Cav3.3 Down-Regulates CaMKII Beta to Ameliorate Neurotoxic Injury to the DRG Induced by Ropivacaine Hydrochloride

Xianjie Wen (✉ xjwen166@163.com)

The second people's hospital of Foshan city <https://orcid.org/0000-0003-2490-8876>

Yiqun Li

The second people's hospital of Foshan

Zhaoxia Wu

The second people's hospital of Foshan City

Meixin Chen

First People's Hospital of Foshan

Shuxuan Yang

The second hospital of Foshan

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Abstract

Background: The neurotoxicity of local anaesthetics is often reported. The present study aimed to investigate the relationship between Cav3.3 and CaMK β in local anaesthetic neurotoxicity.

Methods: An in vitro ropivacaine-induced model of neurotoxic injury to the dorsal root ganglion neurons was used. After specifically inhibiting the expression of Cav3.3 mRNA in DRG neurons by RNAi, cell viability, CaMK β expression and the intracellular calcium ion level were detected in DRG neurons.

Results: The results showed that the expression of CaMK β decreased after the expression of Cav3.3 was inhibited. At the same time, in a model of ropivacaine hydrochloride-induced neurotoxic injury, inhibiting the expression of Cav3.3 mRNA improved cell viability and reduced the intracellular calcium level.

Conclusions: These results suggest that Cav3.3 may be involved in neurotoxic injury induced by local anaesthetics by regulating the expression of CaMK β . Both Cav3.3 and CaMK β are therapeutic targets for neuronal injury induced by local anaesthetics.

Background

Ultrasound-guided nerve blocks are widely used for clinical anaesthesia and chronic pain treatment (1, 2). With a good analgesic effect and few systemic adverse reactions, they are conducive to the rapid recovery of patients after the operation. However, the neurotoxicity of local anaesthetics, which can cause prolonged pain, numbness and discomfort and even permanent nerve damage in severe cases (3-6), is often reported.

Previous studies have found that a low-voltage-dependent calcium channel, Cav3.3, is associated with neurotoxic damage induced by local anaesthetics (7, 8). The specific inhibition of Cav3.3 expression can reduce apoptosis and alleviate neuronal damage induced by local anaesthetics. In contrast, the upregulation of Cav3.3 expression can aggravate neuronal damage, reduce neuronal viability and increase the apoptosis rate. Extracellular calcium ions enter cells through low-voltage-dependent calcium channels, which will increase the intracellular calcium concentration, activate high-voltage-dependent calcium channels, increase calcium influx and activate Ca²⁺/calmodulin-dependent protein kinase β (CaMK β).

CaMK β is a type of protein kinase with four subtypes: alpha, beta, delta and gamma(9, 10). It has been found that ropivacaine hydrochloride can upregulate the expression of CaMK β in DRG neurons(11). After treatment with ropivacaine hydrochloride, expression of the CaMK β subtypes (alpha, beta, gamma, and delta) increases in a manner that is related to the concentration of and exposure time to ropivacaine hydrochloride. There is a close relationship between Cav3.3 and CaMK β gamma in neurotoxic injury induced by local anesthetics (7). Up-regulation of Cav3.3 expression increases the expression of

CaMK γ in neurons. In contrast, the expression of CaMK γ decreases after inhibition of Cav3.3 expression (8).

CaMK β is widely distributed in neurons, is related to cerebral ischaemia-reperfusion injury, and participates in the processes of learning and memory(12-14). A previous study indicated that Cav3.3 and CaMK β are involved in local anaesthetic neurotoxicity(7, 11). Additionally, CaMK γ can regulate the T-type calcium current density(10, 15). Therefore, we speculate that there may be some connection between Cav3.3 and CaMK β in the neurotoxicity of local anaesthetics. The effect of Cav3.3 expression on CaMK β remains unclear.

In this study, we constructed a pAd-shRNA-Cav3.3-DRG plasmid to investigate the effects of Cav3.3 inhibition on CaMK II β in local anaesthetic neurotoxicity. We evaluated cell viability, the cell apoptosis rate, Cav3.3 and CaMK II β expression and calcium ion levels.

