The levels of cytosolic calcium were recorded using a spectrofluoroscopy system (IonOptix, Westwood, MA, USA) at 340/380 nm.

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Experiments for each treatment group was conducted in triplicate. Statistical analyses were performed using the Student's t-test and P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Vitexin protects against isoflurane-induced neurotoxicity in rat brain tissue slices.** The chemical structure of vitexin is shown in Fig. 1. Since isoflurane treatment of rats results in neurotoxicity, the initial aim of this study was to examine the potential neuroprotective effects of vitexin in isoflurane-treated rats. The number of neuron cells in the control group was markedly higher than that of the isoflurane-induced group (Fig. 2A and B). A notable increase in neuron cells was observed in the isoflurane plus vitexin (10 mg/kg)-treated group compared with the isoflurane-treated group (Fig. 2B and E).

**Vitexin increases the growth of isoflurane-treated PC12 cells.** To determine whether the neuroprotective effects of vitexin following isoflurane treatment involves cell growth, the growth of human PC12 pheochromocytoma neurosecretory cells following treatment with isoflurane and vitexin was investigated. As shown in Fig. 3, a significant increase in the growth of PC12 cells was observed following isoflurane plus 10 or 100 µM vitexin treatment, compared with isoflurane treatment alone (P=0.0079 and 0.0021, respectively).

**Vitexin prevents the activation of isoflurane-induced inflammatory signaling pathways.** Considering the key role of induced neuroinflammation in aged rodents (27), the neuroprotective effects of vitexin in inhibiting the level of pro-inflammatory cytokines, TNF-α and IL-6, in isoflurane-treated PC12 cells was investigated using ELISA. As shown in Fig. 4, treatment of PC12 cells with isoflurane plus 10 or 100 µM vitexin, significantly reduced TNF-α (P=0.0088 and 0.0038, respectively) and IL-6 (P=0.0066 and 0.0049, respectively) protein levels compared with isoflurane treatment alone.

**Vitexin protects against the isoflurane-induced increase in oxidative stress.** Considering the key role of oxidative stress in isoflurane-induced PC12 cells, the neuroprotective effects of vitexin against isoflurane-induced GSH and SOD protein expression levels were investigated. As shown in Fig. 5A and B, isoflurane-induced GSH and SOD concentrations in PC12 cells...
were significantly increased following pretreatment of cells with 10 or 100 µM vitexin (GSH, P=0.0069 and 0.0033; SOD, P=0.0059 and 0.0025 for 10 and 100 µM vitexin, respectively). In addition, as shown in Fig. 5C, PC12 cells treated with isoflurane plus 10 and 100 µM vitexin, demonstrated a significant reduction in ROS levels compared with isoflurane-treated PC12 cells (P=0.0041 and 0.0018, respectively).

**Vitexin protects against isoflurane-induced caspase-3 activation.** In order to investigate the neuroprotective effects of vitexin against isoflurane-induced caspase-3 activation, the protein expression levels of caspase-3 in isoflurane and vitexin-treated PC12 cells were determined using western blot analysis. Caspase-3 protein expression levels in isoflurane-treated PC12 cells were significantly reduced following treatment with 10 and 100 µM vitexin (P=0.0069 and 0.0033, respectively; Fig. 6).

**Vitexin protects against the isoflurane-induced increase in BACE protein expression.** A previous study demonstrated that BACE promotes amyloid beta peptide production and affects the inhibition of hypomnesis (28). Therefore, the neuroprotective effect of vitexin against isoflurane-induced BACE levels was investigated by evaluating BACE protein expression levels in isoflurane plus vitexin-treated PC12 cells using western blot analysis. As demonstrated in Fig. 7, treatment of PC12 cells with 10 or 100 µM vitexin following exposure to isoflurane, was associated with a significant reduction in BACE protein expression levels (P=0.0042 and 0.0018, respectively).

**Vitexin protects against the isoflurane-induced increase in cytosolic calcium levels.** To investigate the role of vitexin in preventing the isoflurane-induced increase in cytosolic calcium levels, the levels of cytosolic calcium in isoflurane-induced PC12 cells were significantly reduced following treatment with 10 and 100 µM vitexin (P=0.0031 and 0.0009, respectively).

**Vitexin protects against the isoflurane-induced increase in TRPV1 expression levels.** In order to further investigate the neuroprotective role of vitexin in isoflurane-treated PC12 cells, TRPV1 protein expression levels were examined in isoflurane-treated PC12 cells following treatment with vitexin using western blot analysis. As shown in Fig. 9, treatment with 10 and 100 µM vitexin significantly suppressed TRPV1 protein expression in isoflurane-treated PC12 cells (P=0.0023 and 0.0005, respectively).
Vitexin protects against the isoflurane-induced increase in NR2B expression levels. The NR2B subunit is a fundamental regulatory subunit of the NMDA receptor and serves an important role in its structure and function (29). Therefore, the final aim of the study was to determine whether NR2B protein expression is involved in mediating the neuroprotective effects of vitexin in isoflurane-treated PC12 cells. As shown in Fig. 10, treatment of isoflurane-induced PC12 cells with 10 and 100 µM vitexin significantly suppressed isoflurane-induced NR2B protein expression levels (P=0.0045 and 0.0012, respectively).

