Dexmedetomidine protects PC12 cells from ropivacaine injury through miR-381/LRRC4/SDF-1/CXCR4 signaling pathway

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Abstract

Introduction: Ropivacaine has been regularly used because of its good anesthetic and analgesic effects, but it may exert neurotoxic effects on neurocyte. Dexmedetomidine has presented special advantages in the fields of neuroprotection, and it also could improve peripheral nerve block combining with ropivacaine. However, if dexmedetomidine could repair neurocyte injury induced by ropivacaine, and the specific mechanism remain unclear.

Methods: Western blotting and qRT-PCR were applied for measuring expression of protein and mRNA, respectively. Flow cytometry was used for assessing apoptosis. Cell proliferation was detected using Cell Counting Kit-8 (CCK-8) and colony formation assays. Transwell assay was applied to measure the migration and invasion of cells. Dual luciferase reporter assay was applied for confirming the binding site between microRNA-381 (miR-381) and Leucine-rich repeat C4 protein (LRRC4).

Results: The viability of PC12 cells increased with raising the concentration of dexmedetomidine (0 μM, 10 μM, 50 μM, 100 μM). Dexmedetomidine reversed role of ropivacaine (0 mM, 0.1 mM, 0.5 mM, 1 mM) by upregulating the expression of miR-381 and suppressing the expression of LRRC4 in PC12 cells. miR-381 can directly interact with target gene LRRC4 and negatively regulate its expression. Dexmedetomidine promoted the proliferation, migration, and invasion and inhibited apoptosis of PC12 cells by suppressing LRRC4 via up-regulating the expressions of miR-381 and further activated SDF-1/CXCR4 signaling pathway.

Conclusions: Dexmedetomidine could protect PC12 cells from ropivacaine injury through miR-381/LRRC4/SDF-1/CXCR4 signaling pathway. This study may provide new therapeutic strategy targeting miR-381/LRRC4/SDF-1/CXCR4 signaling pathway about the prevention of ropivacaine induced neurocyte injury.

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1. Introduction

Postoperative pain greatly affects the patient's physiological and psychological recovery, and may extend hospital stay. In order to alleviate the pain of patients after operation, local anesthetics are regularly used in clinic [1,2]. Ropivacaine, an amide local anesthetic, has been widely used in clinic because of its good anesthetic and analgesic effects [3,4]. However, it has been reported that ropivacaine might exert some neurotoxic effects on neurons [5], and the specific mechanism of its neurotoxicity has not been fully elucidated.

Dexmedetomidine has attracted more attention because of its multi-organ protection effects. Especially, it has shown special advantages in the fields of neuroprotection and cardioprotection [6,7]. Previous clinical studies have shown that dexmedetomidine combined with local anesthetics could improve peripheral nerve block and intraspinal anesthesia [8]. Therefore, ropivacaine combined with dexmedetomidine as an alternative to opioids in perioperative period is a feasible technique. However, reports about the...
molecular mechanism of the protective effect of dexmedetomidine on neurological injury induced by ropivacaine are very limited and need to be further explored.

It has been confirmed that microRNAs (miRNAs) are highly expressed in the brain and play an important role in neurodevelopment, synaptic plasticity, learning and memory [9,10]. Some studies have believed that miRNAs could be viewed as a new target of the central nervous system and cardiovascular system [11–13]. It has been found that dexmedetomidine can up-regulate the expression of microRNA-381 (miR-381) in acute lung injury [7]. miR-381 may play a role in the repair of nerve injury after acute cerebral ischemia by negatively regulating LRRC4 and mediated SDF-1/CXCR4 signaling pathway [14]. The role of SDF-1/CXCR4 axis in promoting neuron migration, promoting angiogenesis, and protecting nerve cells has been confirmed by related studies [15]. However, the role of miR-381 and its target genes in ropivacaine-induced neuronal injury has not been reported.

In the present study, we want to find out whether dexmedetomidine can protect PC12 cells from ropivacaine injury by regulating miR-381 and its target LRRC4, and further mediate SDF-1/CXCR4 signaling pathway. Through investigating the regulation mechanism of dexmedetomidine combined with ropivacaine in PC12 cells, this study may provide a new perspective for the treatment of nerve injury with dexmedetomidine.

2. Methods

2.1. PC12 cells culture

Pheochromocytoma cell line (PC12) was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO2 at 37 °C.

