The mechanism of CaMK2α-MCU-mitochondrial oxidative stress in bupivacaine-induced neurotoxicity

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Abstract

Ca^{2+}/calmodulin dependent protein kinase2α (CaMK2α) is a serine/threonine protein kinase in neurons and leads to neuronal injury when it is activated abnormally. Bupivacaine, a local anesthetic commonly used in regional nerve block, could induce neurotoxicity via apoptotic injury. Whether or not CaMK2α is involved in bupivacaine-induced neurotoxicity and it is regulated remains unclear. In this study, bupivacaine was administered for intrathecal injection in C57BL/6 mice for building vivo injury model and was used to culture human neuroblastoma (SH-SY5Y) cells for building vitro injury model. The results showed that bupivacaine induced mitochondrial oxidative stress and neurons apoptotic injury, promoted phosphorylation of CaMK2α and cAMP-response element binding protein (CREB), and elevated mitochondrial Ca^{2+} uniporter (MCU) expression. Furthermore, it induced CaMK2α phosphorylation at Thr286 which phosphorylated CREB at Ser133 and up-regulated MCU transcriptional expression. Inhibition of CaMK2α-MCU signaling with knock-down of CaMK2α and MCU or with inhibitors (KN93 and Ru360) significantly mitigated bupivacaine-induced neurotoxic injury. Over-expression of CaMK2α significantly enhanced above oxidative injury. Activated MCU with agonist (spermine) reversed protective effect of siCaMK2α on bupivacaine-induced mitochondrial oxidative stress. Our data revealed that CaMK2α-MCU-mitochondrial oxidative stress pathway is a major mechanism whereby bupivacaine induces neurotoxicity and inhibition of above signaling could be a therapeutic strategy in the treatment of bupivacaine-induced neurotoxicity.

Keyword CaMK2α, MCU, Mitochondrial oxidative stress, Bupivacaine, Neurotoxicity

Abbreviations:

CaMK2α, Ca^{2+}/calmodulin dependent protein kinase2α; CREB, cAMP-response element binding protein; MCU, mitochondrial Ca^{2+} uniporter; PBS, phosphate buffer saline; mtROS, mitochondrial reactive oxygen species; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; MMP, mitochondrial membrane potentials;

1. Introduction

Mitochondria, the key organelle of biological oxidation and energy metabolism, produce ATP via oxidative phosphorylation to provide most of the energy needed for cell survival [1]. In addition, mitochondria are also involved in signal transduction, cell growth and differentiation and apoptosis [2, 3]. Mitochondrial dysfunction is closely related to many kinds of human diseases, including neurodegenerative disease, mitochondrial disease and cardiac dysfunction [4-6]. Mitochondria are the main production site of reactive oxygen species (ROS) in cells. Under normal conditions, the appropriate level of ROS can be used as a signal molecule to regulate cell physiological function [7]. However, under stress, the interference of oxidative phosphorylation leads to inhibition of ATP production and ROS over-production which attacks mitochondria resulting in mitochondrial oxidative stress, membrane potential decreased, caspase activation and apoptotic cell injury [8].

With the focus on accelerated rehabilitation therapy, regional nerve block, as one of the key technologies, has attracted much attention [9]. Perioperative neurological injury complications were related to local anesthetic-induced neurotoxicity, such as transient neurologic syndrome and equina nerve syndrome [10, 11]. Studies have shown that local anesthetics-induced nerve damage is positively correlated with concentration and action time [12]. Bupivacaine is one of the most commonly used local anesthetic in clinic [13]. Previous study has confirmed that bupivacaine could uncouple the mitochondrial oxidative phosphorylation, inhibits respiratory chain complexes I and III and enhance ROS production [14]. Bupivacaine induces ROS over-production which activates caspase-3, poly ADP-ribose polymerase degradation and triggers apoptosis in the Schwann cell line [15]. The regulation mechanism of bupivacaine-induced oxidative phosphorylation imbalance, ROS over-production and mitochondrial oxidative stress is not clear.

Calcium/calmodulin dependent protein kinase 2 (CaMK2) is a multifunctional serine/threonine protein kinase, which is encoded by 4 different genes and includes about 12 subunits [16]. CaMK2α subunit is abundant in nervous systems [17].

