Hypaconitine confers protection on ketamine-induced neuronal injury in neonatal rat brain via a mechanism involving PI3K/Akt/Bcl-2 pathway

Junbao Liu, Yubo Hu, Linlin Li, Longyun Li*
Department of Anesthesiology, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, China

*For correspondence: Email: OscarqHarriswy@yahoo.com; Tel: 0086-431-84997222

Original Research Article

Abstract

Purpose: To investigate the neuroprotective effect of hypaconitine against ketamine-induced neuronal injury in the brains of neonatal rats, and the underlying mechanism of action.

Methods: Seven day-old Sprague-Dawley pups weighing 15.0 to 20.0 g (mean weight = 17.5 ± 2.5 g), and aged 7 days were used for this study. The pups were sacrificed, and their forebrains isolated and used to prepare cell suspensions. The isolated cells were treated with ketamine (100 µM) or varied concentrations of hypaconitine (0.1 – 2 µM) or LY294002 (10 µM). The cells were trypsinized and cultured at 37 °C in 10 % fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM) in a humidified incubator containing 5 % CO₂. Cell viability was determined using MTT assay, while TUNEL assay was used to determine the extent of apoptosis in the cells. The expressions of p-Akt, Bcl-2 and caspase-3 were determined using Western blotting.

Results: There were only few viable cells in the ketamine-treated group, and cell viability was significantly and dose-dependently increased in hypaconitine-treated groups (p < 0.05). The extent of apoptosis was significantly higher in ketamine-treated cells than in control cells, but treatment with hypaconitine significantly reduced the number of apoptotic cells (p < 0.05). However, in the presence of LY294002 (a PI3K-specific inhibitor), the effect of hypaconitine on neuronal cell apoptosis was significantly reversed (p < 0.05). The expressions of p-Akt and Bcl-2 were significantly down-regulated while the expression of caspase-3 was significantly upregulated in ketamine-treated neuronal cells, when compared with control group (p < 0.05). However, in cells treated with hypaconitine, the expressions of p-Akt and Bcl-2 were significantly upregulated, while the expression of caspase-3 was significantly down-regulated (p < 0.05). Treatment of neuronal cells with hypaconitine in the presence of LY294002 significantly reversed the effect of hypaconitine on the expressions of p-Akt, Bcl-2 and caspase-3 (p < 0.05).

Conclusion: These results suggest that hypaconitine ameliorates ketamine-induced neuronal injury in neonatal rats via a mechanism involving the PI3K/Akt/Bcl-2 pathway.

Keywords: Hypaconitine, Anesthesia, Apoptosis, Neuroprotection, Expression
INTRODUCTION

Ketamine is a dissociative drug used clinically as an anesthetic in infants during surgical procedures [1]. It is an antagonist of non-competitive N-methyl-D-aspartate (NMDA) receptor, and studies have shown that its use during surgeries causes neuronal injury in neonatal rats [2]. It induces apoptosis in neuronal cell culture in vitro [3]. Exposure of children below 4 years to ketamine causes emotional and behavioral disorders [4]. Ketamine-induced apoptosis is the commonest mechanism involved in neurodegeneration [5]. In neonates and pediatric patients, ketamine is used for the induction of anesthesia, but with a number of cautions. Thus protection against ketamine-induced neuronal injury is required in the development of alternative neuroprotective agents [6]. The past few decades saw the emergence of novel drugs from natural sources with potent neuroprotective effects against ketamine induced neuronal injury [7-9].

Aconite tuber is used in Traditional Chinese Medicine (TCM) as an analgesic, and also for treating cardiovascular dysfunction, neurological disorders, rheumatoid arthritis, fever, and as an analgesic [10-13]. This tuber contains several bioactive compounds such as hypaconitine, mesaconitine and aconitine, which are alkaloids reported to confer protection on the heart and central nervous system (CNS) [14-16]. Although these alkaloids are also toxic to some organs and tissues, hypaconitine is a less toxic analogue of aconitine and exhibits it good cardiotoxic property [17]. The present study investigated the neuroprotective effect of hypaconitine against ketamine-induced neuronal injury in brains of neonatal rats, and the underlying mechanism.