2.2. Treatment by ropivacaine and dexmedetomidine

Dexmedetomidine (Precedex®, Hospira Inc., Lake Forest, IL) and ropivacaine (Naropin®, APP Pharmaceuticals, LLC, Schaumburg, IL) were used for this study. A 0.9% normal saline prepared by MilliQ water was used to dilute ropivacaine and dexmedetomidine. To investigate the safe concentration range of dexmedetomidine, it was used to treat PC12 cells with different final concentrations ranging from 0 μM to 200 μM. The cell vitality was measured by CCK-8 assay after 24 h, 48 h, or 72 h. To study the optimum concentration of dexmedetomidine combined with ropivacaine, different final concentrations of dexmedetomidine (0 μM, 10 μM, 50 μM, and 100 μM, respectively) were applied to treat PC12 cells for 24 h. Then different final concentrations of ropivacaine (0 mM, 0.1 mM, 0.5 mM, 1 mM, respectively) were added to treat cells for another 48 h. The cell vitality and the mRNA expression of both miR-381 and LRRC4 were measured.

2.3. Cell transfection

GenePharma Co., Ltd (Shanghai, China) designed and synthesized miR-381 mimic, miR-381 inhibitor, LRRC4, and scrambled controls. Non-specific microRNA mimic, inhibitor, and vector molecules were used as negative controls (mimic-NC, inhibitor-NC, and Vector, respectively). For these analyses, PC12 cells were seeded into 24-well plates and cultured to 70% confluence. Thereafter, cells were transfected with 100 nM LRRC4, 50 nM mimic, or 100 nM inhibitor using Lipofectamine 2000 Transfection Reagent (Invitrogen, Waltham, MA, USA), according to the manufacturer’s instructions. After 6 h, the transfection medium was replaced with DMEM/F-12 supplemented with 10% FBS, and cells were cultured at 37 °C in a humidified 5% CO2 atmosphere. Cells were harvested at 24 and 48 h for qRT-PCR assay and Western blot analysis, respectively.

2.4. Total mRNA extraction and quantitative real-time-PCR (qRT-PCR)

After transfection for 48 h, the total RNA was extracted from PC12 cells with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA). 500 ng RNA was reverse-transcribed into cDNA with Primer Script RT reagent kit (Takara Bio, Inc., Otsu, Japan). The mRNA expression was measured using a SYBR-Green qRT-PCR assay (Takara Bio, Inc.). The cycle number at which the reaction crossed an arbitrarily placed threshold was measured for each gene. GAPDH was used as a control for standardization. The primer sequences were as follows:

miR-381 (forward: 5'-AGTCTTATACAAGGCAAGCCTC-3' and reverse: 5'-ATCCATACAGATCCCTACCG-3');

LRRC4 (forward: 5'-AAGCTTCTTCGCTAACCTGTCC-3' and reverse: 5'-TCATATTGGATCCGTACCTTCC-3');

GAPDH (forward: 5'-ACATGCGCTCAACAGCTTCT-3' and reverse: 5'-CCCTGAAAACAGCTATATGC-3');

U6 (forward: 5'-GTAACACCTTATTCGACATCCACT-3' and reverse: 5'-CTTCTGAAAACAGCTATATGC-3'). Each group had three replicates, and the mean value of each experiment was calculated.

2.5. Protein extraction and Western blot analysis

Total proteins of cells were prepared using lysis buffer. Equal amount of protein was loaded on an SDS-PAGE and then transferred to a PVDF membrane (Millipore, USA). After the membrane was transferred, samples were blocked with TBST containing 5% bovine serum albumin for 1 h, then the membranes were incubated with primary antibody (1:1000) overnight at 4 °C with transfected membrane face up. Primary antibodies employed in this study including LRRC4(sc-376475), Bcl-2(#15071), Cleaved-Caspase-3(#9662), GAPDH(#2118), SDF-1(#3530), and CXCR4(#85578) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA, USA). After washing and incubation, the membranes were incubated with secondary antibody (1:2000) in TBST. The proteins were visualized with ECL-plus reagents. The density of the bands was analyzed by Image J software.