CaMK2 can be activated by Ca^{2+} and calmodulin, resulting in autophosphorylation. CaMK2 phosphorylation activates signaling molecules and several other transcription factors including cAMP-response element binding protein (CREB) [18, 19]. CaMK2 induces oxidative stress to participate in pathophysiological process and results in cell damage [20]. However, the mechanism whereby CaMK2 mediates mitochondrial oxidative stress remains unclear. Mitochondrial calcium uniporter (MCU), an important structure in mitochondrial intima, is involved in mitochondrial Ca^{2+} uptake which plays a vital role in cells growth and metabolism via regulating mitochondrial ROS (mtROS) generation, oxidative phosphorylation and mitochondrial apoptosis pathway [21-23]. When over-activated, MCU could lead to cell injury and death [24]. It has confirmed that MCU is involved in glutamate-induced neurotoxicity, and inhibition of MCU activity can protect the mitochondrial function and subsequently reduce the neuron death [25]. Ca^{2+} signaling regulates mitochondrial metabolism via Ca^{2+}-dependent activation of CREB which controls MCU expression and mitochondrial Ca^{2+} uptake capability [26]. Whether CaMK2α controls MCU function via activating CREB signaling and mediates mitochondrial oxidative stress has not been reported.

In this study, models of bupivacaine-induced neurotoxicity in spinal cord of C57BL/6 mouse and human neuroblastoma (SH-SY5Y) cells line were established. It is predicted that bupivacaine could induce mitochondrial oxidative stress though phosphating CaMK2α which activates CREB-MCU signaling. This study may elucidate the mechanism of CaMK2α in mediating mitochondrial oxidative stress and its role in bupivacaine-induced neurotoxicity.

2. Material and Method

2.1. Antibodies and Reagents

Anti-CaMK2α (sc-13141), anti-phospho-CaMK2α (sc-12886) and anti-β-actin (sc-47778) were from Santa Cruz Biotechnology; anti-CREB (ab31387), anti-phospho-CREB (ab194687), anti-MCU (ab219827) and anti-COXIV (ab202554) were from Abcam. Anti-cleaved caspase-3 (9661) was from Cell Signaling

Technology; Bupivacaine hydrochloride (B5247), Mito-TEMPO (SML0737), Ru360 (557440), dimethyl sulfoxide (DMSO) (156914), KN93 (422708) and spermine (S4264) were from Sigma-Aldrich; Secondary antibodies were from Jackson ImmunoResearch.

2.2. Study Approval and Groups Assignment of Mice.

The study was performed in accordance with the guide for the Care and Use of Laboratory Animals (NRC1996) and was approved by the Animal Research Center of Southern Medical University (protocol number: SYXK-2016-0167, Guangzhou, China). Animal experiments were conducted in male C57BL/6 mice (8-9 weeks old, 18-21g) from Animal Research Center of Southern Medical University. For building bupivacaine-induced mouse spinal cord injury model, 0.75% bupivacaine was administered for intrathecal injection twice at an interval of 1 hour. The method of intrathecal injection was performed as previously described [27]. 0.75% bupivacaine hydrochloride (dissolved in 0.9% saline), 45 nM KN93 (dissolved in 0.9% saline, concentration application according to previous study [28]) or 0.9% saline was injected intrathecally at the L_5-L_6 intervertebral space using a 25 gauge needle. The volume of the single intrathecal injection was 5 µl.

For determining bupivacaine-induced neurotoxicity in spinal cord, mice were divided into four groups: group sham (mice of intrathecal injection 0.9% saline 5µl for twice at an interval of 1 hour, n= 6), group KN93 (mice of intrathecal injection 45 nM KN93 5 µl and 0.9% saline 5µl at an interval of 1 hour, n= 6), group Bup (mice of intrathecal injection 0.75% bupivacaine 5µl for twice at an interval of 1 hour, n= 6), and group KN93+Bup (mice of intrathecal pre-injection 45 nM KN93 5µl and intrathecal injection 0.75% bupivacaine 5µl for twice at an interval of 1 hour, n= 6). Following, all mice spinal reflex function was tested in different times (before drugs injection, 1^{th} day after drugs injection, 2^{th} day after drugs injection and 4^{th} day after drugs injection). Mice spinal cord tissues on drugs injection 4^{th} day (about 5 mm intumescentia lumbalis) were prepared as described [29].

2.3. Spinal Reflex Function Testing

Spinal reflex function was tested by evaluating mice withdrawal reflex responses to mechanical and thermal stimulation. Mechanical stimulation was determined by probing the plantar aspect of the hind paw with calibrated von Frey filaments for calculating the paw withdrawal threshold (PWT, g) according to the method as previously described [30]. Thermal stimulation was measured using the tail flick latency test which measures the tail withdrawal latency (TWL, s) from a heat source [31]. Latencies baseline was 2-3 s and cutoff time was 15 s for preventing tail injury.

2.4. Cells Culture, Small Interfering RNA Transfection and Lentiviral Vector Infection.

SH-SY5Y cell line was purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in Dulbecco´s Modified Eagle´s Medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 50 U/ml penicillin and 50 g/ml streptomycin solution (Gibco, Grand Island, NY) in an incubator with 5% CO_2 at 37 °C. The medium was changed every 2 days and cells were split using Trypsin-EDTA solution (Gibco, Grand Island, NY). CaMK2α siRNAs (sc-29900), MCU siRNAs (sc-90666) and silencer negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Transfection was performed according to manufacturer instructions. Lentiviral vector encoding CaMK2α gene with puromycin resistance (Ubi-MCS-3FLAG-CMV-EGFP) was from Genechem Company (Shanghai, China). Viruses were amplified and titrated in SH-SY5Y cells according to manufacturer's instructions. Lentiviruses containing empty plasmids (null) was used as negative control.

