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Role of CaMK Ⅱα in the Injury of Dorsal Root Ganglion Neurons Induced by Ropivacaine Hydrochloride in the Dorsal Root Ganglion of Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author WX designed and implemented the experiments and wrote the paper. Author LIY collected the data and performed the statistical analysis. Authors LY, WZ, CM and YS implemented the experiments. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To investigate whether CaMKⅡα participates in the dorsal root ganglion neurotoxicity induced by ropivacaine hydrochloride.
Methods: DRG neurons were isolated from 1-day-old SD rats and cultured in vitro. pAd-shRNA-CaMKⅡα-DRG cells were constructed by RNA interference technique to inhibit the expression of CaMKⅡα. The experiment was divided into six groups: DRG group (DRG group), vector DRG group (vector group), pAd-shRNA- CaMKIIα-DRG group (pAd-shRNA group), DRG + ropivacaine group (DRG + R group), vector DRG + ropivacaine group (vector + R group), pAd-shRNA-CaMKII α - DRG + ropivacaine group (pAd-shRNA + R group), and the last three groups were treated with 3 mM ropivacaine hydrochloride for 4 hours. MTT assay was used to detect cell viability, flow cytometry was used to detect cell apoptosis rate, laser confocal microscopy was used to detect

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intracellular calcium level, and real-time PCR was used to detect the mRNA expression of CaMKⅡα, Cav3.2 and Cav3.3.

Results: The cell viability of DRG+R group, vector+R group and pAd-shRNA+R group decreased significantly after 3 mM ropivacaine hydrochloride treatment for 4 h. Compared with DRG+R group, the cell viability of pAd-shRNA+R group was significantly higher. After 3 mM ropivacaine hydrochloride treatment for 4 h, the apoptosis rate of DRG + R group, vector + R group and pAd-shRNA + R group increased significantly. Compared with DRG+R group, the apoptosis rate in pAd shRNA+R group was significantly lower. After 3 mM ropivacaine hydrochloride treatment for 4 h, the intracellular calcium levels in DRG + R group, vector + R group and pAd-shRNA + R group were significantly increased, and the intracellular calcium levels in pAd-shRNA + R group were significantly lower than those in DRG + R group. The mRNA expressions of CaMKⅡα, Cav3.2 and Cav3.3 were significantly decreased in pAd- shRNA group. The mRNA expressions of CaMK Ⅱ α, Cav3.2 and Cav3.3 were up-regulated in DRG + R group, vector + R group and pAd-shRNA + R group after 3 mm ropivacaine treatment for 4 h. The mRNA expressions of CaMKⅡα, Cav3.2 and Cav3.3 in pAd-shRNA + R group were significantly lower than those in DRG + R group.

Conclusion: Inhibition of CaMKⅡα expression can down regulate the expression of Cav3.2 and Cav3.3 mRNA, increase cell viability of DRG neurons, reduce the apoptosis rate, and improve the dorsal root ganglion neurotoxicity induced by ropivacaine hydrochloride.

Keywords: Dorsal root ganglion neurons; neurotoxicity; ropivacaine hydrochloride; CaMKⅡα.

1. INTRODUCTION

Neurotoxic injury of local anesthetics is a common complication in regional block, which often causes discomfort such as paresthesia, pain and numbness [1,2]. Ropivacaine hydrochloride is one of the amide local anaesthetics and widely used in clinical anaesthesia or pain management and some neurotoxicity cases are reported with the use of ropivacaine hydrochloride. The mechanism of neurotoxic injury induced by local anesthetics is still unclear, which may be related to intracellular calcium overload, activation of low voltage dependent calcium channels (Cav3.2, Cav3.3), apoptosis and activation of p38 MAPK signaling system [3,4]. Calcium/calmodulin-dependent protein kinase II (CaMK II) is a multifunctional serine/ threonine kinase to various substrates and consisted of twelve subunits. There are four subtypes of CaMK II as following: CaMK IIa, CaMK IIb, CaMK IId, CaMK IIg, respectively. CaMKⅡα is widely distributed in nerve tissue and closely related to various physiological functions of neurons [5]. Previous studies showed that the expression of CaMKⅡα and T-type calcium channel (Cav3.2, Cav3.3) mRNA increased after ropivacaine hydrochloride treatment of rat DRG neurons, suggesting that ropivacaine hydrochloride may be related to CaMKⅡα [6]. So we supposed that inhibiting the expression of CaMKⅡα can improve the damage of DRG induced by ropivacaine hydrochloride. In this study, we investigated the effects of knockdown CaMKII α mRNA expression on the dorsal root ganglion neurotoxicity induced by ropivacaine hydrochloride, as well as detected the Cav3.2 and Cav3.3 expression of the DRG neurons with CaMKII α mRNA knockdown.

