Collapsin Response Mediator Protein-2 Ameliorates Sevoflurane-Mediated Neurocyte Injury by Targeting PI3K-mTOR-S6K Pathway

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Background: Collapsin response mediator protein-2 (CRMP-2) is the first member of the CRMP family that has been identified in primary neuronal cells; it was originally found and identified in the regulation of microtubule dimerization into microtubules.

Material/Methods: In the present study, we aimed to investigate the roles and mechanisms of CRMP-2 in sevoflurane-induced neurocyte injury. Cell viability, proliferation, and apoptosis were measured by Cell Counting Kit-8 (CCK-8) assay and flow cytometry. Colorimetry was performed to measure the activity of caspase-3. Western blot and quantitative real-time reverse transcription assays were used to evaluate the related mRNAs and proteins expression.

Results: We found that CRMP-2 reversed the inhibitory effect of sevoflurane on the viability of nerve cells. Moreover, CRMP-2 accelerated the proliferation and suppressed the apoptosis of sevoflurane-induced nerve cells. CRMP-2 modulated the expression levels of apoptosis-associated protein in sevoflurane-induced nerve cells. Furthermore, it was demonstrated that CRMP-2 impacted the PI3K-mTOR-S6K pathway.

Conclusions: CRMP2 ameliorated sevoflurane-mediated neurocyte injury by targeting the PI3K-mTOR-S6K pathway. Thus, CRMP2 might be an effective target for sevoflurane-induced neurocyte injury therapies.

MeSH Keywords: Neurocysticercosis • Phosphatidylinositol 3-Kinases • TOR Serine-Threonine Kinases

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Background

Sevoflurane (SEV) was first discovered by Terrell and was synthesized by Regan in 1968 [1,2]. It is well known that SEV is commonly used in pediatric anesthesia [3]. However, at present, the cytoprotective effect or toxicity effect of SEV on pediatric patients remains controversial. A previous investigation demonstrated that SEV ameliorated the cognitive functions of 4- to 5-month-old rats [4]. It has also been reported that SEV affected the spatial reference memory and spatial working memory of P7 rats [5]. Another study found that repeated exposure to volatile anesthetics, such as isoflurane and SEV, enhanced the GABA receptor activity and increased glutamate levels during subsequent neurodevelopmental stages, suggesting that it is a compensatory response [6,7]. However, the accurate pathogenesis of SEV-induced neurocyte injury is still unclear.

The PI3K/mTOR/S6K pathway, a crucial signaling pathway, combines inputs from at least 5 major intracellular and extracellular cues, involving amino acids, oxygen, energy status, stress, and growth factors, which further modulates major progressions such as lipid and protein synthesis and autophagy [8]. It has been demonstrated previously that the PI3K/mTOR pathway participated in the progression of anticancer, chemosensitization, and radiosensitization [9]. Additionally, the PI3K/mTOR pathway has been proven to be critical for hepatocyte proliferation and the apoptosis of breast cancer cells [10,11]. However, there has been limited investigations regarding the effects of the PI3K/mTOR/S6K pathway on the nerve cells induced by SEV.

Collapsin response mediator proteins (CRMPs) represent a novel family of microtubule-associated proteins (MAPs), which consist of 5 highly conserved phosphorylated proteins (CRMP1-5) [12]. CRMP-2 is the first member of the CRMPs family that has been identified in primary neuronal cells, and it was originally found and identified in the regulation of microtubule dimerization into microtubules [13]. Recently, CRMP-2 expression has also been found in non-neuronal cells such as leukocytes [14], fibroblasts [15], and neuroblastoma [16]. In addition, it has been suggested that CRMP-2 could modulate neuronal viability through the PI3K/mTOR/S6K pathway [17]. However, the exact roles and mechanisms of CRMP-2 in the protection of SEV-induced neurocyte injury have not been studied yet.