EXPERIMENTAL

Materials and reagents

Sprague-Dawley pups were procured from Changzhou Cavens Laboratory Animal Co., Ltd. Microplate reader (ELX-800) was purchased from Bio-Tek Instruments (USA). Bicinchoninic acid (BCA) assay kit was a product of Sangon Biotech Co., Ltd., while chemiluminescence liquid and autoradiography film were purchased from Bio-Rad Laboratories Inc. (USA). Trizol reagent was a product of Thermo Fisher Scientific Inc. (USA).

Experimental rats

Sprague-Dawley pups weighing 15.0 to 20.0 g (mean weight = 17.5 ± 2.5 g), and aged 7 days were used for this study. The pups were maintained under standard conditions of care, and were exposed to 12 h light/dark cycles and maintained at 25 °C and 48 % humidity. The study protocol was approved by The Institutional Animal Care and Use Committee of China-Japan Union Hospital of Jilin University, China (approval no. IACUC/C-JUH/JU/2017/05).

Experimental design

The Sprague–Dawley pups were sacrificed. Their forebrains were isolated and used for preparing cell suspensions. The cells were trypsinized and cultured at 37 °C in 10 % FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) in a humidified incubator containing 5 % CO₂. After 48 h of incubation, the medium was replaced with neurobasal medium supplemented with B27. The medium was changed every two days until the cells attained 80 % confluency.

MTT assay

Cell viability was determined using ation of cell viability. The cells at exponential growth phase were seeded at a density of 2 x 10⁵ cells/well in 96-well plates containing DMEM. After 24 h of incubation, the cells were treated with ketamine (100 µM) or varied concentrations of hypaconitine (0.1 – 2 µM) or LY294002 (10 µM), and cultured for 72 h. This was followed by the addition of 10 µl of 5 mg/ml MTT solution within 4 h, after which the culture medium was changed. Dimethylsulfoxide (DMSO, 200 µl) was added in drops to each well, and the wells were placed on an oscillator for 10 min to completely dissolve the formazan crystals. The control wells contained culture medium, MTT solution and DMSO only, and were treated same way as the sample wells. Each well was incubated in the dark for 2 h and absorbance was measured at 570 nm using synergy II microplate reader. The procedure was performed in triplicate and cell viability was calculated.

TUNEL assay

This was used to determine the extent of apoptosis in neuronal cells. The isolated neuronal cells were washed with phosphate-buffered saline (PBS) and fixed with ice-cold paraformaldehyde (4 %). Endogenous peroxidase activity was quenched at room temperature within 30 min, and the cells were further permeabilized with 0.1 % Triton X-100 in 0.1 % sodium acetate for 5 min at 4 °C. The cells were labelled by treating them with TUNEL reaction mixture at 37 °C for 2 h. Dark brown precipitate was formed on incubating the cells
with diaminobenzidine substrate (DAB). The number of TUNEL-positive cells was determined using Olympus light microscope. Apoptotic index (A) was calculated as in Eq 1.

\[ A = \frac{T}{C} \]  

where \( T \) and \( C \) are no. of TUNEL-positive nuclei and total no. of total cell nuclei, respectively.

**Western blotting**

The cells were washed with PBS and ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor was used to lyse them. The resultant lysate was centrifuged at 12000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (20 - 30 μg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. The blots were incubated overnight at 4 °C with primary antibodies for Akt, p-Akt, Bcl-2, caspase-3 and β-actin at a dilution of 1:500. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an x-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

**Statistical analysis**

Data are expressed as mean ± SEM (n = 10). Statistical analysis was performed using GraphPad prism (6.1). Groups were compared using Duncan multiple test range. Values of \( p < 0.05 \) were considered statistically significant.

**RESULTS**

**Effect of hypaconitine on the viability of neuronal cells**

There were only few viable cells in ketamine-treated group. However, cell viability was significantly and dose-dependently increased in hypaconitine-treated groups (\( p < 0.05 \); Figure 1).