2. MATERIALS AND METHODS

2.1 Isolation and Culture of DRG Neurons

1-day-old Sparague-Dawley rats were anesthetized by intraperitoneal injection of 1% Pentobarbital Sodium 60 mg/kg. The spinal cord and DRG were exposed under microscope. The DRG was separated from the intervertebral foramen and transferred into a 15 ml centrifuge tube. The rats were rinsed twice with PBS buffer. Add 4ml 0.125% trypsin, blow and suspend g DRG, incubate in 37 ℃ incubator for 20min, add 4ml DMEM complete medium to stop digestion, centrifuge at 3000rpm for 2min, and then add 2ml neurobasic medium: containing 4.5g/l D-glucose, 2mmol / L L-glutamine, 1% FBS, 20ml / L B-27 additive, 10ug / ml NGF, penicillin 100u / ml, streptomycin 100ug / ml. After the DRG were fully dispersed, they were filtered by 400 mesh stainless steel mesh and made into cell suspension. The cell suspension was inoculated in the cell culture plate and placed at 37 ℃ and 5% CO2 incubator for 48 hours. The cell culture medium was changed, and the final concentration of 5 mM cytarabine was added to inhibit the proliferation of non-neuronal cells. After 96 hours, the medium was changed to the

medium without cytarabine, and the medium was changed every 3 days.

2.2 Construction of pAd-shRNA-CaMKⅡα-DRG cells

In order to inhibit the expression of CaMKⅡα, we constructed the dorsal root ganglion neurons with low expression of CaMKⅡα. In shortly, according to the Gene bank of rat CaMKⅡαgene (NM 012920.1), the shRNA primers were designed and synthesised (Nanjing Jinsirui Biotechnology Co., Ltd., Nanjing), and the primer sequences were as follow: 5'-TTTGGCCACTGATCCAGAGTTCAAGACGTGC TGGATACAGTTTTTTTTTTTTG-3'; 5'-AGCTCAAAAAAAAAAAGCCACTGATCCAGAG TTCGTTGAATCTGCTGGATACAGTTGGC-3'. After annealing, the recombinant adenovirus (pAd/PL-DEST)was ligated with the recombinant plasmid pYr-1.1 and Lipo2000 system was used to transfect pAd-shRNA into HEK 293 cells for viral amplification and infection of DRG neurons. The expression of CaMKⅡα in pAd-shRNA-CaMKⅡα-DRG neurons was detected by real-time PCR and western blotting.

2.3 Experimental Protocol

The experiment was divided into 6 groups: DRG cell group (DRG group), vector DRG cell group (vector group), pAd-shRNA-CaMKIIα-DRG cell group (pAd-shRNA group). The above three groups were cultured in normal medium in vitro; DRG cell + ropivacaine group (DRG + R group), vector DRG cell + ropivacaine group (vector + R group), pAd-shRNA-CaMKII α - DRG cell + ropivacaine group (pAd-shRNA+R group) The cells in the latter three groups were treated with 3 mM ropivacaine hydrochloride for 4 hours, the concentration of ropivacaine hydrochloride was from the previous study [6].

2.4 Cell Viability Detection

The cells in every group were seeded on 96 well plate at the rate of 2 × 10^5 cells / ml. after treatment with the experimental protocol, the cell viability of the cells was detected by MTT method. In short, the normal medium was used to terminate the treatment with ropivacaine hydrochloride. After incubation with 5 mg / ml MTT solution 20 μ L for 4 h, the supernatant was discarded, and 150 μl DMSO was added to each well. After full shaking, the absorbance (OD570 and OD630) at 570 nm and 630 nm were measured on the microplate, The differences between OD570 and OD630 of normal cultured DRG cells was 100%. The cell viability of the other groups was calculated by the ratio of OD570 and OD630. The average value of 6 wells in each group was calculated.