In the current research, we analyzed the correlation between CRMP-2 and the neurocytes injury induced by SEV. Furthermore, it was also fascinating to explore the exact roles and mechanisms of CRMP-2 and PI3K/mTOR/S6K pathway in the protection of SEV-induced nerve cells.

Material and Methods

Animals

Pregnant Sprague-Dawley (SD) rats (2 months old, pregnancy cycle 18 days, weight 180–200 g) were kept in cages at 22±1°C. The rats had free access to food and water. The light/dark cycle of the animal house was 12 hours/12 hours, and the light time was 8 am to 8 pm. The animals used for experimental research had been approved by the ethics committee of the Hanzhong Central Hospital (Shaanxi, China), and the associated permit number was 170621.

Acquisition of hippocampal neuron cells

The following extraction and cultivation methods have been described in detail in previous articles [18,19]. The pregnant SD rats were anesthetized by ether, and the fetus removed. The fetuses were sterilized in culture dishes which contained 75% ethanol. The heads were cut using an animal breaker (Ugo Basile, #7950; Guangzhou, Guangdong, China). Afterwards, the hippocampus was removed and digested with 0.125% trypsin (Beyotime, Shanghai, China) for 10 min. The supernatant was discarded, and then Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) was added followed by gently shaking. The obtained nerve cells were then maintained in a 5% CO₂ atmosphere at 37°C. The morphology of the cells was observed under the TMM-220 inverted microscope (TX; Shanghai, China).

Cell transfection and grouping

The CRMP-2 mRNA (GTCTTCACCACCATGGAGAAGGCTGG) was cloned into pcDNA3.1(+) empty vector (Invitrogen, Carlsbad, CA, USA). Nerve cells were transfected with pcDNA3.1(+) CRMP-2 and pcDNA3.1(+) plasmids by lipofectamine 2000 (Sxbio, Shanghai, China). There were 6 treatment groups included in the current study: the control group (nerve cells with no treatment), the NC group (nerve cells transfected with empty vector), the CRMP-2 group (nerve cells transfected with CRMP-2), the SEV group (nerve cells treated with 3% SEV (Sealia Precision; Wuhan, Hubei, China) mixed gas for 12 hours) [20], the NC+SEV group (nerve cells transfected with empty vector, and then treated with 3% SEV), the CRMP-2+SEV group (nerve cells transfected with CRMP-2, and then treated with 3% SEV mixed gas for 12 hours), and the CRMP-2+SEV group (nerve cells transfected with CRMP-2, and then treated with 3% SEV mixed gas for 12 hours).

Cell viability analysis

Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) assay was performed to measure cell viability. About 6×10⁴ cells/mL of nerve cells in the logarithmic phase were seeded into 96-well plates and maintained in an incubator (37°C, 5% CO₂) for 12
hours. After that, cells were treated as previously described. Cells were then maintained in the incubator (37°C, 5% CO₂) for 6 hours, 12 hours, and 24 hours, respectively. Then 10 μL of CCK8 reagent was added to each well. Cells were then maintained with 5% CO₂ at 37°C for 3 hours. Microplate reader (Bio-Rad Laboratories, Inc., USA) was used to read the absorbance at 450 nm. Cell viability was evaluated by the percentage of cell survival compared with the control.

**Flow cytometry (FCM)**

Cultured nerve cells were treated with 0.25% trypsin (Beyotime). The supernatant was removed, and the nerve cells were suspended in the incubation buffer at a density of 1×10⁵ cells/mL. Nerve cells were then maintained with Annexin V-FITC and propidium iodide (PI) (XilongScientific) in the dark at room temperature for 15 min. Finally, FACSCalibur (BD Biosciences, San Diego, CA, USA) was performed to measure cell proliferation and apoptosis.

