Original Article

Inhibition of CD38/Cyclic ADP-ribose Pathway Protects Rats against Ropivacaine-induced Convulsion

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Abstract

Background: The CD38/cyclic ADP-ribose (cADPR) pathway plays a role in various central nervous system diseases and in morphine tolerance, but its role in local anesthetic intoxication is unknown. The aim of this study was to determine the role of the CD38/cADPR pathway in ropivacaine-induced convulsion.

Methods: Forty male Sprague-Dawley rats were randomly divided into five groups (n = 8 per group): sham group, ropivacaine group, ropivacaine+8-Br-cADPR (5 nmol) group, ropivacaine+8-Br-cADPR (10 nmol) group, and ropivacaine+8-Br-cADPR (20 nmol) group (no rats died). Rats were intracerebroventricularly injected with normal saline or 8-Br-cADPR 30 min before receiving an intraperitoneal injection of ropivacaine. Electroencephalography and convulsion behavior scores were recorded. The hippocampus was harvested from each group and subjected to nicotinamide adenine dinucleotide and cADPR assays, Western blotting analysis, and malondialdehyde (MDA) and superoxide dismutase (SOD) assays.

Results: Intraperitoneal injection of ropivacaine (33.8 mg/kg) induced convulsions in rats. CD38 and cADPR levels increased significantly following ropivacaine-induced convulsion (P = 0.031 and 0.020, respectively, compared with the sham group). Intraventricular injection of 8-Br-cADPR (5, 10, and 20 nmol) significantly prolonged convulsion latency (P = 0.037, 0.034, and 0.000, respectively), reduced convulsion duration (P = 0.005, 0.005, and 0.005, respectively), and reduced convulsion behavior scores (P = 0.015, 0.015, and 0.000, respectively). Intraventricular injection of 8-Br-cADPR (10 nmol) also increased the B-cell lymphoma-2 (Bcl-2)/Bcl-2-associated X protein ratio (P = 0.044) and reduced cleaved Caspase 3/Caspase 3 ratio, inducible nitric oxide synthase, MDA and SOD levels (P = 0.014, 0.044, 0.001, and 0.010, respectively) compared with the ropivacaine group.

Conclusions: The CD38/cADPR pathway is activated in ropivacaine-induced convulsion. Inhibiting this pathway alleviates ropivacaine-induced convulsion and protects the brain from apoptosis and oxidative stress.

Key words: CD38; Convulsion; Cyclic ADP-ribose; Nicotinamide Adenine Dinucleotide; Ropivacaine

Introduction

Ropivacaine is recognized as one of the safest amino-amide local anesthetics.[1] However, toxic symptoms have been reported in the central nervous system (CNS) and the cardiovascular system.[2] Serious convulsions have also been documented, and these are generally caused by drug overdose and/or accidental intravascular injection.[1] According to previous studies, the mechanism of local anesthetic-induced convulsion may involve the γ-aminobutyric acid type A receptor,[3,4] N-methyl-D-aspartate receptor, and potassium channels.[5-7] Ropivacaine-induced potential bursts were associated with phospholipase C activity in neurons, and the seizure discharge emanated mainly from the amygdala and hippocampus.[8,9] However, the mechanism of ropivacaine-induced convulsion remains unclear.

CD38 is the main nicotinamide adenine dinucleotide (NAD^+)-glycohydrolase in mammals. CD38 catalyzes both the synthesis and degradation of cyclic ADP-ribose (cADPR) from NAD^+.[10] cADPR is an important second messenger

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that targets Ca^{2+}-induced Ca^{2+} release mediated by ryanodine receptors (RyRs) to mobilize Ca^{2+} outflow, as well as activates Ca^{2+} influx by stimulating the transient receptor potential melastatin 2 channel in the plasma membrane.[11]

The CD38/cADPR pathway plays a role in various physiological and pathological conditions in the CNS. CD38 is an important regulator of oxytocin release, which is necessary for the regulation of pair cognition and nurturing behaviors in mice.[12] CD38 is highly expressed in the brain during development and is a positive regulator of astrocyte development and oligodendrocyte differentiation.[13] In mice, cADPR inhibits the mTOR signaling pathway downstream of dopamine receptors in the striatum.[14] Hull *et al*.[15] reported that the antinociceptive potency of morphine was increased by intracerebroventricular injection of the CD38 substrate, NAD, in mice. Furthermore, morphine tolerance was reversed by intracerebroventricular administration of a cADPR inhibitor. The antinociceptive action of morphine was also less potent in CD38 knockout mice compared to wild-type mice. These findings indicate that the CD38/cADPR pathway plays a role in the mechanistic actions of anesthesia. This study aimed to determine if the CD38/cADPR pathway plays a role in ropivacaine-induced convulsion, as well as to explore the underlying mechanism.