2.5 Cell Apoptosis Rate

The cells in every group were inoculated on 24 well plates with a concentration of 2 × 10^5 / ml. After treatment according to the experimental protocol, the cells were digested and collected. The apoptosis rate was detected by flow cytometry according to the instructions of apoptosis detection kit (Nanjing Kaiji biological, Nanjing). In short, the cell collection solution was centrifuged at 3000rmp for 3min, the supernatant was discarded, 1ml of 1 × binding buffer was added, the supernatant was centrifuged at 3000rmp for 3min, 100 μ L of 1:20 Annexin V antibody was added, incubated in dark for 15min at room temperature, 1ml of 1 × binding buffer was added, the supernatant was discarded, and propidium iodide (PI, 50ug/ ml) 5 μL was added. The cells were incubated in dark for 30 min. after adding 190 μ L1 × binding buffer, the apoptosis rate was detected by flow cytometry. Each group was repeated 3 times, and the average value was taken.

2.6 Intracellular Calcium Detection

The cells in every group was seeded with 2 × 10^4 cells on the 12 well plate, treated for 4 h according to the experimental protocol, then the medium was discarded and washed with PBS; the final concentration of 5 μ m rhode-2 / am (Thermo Scientific, USA) was added into the cell plate, and incubated in 5% CO2 incubator at 37 ℃ for 30 min; Then the cells were rinsed twice with PBS, and the cells were observed under confocal microscope (Leica Sp8, German).The fluorescence intensity (excitation wavelength 549 / emission wavelength 578 nm) of the cells in every group was observed under the step laser scanning confocal microscope. Three fields with the same multiple were selected to analyze the area of all cells in the field, and then the total fluorescence intensity of all cells in the field was analyzed.

2.7 Expression of CaMKⅡα, Cav3.2 and Cav3.3 mRNA

After treated with the experimental protocol, the cells were collected, 200μL Trizol was used to lyse the cells at 44 ℃, 5 times of the volume of chloroform was added, and centrifuged at 14000

rpm at 4 ℃ for 15min. The upper layer of RNA was removed, and the supernatant was centrifuged after precipitation with equal volume of isopropanol. The supernatant was washed with 500 μ l 70% ethanol, and the RNA was dissolved with 30 μL nuclear emzyme free water. Reverse transcription reaction as following: RNA 5 μ g, 2 mM dNTP 5 μ L, radom primer 1μL, add DEPC water to 37μL, 65℃ for 5 min, 5×first stand buffer 10μL, 0.1M DTT 2μL, MLV reverse transcriptase 1μL, total volume 50μL, 42 ℃ for 1 h, 70℃ for 15 min were added. The product cDNA was used for PCR reaction. 5μL cDNA product, 6μL sybergreen, 10 pM/L CaMKⅡα, Cav3.2, CaV3.3 mRNA up and down-primer (Nanjing Kingsley Biotechnology Co., Ltd., Nanjing), 0.5μL, the total volume was 12μL, and set 40 cycles (95℃ 15s, 60℃ 30s, 95 ℃ 15s) reaction to collect fluorescence signal, draw amplification curve and melting curve respectively, $2^{-\Delta\Delta\ CT}$ (CT represents cycle threshold) represents gene expression, ΔΔ CT = [CT (target gene of test group) - CT (β - actin)] - [CT (target gene of control group) - CT (β - actin)].

2.8 Statistical Analysis

The data are expressed as the Mean±SD. Normality and variance homogeneity were determined with SPSS17.0. For the normality and variance homogeneity data, one-way analysis of variance (one-way ANOVA) was used for comparisons among groups, and the LSD method was used for multiple comparisons.

3. RESULTS

3.1 Cell Viability

The cell viability of the cells in DRG group, vector group and pAd-shRNA group was (100 ± 0.0)%, (100 ± 4.0)% and (99.8 ± 2.5)%, respectively. Compared with the above three control groups, the cell viability of DRG+R group, vector+R group and pAd-shRNA + R group was significantly decreased by (49.8 ± 3.2)%, (47.8 ± 5.9)%, (70.3 ± 4.1)%, respectively. While pAd-shRNA + R group had significantly higher cell viability than those in DRG + R group and vector + R group respectively, as shown in Table 2.