2.6. Dual luciferase reporter assay

The dual luciferase reporter assay was conducted as described before [14]. LRRC4-3'-untranslated region-wild type (LRRC4-3'- UTR-wt), LRRC4-3'-untranslated region-mutant type (LRRC4-3'- UTR-mut) containing miR-381 binding site were constructed and cloned into a luciferase reporter vector. Luciferase activity was measured using a dual luciferase reporter gene assay reagent (E1910, Promega Corporation, Madison, WI, USA). Cells were seeded into a 24-well plate 24 h before transfection. Plasmids of pMIR-REPORT™ beta-gal (control vector) (Ambion, Shanghai, China) or pMIR-REPORT™ − wt/mut 3' UTR LLRRC4 and the miR-381 mimic was co-transfected into cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase assays were performed 24–48 h after transfection using the Luciferase Reporter Assay System (Promega, Shanghai, China). Firefly luciferase activity was standardized to Renilla luciferase activity for every transfected well.
2.7. Cell proliferation test by CCK8

The Cell Counting Kit-8 (CCK-8) proliferation assay was obtained from Beyotime Biotechnology Company (Haimen, China). Different groups were seeded in 96-well plates at 5000 cells/well. After different treatments for 24, 48, and 72 h, the CCK-8 reagent was added to the cells (10 μL/well). After incubation for 1 h at 37 °C the cells were measured at 450 nm by a Microplate Reader (Bio-Rad 680).

2.8. Cell apoptosis test by flow cytometry

Flow cytometry was used to detect cell apoptosis using Annexin V-fluorescein isothiocyanate (FITC) apoptosis measurement kit (BD Biosciences, United State). Cells were collected and washed twice by cold PBS. 10^6 cells were suspended in 200 μL binding buffer containing 5 μL propidium iodide and 10 μL Annexin V-FITC. Then the cells were incubated in the dark for 30 min, and detected through flow cytometry analysis (Mofo XDP, USA).

2.9. Colony formation assay

Cells (2 x 10^3 per well) were seeded in 6-well plates and were cultured in complete media for 2 weeks. Then the media was removed, and cells were washed two times with PBS and stained by crystal violet (Sigma–Aldrich, MO, USA) for 60 min at room temperature. Colonies of >50 μm in size were counted by quantity one software (Bio-Rad, Richmond, USA).

2.10. Transwell assay

Cell invasion and migration was measured using polycarbonate membrane Boyden chambers with transwell apparatus (Costar, USA). After different treatments, cells were suspended in serum free medium, and 10^5 cells were added into the top chamber. The lower chamber was filled with 2 mL DMEM containing 10% FBS. The migration and invasion tests were conducted by the same steps except that the transwell chambers were coated with matrigel (BD Biosciences, San Jose, CA, USA) for invasion assay. After incubation of cells for 24 h. Cells remaining on the top surface were removed and cells migrated to the lower surface of the membrane were fixed with 100% methanol and stained with 0.5% crystal violet (Sigma, St Louis, MO, USA) for 2 min. Finally, those invasive cells were counted, and related images were captured. The average value was obtained by counting three randomly selected areas.

2.11. Statistical analysis

GraphPad Prism 6.0 software (USA) was used for statistical analysis. All experiments were conducted in triplicate and repeated at least three different times, with one representative experiment presented. The measurement data were presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for analysis among multiple groups. Comparisons between two groups were analyzed using the t test. P < 0.05 was considered statistically significant.

3. Results

3.1. Safe concentration range of dexmedetomidine for PC12 cells

Dexmedetomidine has shown special advantages in the fields of neuroprotection, and can induce cell proliferation in nerve cells [6,7]. To investigate the safe concentration range of dexmedetomidine, we measured the vitality of PC12 cells treated by different concentrations of dexmedetomidine with CCK8 assay. From 0 μM to 100 μM, the cell viability increased with the increase of dexmedetomidine concentration. However, the viability decreased significantly with the increase of dexmedetomidine concentration ranging between 150 and 200 μM (Fig. 1 A). Then, we investigated the viability changes of PC12 cells after treatment with 100 μM and 200 μM dexmedetomidine, the viability decreased significantly with the dexmedetomidine concentration of 200 μM compared with 100 μM (Fig. 1 B).