**Western blot analysis**

After cell treatments, the cells were washed with PBS 3 times, and then lysed by RIPA (Balb.biomart.cn; Beijing, China). Protein concentration was measured by BCA protein assay kit (Enyi; Shanghai, China). Protein lysates were separated in electrophoretic fluid by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% SDS-PAGE) using electrophoresis tank (Baygene; Beijing, China) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blotting was carried out on specific antibodies (anti-CRMP-2 1: 10 000, Abcam, ab129082 rabbit anti-rat; anti-activated-caspase-3 1: 500, Abcam, ab13847 rabbit anti-rat; anti-Bax 1: 1000, Abcam, ab32503 rabbit anti-rat; anti-Bcl-2 1: 1000, Abcam, ab59348 rabbit anti-rat; anti-synapsin-I 1: 1000, Abcam, ab18814, rabbit anti-rat; anti-p-mTOR 1: 1000, Abcam, ab84400, rabbit anti-rat; anti-mTOR 1: 2000, Abcam, ab2732, rabbit anti-rat; anti-p-S6K 1: 1000, Abcam, ab59348, rabbit anti-rat; anti-p-S6 1: 1000, Abcam, ab109393, rabbit anti-rat; anti-S6 1: 1000, Abcam, ab131526, rabbit anti-rat; anti-actin 1: 5000, Abcam, ab179467, rabbit anti-rat; anti-GAPDH 1: 2500, Abcam, ab9485, rabbit anti-rat). Horseradish peroxidase-conjugated secondary antibodies (1: 1000, bs-0293M; BIOSS, Beijing, China) were then added in the incubator and held at 4°C. Actin or GAPDH were used as control of the input RNA level. The primers used in this analysis were designed by Invitrogen (Shanghai, China) and were as follows: CRMP-2, forward: 5′-GGCTGTGGGGAAGGATAACT-3′ and reverse: 5′-CTTCACACTGTGAGGTCC-3′ (product: 233 bp); caspase-3, forward: 5′-TGTCATGACGCTGCACT-3′ and reverse: 5′-CGAGTAGTGGCCCTGAAAGA-3′ (product: 241 bp); Bax, forward: 5′-GAGACACCTGGATGACCTTT-3′ and reverse: 5′-CGTCTGAAACATGGTCAGCT-3′ (product: 187 bp); Bcl-2, forward: 5′-AAGCTTCAAGGATGGGT-3′ and reverse: 5′-GTGCTGAGCTGATTGCTG-3′ (product: 209 bp); actin, forward: 5′-CAACATGGATGAGCGGAAGG-3′ and reverse: 5′-GCAGTGTAGCAGCATCGAAA-3′ (product: 233 bp); GAPDH, forward: 5′-AGTCTACGGCGTCTTCC-3′ and reverse: 5′-CCACGATGCAAAATGGTGTA-3′ (product: 225 bp). The formula 2−ΔΔCT was executed to count the expression levels of each mRNA.

**Statistical analysis**

IBM SPSS statistics 20 software was used in the statistical analysis of this study. Results of our study are shown as mean ±SD of at least 3 independent experiments. The experimental data was analyzed by one-way analysis of variance (ANOVA), Kruskal-Wallis, and Tukey’s test. The statistical significance was defined as P<0.05.

**Results**

**Authentication of the rat primary-cultured hippocampal neurons**

In the present study, the hippocampus was separated from SD fetal rats and the primary nerve cells were cultured with DMEM medium containing 10% FBS. After incubating for 48 hours, the neurons were observed by inverted microscope. As shown by the arrowhead (Figure 1A), 2 to 3 protuberances could be observed in each nerve cell, and the length of protuberances was longer than before. Moreover, a fraction of glial cells with flat polygonal shape were observed (Figure 1A). Nevertheless, after incubating for 6 days, as shown by the arrowhead, the morphology...
Figure 1. Identification of the rat neurocytes. Primary cultured rat neurocytes for 2 days (A) and 6 days (B) were observed by inverted fluorescence microscope. Magnification 200×; n=3.