2.5. Malondialdehyde (MDA) and 8-Hydroxydeoxyguanosine (8-OHdG) using ELISA in Mice Spinal Cord

Spinal cord tissues DNA was extracted by using DNeasy Blood & Tissue Kit (69506, Qiagen, Germany) according to manufacturer instructions. MDA and 8-OHdG levels were determined using an ELISA kit (CSB-E08559m and CSB-E10527m, CUSABIO, China). Chemiluminescent absorbance was measured with a microplate reader (Bio-Rad, USA). MDA and 8-OHdG concentration per microgram

of protein for each group was calculated.

2.6. Cell Viability Assay

Cells viability was determined with the MTT assay (ST316, Beyotime Biotechnology, China). Spent medium was removed and 10 µl MTT solution (5 mg/ml) was added to 100 µl of respective growth medium without phenol red, and plates were incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. Next, formazan crystals formed by mitochondrial reduction of MTT were solubilized in DMSO (100 µl/well) and absorbance was read at 540 nm using a microplate reader (BioRad, Hercules, CA).

2.7. Detection of mtROS

Measurement of mtROS was performed using MitoSOX (M36008, Invitrogen, CA). MitoSOX selectively reacts with superoxide in the mitochondria and is used to measure specific mitochondrially generated ROS. Briefly, after cultured in six-well plates and treated for the mtROS measurement, cells were incubated with 5 µM MitoSOX for 15 min, then washed with phosphate buffer saline (PBS). The concentration of MitoSOX at 5 uM was chosen based on previous study conducted in SH-SY5Y cells [32], and our preliminary experiments which showed that the suitability to catch oxidative stress in our experimental settings. Measurements were performed using flow cytometry at excitation/emission wavelengths of 510/580 nm.

2.8. Western Blot Assay

The methods of cell lysates, measurement of protein concentration, electrophoresis and immunoblotting were performed as described previously [33]. They were immunoblotted with anti-CaMK2α (1:500), anti-phospho-CaMK2α (Thr286) (1:500), anti-CREB (1:1000), anti-phospho-CREB (Ser133), anti-MCU (1:500), anti-cleaved caspase-3 (1:500), or anti-β-actin (1:1,000) diluted in blocking solution containing 5% nonfat dry milk and 0.1% Tween-20 in Tris-HCl-buffered saline overnight at 4 °C. After rinsed, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin at 1:1,000 for 1 h. Band densities were measured using a densitometer and analyzed with Quantity One analysis

software (Bio-Rad, Hercules, CA). MCU and cleaved caspase-3 protein expression were normalized to corresponding β-actin bands. CaMK2α or CREB activation was determined by the ratio of pCaMK2α to CaMK2α or pCREB to CREB. All data is converted into folds to group control.

2.9. Immunofluorescent Staining

The method of immunofluorescence staining for detecting pCaMK2α nuclear expression and co-localization of mitochondria and MCU was performed as described [34]. After drugs treatment, cells were washed with PBS and fixed with 4% paraformaldehyde, followed permeabilization with 0.2% Triton X-100. After blocked with 10% goat serum, cells were incubated with anti-pCaMK2α (1:200), or anti-MCU (1:200) and anti-COXVI (1:200), followed exposure to fluorescent labeled secondary antibodies and DAPI. Next, cells were photographed by LSM imaging software (Zeiss, Oberkochen, Germany). Thirty cells were randomly selected from each group in three independent experiments for analyzing densities of overlay in MCU and COXVI in every cell with Image J (https://imagej.nih.gov/ij/). The same method was used for determining pCaMK2α nuclear translocation. Folds to group NC of densities was calculated for representing mitochondrial MCU expression of each group and pCaMK2α nuclear translocation.

2.10. ATP Production Detection

ATP level was detected using ATP Assay Kit (S0026, Beyotime Biotechnology, China) according to manufacturer instructions. After drugs treatment, cells were lyzed and centrifuged. Next, 100 μl ATP reagent was added into each well for 5 min. After 20 μl sample added to the each well, RLU value was determined by luminometer. ATP concentration was calculated according to the standard curve.

2.11. Mitochondrial Membrane Potentials (MMP) Assay

MMP loss was measured by JC-1 assay kit (T-3168, Life Technologies Corporation, USA) as described [35]. After drugs treatment, cells were washed with PBS and incubated with 1 ml JC-1(10 μg/ml) staining solution for 20 min. Finally, MMP was detected by flow cytometry (BD FACS Calibur, BD Biosciences, USA).