3.2 Cell Apoptosis Rate

The apoptosis rates of the cells in DRG group, vector group and pAd-shRNA group were (15 ± 2)%, (16 ± 2)%, (14 ± 3)%, respectively, and there were no significant difference among the three groups. Compared with the above three control groups, the apoptosis rate of the cell in DRG+R group, vector + R group and pAd-shRNA+R group increased significantly after 3 mM ropivacaine hydrochloride treatment for 4 h, which were (41±2)%, (40±4)%, (27±5)%, respectively. Compared with the cells in DRG+R group, the apoptosis rate of the cells in vector+R group had no significant difference, but the apoptosis rate of pAd-shRNA + R group cells was significantly lower than that in DRG + R group and vector + R group respectively, as shown in Table 2.

3.3 Intracellular Ca^{2+} Level

The intracellular calcium levels of the cells in DRG group, vector group and pAd-shRNA group were 2.50 ± 0.20, 2.45 ± 0.38 and 2.53 ± 0.15, respectively. Compared with the the cells in above three control groups, the intracellular calcium levels of DRG +R group, vector +R group and pAd-shRNA + R group were significantly increased after 3 mM ropivacaine hydrochloride treatment for 4 h, which were 4.03 ± 0.38, 4.10 ± 0.36 and 3.10 ± 0.21, respectively. Compared with that in DRG + R group, there was no significant difference in intracellular calcium level of the cells in vector+R group. The intracellular calcium level in pAd-shRNA+R group was significantly lower than that in DRG+R group. Compared with vector+R group, the intracellular calcium of pAd-shRNA + R group was significantly decreased, as shown in Table 2.

3.4 mRNA Expression

The mRNA expressions of CaMKⅡα, Cav3.2 and Cav3.3 of the cells in DRG group and vector group were not significantly different. Compared with the above two groups, the mRNA expressions of CaMKⅡα, Cav3.2 and Cav3.3 of the cells were significantly decreased in pAd-shRNA group and up-regulated in DRG+R group, vector+R group, pAd-shRNA+R group after 3 mM ropivacaine hydrochloride treatment for 4 h. Compared with DRG+R group, the expression of CaMKⅡα, Cav3.2 and Cav3.3 mRNA in vector + R group was not significantly different, while the expression of CaMKⅡα, Cav3.2 and Cav3.3 mRNA in pAd-shRNA+R group was significantly lower than that in DRG+R group. Compared with vector + R group, the mRNA expressions of CaMKⅡα, Cav3.2 and Cav3.3 in pAd-shRNA + R group were significantly decreased, as shown in Table 3.

Table 1. Primer sequences of CaMKⅡα, Cav3.2 and Cav3.3 mRNA

Gene	Primer	Product size
β-actin	F : 5`-CACGATGGAGGGGCCGGACTCATC -3` R : 5`-TAAAGACCTCTATGCCAACACAGT-3`	240bp
CaMKⅡα	F: 5`- GAAGATGTGCGACCCTGGAA-3` R: 5`- GGTACTGAGTGATGCGGATGTAG-3`	203 bp
Cav3.2	F:5`-GGAGTTTGATGATGACATAGAGG-3` R:5`-GGAAGATGAAGACAAGGACCAC-3`	197bp
Cav3.3	F :5`-GACCAGCAGCCAGTGACGAA-3` R:5`-CACGACCACGCCCACAAACA-3`	108bp

Table 2. Comparison of cell viability, apoptosis rate and intracellular Ca2 + level in each group (mean ± SD, n = 3)

Item	DRG group	vector group	pAd-shRNA group	DRG+R group	vector+R group	pAd-shRNA+R group
Cell viability (%, n=6)	100.0±0.0	100.0±4.0	99.8±2.5	49.8±3.2^{ab}	47.8±5.9^{ab}	70.3±4.1^{abc}
apoptosis rate (%, n=3)	15±2	16±2	14±3	41±2 ^{ab}	40±4 ^{ab}	27±5 ^{abc}
Ca^{2+} level (n=3)	2.50±0.20	2.45±0.38	2.53±0.15	4.03±0.38	4.10±0.36	3.10±0.21

Compared with DRG group, $^{a}P < 0.05$, compared with pAd-shRNA group, $^{b}P < 0.05$, compared with DRG + R group, $^{c}P < 0.05$.