Methods

Isolation of DRG neurons

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (16). The protocols for animal use were approved by the ethics committee of the Affiliated Foshan Hospital of the Southern Medical University. After 1–3 days of acclimatization and sevoflurane (2% vol) inhalation anesthesia, their spinal cords and ganglia of the rats were exposed rapidly at 4°C, and the ganglia were separated. After digestion with 0.125% trypsin for 20 min, the samples were centrifuged at 2000 rpm for 2 min, and the supernatant was discarded. Neurobasal medium (containing 4.5 g/L D-glucose, 2 mmol L-glutamine, 1% FBS, 20 ml/L B-27 supplement, 10 μ g/ml NGF, 100 U/ml penicillin, and 100 μ g/ml streptomycin) was added to the separated DRG samples. The samples were filtrated with 400 mesh stainless steel mesh, seeded into a cell culture plate and incubated at 37°C and 5% CO₂. Cytarabine (5 mM final concentration) was added to inhibit the proliferation of nonneuronal cells. The cell density was seeded with 2×10^5 / L. After 96 h, the medium was replaced with cytarabine-free culture solution, and the medium solution was changed once every 3 days.

Construction of pAd-shRNA-Cav3.3-DRG neurons

To observe the effects of Cav3.3 on CaMK β , we constructed pAd-shRNA-Cav3.3-DRG neurons with low levels of Cav3.3 expression(16). In brief, shRNA primers were designed based on the rat Cav3.3 gene (NM_020084.3). The primer sequences were 5'-TTTGGCCAGGAAGTTCAACAGTATTCAAGACGTACTGTTGAACTTCCTGGCTTTTTTG-3' and 5'-AGCTCAAAAAGCCAGGAAGTTCAACAGTACGTCTTGAATACTGTTGAACTTCCTGGC-3' (the underlined sequences are the interference sequences). The above primers formed double chains after annealing and were ligated into the pYr-1.1 vector. The conjugates were amplified and sequenced. The pAd/PL-DEST

adenovirus expression vector was ligated to the interfering sequence to form the pAd-Cav3.3-shRNA adenovirus.

To construct pAd-shRNA-Cav3.3-DRG neurons, the pAd-Cav3.3-shRNA adenovirus (DNA:lipid=1:2) was transfected into HEK 293 cells with the Lipo 2000 system to amplify the virus, which was then used to infect the DRG neurons. The expression of Cav3.3 in the pAd-shRNA-Cav3.3-DRG neurons was identified by real-time PCR and western blotting. Additionally, we constructed empty vector DRG cells as the control group.

Experimental protocol

There were six groups in this study. In the normal group, DRG cells were cultured with normal medium; in the vector group, empty vector DRG cells were treated with normal medium; in the pAd-shRNA-Cav3.3-DRG group, pAd-shRNA-Cav3.3-DRG cells were treated with normal medium; in the normal +R group, DRG cells were cultured with 3 mM ropivacaine hydrochloride for 4 h; in the vector+R group, empty vector DRG cells were treated with 3 mM ropivacaine hydrochloride for 4 h; in the pAd-shRNA-Cav3.3-DRG +R group, the pAd-shRNA-Cav3.3-DRG cells were treated with 3 mM ropivacaine hydrochloride for 4 h. Cell viability, cellular ultrastructure changes, the expression of Cav3.3 and CaMK β , and the intracellular calcium ion level were detected. The concentration (3 mM) and exposure time (4 h) to ropivacaine hydrochloride were based on a previous study (7,11). The titration pH value of the cell culture medium in each group was 4.0–6.0, which was consistent with that of ropivacaine hydrochloride.

Cell viability as detected by an MTT assay

Cell viability was detected using an MTT assay. In brief, after treatment with or without ropivacaine hydrochloride, the cells from each group were collected, MTT solution (5 mg/ml) was added and the samples were incubated for 4 h. The supernatant was discarded, and DMSO was added to the samples. After the purple crystals dissolved, the absorbance at 570 and 630 nm (OD570 and OD630) was measured. The ratio of OD570 to OD630 of the cells in the normal group was regarded as 100%. The difference in the OD570/OD630 ratio between the cells in the normal group and other groups represented the cell viability.

Cellular ultrastructure changes observed by electron microscopy

Cells from each group were collected and fixed at 4°C. Then, the cells were dehydrated with 50%, 70% and 90% acetone overnight at room temperature with an embedding agent. The cell mass was moved into the centre of the bottom of the capsule for embedding. Ultrathin slices of 50–70 nm were cut with an ultrathin microtome. The slices were stored in a drying vessel for dyeing and cleaned with sodium acetate and lead citrate. After dyeing, the slices were dried, and the mitochondrial structure of the neurons was observed under a transmission electron microscope (Hitachi, HT7700, Japan).

mRNA detected by real-time PCR

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After treatment with or without ropivacaine hydrochloride, the cells were collected to detect Cav3.3 and CaMKβ mRNA expression. In brief, total RNA was isolated by the TRIzol method. After lysis, isolation, and washing, the samples were air-dried, and 30 µl of nuclease-free water was used to dissolve the RNA. The reaction for generating cDNA contained 5 µg of total RNA, 5 µl of 2 mM dNTPs, 1 µl of random primer 1, and DEPC H₂O was added to reach a final volume of 37 µl. The mixture was incubated at 65°C for 5 min. Then, 10 µl of 5× first strand buffer, 2 µl of 0.1 M DTT, and 1 µl of MLV reverse transcriptase was added to the reaction. The total volume was 50 µl, and the mixture was incubated at 42°C for 1 h and then at 70°C for 15 min. The product was cDNA.