Mitochondrial depolarization was represented with a decrease in ratio of red to green fluorescence intensity.

2.12. Apoptosis Assay by Flow Cytometry

Apoptotic cells were measured using Annexin V-FITC Apoptosis Detection Kit (KGA106, KeyGEN, China) as previously described [36]. Briefly, cells were rinsed using PBS and resuspended in 500 μl binding buffer after bupivacaine treatment. Annexin V-FITC 1.25 μl and propidium iodide 10 μl was added to cell suspension. After incubation for 10 min, apoptotic cell was determined by flow cytometry (BD FACS Calibur, BD Biosciences, USA). Apoptosis are Annexin V-FITC positive and PI-negative.

2.13. Mitochondrial Ultrastructure with Transmission Electron Microscopy.

After treatment, cells were washed with PBS, fixed in 2.5% glutaraldehyde at 4◦ C for 1h, postfixed with 1% osmic acid for 30 min, and stained with lead uranium. Mitochondrial ultrastructure was observed with a transmission electron microscope (Hitachi-600, Japan).

2.14. Statistical Analysis

Statistical analysis was conducted using SPSS soft-ware 13.0 (SPSS Inc., Chicago, IL). Data are presented as means ± standard of the means (SDs). Statistical differences of multiple groups were calculated by multiple comparisons with variance analysis, followed by Turkey's post hoc test. Differences between two groups were calculated by two tailed unpaired or paired Student´s t test. Statistical significance was set at $P< 0.05$.

3. Results

3.1. Inhibition of CaMK2α attenuated bupivacaine-induced mice spinal inflex dysfunction.

KN93, an inhibitor of CaMK2 (chemical name, N-[2-[[[(2E)-3-(4-chlorophenyl)-2-propen-1-yl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy-phosphate), was reported to inhibit CaMK2 phosphorylation and improve spinal cord injury in mice neuropathic pain [28]. So, bupivacaine or KN93 were administrated

with intrathecal injection to determine the role of CaMK2α on bupivacaine-induced mice spinal inflex dysfunction. KN93 dosage was administrated as described previously [28]. As showed in Fig.1A &1B, compared with sham group, the PWT and TWL values were significantly increased on the 1^{th}, 2^{th} and 4^{th} day in group Bup ($P< 0.05$); Compared with pre-drug (baseline), PWT and TWL values in group Bup were significantly increased on the 1^{th}, 2^{th} and 4^{th} day ($P< 0.05$). Compared with sham group, the PWT and TWL values were not significantly different on the 1^{th}, 2^{th} and 4^{th} day in group KN93 ($P> 0.05$). As compared with group Bup, the PWT and TWL values were significantly decreased on the 1^{th}, 2^{th} and 4^{th} day in group KN93+Bup ($P< 0.05$). And, the PWT and TWL values of group KN93+Bup were not significantly different on the 2^{th} and 4^{th} day as compared with pre-drug ($P> 0.05$). The results suggested that CaMK2α be involved in bupivacaine-induced mice spinal inflex dysfunction.

3.2. Inhibition of CaMK2α down-regulated MCU expression and attenuated bupivacaine-induced oxidative damage in mice spinal cord

As showed in Fig.2A-2F, compared with group sham, phosphorylation of CaMK2α and CREB and MCU expression were significantly increased in group Bup ($P< 0.05$). Compared with group Bup, phosphorylation of CaMK2α and CREB and MCU expression were significantly decreased in group KN93+Bup ($P< 0.05$). Malondialdehyde (MDA) and 8-Hydroxydeoxyguanosine (8-OHdG) were detected with ELISA for investigating oxidative damage in mice spinal cord. Cleaved caspase-3 was measured with western blotting for detecting spinal cord apoptotic injury. As showed in Fig.2G-2J, compared with group sham, the expression of MDA, 8-OHdG and cleaved caspase-3 were significantly increased in group Bup ($P< 0.05$). As showed in Fig.1F& 1G, compared with group Bup, the expression of MDA, 8-OHdG and cleaved caspase-3 were significantly decreased in group KN93+Bup ($P< 0.05$). The results suggested that bupivacaine induce mice spinal cord oxidative injury via activating CaMK2α-CREB-MCU signaling.

3.3. Bupivacaine activated CaMK2α-CREB-MCU signaling and inhibition of

CaMK2α-MCU-mitochondrial oxidative stress attenuated bupivacaine-induced cells injury.

