Activation of the Akt signaling pathway alleviates ropivacaine-induced myelination impairment of the spinal cord and hypoalgesia in neonatal rats

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Research Article

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Abstract

Prolonged exposure to local anesthetics (LAs) or intrathecal administration of high doses of LAs can cause spinal cord damage. Intraspinal administration of LAs is increasingly being used in children and neonates. Therefore, it is important to study LA-related spinal cord damage and the underlying mechanism in developmental models. First, neonatal Sprague–Dawley rats received three intrathecal injections of 0.5%, 1%, or 2% ropivacaine or saline (90 min interval) on postnatal day (P)7. The electron microscopy and behavioral tests were performed to evaluate the neurotoxic effects of ropivacaine at different concentrations on spinal cord in rats. Western blot analysis was performed to measure the expression levels of p-Akt, Akt, myelin gene regulatory factor (MYRF) and myelin basic protein (MBP) in the spinal cord following administration of 2% ropivacaine. Our results showed that ropivacaine impaired myelination in the spinal cord and induced hypoalgesia in a dose-dependent, whereas 0.5% ropivacaine did not cause spinal cord damage in neonatal rats. Moreover, 2% ropivacaine decreased the expression of p-Akt, MYRF and MBP in the spinal cord. Then, the Akt-specific activator (SC79) was intraperitoneally injected 30 min before 2% ropivacaine treatment. Interestingly, SC79-mediated activation of the Akt signaling pathway alleviated ropivacaine-induced the impairment of myelination in the spinal cord and hypoalgesia. Overall, the results showed that ropivacaine caused spinal cord damage in a dose-dependent manner in neonatal rats and that activation of the Akt signaling pathway alleviated these ropivacaine-induced changes. These data provide insight into the damage to the developing spinal cord caused by LAs.

Introduction

Local anesthetics (LAs) are extensively used in epidural and spinal anesthesia. However, prolonged exposure to LAs or administration of high doses of LAs can cause neuronal damage, manifested as transient nervous system syndrome, cauda equina syndrome, and delayed sacral nerve disorder[1–3]. Studies in animal models have also demonstrated that LAs exert neurotoxic effects on the spinal cord, with the major histological change being myelin damage [4–7]. Previous studies on the toxic effect of LAs on the spinal cord have mainly focused on adults or adult rodent models, but there are few studies on the toxic effect of LAs on the developing spinal cord. As epidural and spinal anesthesia is widely used in children and newborns [8], it is necessary to elucidate the toxic effect of LAs during early development and the underlying mechanisms.

Myelin damage is the most serious histological change in the spinal cord following LA administration [4–6]. However, the mechanism by which LAs cause myelin damage remains unclear. Myelin gene regulatory factor (MYRF) is a transcription factor that is specifically expressed in oligodendrocytes in the central nervous system and plays a key role in axonal myelination and remyelination [9–11]. MYRF can directly bind to the enhancer sequences of myelin genes, such as myelin basic protein (MBP) and proteolipid protein (PLP), to induce their expression, thereby promoting the maturity of oligodendrocytes and eventually inducing axonal myelination and remyelination[9–12]. Importantly, we found that in adult rats, lidocaine downregulates MYRF expression and blocks remyelination in spinal axons [6]. In vitro and in
vivo studies have demonstrated that LAs cause neurotoxicity by decreasing the activation of Akt signaling [13–15]. More importantly, repeated intrathecal injection of ropivacaine significantly reduces phosphorylated Akt (p-Akt) expression in the rat spinal cord for up to 28 days[13]. Prior studies have shown that the Akt signaling pathway plays a pivotal role in myelination and remyelination in the central nervous system[16]. One possible mechanism is Akt signaling pathway-mediated regulation of MYRF and myelin gene (e.g., MBP, PLP and MOG) expression [17–20]. In accordance with these findings, we hypothesized that the Akt signaling pathway and MYRF may be involved in myelin damage induced by LAs. However, whether they are involved in the toxic effect of LAs on the spinal cord in developmental models remains to be further studied.

Using neonatal rat models, we investigated spinal cord damage by electron microscopy and behavioral tests after intrathecal injection of 0.5%, 1% or 2% ropivacaine during infancy. The results showed that intrathecal administration of 1% or 2% ropivacaine impaired myelination and induced hypoesthesia in neonatal rats. In addition, the expression levels of P-Akt, MYRF and MBP in the spinal cords of newborn rats treated with 2% ropivacaine were determined by western blotting. The results showed that 2% ropivacaine significantly downregulated the protein expression of P-Akt, MYRF and MBP in the spinal cords of neonatal rats. Moreover, activation of the Akt signaling pathway alleviated 2% ropivacaine-induced impairment of myelination in the spinal cord and hypoesthesia. Our studies provide insight into the toxic effect of LAs on the spinal cord.