Figure 2. Sevoflurane (SEV) restrained the cell viability of nerve cells, and collapsin response mediator protein-2 (CRMP-2) was overexpressed in nerve cells transfected with CRMP-2. (A) CCK-8 assay was carried out to measure the cell viability of nerve cells treated with different concentration of SEV mixed gas (0.5%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%). qRT-PCR (B) and western blot (C) assays were performed on the expression level of CRMP-2 in nerve cells, nerve cells transfected with empty vector, nerve cells transfected with CRMP-2, nerve cells treated with 3% SEV mixed gas, nerve cells transfected with empty vector and then treated with SEV, and nerve cells transfected with CRMP-2 and then treated with SEV. * P<0.05 and ** P<0.01 versus NC; ## P<0.01 versus NC+SEV; n=3.
of nerve cells was more typical. The protuberances of nerve cells linked into a dense network and the cell bodies were fuller. Visible halo could be noted around the nerve cells (Figure 1B). The nerve cells were harvested and used for the later studies.

The cell viability of SEV-induced nerve cells SEV was enhanced by CRMP-2

In order to confirm the accurate concentration of SEV that was used to establish the neurocyte injury model, we evaluated the cell viability of nerve cells treated with different concentration of SEV mixed gas. According to the CCK-8 data, it was noted that cell viability of nerve cells was evidently reduced when the concentration of SEV mixed gas was over 3.0% (Figure 2A). Hence, we selected the 3.0% SEV mixed gas as the concentration of SEV that was used to establish the neurocyte injury model in our study. Furthermore, the CRMP-2 overexpression plasmid, together with its empty vector, was used in the current investigation. qRT-PCR and western blot results indicated that compared with other groups, the expression level of CRMP-2 in nerve cells transfected with CRMP-2 was significantly upregulated (Figure 2B, 2C). To further explore whether CRMP-2 impacted the cell growth of nerve cells, the cell viability of nerve cells treated with CRMP-2 together with its empty vector and 3.0% SEV mixed gas

Figure 3. Collapsin response mediator protein-2 (CRMP-2) enhanced the cell viability of nerve cells suffered from sevoflurane (SEV). CCK-8 assay was carried out to measure the cell viability of nerve cells, nerve cells transfected with empty vector, nerve cells transfected with CRMP-2, nerve cells treated with 3% SEV mixed gas, nerve cells transfected with empty vector and then treated with SEV, and nerve cells transfected with CRMP-2 and then treated with SEV. * P < 0.05 versus NC; # P < 0.01 versus NC+SEV; n=3.

Figure 4. The cell proliferation of sevoflurane (SEV)-induced nerve cells was promoted by transfecting with collapsin response mediator protein-2 (CRMP-2). Flow cytometry was performed on the cell proliferation of nerve cells, nerve cells transfected with empty vector, nerve cells transfected with CRMP-2, nerve cells treated with 3% SEV mixed gas, nerve cells transfected with empty vector and then treated with SEV, and nerve cells transfected with CRMP-2 and then treated with SEV. * P<0.05 versus NC; * P<0.05 versus NC+SEV; n=3.
was measured. As the CCK-8 data showed, compared with other groups, SEV treatment inhibited cell viability of nerve cells. However, in the groups that were transfected with CRMP-2, the cell viability of SEV-induced nerve cells was enhanced (Figure 3). These results suggested that CRMP-2 possessed the ability to accelerate the cell viability of nerve cells induced by SEV.

**CRMP-2 accelerated the proliferation of SEV-induced nerve cells**

CRMP-2 reversed the ability of SEV to inhibit the viability of neural cells according to CCK-8 data, we thereby conjectured that CRMP-2 might also affect the cell proliferation of nerve cells suffered from SEV treatment. Hence, flow cytometry (FCM) was carried out to assess the proliferation capacity of nerve cells from each treatment group. The FCM results revealed that the proliferation number of nerve cells was significantly reduced by SEV treatment. However, the proliferation number of nerve cells were distinctly increased in CRMP-2+SEV group (Figure 4). These results suggested that SEV reduced the proliferation capacity of nerve cells, while CRMP-2 could evidently promote the cell proliferation of SEV-induced nerve cells. Thus, it was determined that CRMP-2 accelerated the proliferation of nerve cells induced by SEV.