As showed in Fig.3A&3B, compared with group control (baseline), cell viability was significantly reduced in different doses bupivacaine treatment (0.5 mM, 2.0 mM or 8.0 mM) for 6 h and different times (6 h, 12 h or 24 h) after 2.0 mM bupivacaine treatment ($P< 0.05$). As showed in Fig.3C-3F, compared with group control, CaMK2α and CREB phosphorylation and MCU expression were significantly elevated in 6 h, 12 h or 24 h after 2.0 mM Bupivacaine treatment ($P< 0.05$). As showed in Fig.3H & 3I, compared to cells with bupivacaine treatment only, cells viability was significantly elevated in cells precultured with different concentration KN93 or Ru360 (an inhibitor of MCU) and bupivacaine treatment ($P< 0.05$). At the same time, mito-TEMPO (a mitochondria-targeted antioxidant) was used to confirm the role of mitochondrial oxidative stress in bupivacaine-induced neurotoxic injury and the results showed that it could protect cells against above injury (Fig.3G, $P< 0.05$).

3.4. siCaMK2α inhibited bupivacaine-induced activation of CREB-MCU signaling.

We knocked down CaMK2α expression with a specific small interfering RNA (siCaMK2α) for determining the its role in bupivacaine-induced neurotoxicity. As showed in Fig.4A&4B, compared with group control after siRNA transfection, CaMK2α expression were significantly decreased in group siCaMK2α ($P< 0.05$). As showed in Fig.4C&4D, compared with group NC, nuclear translocation of pCaMK2α was significantly elevated in group Bup ($P< 0.05$); Compared with group Bup, nuclear translocation of pCaMK2α was significantly decreased in group siCaMK2α +Bup ($P< 0.05$). As showed in Fig.4E-4G, compared with group NC, CREB phosphorylation and MCU expression were significantly decreased in group siCaMK2α ($P< 0.05$), but significantly increased in group Bup ($P< 0.05$); Compared with group Bup, CaMK2α phosphorylation and MCU expression were significantly decreased in group siCaMK2α+Bup ($P< 0.05$).

3.5. siCaMK2α attenuated bupivacaine-induced mitochondrial oxidative damage

and cells apoptotic injury.

Mitochondrial membrane potentials (MMP) loss was detected by JC-1 assay kit. As showed in Fig.5, bupivacaine caused significant increase of mtROS (Fig. 5A&5B) that was concomitant with a significant reduction in ATP production and MMP (Fig. 5C-5E) and increases in apoptotic cell death (Fig. 5F&5G), while these adverse changes caused by bupivacaine were significantly attenuated by siCaMK2α (all $P< 0.05$, siCaMK2α+Bup vs. Bup). Spermine, an agonist of MCU [37], was used for further determination of the role of MCU in CaMK2α-mediated mitochondrial oxidative stress. Compared with group siCaMK2α+Bup, ATP production inhibition, mtROS over-production and cell apoptosis were significantly aggravated in group siCaMK2α+sp+Bup ($P< 0.05$). The results demonstrated that siCaMK2α could inhibit bupivacaine-induced mitochondria oxidative stress and apoptosis, and spermine reduced protective effect of siCaMK2α on bupivacaine-induced neurotoxicity. It should be noted that despite MitoSOX is among the most commonly used probe for detecting cellular ROS, in particular mitochondrial oxidant stress, in experimental settings, high concentration of MitoSOX may potentially cause mitochondrial dysfunction or result in non mitochondrial specific detection [38]. Furthermore, we observed mitochondrial ultrastructure with transmission electron microscopy and found that siCaMK2α could protect cells against bupivacaine-induced mitochondrial damage such as mitochondria with vacuoles and loss of internal membrane structure.

3.6. Over-expression of CaMK2α elevated activation of CREB-MCU signaling and enhanced bupivacaine-induced cells apoptotic injury.

Lentiviral vector expressing CaMK2α gene was used to infect SH-SY5Y cells. As showed in Fig.6A&6B, CaMK2α expression was not different significantly between group Con and group null ($P> 0.05$); Compared to group null, CaMK2α expression was significantly increased in group LV-CaMK2α ($P< 0.05$). As showed in Fig.6C-6F, compared to group Bup, CaMK2α and CREB phosphorylation, and MCU expression were significantly increased in group LV-CaMK2α+Bup ($P< 0.05$). As showed in Fig.6G-6I, compared to group Bup, elevation of mtROS and cleaved caspase-3

expression were significantly enhanced in group LV-CaMK2α+Bup ($P< 0.05$).

3.7. Inhibition of MCU with Ru360 or with siMCU attenuated bupivacaine-induced mitochondrial oxidative damage and apoptotic injury.

We knocked down MCU expression with siMCU and found that it inhibited bupivacaine-induced up-regulation of mitochondrial MCU expression ($P< 0.05$, Fig. 7A-7D). Ru360 or siMCU were used to confirm the role of MCU in bupivacaine-induced neurotoxicity. Bupivacaine-induced ATP production inhibition, mtROS over-production and cell apoptosis were significantly attenuated by siMCU and Ru360 ($P< 0.05$, Fig. 7E-7H).