**Methods**

**Animals**

The experimental procedures and protocols of this study were approved by the Institutional Animal Care and Use Committee of Hunan Children's Hospital, Changsha, China. The female and male Sprague–Dawley rat pups used in the experiment were provided by Hunan SJA Laboratory Animal Co., Ltd. (China). The experimental procedure was performed in strict accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines. All rats were housed in an animal care facility at a temperature of 20°C on a 12-h light-dark cycle.

**Animal grouping and treatment**

The animal experiment was divided into two parts. All pups received percutaneous intrathecal injection into the L4-L5 or L5-L6 intervertebral space using a 30-gauge needle connected to a microinjector and Hamilton syringe under 3% isoflurane anesthesia as previously described[21]. In the first part of the experiment, postnatal day (P)7 rats were randomly divided into the NS, 0.5% R, 1% R and 2% R groups. Rats in these groups received 3 injections of saline (0.5 µl/g) or 0.5%, 1%, or 2% ropivacaine (Macklin, China), respectively, at 90-min intervals for a duration of 4.5 h (Fig. 1a)[13, 22]. In the second part of the experiment, P7 rats were randomly divided into the NS + vehicle, 2% R + vehicle and 2% R + SC79 groups. The pups in the 2% R + SC79 group were intraperitoneally injected with 0.04 mg/g SC79 (Beyotime, China) 30 min before intrathecal administration of 2% ropivacaine. SC79 was dissolved in
dimethylsulfoxide (DMSO) (Beyotime, China). The pups in the other 2 groups received the same volume of vehicle (DMSO) (Fig. 4a). The researchers involved in data collection and analysis were blinded to the experimental groups.

**Behavioral tests**

Baseline behavioral measurements were performed before intrathecal injection on P7. Then, behavioral tests were performed daily after intrathecal injection until P21.

**Mechanical withdrawal threshold (MWT).** After the pup rats were allowed to acclimate to an elevated mesh platform, von Frey filaments (Yuyan Instruments, China) were used to stimulate the middle surface of the hindpaw. Filaments with an increasing bending force were applied until the first foot withdrawal response. The bending force in grams of the filament that first evoked a withdrawal response was recorded. The MWT was calculated as the average of three consecutive measurements for each hindpaw.

**Thermal withdrawal latency (TWL).** The TWL was measured with a Hargreaves box (Yuyan Instruments, China). The rat pups were placed on a glass plate and allowed to acclimate, and then an infrared light was focused on the mid-plantar surface of the hindpaw. The intensity of the infrared light and duration of infrared light stimulation were recorded. To avoid scalding the hindpaw, the cutoff time was set to 20 s. The TWL was calculated as the average of three measurements for each hindpaw.

**Molecular biology analysis**

Western blot analysis of spinal cord tissues was performed on P10, P14 and P21. After the rats were euthanized, the L2-L6 spinal cord tissues of the rats were quickly placed on ice. Then, total protein was extracted from the L2-L6 spinal cord tissues using cold RIPA buffer containing a protease inhibitor (Beyotime, China). The protein concentration was measured using the BCA Protein Assay Kit. The proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% bovine serum albumin in TBS and then incubated with the primary antibodies at 4°C overnight, followed by incubation with secondary antibody (Absin, Shanghai, China) for 1 h at room temperature. The following primary antibodies were used: rabbit anti-MYRF, rabbit anti-MBP, rabbit anti-Akt, rabbit anti-p-Akt and rabbit anti-GAPDH (Abcam, MA, USA). Super ECL Western Blot Detection Reagents (Millipore, USA) were used to detect the immune complexes. The intensity of each protein band was determined by ImageJ software (National Institutes of Health, United States).

**Histomorphometry analysis**

An electron microscope was used to observe the posterior white matter of the spinal cord on P14 and P21. After being euthanized, the animals were perfused with fixative (2% paraformaldehyde/2.5% glutaraldehyde) in phosphate buffer (pH 7.4). Next, the L2-L6 spinal cord tissue was removed and placed in the same fixative overnight. The spinal posterior white matter was cut into 1-mm³ coronal sections. All tissues were postfixed in 1% osmium tetroxide, dehydrated in graded acetone solutions, and embedded in Embed 812. The resin blocks were cut into 60–80-nm ultrathin slices by an ultramicrotome. The ultrathin
sections were stained with uranyl acetate and observed and analyzed under an electron microscope. The g-ratio was measured using Image-Pro Plus 6.0 image processing software (Media Cybernetics, USA) by an investigator blinded to the groups. The g-ratio is the ratio of the inner diameter of the axon to the outer diameter of the axon and was used to assess axonal myelination. At least 200 axons from four rats per group were assessed to determine the g-ratio. The g-ratios of at least 200 axons in the spinal cord of each rat were measured, and at least 4 rats from each group were assessed.