The PCR mixture included 5 µl of cDNA, 6 µl of SYBRGreen, and 1 µl of 10 pmol/l CaMKβ and Cav3.3 mRNA forward-and-reverse primers (Table 1). The PCR parameters were as follows: 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s for forty cycles, then 72°C for 10 min. The fluorescence signals were detected and amplification and melting curves were created. The expression of the genes was calculated by 2^{-ΔCt} (Ct stands for the cycle threshold). Ct=[Ct (target gene in the test group) – Ct (β-actin)] – [Ct (target gene in the control group) – Ct (β-actin)] – [Ct (target gene in the control group)].

Tab 1 CaMKβ and Cav3.3 mRNA primers

Gene	Primer	Product size
CaMKβ	F: 5- <i>CGAC T TGAGGC TATGCG</i> - 3 R:5- <i>ACGTGCGGG T CAGGATAGTG</i> - 3	165 bp
Cav3.3	F:5- <i>GACAGCAGCAGTGACG</i> - 3 R:5- <i>CACGACACGC CAC</i> - 3	108 bp
β-actin	F: 5- <i>CACGATGGAGGGGC GGACTCATC</i> - 3 R: 5- <i>T</i> - 3	240 bp

Cav3.3 and CaMKβ protein expression

The cells were inoculated on coverslips in 12-well plates. After treatment with or without ropivacaine hydrochloride, cells from each group were fixed with 4% polyformaldehyde for 15 min and washed 3 times with PBS. Normal goat serum was added to the slides at room temperature for 30 min. Cav3.3 and CaMKβ antibodies (1:100) were added, and the slides were incubated at 4°C overnight. A fluorescent-labelled (Cy3) sheep anti-rabbit IgG (1:100) antibody was added to the slides, and the slides were

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ated with DAPI for 5 min. The coverslips were sealed with an

antifluorescence quenching agent, and fluorescence images were collected with a fluorescence microscope. Image analysis was carried out with ImageJ 5.0 software. The ratio of the integrated density to the visual field area was calculated as the mean integrated density. The samples from each group were measured three times.

Detection of intracellular calcium ion level by laser confocal microscopy

The cells were incubated in 12-well migration plates. After treatment, the cells from each group were washed with PBS and incubated in a 5% CO₂ incubator at 37°C for 30 min with Rhod-2 AM (final concentration, 5 µM). The cells were washed twice with PBS and observed under a laser scanning confocal microscopy (Leica SP8 STED). The fluorescence intensity (excitation wavelength of 549 nm and emission wavelength of 578 nm) was recorded. ImageJ 5.0 software was used for image analysis. The total fluorescence intensity of all cells in the field of vision was analysed. The total fluorescence intensity/the total cell area was regarded as the average optical density.

Statistical analysis

The data are expressed as the Mean±SD. A factorial design was adopted for the statistical analysis. 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.

Results

Cell viability

There was no difference in the cell viability among the cells from the normal, vector and pAd-shRNA-Cav3.3-DRG groups. However, compared with that of the cells from the normal group, the cell viability of the cells from the normal +R group, the vector +R group and the pAd-shRNA-Cav3.3-DRG+R group were decreased. As well as, compared with that of the cells from the normal +R group, the cell viability of the cells from the pAd-shRNA-Cav3.3-DRG+R group was increased. These data suggest that the inhibition of Cav3.3 expression improved cell viability, as shown in Figure 1.

Cellular ultrastructure changes

A large number of nerve filaments were observed in the normal group cells by electron microscopy, as well as, the shape of the mitochondria was regular, and the structure of the mitochondria was complete. There were no significant differences between the cells from the vector group and the pAd-shRNA-Cav3.3-DRG group.

However, after treated with ropivacaine hydrochloride, the mitochondria of the cells from the normal +R group, the vector +R group and the pAd-shRNA-Cav3.3-DRG+R group, were destroyed and vacuolated, the

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nt and bilateral membranes were observed in the organelles.