3.2. Dexmedetomidine protects PC12 cells from ropivacaine injury by upregulating the expression of miR-381 and suppressing the expression of LRRC4

In order to observe the influence of dexmedetomidine combined with ropivacaine on vitality of PC12 cells, different concentrations of dexmedetomidine (0 μM, 10 μM, 50 μM, 100 μM) and ropivacaine (0 mM, 0.1 mM, 0.5 mM, 1 mM) were applied. The cell vitality increased significantly with the increase of dexmedetomidine on the condition that the concentration of ropivacaine ranged from 0.1 mM to 1 mM. Meanwhile, the cell vitality decreased remarkably with the increase of ropivacaine (Fig. 2 A). The mRNA expression of miR-381 decreased remarkably after treatment with ropivacaine, but treatment with ropivacaine combined with dexmedetomidine reversed this trend and markedly increased the level of miR-381 (Fig. 2 B). Meanwhile, we found that ropivacaine significantly increased the expression of LRRC4, but ropivacaine combined with dexmedetomidine remarkably inhibited the expression of LRRC4 (Fig. 2 C).

3.3. MiR-381 could directly interact with target gene LRRC4 and negatively regulate its expression

A recent study demonstrated that LRRC4, is a direct target of miR-381. In this study, we further verified this interaction in 293T cells (Fig. 3 A). Then we confirmed that LRRC4 is a common bona fide target of miR-381 by performing dualluciferase reporter assays (Fig. 3 B). Meanwhile, we found that transfection of miR-381 into PC12 cells led to a significant reduction of LRRC4 at both mRNA and protein levels (Fig. 3 C and D). These findings might indicate that miR-381 could directly bind to LRRC4 3’UTR and negatively regulate LRRC4 expression.

3.4. Dexmedetomidine could reverse ropivacaine-mediated effect of PC12 cells proliferation and apoptosis by upregulating miR-381 and inhibiting its target LRRC4

The cell proliferation and apoptosis of PC12 cells were measured after different treatments. Ropivacaine (1 mM) and dexmedetomine (100 μM) were applied in this assay. The results of cell clone formation assay and CCK8 assay were similar (Fig. 4 A–C). Ropivacaine significantly decreased the cell proliferation ability compared with control, but group RD (Ropivacaine combined with dexmedetomine) markedly reversed this trend and promoted proliferation. RD + miR-381 inhibitor remarkably suppressed the cell proliferation compared with group RD + inhibitor –NC. Meanwhile, RD + LRRC4 significantly inhibited the proliferation of PC12 cells compared with group RD + vector. The results of flow cytometry indicated that Ropivacaine significantly increased apoptosis, but group RD markedly suppressed the apoptosis of PC12 cells. Meanwhile, transfection with miR-381 inhibitor and LRRC4 both could markedly increase the level of apoptosis (Fig. 4 D and E). Caspase-3 gene plays an important role in apoptosis, and the expression intensity of Caspase-3 protein reflects the degree of apoptosis of cells. The Bcl-2 gene is an important apoptosis...
inhibitory gene, which can prevent the occurrence of apoptosis and prolong the survival of cells. There was a negative correlation between Bcl-2 and Caspase-3 protein expression. The expression of apoptosis related proteins, Bcl-2 and Cleaved-Caspase-3, were also measured by western blotting after different treatments (Fig. 4F and G). Ropivacaine significantly increased Cleaved-Caspase-3 and decreased Bcl-2 compared with control group, but treatment with RD markedly reverse these changes. Meanwhile, group RD + miR-381 inhibitor and group RD + LRRC4 shown similar influence on the expression of apoptosis related proteins, they both decreased the expression of Bcl-2 and increase the expression of Cleaved-Caspase-3 (Fig. 4F and G).

3.5. Dexmedetomidine could reverse ropivacaine-mediated effect of PC12 cells migration and invasion through miR-381/LRRC4/SDF-1/ CXCR4 signaling pathway

We further investigated the migration and invasion of PC12 cells after different treatments through transwell assay. The result of migration was in line with invasion experiment (Fig. 5A–D). The migration and invasion of cells were significantly inhibited after treatment with ropivacaine, but ropivacaine (1 mM) combined with dexmedetomidine (100 μM) markedly decreased this inhibition. Meanwhile, both RD + miR-381 inhibitor and RD + LRRC4 could remarkably suppress the migration and invasion of PC12 cells. We also proved that ropivacaine could significantly reduce the expression of SDF-1 and CXCR4, but increase LRRC4. However, ropivacaine combined with dexmedetomidine could markedly reverse the effects induced by treatment with ropivacaine only. Meanwhile, comparing with relative controls, RD + miR-381 inhibitor and RD + LRRC4 remarkably reduced the expression of SDF-1 and CXCR4, but upregulated the level of LRRC4 (Fig. 5E and F). These results suggest that dexmedetomidine could effectively regulate SDF-1/CXCR4 pathway-related proteins. The protective effect of dexmedetomidine on ropivacaine-induced cytotoxicity is related to the miR-381/LRRC4/SDF-1/CXCR4 signaling pathway.