Table 3. mRNA expression of CaMKⅡα, Cav3.2 and Cav3.3 in each group (mean ± SD, n = 3)

Item	DRG group	vector group	pAd-shRNA group	DRG+R group	vector+R group	pAd-shRNA+R group
CaMKⅡα	1.09±0.11	1.06±0.12	0.21±0.04^{a}	1.90±0.10^{ab}	1.97±0.21^{ab}	1.03±0.19^{bc}
Cav3.2	0.39±0.03	0.38±0.03	0.20±0.02^{a}	0.74±0.05^{ab}	0.79±0.02^{ab}	0.57±0.02^{abc}
Cav3.3	0.54±0.04	0.53±0.04	0.20±0.02^{a}	0.82±0.03^{ab}	0.81±0.04^{ab}	0.51±0.03^{bc}

Compared with DRG group, $^{a}P < 0.05$, compared with pAd-shRNA group, $^{b}P < 0.05$, compared with DRG + R group,$^{c}P < 0.05$

4. DISCUSSION

The present study demonstrated the effects of knockdown CaMKIIα mRNA expression on the dorsal root ganglion neurotoxicity induced by ropivacaine hydrochloride. In this study, we used RNAi to specifically inhibit the expression of CaMKⅡαmRNA, and found that it can alleviate the cell damage caused by ropivacaine hydrochloride, which showed that the cell viability increased, the apoptosis rate decreased, and the intracellular calcium level decreased. At the same time, the expressions of Cav3.2 and Cav3.3 mRNA were down regulated after the inhibition of CaMKⅡαmRNA expression.

Ropivacaine hydrochloride is one of the commonly used local anesthetics in clinic. In this study, referring to previous studies, we used 3 mM ropivacaine hydrochloride to treat rat DRG neurons for 4 h to prepare a local anesthetic toxic nerve injury model [6]. The results showed that the viability of DRG cells was significantly decreased, the apoptosis rate was increased and the level of intracellular calcium ion was increased after 3 mM ropivacaine treatment for 4 h, suggesting that 3 mM ropivacaine treatment of rat DRG neurons could significantly cause damage to DRG cells.

CaMKⅡis a protein kinase, which is widely distributed in the nervous system and plays an important role in regulating intracellular calcium homeostasis. Ca^{2+} binds with calmodulin to form protein complex, which phosphorylates CaMKⅡand activates CaMKⅡ. After activation of CaMKⅡ, it can regulate intracellular Ca^{2+}

rebalancing, thus regulating intracellular pathophysiological activities [7,8]. CaMKⅡα is a subtype of CaMKⅡ, which is widely distributed in the nervous system. Previous studies showed that the mRNA expression of CaMKⅡα and T-type calcium channels (Cav3.2, Cav3.3) increased after ropivacaine hydrochloride treatment of rat dorsal root ganglion neurons, suggesting that ropivacaine hydrochloride may be related to CaMK Ⅱα [6]. Studies have shown that CaMKⅡ is closely related to T-type calcium channel. Up regulation of CaMK Ⅱ can activate low voltage dependent calcium channel (T-type calcium channel) and enhance T-type calcium current. Conversely, up regulation of T-type calcium current can activate CaMKⅡ protein and up regulate CaMKⅡ expression [9,10]. In this study, we used RNAi to specifically inhibit the expression of CaMK ⅡαmRNA, and found that it can alleviate the cell damage caused by ropivacaine hydrochloride, which showed that the cell viability increased, the apoptosis rate decreased, and the intracellular calcium level decreased. At the same time, the expressions of Cav3.2 and Cav3.3 mRNA were down regulated after the inhibition of CaMKⅡαmRNA expression. The results showed that CaMKⅡα may participate in the nerve injury induced by ropivacaine hydrochloride by regulating the expression of Cav3.2 and Cav3.3 mRNA.

The results suggesting that 3 mM ropivacaine treatment of rat DRG neurons could significantly cause damage to DRG cells and showed that CaMKⅡα may participate in the nerve injury induced by ropivacaine hydrochloride by regulating the expression of Cav3.2 and Cav3.3 mRNA.

There is some limitation for this study. we observed the changes of the mRNA expression induced by ropivacaine hydrochloride in vitro. However, the results from the cultured cells may be different with the experiment in vivo. In the future, experiment in vivo should be done to test the above results.

5. CONCLUSION

In conclusion, inhibition of CaMKⅡα expression can down regulate the expression of Cav3.2 and Cav3.3 mRNA, increase cell viability of DRG neurons, reduce the apoptosis rate, and improve the dorsal root ganglion neurotoxicity induced by ropivacaine hydrochloride.

CONSENT

It is Not Applicable.

ETHICAL APPROVAL

The protocol was approved by the ethics committee of the second people`s hospital of Foshan.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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