Methods

Ethical statement

This study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (No. 201502034), and performed in compliance with the World Medical Association *Declaration of Helsinki on Ethical Principles for Medical Research Involving Humans for studies involving experimental animals* and Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines.

Materials and reagents

Ropivacaine was purchased from AstraZeneca (London, UK). ADP-ribosyl cyclase, 8-Br-cADPR, alcohol dehydrogenase, resazurin, NAD, nicotinamide, bovine serum albumin (BSA), diaphorase, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), bicine, alkaline phosphatase, riboflavin 5'-mono-phosphate (FMN), NADase, nucleotide pyrophosphatase, and ethylene diamine tetraacetic acid (EDTA)-Na were purchased from Sigma (St. Louis, MO, USA). Phosphodiesterase I was purchased from Worthington Biochemicals (Lakewood, Canada). CD38 antibody and cADPR were purchased from Santa Cruz (Dallas, TX, USA). Antibodies for Caspase 3, cleaved Caspase 3 (C-Caspase 3), and inducible nitric oxide synthase (iNOS) were purchased from Cell Signaling Technology (MA, USA). Antibodies for B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) were purchased from Biyotime (Shanghai, China). The superoxide dismutase (SOD) assay kit was purchased from Neobioscience (Shenzhen, China), and the malondialdehyde (MDA) assay kit was purchased from Solarbio (Beijing, China).

Electroencephalography monitoring

Male Sprague-Dawley rats with a mean weight of 200 g were acclimated for at least 1 week, maintained at 22 ± 2°C on a 12 h light/dark cycle, and fasted overnight before surgery. Electroencephalography (EEG) electrodes were placed in each rat 10 days before intraperitoneal injection of ropivacaine as previously described.[16] Following intraperitoneal anesthesia with 10% chloral hydrate at 350 mg/kg, the rat head was fixed in a stereotaxic apparatus (Narishige Scientific Instrument Lab, Setagaya-ku, Tokyo, Japan). After sterilization, a sagittal incision of 3 cm was made on the top of the head, and 10% lidocaine was sprayed onto the skull surface. After removing the periosteum, three holes were drilled in the skull and a sterilized stainless steel 0.6-mm screw was gently placed in each hole: two at the frontal sinus (10 mm anterior to the anterior fontanelle, 1 mm lateral to the sagittal plane) and one at the somatosensory cortex (2.5 mm posterior to the anterior fontanelle, 2.5 mm right lateral to the sagittal suture). The left electrode was connected to the ground.

Intraventricular injection

Intraventricular injection was performed as previously described[12] with slight modifications. Rats were anesthetized with 2.5 ± 0.2% isoflurane vaporized into a mixture of 30% O_2 and 70% N_2, through a gas anesthesia mask (Model 929-B Rat Gas Anesthesia Head Holder; David Kopf Instruments, Tujunga, CA, USA), and delivered at a flow rate of 5 L/min.[17] Rats were fixed in a stereotaxic apparatus, and holes were drilled (0.8 mm posterior to the anterior fontanelle, 1.5 mm right lateral to the anterior fontanelle) based on a stereotaxic rat brain atlas.[18] A microsyringe was placed in the hole toward the right ventricle until the needle had penetrated and was withdrawn with cerebrospinal fluid. Then, 10 μl of solution was injected over 10 min.