4. Discussion

The transient neurological syndrome and the cauda equina syndrome may occur after local anesthesia, especially spinal anesthesia. Above-mentioned symptoms are closely related to continuous spinal anesthesia or some patients suffer from hypersensibility of the local anesthetic-induced neurotoxicity [11, 39]. Accumulating evidence has demonstrated that ROS over-production plays a crucial role in local anesthetic-induced oxidative stress and cells apoptosis [14, 15]. However, the related mechanism of regulation pathway remains unclear. In this study, bupivacaine-induced mice spinal cord injury model was established by intrathecal injection of 0.75% bupivacaine twice to imitate clinical anesthesia performance. The results showed that continuous administrating bupivacaine could induce mice spinal cord oxidative damage and spinal reflex dysfunction. At the same time, CaMK2α phosphorylation and MCU expression were elevated in the above model. KN93, a selective inhibitor of CaMK2, could attenuate CaMK2-mediated neuronal cell injury [28, 40]. Here, KN93 improved bupivacaine-induced mice spinal cord oxidative damage and spinal reflex dysfunction, and decreased CaMK2α phosphorylation and MCU expression. This suggested that CaMK2α-MCU signaling be involved in bupivacaine-induced spinal cord oxidative damage. Next, the model of bupivacaine-induced neurotoxicity in SH-SY5Y cells was established. The results demonstrated that bupivacaine increased phosphorylation of CaMK2α, decreased of ATP production, and increased mtROS

over-production and cells injury. KN93 and mito-TEMPO could restore cells viability via improving above abnormal changes. How could CaMK2α regulate mitochondrial oxidative stress in bupivacaine-induced neurotoxicity?

Oxidative stress is thought to be the main cause of neuronal death in neurodegenerative diseases [41]. MCU is an important cell element that transports Ca^{2+} into mitochondria, which plays an important buffer role in maintaining the homeostasis of Ca^{2+} in physiological state [22]. Under stress, over-activation of MCU can lead to mitochondrial Ca^{2+} overload, interfere with oxidative phosphorylation, ROS over-production, decrease of mitochondrial membrane potential and the release of cytochrome c, which results in apoptosis [42]. We have confirmed that high glucose could enhance bupivacaine-induced neurotoxicity via elevating MCU expression and mitochondrial Ca^{2+} accumulation [33]. Whether CaMK2α mediate mitochondrial oxidative stress via regulating MCU signaling in bupivacaine-induced neurotoxicity remains unknown. Here, MCU expression was increased in bupivacine-induced neurotoxicity and Ru360 could restore SH-SY5Y cells viability. Ru360 or siMCU could inhibit bupivacaine-induced mitochondrial oxidative stress and cells apoptotic injury. Down-regulation of CaMK2α expression could lead to decrease of MCU expression and inhibition of mitochondrial oxidative stress. Up-regulation of CaMK2α expression could enhanced bupivacaine-induced increase of MCU expression and mitochondrial oxidative stress. To determine MCU role in CaMK2α-mediated mitochondrial oxidative stress, spermine, an agonist of MCU, was used to pretreat SH-SY5Y cells transfected with siCaMK2α. The results showed that spermine could weaken siCaMK2α protective effect on bupivacaine-induced mitochondrial oxidative damage. Above evidence confirmed that CaMK2α mediated mtROS over-production and mitochondrial oxidative stress via MCU signaling. In the current study, we mainly wanted to explore the role of MCU in CaMK2α-mediated mitochondrial oxidative stress. Future study is needed to directly measure the changes of mitochondrial Ca^{2+} during neurons exposed to bupivacaine to confirm role of mitochondrial Ca^{2+} in bupivacaine-induced oxidative injury.

CREB, location in the nucleus, is a member of the nuclear transcription factor family. Under stress, CREB is activated and promotes transcription and expression of related genes by phosphorylated at Ser133 [43]. Ca^{2+} signal transduction system is activated to enhance CaMK2 phosphorylation. Activated CaMK2 promotes CREB phosphorylation and enhances its gene transcriptional function [44]. Whether CaMK2α could promote MCU expression via enhancing CREB function remains unclear. Here, we found that CREB phosphorylation was elevated in bupivacaine-induced SH-SY5Y cells oxidative damage. Inhibition of CaMK2α activity with siCaMK2α or with KN93 could reduce CREB phosphorylation and MCU expression. Up-regulation of CaMK2α activity with lentiviral vector infection could enhance CREB phosphorylation and MCU expression. Those results demonstrated that MCU gene transcription was regulated by CaMK2α-CREB signaling in bupivacaine-induced neurotoxicity. Oxidative stress reportedly triggers formation of the mitochondrial permeability transition pore (mPTP) which is a channel modulated by cyclophilin D (CypD) and related to mitochondrial dysfunction [45]. Study confirms that MCU is involved in modulating mPTP pore opening via regulating mitochondrial Ca^{2+} influx [46]. Although elevation of mitochondrial Ca^{2+} is the crucial trigger for mPTP formation, oxidative stress has also been implicated in regulating it opening [47]. So, the regulation of CypD-mPTP and MCU in bupivacaine-induced mitochondrial oxidative stress needs to be further explored in future studies.