**CRMP-2 suppressed the SEV-mediated apoptosis of nerve cells**

Additionally, we also evaluated the apoptosis ability of nerve cells treated with CRMP-2 and SEV. Based on the FCM data, the percentage of apoptosis nerve cells in the SEV group was 24.57%, which was markedly higher than the control group (6.90%). Nevertheless, when the cells were co-processed by CRMP-2 and SEV, the apoptosis rate of SEV-induced nerve cells was decreased from 28.44% to 17.21% (Figure 5). According to the FCM results, we noted that the percentage of apoptosis nerve cells in the CRMP-2+SEV group was distinctly lower than that of the SEV+NC group, which suggested that CRMP-2 could lessen the apoptosis capacity of SEV-induced nerve cells.

**CRMP-2 modulated the expression levels of apoptosis-associated protein in SEV-induced nerve cells**

In order to further investigate the molecular mechanism of CRMP-2 inhibition of SEV-induced neuronal apoptosis, the apoptosis-related factors were assessed. We first detected the activity of caspase-3 in nerve cells from all of the treatment groups. It was revealed that SEV obviously strengthened the caspase-3 activity in nerve cells, while CRMP-2 could distinctly reduce...
the caspase-3 activity in SEV-induced nerve cells (Figure 6A). Moreover, the mRNA and protein levels of apoptosis-associated proteins, involving caspase-3, B-cell lymphoma 2-associated x protein (Bax), and B-cell lymphoma 2 (Bcl-2) in nerve cells were measured in the current investigation. On the basis of qRT-PCR data, we found that the expression levels of caspase-3 and Bax in nerve cells treated with SEV were obviously upregulated. However, in the groups that were transfected with CRMP-2, decreases in the expression levels of caspase-3 and Bax in SEV-induced nerve cells were evidently observed. Moreover, it was also revealed that SEV treatment markedly lessened the Bcl-2 expression in nerve cells, while CRMP-2 transfection distinctly upregulated the Bcl-2 expression in SEV-induced nerve cells (Figure 6B). At the same time, western blot data also showed similar trends of caspase-3, Bax, and Bcl-2 expression in nerve cells from the treatment groups (Figure 6C). Based on these findings it was confirmed that CRMP-2 suppressed the apoptosis of SEV-induced nerve cells through modulating the expression levels of caspase-3, Bax, and Bcl-2.

### CRMP-2 impacted the PI3K-mTOR-S6K pathway

On the basis of cell proliferation and apoptosis results, we demonstrated that CRMP-2 promoted the proliferation capacity and suppressed the apoptosis of nerve cells induced by SEV. In order to further investigate the accurate mechanisms of CRMP-2 on the protection of nerve cells against SEV, we detected the related signaling pathway. Western blot results suggested that the expression levels of synapsin-I, p-mTOR, p-S6K, and p-S6 were markedly downregulated by treating with SEV. Nevertheless, when cells were treated with SEV and CRMP-2 together, evident increases in synapsin-I, p-mTOR, p-S6K, and p-S6 expression were observed, relative to the NC+SEV group (Figure 7). However, no significant difference was observed in the expression levels of mTOR, S6K, and S6 in nerve cells from all of the treatment groups. Thus, it was affirmed that CRMP-2 could enhance the phosphorylation of mTOR, S6K, and S6 in nerve cells induced by SEV, which indicated that CRMP-2 could impact the PI3K-mTOR-S6K pathway in nerve cells treated with SEV.
The hippocampus is the limbic system of the brain, and it is related functions of the central nervous system such as learning and memory [21,22]. Meanwhile, hippocampal tissue is composed of a large number of neural stem cells, with structure of neat, relatively independent cells. Therefore, the model of cultured hippocampal neurons in vitro is considered an ideal experimental model. The hippocampus of rodents, especially newborn mice (24 hours), is easy to locate and removed and thus is often used [23–25]. Hence, we extracted hippocampal neurons cells from neonatal rats as a model.