4. Discussion

Peripheral nerve block is a common regional anaesthetic technique and is regularly used for a broad spectrum of interventional, surgical, or diagnostic procedures [16,17]. Ropivacaine, a kind of long-acting blocks with local anaesthetics, is believed to be beneficial for improving postoperative pain therapy. However, it has been found that ropivacaine could damage neurons to some extent [5], and the specific mechanism of its neurotoxicity has not been fully elucidated.

Dexmedetomidine, a kind of α2 adrenergic receptor agonist, has always been considered as an adjuvant of ropivacaine to prolong the nerve block [18]. Several studies have reported that dexmedetomidine plays an important role against the injury of neural related cells [19−21]. PC12 cells are derived from the pheochromocytoma of the rat adrenal medulla, and they are often used for study as a common neuronal cell line [21,22]. In this study, we found that dexmedetomidine could promote the proliferation of PC12 cells within a specific concentration range. The safe concentration range of dexmedetomidine for PC12 cells is 0 μM−100 μM. Meanwhile, the proliferation of PC12 cells was inhibited significantly with the concentration increasing of ropivacaine (from 0 mM to 1 mM).
to 1mM), which was in line with previous reports that ropivacaine could damage neurocytes [5]. Therefore, dexmedetomidine might protect PC12 cells from ropivacaine injury.

It has been confirmed that miR-381 was upregulated in normal hippocampal neurons and mature brain [14]. Meanwhile, inhibition of cell proliferation and invasion was accevious reports suggested that LRRC4 was highly specific to brain tissue [23,24], and it is a regulator in the pathogenesis of malignant gliomas [25,26]. In the present study, we found the direct interaction between miR-381 and LRRC4, and LRRC4 could be negatively regulated by miR-381. Therefore, dexmedetomidine might protect PC12 cells from ropivacaine-induced injury by increasing miR-381 and inhibiting its target LRRC4. We further proved that treatment with dexmedetomidine could reverse ropivacaine-mediated effects and significantly increased the proliferation, migration, and invasion of cell, and inhibited the apoptosis comparing with treatment with ropivacaine only. However, transfection with miR-381 inhibitor or LRRC4 could markedly reverse those trends described above, which indicates that both miR-381 and LRRC4 might be the downstream regulator of dexmedetomidine. It was reported that miR-381 repair nerve injury through regulation of the SDF-1/CXCR4 signaling pathway via LRRC4 [14]. Therefore, we finally investigated if dexmedetomidine protect PC12 cells from ropivacaine injury through the SDF-1/CXCR4 signaling pathway. Our findings indicate that both RD + miR-381 inhibitor and RD + LRRC4 could remarkably reduce the expression of SDF-1/CXCR4, but upregulate the level of LRRC4. Therefore, dexmedetomidine may protect PC12 cells from ropivacaine-induced cytotoxicity through miR-381/LRRC4/SDF-1/CXCR4 signaling pathway, consolidating the mechanism of dexmedetomidine in ropivacaine induced cell injury.

The present study aims to investigate the mechanism and neuroprotection of dexmedetomidine on nerve injury induced by
Fig. 4. Dexmedetomidine regulated the proliferation and apoptosis of PC12 cells by upregulating miR-381 and inhibiting its target LRRC4. Representative pictures of clone formation assay (A). The amount of colony formation was counted (B). The proliferation of PC12 cells was detected by CCK8 assay (C), Representative pictures of cell apoptosis measured by flow cytometry (D). Quantification of the flow cytometry results (E). Measurement of apoptosis related proteins expression by western blotting (F). Quantification of apoptosis related proteins expression after different treatments (G). All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001.
ropivacaine in neuronal PC12 cells. Our findings suggested that dexmedetomidine could be used as a potential therapeutic drug for anesthetic toxicity.

5. Conclusions

Our study demonstrates the safe concentration range of dexmedetomidine for PC12 cells, and identifies the protection effect of dexmedetomidine on PC12 cells form ropivacaine injury. Meanwhile, we prove that dexmedetomidine plays the protective effect through miR-381/LRRC4/SDF-1/CXCR4 signaling pathway. This study may provide new insight targeting miR-381/LRRC4/SDF-1/CXCR4 signaling pathway on the prevention of neurocyte injury.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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None.

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