Rats were randomly divided into five intervention groups (n = 8 per group): sham group (one rat died), ropivacaine group (one rat died), ropivacaine+8-Br-cADPR (5 nmol) group (no rats died), ropivacaine+8-Br-cADPR (10 nmol) group (no rats died), and ropivacaine+8-Br-cADPR (20 nmol) group (no rats died). The dose ranges of 8-Br-cADPR were chosen according to a previous study.[11] Sham and ropivacaine groups were intraventricularly injected with 10 μl normal saline solution. Ropivacaine+8-Br-cADPR groups were injected with 5, 10, and 20 nmol of 8-Br-cADPR dissolved in 10 μl normal saline solution, respectively. Rats were anesthetized and sacrificed 60 min after ropivacaine injection. The hippocampus was dissected according to the rat brain atlas[18] and frozen in liquid nitrogen.

Rat models of ropivacaine-induced convulsion

According to a previous pharmacological study, the maximum convulsive dose of ropivacaine is 33.8 mg/kg in rats.[19] Therefore, 30 min after intraventricular injection, rats in the ropivacaine and ropivacaine+8-Br-cADPR groups

were intraperitoneally injected with 33.8 mg/kg of 0.5% ropivacaine dissolved in 10 ml of normal saline solution. The sham group was intraperitoneally injected with 10 ml of normal saline solution.

Convulsion behavior scoring

Convulsion behavior was continually observed for 60 min and scored with a convulsive behavior score as previously described:[19,20] 0 = normal; 1 = increased excitability, nodding-like jitter, facial muscle spasm (rhythmical blinking, chewing, beard shaking, etc.); 2 = convulsion (nodding or whipping); 3 = clonicity of one front paw; 4 = clonicity of both front paws, but able to stand; and 5 = generalized clonic seizures, imbalance, unable to stand, and impaired righting reflex. Latency was measured from the time of intraperitoneal ropivacaine injection to the occurrence of level 4 convulsion behavior, and the duration was measured from the occurrence of level 4 convulsion behavior to the disappearance of convulsion behavior.

Nicotinamide adenine dinucleotide assay

Intracellular NAD levels were determined as previously described.[21,22] Briefly, the samples were lysed in 100 mmol/L HCl on ice for 10 min, and the supernatants were neutralized with 100 mmol/L KOH. Samples were mixed with a freshly prepared reaction medium (100 mmol/L bicine, 1.66 mmol/L phenazine ethosulfate 0.83 mg/ml of BSA, 0.5 mol/L ethanol, 0.42 mmol/L of MTT, 4.17 mmol/L EDTA-Na, and 2 U alcohol dehydrogenase) in 96-well plates and incubated at 30°C for 30 min in the dark. Absorbance at 550 nm was measured with purified NAD as the standard.

Cyclic ADP-ribose assay

cADPR levels were measured as previously described.[22,23] Briefly, samples were extracted with 0.6 mol/L perchloric acid and then with an organic solution of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine (3:1) on ice. The aqueous layer was collected and a reagent (0.44 U/ml nucleotide pyrophosphatase, 0.0625 U/ml NADase in 2.5 mmol/L $MgCl_2$, 12.5 U/ml alkaline phosphatase, and 20 mmol/L sodium phosphate pH 8.0) was added and incubated overnight at 37°C. The samples were filtered with Multiscreen Assay System filtration plates. Then, 50 μl of the next reagent (30 mmol/L nicotinamide, 0.3 μg/ml ADP-ribosyl cyclase, and 100 mmol/L sodium phosphate, pH 8.0) and 100 μl of cycling reagent (100 μg/ml alcohol dehydrogenase, 0.1 mg/ml BSA, 2% ethanol, 20 μmol/L resazurin, 10 μmol/L of FMN, 10 μg/ml diaphorase, 10 mmol/L nicotinamide, and 100 mmol/L sodium phosphate, pH 8.0) were sequentially added to 100 μl of each samples and incubated for 15 min and 4 h, respectively, at room temperature. Fluorescence was measured with a fluorescence plate reader at an excitation of 544 nm and an emission of 590 nm, with purified cADPR as the standard.

Western blotting analysis

Tissues were chopped into small pieces, lysed with radioimmunoprecipitation assay buffer, and sonicated on ice. The lysates were centrifuged and the supernatants were collected. Samples were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min, electrophoresed on 10% SDS-PAGE, and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were probed with primary antibodies overnight at 4°C and then with HRP-conjugated secondary antibodies. Bands were visualized using ECL reagent (GE Healthcare, Marlborough, MA, USA).