**Effect of hypaconitine on neuronal cell apoptosis**

As shown in Figure 2, the extent of apoptosis was significantly higher in ketamine-treated cells than in control cells, but treatment with hypaconitine significantly reduced the number of apoptotic cells (\( p < 0.05 \)). However, in the presence of LY294002 (a PI3K-specific inhibitor), the effect of hypaconitine on neuronal cell apoptosis was significantly reversed (\( p < 0.05 \)).

**Effect of hypaconitine on the expressions of p-Akt, Bcl-2 and caspase-3**

The expressions of p-Akt and Bcl-2 were significantly down-regulated, and the expression of caspase-3 was significantly upregulated in ketamine-treated neuronal cells, when compared with control group (\( p < 0.05 \)). However, in cells treated with hypaconitine, the expressions of p-Akt and Bcl-2 were significantly upregulated, while the expression of caspase-3 was significantly down-regulated (\( p < 0.05 \)).
Treatment of neuronal cells with hypaconitine in the presence of LY294002 significantly reversed the effect of hypaconitine on the expressions of p-Akt, Bcl-2 and caspase-3 (p < 0.05). These results are shown in Figure 3.

Viability of neuronal cells after treatment with ketamine, hypaconitine and LY294002

Ketamine significantly reduced viability of neuronal cells, when compared with control group, but the number of viable cells were significantly increased in the hypaconitine-treated group (p < 0.05). However, in the presence of LY294002, the effect of hypaconitine on ketamine-induced decreases in viability of neuronal cells was significantly reversed (p < 0.05; Figure 4).

DISCUSSION

Ketamine, an analgesic, is widely used for induction of anesthesia during surgery [1]. It is reported to cause neuronal injury in the developing brain [2]. The present study investigated the neuroprotective effect of hypaconitine on ketamine-induced neurodegeneration in brains of neonatal rats, and the underlying mechanism. Ketamine promotes apoptosis in neuronal cells via the down-regulation of p-Akt expression and blockage of N-methyl-D-aspartate (NMDA) receptor [18]. In this study, there were only few viable cells in ketamine-treated group, but cell viability was significantly and dose-dependently increased in the hypaconitine-treated groups, an indication that hypaconitine may protect neuronal cells against ketamine-induced neurodegeneration.
The PI3K pathway plays a key role in the survival of neuronal cells: it activates the phosphorylated form of protein kinase B (p-Akt) [19]. The results of this study showed that treatment with hypaconitine significantly reduced the number of apoptotic cells. However, PI3K-specific inhibitor (LY294002) significantly reversed the effect of hypaconitine on neuronal cell apoptosis. Caspase-3 and Bcl-2 proteins play important roles in the induction of apoptosis [20].

In this study, the expressions of p-Akt and Bcl-2 were significantly down-regulated, while the expression of caspase-3 was significantly upregulated in ketamine-treated neuronal cells, when compared with control group. However, in hypaconitine-treated cells, the expressions of p-Akt and Bcl-2 were significantly upregulated, while the expression of caspase-3 was significantly down-regulated. Treatment of neuronal cells with hypaconitine in the presence of LY294002 significantly reversed the effect of hypaconitine on the expressions of p-Akt, Bcl-2 and caspase-3. These results suggest that LY294001, when used in combination with hypaconitine may reverse hypaconitine-enhanced viability of neuronal cells.

CONCLUSION

The results obtained in this study suggest that hypaconitine ameliorates ketamine-induced neuronal injury in neonatal rats via a mechanism involving the PI3K/Akt/Bcl-2 pathway.

DECLARATIONS

Acknowledgement

The authors acknowledge the support from China-Japan Union Hospital of Jilin University, Changchun, Jilin, China. This study was financed by China-Japan Union Hospital of Jilin University, Changchun, Jilin, China.

Conflict of interest

No conflict of interest is associated with this work. The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the report for publication.

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. The manuscript was written by Junbao Liu, Yubo Hu and Linlin Li collected materials and did statistical analysis. The whole study was designed and supervised by Longyun Li.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/rationale), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES


Trop J Pharm Res, September 2019; 18(9): 1843