Additionally, cytophagosomes were present. However, it is gratifying that those changes of the cells from pAd-shRNA-Cav3.3-DRG+R group, inhibiting the expression of Cav3.3 mRNA, is significant smaller than those from the normal +R group and the vector +R group (Figure 2).

mRNA expression

Cav3.3 mRNA expression

Compared with the cells from the normal group, Cav3.3 mRNA expression in the cells from the pAd-shRNA-Cav3.3-DRG group was decreased. There was no difference between the cells from the normal group and the vector group. After treatment with ropivacaine hydrochloride, Cav3.3 mRNA expression was upregulated in the cells from the normal+R, vector+R, and pAd-shRNA-Cav3.3-DRG+R groups. Compared with the cells from the normal+R group, Cav3.3 mRNA expression in the cells from the pAd-shRNA-Cav3.3-DRG+R group was decreased. There was no difference in Cav3.3 mRNA expression between the cells from the normal+R group and the vector+R group (Figure 3A).

CaMK β mRNA expression

Compared with the cells from the normal group, CaMK β mRNA expression in the cells from the pAd-shRNA-Cav3.3-DRG group was downregulated. Additionally, the cells treated with ropivacaine hydrochloride exhibited upregulated CaMK β mRNA expression. Compared with the cells from the normal+R group, CaMK β mRNA expression in the cells from the pAd-shRNA-Cav3.3-DRG+R group was downregulated (Figure 3B).

Cav3.3 and CaMK β expression detected by immunofluorescence

Cav3.3 protein expression

There was no difference in Cav3.3 expression between the cells from the normal group and the vector group. The inhibition of Cav3.3 mRNA expression downregulated Cav3.3 protein expression. After treatment with ropivacaine, Cav3.3 protein expression was upregulated. Compared with the cells from the normal+R group, the Cav3.3 expression in the cells from the vector +R group was not different; however, the cells from the pAd-shRNA-Cav3.3-DRG+R group exhibited downregulated Cav3.3 expression (Figure 4).

CaMK β

Compared with the cells from the normal group, the CaMK β protein expression in the cells from the vector group was not different, but the CaMK β protein expression in the cells from the pAd-shRNA-Cav3.3-DRG group was downregulated. After treatment with ropivacaine hydrochloride, CaMK β protein expression was upregulated. There was no difference between the cells from the normal+R group and the vector+R group. Additionally, CaMK β protein expression in the pAd-shRNA-Cav3.3-DRG+R group was downregulated (Figure 5)

Calcium ion level, as detected by laser confocal microscopy

Compared with the cells from the normal group, there was no difference in intracellular calcium levels in the cells from the vector group and the pAd-shRNA-Cav3.3-DRG group. After ropivacaine hydrochloride treatment, the intracellular calcium level of the cells increased. Compared with the normal+R group, the intracellular calcium ion level in the vector+R group was not different, while that in the pAd-shRNA-Cav3.3-DRG+R group was decreased (Figure 6).

Discussion

T-type calcium channels are involved in the neurotoxic injury induced by local anaesthetics (7, 17, 18). Extracellular calcium ions enter into cells through low-voltage-dependent calcium channels (T-type calcium channels) and induce the opening of high-voltage-dependent calcium channels and ligand-dependent calcium channels (19-21). Additionally, extracellular calcium ion influx increases intracellular calcium ion levels and causes cell injury. Cav3.2 and Cav3.3 calcium channels are mainly distributed in dorsal root ganglion cells (22). Both are involved in the spinal neurotoxicity of local anaesthetics. Inhibiting the expression of Cav3.2 and Cav3.3 calcium channels can alleviate neurotoxic injury induced by local anaesthetics (7). The mutual regulation of CaMK γ and Cav3.3 participates in the neurotoxic damage induced by local anaesthetics (8). The calcium ion level increase induced by their positive regulation is one of the mechanisms by which neurotoxic injury is induced by local anaesthetics (23).

CaMK β , a subtype of CaMK, is widely distributed in the central nervous system and is related to cerebral ischaemia-reperfusion injury, learning and the formation of memories(24-27). Ropivacaine hydrochloride can upregulate the expression of CaMK β in the spinal cord of rats in a manner that is related to the concentration of and exposure time to ropivacaine hydrochloride (11). As well as, our previous study show that inhibition of CaMK β expression can down-regulate Cav3.3 expression and reduce the neurotoxicity induce by ropivacaine hydrochloride(28).This study showed that inhibiting the expression of Cav3.3 mRNA in the spinal cord results in downregulation of the expression of CaMK β , which can alleviate neurotoxicity injury induced by ropivacaine hydrochloride. The mechanism may be related to the decrease of extracellular calcium influx and the decrease of CaMK β activity induced by the Cav3.3 mRNA downregulation. The cells exhibited an increase in cell viability and an intracellular calcium ion level decrease. These results suggested that Cav3.3 and CaMK β are involved in the neurotoxic injury induced by ropivacaine hydrochloride. The Cav3.3 - Ca²⁺/CaMK β pathway may be one of the important mechanisms regulating the neurotoxicity induced by ropivacaine hydrochloride.