Tissue malondialdehyde and superoxide dismutase assays

Tissue MDA levels and SOD activity were measured with commercial kits following the manufacturer's instructions. Absorbance at 450 nm (SOD) and 532 nm (MDA) was measured with a microplate reader (Bio-Tek, Shanghai, China). Concentrations were calculated according to the manufacturer's instructions and normalized for total protein concentration.

Statistical analysis

Data were expressed as mean ± standard deviation, which satisfied the normal distribution with homogeneous variances. Differences between groups were analyzed by one-way analysis of variance and *post hoc* analysis using SPSS 19.0 software (IBM, Armonk, NY, USA). A $P < 0.05$ was considered statistically significant.

Results

Intraventricular injection of 8-Br-cyclic ADP-ribose reduces convulsion behavior scores, prolongs latency, and shortens duration of ropivacaine-induced convulsion

Convulsion behavior scores were >4 following intraperitoneal injection of ropivacaine (33.8 mg/kg) in all rats in the ropivacaine group. In ropivacaine+8-Br-cADPR (5 nmol and 10 nmol) groups, we only observed convulsion scores >4 in one rat in each dose group. In the ropivacaine+8-Br-cADPR (20 nmol) group, we did not observe any convulsion score >4. The convulsion behavior scores were significantly reduced in the ropivacaine+8-Br-cADPR (5, 10, and 20 nmol) groups compared to the ropivacaine group ($P = 0.015$, 0.015, and 0.000, respectively). Convulsion latency was prolonged ($P = 0.037$, 0.034, and 0.000, respectively), and the duration was shortened ($P = 0.005$, 0.005, and 0.005, respectively) in the ropivacaine+8-Br-cADPR (5, 10, and 20 nmol) groups compared to the ropivacaine group [Figure 1a–1c].

Intraventricular injection of 8-Br-cyclic ADP-ribose reduces electroencephalography spike waves

EEG was continually monitored from the time of intraperitoneal ropivacaine injection to the disappearance of convulsion. If convulsion was not observed, EEG was monitored for 60 min. After intraperitoneal injection of ropivacaine, we detected spike waves on the EEG. As shown in the representative EEGs of sham, ropivacaine, and ropivacaine+8-Br-cADPR groups, spike waves were significantly reduced in the ropivacaine+8-Br-cADPR groups [Figure 1d].