It should be noted that bupivacaine is more widely used for spinal anesthesia than the other local anesthetics [11]. Severe neurological complications such as cauda equina syndrome were associated with bupivacaine spinal anesthesia which application concentration is 0.5%-0.75% [48]. So, we developed bupivacaine-induced mice spinal cord injury model with intrathecal injection of 0.75% bupivacaine, which was the maximal clinical concentration [48]. However, in SH-SY5Y cells injury model, bupivacaine treatment concentration was 2 mM, which was equal to 0.06% clinical concentration. According to previous study, if clinical concentration of bupivacaine is used to treat SH-SY5Y cells, almost all cells will be killed [15]. At the

same time, the axons in the nerve roots of the cauda equina suffer from the brunt of bupivacaine injection which is greater than the cell experimental concentration. So, bupivacaine-induced neurotoxicity can be confirmed by the above concentration.

5. Conclusions

In summary, our study has confirmed that CaMK2α was activated and promoted MCU transfer function via phosphorylating CREB. CaMK2α mediated mitochondrial oxidative stress via controlling MCU function in bupivacaine-induced neurotoxicity. So, inhibition of CaMK2α-MCU signaling might be instrumental in combating bupivacaine-induced neurotoxicity.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends:

Fig.1. Inhibition of CaMK2α with KN93 attenuated bupivacaine-induced mice spinal inflex dysfunction. Experiments were conducted in male C57BL/6 mice (8-9 weeks old, 18-21g) and the volume of the single intrathecal injection was 5 μl. sham: mice of intrathecal injection 0.9% saline 5μl twice at an interval of 1 hour; KN93: mice of intrathecal injection 45 nM KN93 5μl and 0.9% saline 5μl at an interval of 1 hour; Bup: mice of intrathecal injection 0.75% bupivacaine 5μl twice at an interval of 1 hour; KN93+Bup: mice of intrathecal injection 45 nM KN93 5μl for 30 min before intrathecal injection 0.75% bupivacaine 5μl twice at an interval of 1 hour. **A, B**: PWT and TWL were tested in different times (before drugs injection, 1^{th} day after drugs injection, 2^{th} day after drugs injection and 4^{th} day after drugs injection). Values are the mean± SD of n = 6; *: $P< 0.05$ compared with pre-drug; #: $P< 0.05$ compared with the group Bup; &: $P< 0.05$ compared with the group sham.

Fig.2. Inhibition of CaMK2α with KN93 attenuated bupivacaine-induced mice spinal cord oxidative injury. sham: mice of intrathecal injection 0.9% saline 5μl twice at an interval of 1 hour; KN93: mice of intrathecal injection 45 nM KN93 5μl and 0.9% saline 5μl at an interval of 1 hour; Bup: mice of intrathecal injection 0.75%

bupivacaine 5μl twice at an interval of 1 hour; KN93+Bup: mice of intrathecal injection 45 nM KN93 5μl for 30 min before intrathecal injection 0.75% bupivacaine 5μl twice at an interval of 1 hour. **A-H**: CaMK2α phosphorylation, MCU and cleaved caspase-3 expression were measured with western blotting on 4th day after drugs injection; Values are the mean± SD of n = 6; *: $P< 0.05$. **I, J**: MDA and 8-OHdG were tested with ELISA on 4th day after drugs injection; Values are the mean± SD of n = 6; *: $P< 0.05$.

Fig.3. Bupivacaine induced SH-SY5Y cells damage and activated CaMK2α-CREB-MCU signaling. **A**: cells viability was assessed using the MTT assay after treated with bupivacaine in different concentration (0.5 mM, 2.0 mM or 8.0 mM) for 6 h; Values are the mean± SD of n = 6; *: $P< 0.05$ compared with untreated cells; #: $P< 0.05$ compared with the cells treated with 2.0 mM Bupivacaine. **B**: cells viability was assessed using the MTT assay in different times (6 h, 12 h or 24 h) after 2.0 mM bupivacaine treatment; Values are the mean± SD of n = 6; *: $P< 0.05$ compared with untreated cells; #: $P< 0.05$ compared with group 12 h. **C-F**: CaMK2α and CREB phosphorylation and MCU expression were determined with western blotting in different times (6 h, 12 h or 24 h) after cells treated with 2.0 mM bupivacaine for 6 h. Values are the mean± SD of n = 3; *: $P< 0.05$ compared with untreated cells; #: $P< 0.05$ compared with group 12 h. **G**: cells viability was assessed using the MTT assay in cells precultured with different concentration (5, 25 or 100 nM) mito-TEMPO for 60 min or/and treated with 2.0 mM bupivacaine for 6 h; **H**: cells viability was assessed using the MTT assay in cells precultured with different concentration (5, 10 or 20 μM) Ru360 for 30 min or/and treated with 2.0 mM bupivacaine for 6 h; **I**: cells viability was assessed using the MTT assay in cells precultured with different concentration (1, 5 or 25 μM) KN93 for 30 min or/and treated with 2.0 mM bupivacaine for 6 h; Con: untreated cells; Values are the mean± SD of n = 6; *: $P< 0.05$.