**Statistical analysis**

Unless otherwise stated, all data are shown as the mean ± standard deviation (SD). GraphPad Prism software (version 8.0; GraphPad Software, Inc., USA) was used for data analyses and graph production. An unpaired t test was used to compare means between two groups. One-way ANOVA followed by Tukey's post hoc test was used for comparisons of means between more than two groups. Two-way repeated-measures ANOVA followed by Tukey's post hoc test was used for comparisons of behavioral data. Statistical significance was defined as P < 0.05.

**Results**

**Ropivacaine impaired myelination and induced hypoalgesia in a dose-dependent manner**

To investigate spinal cord damage in neonatal rats after intrathecal injection of ropivacaine, we examined myelin sheaths in the posterior white matter of the spinal cord on P14 and P21 using electron microscopy. The myelin thickness in the posterior white matter of the spinal cord was decreased in the rats treated with 1% or 2% ropivacaine compared with rats in the NS group on P14 and P21 (Fig. 1b). Quantitative analysis of the g-ratio showed that the g-ratio in the posterior white matter of the spinal cord increased as the ropivacaine concentration increased (Fig. 1c and d). However, there was no significant difference in the g-ratio in the posterior spinal cord between the 0.5% ropivacaine group and the NS group on P14 or P21 (Fig. 1c and d). In addition, behavioral tests were performed in our study. Similarly, we found that ropivacaine induced hypoalgesia in a dose-dependent manner. The results showed that compared with NS group, the 2% R group showed the greatest increase in the MWT and TWL, followed by the 1% R group, while the 0.5% R group showed no significant increase in these values (Fig. 2a and b).

**Ropivacaine inhibited Akt signaling and downregulated MYRF and MBP expression**

To investigate the molecular mechanism by which ropivacaine impairs myelination in the neonatal rat spinal cord, we measured the protein expression levels of p-Akt, MYRF and MBP in spinal cord tissues from rats treated with 2% ropivacaine on P10, P14 and P21. The results showed that the expression of p-Akt, MYRF and MBP in the spinal cord was reduced in rats in the 2% R group compared to those in the NS group on P10 (Fig. 3b) and P14 (Fig. 3c), but there was no significant difference in the expression of these proteins between the two groups on P21 (Fig. 3d).
Akt activation reversed the decrease in MYRF and MBP expression induced by ropivacaine

To explore the role of the Akt signaling pathway in ropivacaine-induced myelination impairment, we induced Akt signaling pathway activation in this model. In this study, we used an Akt-specific activator (SC79) to activate the Akt signaling pathway. Western blotting showed that the expression levels of p-Akt, MYRF, and MBP in the spinal cord were significantly decreased in the 2% R + vehicle group compared with the NS + vehicle group on P10 and P14 (Fig. 4b and c). However, the expression of p-Akt, MYRF, and MBP in the spinal cord was significantly increased in the 2% ropivacaine- and SC79-cotreated rats (2% R + SC79 group) compared with the 2% R + vehicle group rats on P10 and P14 (Fig. 4b and c).

Akt activation attenuated ropivacaine-induced myelination impairment in the spinal cord and hypoalgesia

Electron microscopy showed that the 2% ropivacaine- and vehicle-cotreated rats (2% R + vehicle group) exhibited a significantly thinner myelin sheath in the posterior white matter of the spinal cord than rats in the NS + vehicle group at P14 and P21 (Fig. 5a). Similarly, the g-ratio in the posterior white matter of the spinal cord was significantly increased in the rats in the 2% R + vehicle group compared with those in the NS + vehicle group on P14 and P21 (Fig. 5b and c). Interestingly, the myelin sheath thickness in the posterior white matter of the spinal cord was significantly increased in the 2% R + SC79 group compared with the 2% R + vehicle group (Fig. 5a), and the g-ratio value was significantly decreased on P14 and P21 (Fig. 5b and c). The MWT and TWL of rats in the 2% R + SC79 group were significantly decreased compared with those of rats in the 2% R + vehicle group on P14 and P21 (Fig. 6a and b).