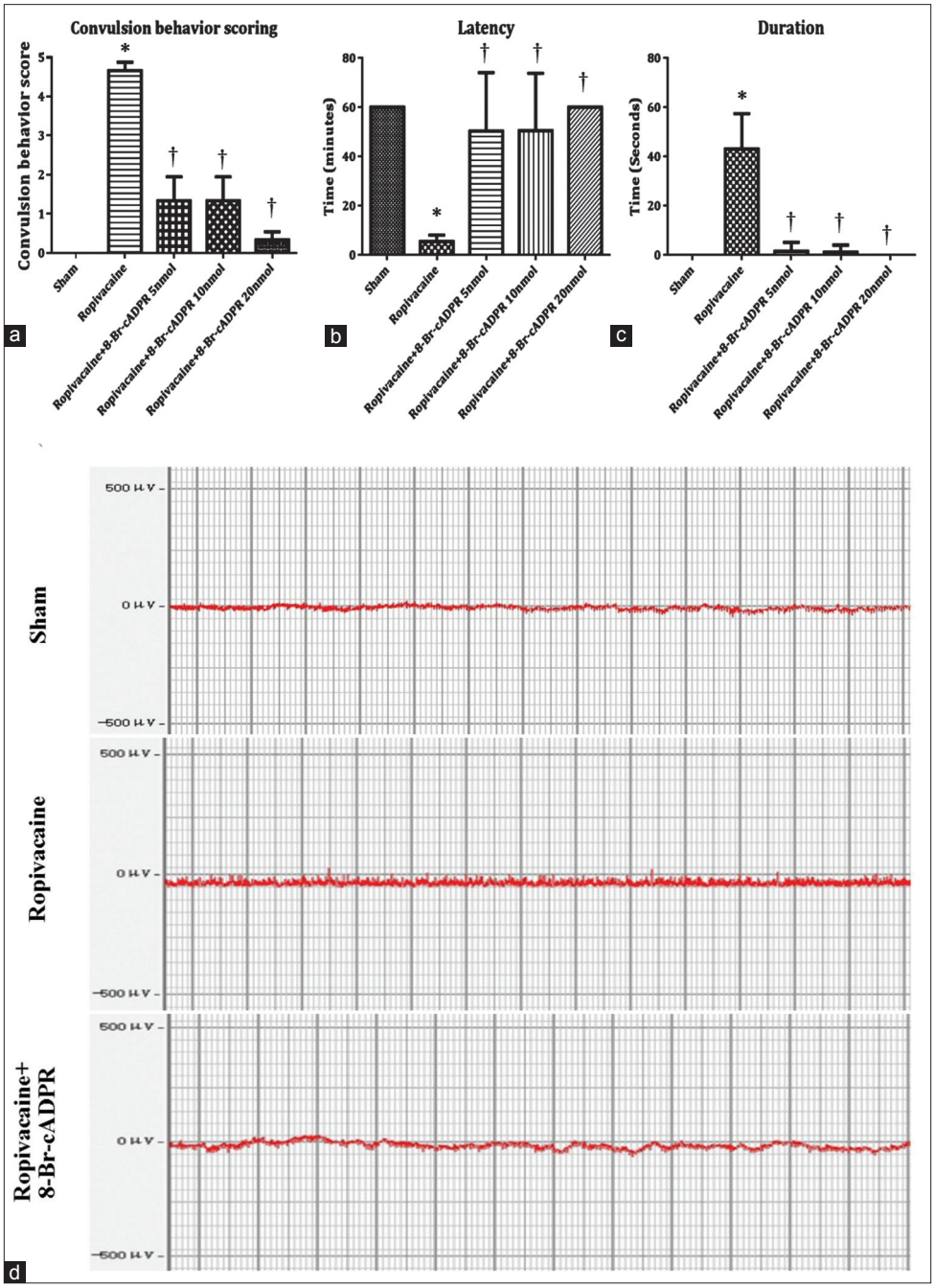

Figure 1: Convulsion behavior and electroencephalography analysis of rats (n = 6 per group). (a) Convulsion behavior scores in the different groups. (b) Convulsion latency. (c) Convulsion duration. (d) Respective electroencephalography images: sham group, ropivacaine group, and ropivacaine+8-Br-cyclic ADP-ribose (10 nmol) group. *P < 0.05 compared to the sham group; †P < 0.05 compared to the ropivacaine group.

Intraventricular injection of 8-Br-cyclic ADP-ribose reduces CD38 and cyclic ADP-ribose levels following ropivacaine-induced convulsion

CD38 and cADPR levels in the hippocampus increased significantly in the ropivacaine group compared to the sham group (P = 0.031 and 0.020, respectively). Intraventricular injection of 8-Br-cADPR (5, 10, and 20 nmol) significantly reduced intracellular cADPR levels compared to the ropivacaine group (P = 0.026, 0.035, and 0.016, respectively). CD38 expression was significantly reduced in the ropivacaine+8-Br-cADPR (20 nmol) group compared to the ropivacaine group (P = 0.025) [Figures 2 and 3].

Intraventricular injection of 8-Br-cyclic ADP-ribose inhibits apoptosis following ropivacaine-induced convulsion

Western blotting analysis showed that the Bcl-2/Bax ratio decreased and the C-Caspase 3/Caspase 3 ratio increased in the ropivacaine group compared to the sham group (P = 0.001 and 0.042, respectively). Intraventricular injection of 8-Br-cADPR (10 nmol) significantly increased the Bcl-2/Bax ratio (P = 0.044). Intraventricular injection of