Recent studies have shown that exposure to clinically relevant doses of narcotic drugs, such as isoflurane and SEV, can cause nerve structural disorder in rats, change hippocampal synapses to reduce the density of dendritic spines in prefrontal cortex of rats, decrease the expression of related proteins involved in the development of connections and axons, causing cortical axons needle disorder [26–28]. SEV anesthesia has been proven to led to more death of hippocampal neurons [29]. In addition, SEV has been used as an inducer to successfully construct a model of nerve cell injury, which makes cell proliferation decrease and apoptosis increase [30]. Hence, in the present study, the hippocampal neurons separated from 18-day SD fetal rats were chosen to establish the SEV-induced neurocyte injury model. Similarly, we found that SEV could markedly inhibit the proliferation of hippocampal neurons and promote apoptosis.

Because CRMP-2 has been suggested to possess multiple functions in the modulation of hippocampal neurons growth, we thereby selected CRMP-2 as the object of our study on SEV-induced neurocyte injury [31]. After transfecting with CRMP-2 and its empty vector, the data indicated that CRMP-2 could reduce the viability of SEV-suppressed nerve cells. Additionally, some researchers have found that CRMP-2 involves the development of the nervous system, as well suppresses apoptosis of various cancer cells [16,32–34]. Hence, we suspected that CRMP-2 also plays a role in the apoptosis

Discussion

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of nerve cells. Our results showed that CRMP-2 obviously suppressed the apoptosis of SEV-induced nerve cells. Furthermore, the related apoptosis factors were also investigated in our study. According to the experimental data, we found that CRMP-2 distinctly downregulated the expression levels of caspase-3 and Bax, while enhancing the Bcl-2 expression in SEV-induced nerve cells. These results suggested that CRMP-2 might suppress the apoptosis of SEV-induced nerve cells by modulating the expression levels of caspase-3, Bax, and Bcl-2.

Previous investigations have confirmed that the PI3K-mTOR-S6K pathway plays a crucial role in the proliferation and apoptosis of tumor cells [35]. Furthermore, recent studies have confirmed that CRMP-2 regulates neuronal growth via controlling the PI3K-mTOR-S6K pathway [17]. Thus, the roles and mechanisms of the PI3K-mTOR-S6K pathway in the proliferation and apoptosis of SEV-induced nerve cells affected by CRMP-2 were explored in our research. Based on the western blot data, it was noted that CRMP-2 significantly upregulated the expression level of synapsin-I and enhanced the phosphorylation of mTOR, S6K, and S6 in nerve cells induced by SEV. However, no significant difference was observed in the expression levels of mTOR, S6K, and S6 in nerve cells. Hence, we could affirm that CRMP-2 might modulate the proliferation and apoptosis of SEV-induced nerve cells via the regulation of the PI3K-mTOR-S6K pathway.

Altogther, our study suggested that CRMP-2 alleviated neurocyte injury induced by SEV through the modulation of the PI3K-mTOR-S6K pathway. And the study results provide a new thread for understanding the pathogenesis of neurocyte injury and afford a fascinating approach for the therapy of neurocyte injury.

Conclusions

Collectively, our current work highlights that CRMP-2 alleviated neurocyte injury induced by SEV through the modulation of the PI3K-mTOR-S6K pathway. These findings have a momentous impact on the understanding of the mechanisms of CRMP-2 in rat nerve cells. It indicates that CRMP-2 might be an effective target for the therapy of neurocyte injury.

Conflict of interest

None.

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