Discussion

Ropivacaine is a commonly used LA for pediatric spinal anesthesia. Our study showed that ropivacaine impaired myelination in the spinal cord and induced hypoalgesia in a dose-dependent manner in neonatal rats. Moreover, 2% ropivacaine significantly downregulated the expression of P-Akt, MYRF and MBP in neonatal rat spinal cord tissue. Prophylactic administration of an Akt activator (SC79) alleviated ropivacaine-induced impairment of myelination and sensory dysfunction and upregulated the expression of MYRF and MBP in the spinal cord.

Past studies have indicated that intrathecal injection of LAs can cause spinal cord damage in adult rabbits and rodents, but different types and different concentrations of LAs have different effects. Moreover, the toxic effect of most LAs on the spinal cord is concentration dependent. Treatment with 10% lidocaine results in axonal degeneration in the spinal cord and myelin changes [6, 23], but the equivalent dose of bupivacaine does not [24]. Treatment with 0.6 mg/kg 5% bupivacaine does not cause histological changes in the spinal cord in adult rats[25], whereas a higher dose (2.8 mg/kg) induces spinal cord white matter damage[4]. Intrathecal injections of 10% lidocaine, tetracaine, 2% bupivacaine, and 2% ropivacaine result in vacuolation and degeneration of gray matter neurons[5, 23]. However, there have been few
studies on the toxic effect of LAs on the spinal cord during development. Hamurtekin E et al. showed that a single administration of LA at close to the clinical concentration (0.5% bupivacaine) has no effect on the spinal nerves of developing rats[26]. However, the researchers noted that rat pups received a lower dose per body weight (2.5 mg/kg) and were exposed to a lower drug concentration because of both the injectate concentration and further dilution due to the relatively greater cerebrospinal fluid volume in pups[26]. The researchers could not rule out toxicity from high concentrations and repeated exposure [26]. In our study, we administered 0.5%, 1% or 2% ropivacaine by intrathecal injection 3 times (0.5 µl/g each, 90 min apart) and surprisingly found that ropivacaine impaired myelination in the spinal cord and induced hypoalgesia in a dose-dependent manner in neonatal rats. However, at a concentration of 0.5% (similar to the clinical concentration), ropivacaine did not cause spinal cord damage in neonatal rats, similar to previous studies. Myelination impairment is an important factor leading to sensory dysfunction[27], which is consistent with our results, suggesting that ropivacaine may cause hypoalgesia in neonatal rats by impairing myelination. Due to the short observation period, it is unclear whether the ropivacaine-induced impairment of spinal myelination and hypoalgesia are completely alleviated when rats reach adulthood.

Previous studies on the neurotoxic mechanisms of LAs have mainly assessed its effects on apoptosis, oxidative stress, and mitochondrial damage [28–31]. The molecular mechanism of LA-induced myelin changes has rarely been studied. Our previous study showed that 10% lidocaine results in demyelination and downregulated MYRF expression in the spinal cords of adult rats[6]. In addition, decreased expression of MYRF blocks remyelination[6]. MYRF regulates MBP expression and promotes oligodendrocyte maturation, which plays an important role in myelination and remyelination in the central nervous system [9–12]. In this neonatal rat model, we observed downregulation of MYRF and MBP expression on days 3 and 7 after repeated intrathecal injection of 2% ropivacaine, and on day 14, the levels tended to be normal. Intrathecal injection of 2% ropivacaine decreased MYRF and MBP expression in the neonatal rat spinal cord for at least one week. In this study, we also found a significant decrease in Akt phosphorylation, similar to previous studies [13–15]. In addition, the expression of MYRF and MBP was similar to that of p-Akt in this model. Prior studies have suggested that the Akt signaling pathway regulates MYRF, MBP and PLP and plays a vital role in myelination and remyelination[16–20]. Therefore, in this study, SC79 (an activator of Akt) was used to reverse the ropivacaine-induced decline in p-Akt expression in the neonatal rat spinal cord. Interestingly, following activation of the Akt signaling pathway (increased p-Akt expression), the expression of MYRF and MBP was upregulated, and myelination impairment and hypoalgesia were significantly alleviated. Hence, Akt signaling pathway-mediated regulation of MYRF and MBP expression may be involved in ropivacaine-induced myelination impairment in the spinal cord and hypoalgesia in neonatal rats.