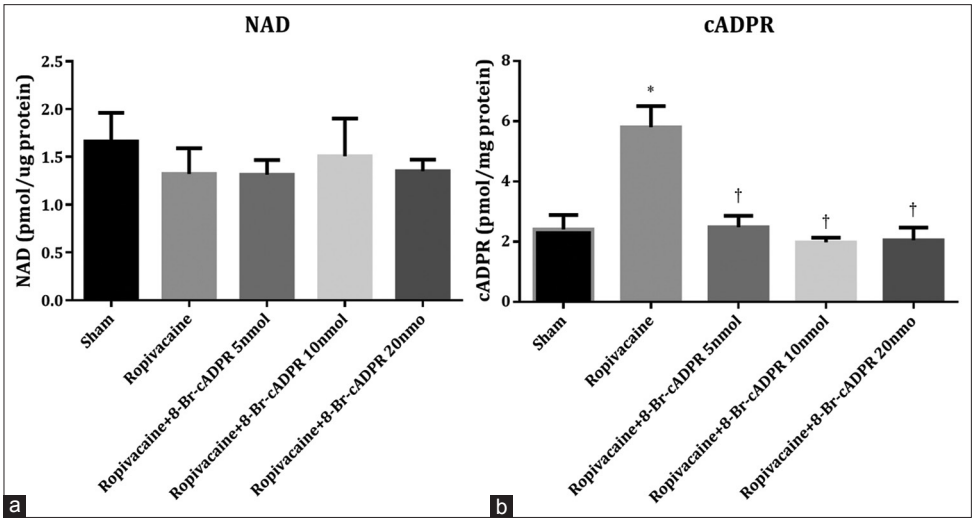

Figure 2: Intracellular NAD^+ (a) and cyclic ADP-ribose (b) levels in the brain ($n = 3$ per group). *$P < 0.05$ compared to the sham group; †$P < 0.05$ compared to the ropivacaine group. NAD: Nicotinamide adenine dinucleotide.