After the expression of Cav3.3 mRNA was reduced, calcium ions entered the cells through fewer Cav3.3 channels. Thus, the intracellular calcium ion level was reduced. Therefore, calcium-dependent calcium release mediated by Ca²⁺/CaMK β was inhibited, which reduced the intracellular calcium overload and alleviated the neuronal damage caused by local anaesthetics.

On the other hand, we found that ropivacaine hydrochloride can cause mitochondrial damage in DRG cells. Mitochondrial membrane injury inhibits oxidative phosphorylation and reduces ATP production, which causes a shortage of energy supply for Ca^{2+} -ATPases and promotes Ca^{2+} overload (29). An insufficient energy supply for Na^{+} -ATPases in the cell membrane also significantly increases the intracellular sodium ion concentration, which activates the Na^{+} - Ca^{2+} exchange proteins and increases the Ca^{2+} influx (30). In fact, our previous study shows that Cav3.3 is also closely related to CaMK β in the neurotoxicity of ropivacaine hydrochloride, and inhibition of Cav3.3 can down regulate the expression of CaMK β to reduce the neurotoxicity (31).

This study found a close relationship between Cav3.3 and CaMK β in local anaesthetic neurotoxicity, but there were also some limitations. This study only explored the effect of the downregulation of Cav3.3 on CaMK β in neurotoxic injury induced by local anaesthetics. The effects of the upregulation of CaMK β needs to be further studied.

Conclusion

In this study, we observed the effects of the inhibition of Cav3.3 mRNA expression on CaMK β in a model of local anaesthetic neurotoxicity. The data showed that the inhibition of Cav3.3 resulted in a decrease in CaMK β expression and alleviated the injury induced by ropivacaine hydrochloride. On the contrary, inhibition of CaMK β expression can down regulate Cav3.3 expression and reduce nerve injury induced by ropivacaine hydrochloride. The interaction between Cav3.3 and CaMK β is involved in the neurotoxicity of ropivacaine hydrochloride.

Abbreviations

CaMK: Ca^{2+} /calmodulin-dependent protein kinase; DRG: Dorsal root ganglion; RNAi: RNA interference; PCR: Polymerase Chain Reaction.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of *the Second People's Hospital of Foshan City*.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have read and approved the manuscript, WX designed and implemented the experiments and wrote the paper; WZ, LY, collected the data and performed the statistical analysis; WZ, LY, WX, CM, and YS implemented the experiments.

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Not Applicable

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Figures

Figure 1

Cell viability of the cells from each group (mean $\pm$ sd, n=6). Compared with the cells from the normal group, aP<0.05; Compared with the cells from the normal +R group, bP<0.05.

Normal

vector

pAd-shRNA-Cav3.3-DRG

Figure 2

Changes in the cells as observed by electron microscopy (200 W, 5000×).

Figure 3

Cav3.3 and CaMKIIβ (CaMKIIβ) mRNA expression in the cells from each group (mean±sd, n=3). A: Cav3.3 mRNA expression. B: CaMKIIβ (CaMKIIβ) mRNA expression. Compared with the cells from the normal group, aP<0.05. Compared with the cells from the pAd-shRNA-Cav3.3-DRG group, bP<0.05. Compared with the cells from the normal+R group, cP<0.05.

A:

B:

Figure 4

A: Protein expression, as detected by immunofluorescence (400×). B: The expression of Cav3.3 protein (mean±sd, n=3). Compared with the cells from the normal group, aP<0.05. Compared with the cells from the pAd-shRNA-Cav3.3-DRG group, bP<0.05. Compared with the cells from the normal+R group, cP<0.05.

A:

B:

Figure 5

A: Protein expression, as detected by immunofluorescence (400×). B: The expression of CaMKβ (mean±sd, n=3). Compared with the cells from the normal group, aP<0.05. Compared with the cells from the pAd-shRNA-Cav3.3-DRG group, bP<0.05. Compared with the cells from the normal+R group, cP<0.05.

Figure 6

A: Calcium ion level, as detected by immunofluorescence (400×). B: The calcium ion level (mean±sd, n=3). Compared with the cells from the normal group, aP<0.05. Compared with the cells from the pAd-shRNA-Cav3.3-DRG group, bP<0.05. Compared with the cells from the normal+R group, cP<0.05.