In summary, the toxic effect of ropivacaine on the developing spinal cord was dose-dependent and manifested as myelination impairment in the spinal cord and hypoalgesia. Moreover, 2% ropivacaine significantly downregulated p-Akt, MYRF and MBP expression in the neonatal rat spinal cord, and activation of the Akt signaling pathway alleviated 2% ropivacaine-induced myelination impairment in the
spinal cord and hypoalgesia. These data provide insight into the toxic effect of LAs on the spinal cord during development.

Declarations

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Data Availability All data generated or analyzed during this study are included in this published article.

Author Contribution All authors contributed to the study conception and design. Study design: Lei Wu, Shuangquan Qu and Zhen Du. Study conduct: Lei Wu, Siwei Wei, Zhen Xiang, Eryou Yu, Zhen Chen, Dongjie Pei and Yiyi Yao. Data analysis and writing the manuscript: Lei Wu and Siwei Wei. Revision of the manuscript: Lei Wu, Shuangquan Qu and Zhen Du.

Ethics approval This study has been approved by the Animal Ethics Committee of Hunan Children's Hospital.

Conflict of Interest The authors declare no competing interests.

Consent to participate Not applicable.

Consent for Publication Not applicable.

References


Figures

Figure 1
Ropivacaine impaired myelination in the spinal cord of neonatal rats in a dose-dependent manner. a Schematic describing the workflow of the experiment. b Electron microscope images of the myelin sheath in the spinal posterior white matter of each group at P14 and P21. (c, d) Quantification of the g-ratio at P14 (c) and P21 (d). Compared with the NS group, the g-ratio value of the 2%R group increased the most, while the g-ratio value of the 0.5%R group did not increase significantly. *P<0.05, **P<0.01 and ***P<0.001 vs. NS group, one-way ANOVA followed by a Tukey post hoc test.

Figure 2

Ropivacaine induced hypalgesia in neonatal rats in a dose-dependent manner. (a, b) There was no difference in the mechanical withdrawal threshold or thermal withdrawal latency in each group before injection of drugs. After injection, the mechanical withdrawal threshold (a) and thermal withdrawal latency (b) were significantly increased in the 1%R and 2%R groups on P10, P14 and P21 compared with the NS group but not in the 0.5%R group. Among them, the mechanical withdrawal threshold and thermal withdrawal latency increased the most in the 2%R group. ***P<0.001 vs. NS group, two-way repeated-measures ANOVA followed by the Tukey post hoc test.
Ropivacaine (2%) downregulated P-Akt, MYRF and MBP expression in the spinal cord. **a** Representative Western blots of each protein. **b-d** Relative quantitative analysis of each protein. The protein expression of p-Akt, MYRF and MBP in the spinal cord of rats in the 2% R group was significantly decreased on P10 (**b**) and P14 (**c**) compared with that in the NS group. However, there was no significant difference in
protein expression in each group on P21 (d). **P<0.01 and ***P<0.001 vs. NS group, one-way ANOVA followed by a Tukey post hoc test.

Figure 4

Akt activation (SC79) reversed the decreased expression of MYRF and MBP induced by ropivacaine. a Schematic describing the workflow of the experiment. b Representative Western blots of each protein. c
Relative quantitative analysis of each protein. The expression of P-Akt, MYRF and MBP in the spinal cord of the 2%R+SC79 group was significantly increased on P10 (b) and P14 (c) compared with that in the 2%R+vehicle group. **P<0.01 and ***P<0.001 vs. NS+vehicle group, ##P<0.01 and ###P<0.001 vs. 2% R+vehicle group, one-way ANOVA followed by a Tukey post hoc test.

Akt activation (SC79) attenuated ropivacaine-induced myelination impairment in the spinal cord. a Electron microscope images of the myelin sheath in the spinal posterior white matter of each group at P14 and P21. (b, c) Quantification of the g-ratio at P14 (b) and P21 (c). The g-ratio value of posterior
spinal white matter in the 2%R+SC79 group was significantly decreased compared with that in the 2%R+vehicle group on P14 and P21. ***P<0.001 vs. NS+vehicle group, #P<0.05 and ###P<0.001 vs. 2% R+vehicle group, one-way ANOVA followed by a Tukey post hoc test.

Figure 6

Akt activation (SC79) attenuated ropivacaine-induced hyperalgesia. (a, b) The mechanical withdrawal threshold (a) and thermal withdrawal latency (b) in the 2%R+SC79 group were significantly reduced compared with those in the 2%R+Vehicle group on P14 and P21. ***P<0.001 vs. NS+vehicle group, #P<0.05, ##P<0.01 and ###P<0.001 vs. 2% R+vehicle group, two-way repeated-measures ANOVA followed by the Tukey post hoc test.