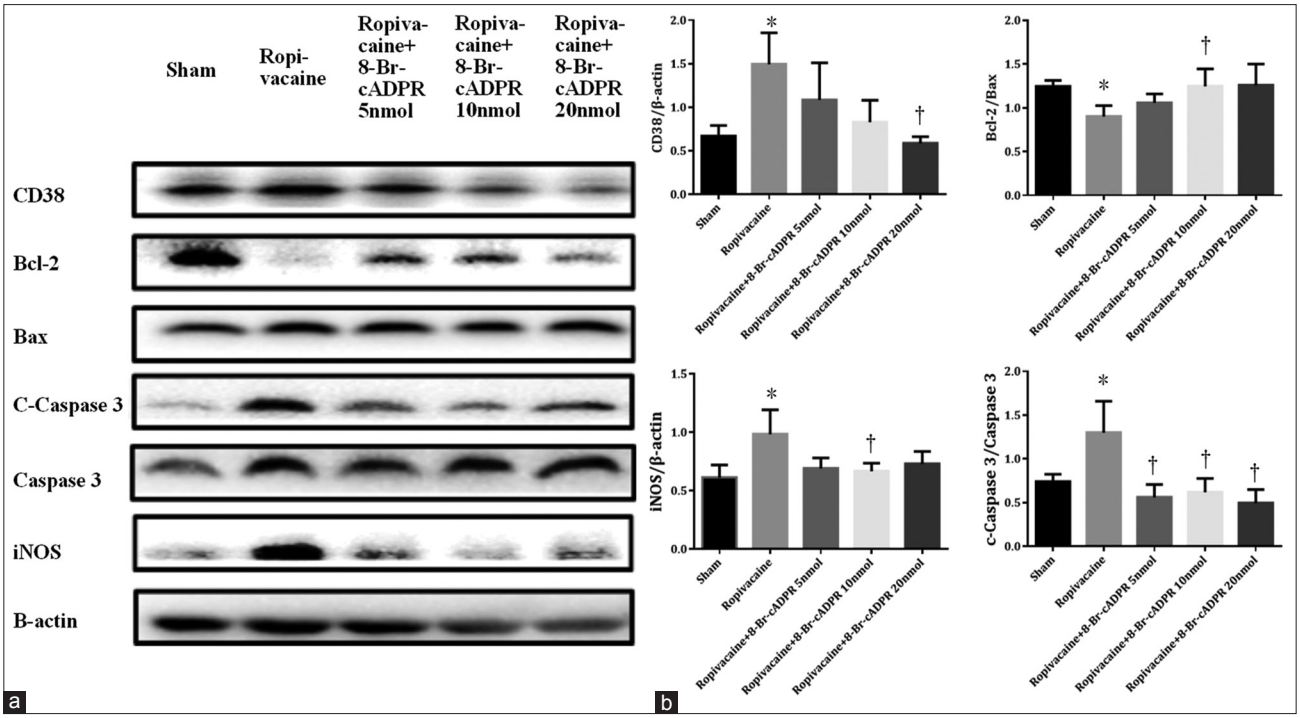

Figure 3: CD38, Bcl-2, Bax, iNOS, Caspase 3, and cleaved Caspase 3 (C-Caspase 3) expression in the brain. (a) Western blotting analysis of CD38, Bcl-2, Bax, iNOS, Caspase 3, C-Caspase 3, and β-actin. (b) Densitometry analysis of CD38/β-actin, Bcl-2/Bax, iNOS/β-actin, C-Caspase 3/Caspase 3 ($n = 4$ per group). *$P < 0.05$ compared to the sham group; †$P < 0.05$ compared to the ropivacaine group. Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein; iNOS: Inducible nitric oxide synthase.

8-Br-cADPR (5, 10, and 20 nmol) significantly decreased the C-Caspase 3/Caspase 3 ratio ($P = 0.009$, 0.014, and 0.005, respectively) [Figure 3].

Intraventricular injection of 8-Br-cyclic ADP-ribose reduces inducible nitric oxide synthase, superoxide dismutase activity, and malondialdehyde levels following ropivacaine-induced convulsion

Intraperitoneal injection of ropivacaine significantly increased iNOS expression in the hippocampus compared to the sham group ($P = 0.029$). Intraventricular injection of 8-Br-cADPR (10 nmol) significantly reduced iNOS expression ($P = 0.044$ vs. ropivacaine group) [Figure 3]. Intraperitoneal injection of ropivacaine also significantly increased SOD activity ($P = 0.017$ vs. the sham group) and MDA levels ($P = 0.012$ vs. the sham group), while 8-Br-cADPR groups (5, 10, and 20 nmol) reduced SOD activity ($P = 0.009$, 0.010, and 0.019, respectively) and MDA levels ($P = 0.007$, 0.001, and 0.003, respectively) compared to the ropivacaine group [Figure 4].

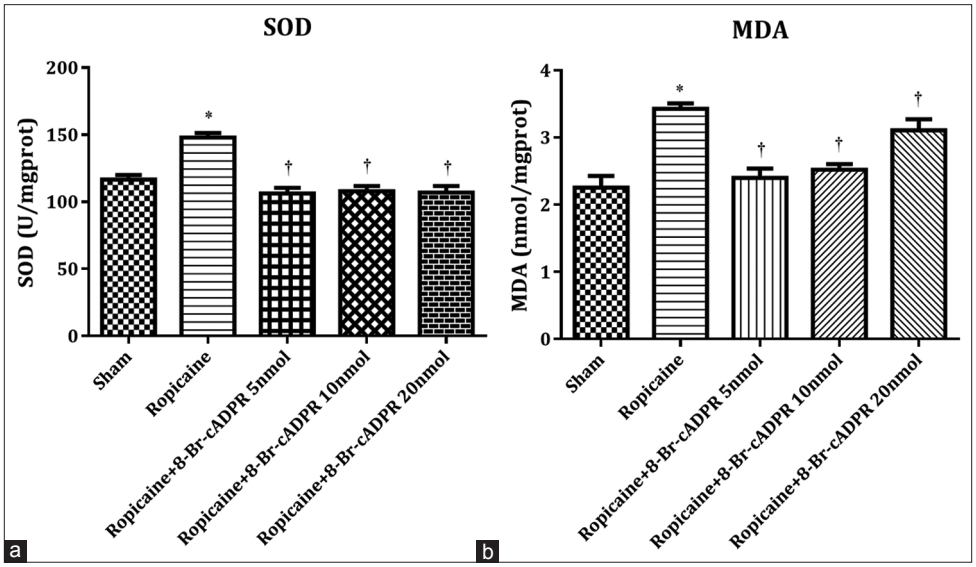

Figure 4: SOD activity (a) and MDA levels (b) in the brain ($n = 3$ per group). $^{*}P < 0.05$ compared to the sham group; $^{\dagger}P < 0.05$ compared to the ropivacaine group. SOD: superoxide dismutase; MDA: malondialdehyde.