Discussion

Consistent with other inhaled anesthetics of the halogen family, isoflurane-mediated hyperpolarization of neurocytes decreases the excitability of the neural network (30). Inhalation anesthetics at low concentrations can noticeably inhibit the function of nicotinic receptors (31). In the present study, vitexin visibly increased the number of neuron cells in isoflurane-treated rats. In addition, vitexin increased the growth and reduced isoflurane-induced TNF-α, IL-6, GSH and SOD levels in isoflurane-treated PC12 cells. Using a rat pup model, Min et al. (22) demonstrated that vitexin reduces hypoxia-ischemia neonatal brain injury. Consistent with these observations, Dong et al. (32) demonstrated that vitexin protects against myocardial ischemia/reperfusion injury through attenuating the inflammatory response. Furthermore, Borghi et al. (33) observed that vitexin inhibits inflammation-associated pain through TRPV1 and oxidative stress.

Inhalation anesthetics used at concentrations higher than the clinical range may induce the following effects: Inhibition of the voltage susceptibility of Na+, K+ and Ca2+; thereby reducing the transmission of harmful ostensive stimuli; promote the hyperpolarization of the cell membranes; relieve the overload of Ca2+; analgesia; anesthesia; and protective cerebral functions (34). Whether neurocytes can be protected against neurotoxicity, and the potential molecular and physiological mechanisms involved, has drawn substantial attention (35). A previous study demonstrated that isoflurane can induce cytotoxicity in different neurocytes at different concentrations and exposure times (36). The results of the present study demonstrated that vitexin significantly downregulated caspase-3 and BACE protein expression levels, and reduced ROS and cytosolic calcium levels in isoflurane-induced PC12 cells. Yang et al. (23) provided evidence to suggest that vitexin protected the PC12 cells against 20 h of reoxygenation-induced
injury, through a reduction in ROS production and caspase 3/7 activities.

Ca$^{2+}$ influx, mediated by the voltage-gated calcium channel, is an important mechanism for activating the presynaptic membrane (37). The TRPV4 receptor is a type of Ca$^{2+}$channel (24). A previous study reported that isoflurane-induced neurotoxicity in rats was associated with increased TRPV4 protein expression, which may have led to the over-activation of the TRPV4 receptor (38). Furthermore, a TRPV4-mediated Ca$^{2+}$ influx was observed. Treatment with a TRPV4 receptor agonist was associated with increased Ca$^{2+}$ and enhanced excitability of cells. An increase in the cellular excitability and the extension of depolarization may be responsible for increasing Ca$^{2+}$ influx further (39). Through the influx of Ca$^{2+}$, the TRPV4 receptor activity may promote the release of presynaptic glutamic acid. Moreover, additional receptors, such as the metabotropic glutamate receptor and nicotine acetylcholine receptor, also participate in regulating the release of glutamic acid from the presynaptic membrane (40). In the present study, vitexin significantly suppressed the expression levels of TRPV1 protein in isoflurane-induced PC12 cells. In addition, Borghi et al. (33) demonstrated that vitexin inhibits inflammatory pain by targeting TRPV1 and oxidative stress in mice.

In the nervous tissues of mammals, the functional N-methyl-D-aspartate (NMDA) receptor consists of NR1 and NR2 subunits (41). The NR2B subunit is a fundamental regulatory subunit of the NMDA receptor and serves an important role in its structure and function (29). Phosphorylation of NR2B may increase the opening rate and time of the NMDA receptor, resulting in increased ion influx (42). Selective NR2B subunit inhibitors prevent hypotonic-stimulated enhancement of NMDA receptor activity whereas, selective NR2A subunit inhibitors do not demonstrate any obvious alterations of the NMDA receptor following hypotonic stimulation (43). This suggests that the NR2B subunit may be an important target for the regulation of the NMDA receptor through the TRPV4 receptor (44). In the present study, vitexin significantly suppressed isoflurane-induced NR2B protein expression levels in PC12 cells. Consistent with these observations, Yang et al. (45) reported that vitexin inhibited NMDA receptor activity in cultured cortical neurons.

In conclusion, the present study demonstrated that vitexin mediates neuroprotective effects against isoflurane-induced neurotoxicity by targeting the TRPV1 and NR2B signaling pathways. This suggests that vitexin may be a strong candidate as a neurotoxicity drug. However, these results require validation in further studies.

References