Fig.4. Inhibition of CaMK2α with siCaMK2α could inhibit bupivacaine-induced activation of CaMK2α-CREB and elevation of MCU expression. NC: cells transfected

with silencer negative control siRNA; siCaMK2α: cells transfected with CaMK2α siRNA; Bup: cells transfected with silencer negative control siRNA and treated with 2.0 mM bupivacaine for 6 h; siCaMK2α+Bup: cells transfected with CaMK2α siRNA and treated with 2.0 mM bupivacaine for 6 h. **A, B**: expression of CaMK2α after cells transfected with CaMK2α siRNA or silencer negative control siRNA; **C, D**: Detection of pCaMK2α nuclear translocation with immunofluorescence nuclear localization; Bar: 10 μm. **E-G**: CREB phosphorylation and MCU expression were determined with western blotting. Values are the mean± SD of n = 3; *: $P< 0.05$.

Fig.5. siCaMK2α could inhibit bupivacaine-induced mitochondrial oxidative stress and cell apoptotic injury. NC: cells transfected with silencer negative control siRNA; Bup: cells transfected with silencer negative control siRNA and treated with 2.0 mM bupivacaine for 6 h; siCaMK2α+Bup: cells transfected with CaMK2α siRNA and treated with 2.0 mM bupivacaine for 6 h. siCaMK2α+sp+Bup: cells transfected with CaMK2α siRNA and precultured with 10 μM spermine, and treated with 2.0 mM bupivacaine for 6 h. **A, B**: mtROS detection with flow cytometry to observe MitoSOX fluorescence intensity; Bar: 20 μm; **C, D:** MMP detection with JC-1 by flow cytometry to calculation ratio of polymer to monomer; Bar: 100 μm. **E**: ATP production of every group; **F, G**: apoptotic level was detected by flow cytometry; **H**: mitochondrial morphologic changes of cells detected by transmission electron microscopy; Bar: 500 nm. Values are the mean± SD of n = 3; *: $P< 0.05$.

Fig.6. Over-expression of CaMK2α could enhance bupivacaine-induced activation of CREB, elevation of MCU expression and cell oxidative damage. Con: untreated cells; null: cells infected with lentiviruses containing empty plasmids; Bup: cells infected with lentiviruses containing empty plasmids and treated with 2.0 mM bupivacaine for 6 h; LV-CaMK2α+Bup: cells infected with lentiviruses expressing CaMK2α and treated with 2.0 mM bupivacaine for 6 h. **A, B**: expression of CaMK2α after cells infected with lentiviruses expressing CaMK2α or with lentiviruses containing empty plasmids. **C-F**: CaMK2α and CREB phosphorylation, and MCU expression were

determined with western blotting; **G**: mtROS detection with flow cytometry to observe MitoSOX fluorescence intensity; **H, I**: cells apoptotic injury was determined with cleaved caspase-3 expression by western blotting. Values are the mean± SD of n = 3; *: $P< 0.05$.

Fig.7. Inhibition of MCU with the special inhibitor Ru360 or with siMCU could inhibit bupivacaine-induced mitochondrial oxidative damage and apoptotic injury. NC: cells transfected with silencer negative control siRNA; Bup: cells transfected with silencer negative control siRNA and treated with 2.0 mM bupivacaine for 6 h; siMCU+Bup: cells transfected with MCU siRNA and treated with 2.0 mM bupivacaine for 6 h; Ru360+Bup: cells precultured with 10 μM Ru360 and treated with 2.0 mM bupivacaine for 6 h. **A, B**: co-localization of MCU and COXVI with double immunofluorescent staining for determining mitochondrial MCU expression; Red fluorescence represented MCU and green fluorescence represented COXVI; The fluorescence intensity of overlapping part represents mitochondrial MCU (folds to group NC); Bar: 10 μm. **C, D**: MCU expression after cells transfected with MCU siRNA and bupivacaine treatment; **E:** ATP production of every group; **F**: mtROS detection with flow cytometry to observe MitoSOX fluorescence intensity; G, H: apoptotic level was detected by flow cytometry; Values are the mean± SD of n = 3; *: $P< 0.05$.