Discussion

Convulsion is a highly severe toxic symptom caused by ropivacaine, but the underlying mechanisms of neurotoxicity are poorly understood. According to previous studies, neuronal potential bursts and seizures that discharge from the amygdala and hippocampus are potential mechanisms underlying ropivacaine-induced convulsions.[8,9] The CD38/cADPR pathway is an important regulator of intracellular Ca^{2+} signaling that induces Ca^{2+} outflow from the endoplasmic reticulum Ca^{2+} stores through the RyRs.[11] RyRs abnormalities have been implicated in the generation and maintenance of seizures, and inhibiting RyRs-protected mice against pentylenetetrazole-induced seizures.[24] The CD38/cADPR pathway also plays an important role in potential burst, synaptic transmission, and signal transduction in neurons and glia cells.[12] Therefore, we hypothesized that the CD38/cADPR pathway is involved in ropivacaine-induced convulsion. In this study, convulsions were induced by intraperitoneally injecting a large dose of ropivacaine. The convulsions were accompanied by activation of the CD38/cADPR pathway in the hippocampus. We chose to study the hippocampus because it is the main origin of local anesthetic-induced seizure discharge.[8] Intraventricular injection of a cADPR inhibitor, 8-Br-cADPR, significantly reduced CD38 and cADPR levels, alleviated ropivacaine-induced convulsion (as evidenced by prolonged latency), and reduced duration and convulsion behavior scores. These results indicate that the CD38/cADPR pathway is involved in the pathogenesis of ropivacaine-induced convulsion.

Previous studies have reported that the mechanism of ropivacaine-mediated neurotoxicity involves apoptosis and oxidative stress. Ropivacaine promoted apoptosis in the spinal cord and caused cell death in PC12 and SH-SY5Y cells.[25,26] Ropivacaine dose dependently induced neurotoxicity and triggered apoptosis in the spinal cord after repeated intrathecal administration.[27] Spinal injection of ropivacaine increased MDA, glutathione, and SOD levels in spinal cord, as well as increased the levels of free radicals in the spinal fluid.[28] Therefore, to understand the mechanism by which 8-Br-cADPR alleviates ropivacaine-induced convulsion, we investigated the effect of 8-Br-cADPR on apoptosis and oxidative stress. Existing evidence on the effect of the CD38/cADPR pathway on apoptosis is scant and controversial. A previous study reported that inhibiting the CD38/cADPR pathway reduced lipopolysaccharide/interferon-γ (LPS/IFN-γ)-induced microglia activation and activation-induced cell death.[29] However, Ma *et al*. reported that CD38 plays an important role in cell survival and that CD38 deficiency increased apoptosis.[30,31] In this study, we found that inhibiting cADPR with 8-Br-cADPR reduced apoptosis in the brains of rats following ropivacaine-induced convulsion, which was associated with changes in the Bcl-2/Bax and Caspase 3 pathways.

The CD38/cADPR pathway also plays a role in oxidative stress. iNOS expression and NO production induced by LPS/IFN-γ were significantly reduced in a CD38 knockout model and by 8-Br-cADPR treatment.[29,32] CD38 overexpression enhanced the production of reactive oxygen species (ROS), while CD38 deficiency reduced ROS during neural differentiation of mouse embryonic stem cells.[33] In this study, we found that intraventricular injection of 8-Br-cADPR significantly reduced iNOS, SOD activity, and MDA levels in the brains of rats following ropivacaine-induced convulsion, indicating ropivacaine's protective effect against oxidative stress.

In conclusion, this study showed that intraperitoneal injection of ropivacaine (33.8 mg/kg) induced convulsions

in rats. The CD38/cADPR pathway was activated in the brains of rats following ropivacaine-induced convulsion. Intracerebroventricular injection of a cADPR inhibitor, 8-Br-cADPR, significantly alleviated ropivacaine-induced convulsions and protected the brain from apoptosis and oxidative stress.

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Conflicts of interest

There are no conflicts of interest.

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