In vivo and *in vitro* evidence of the neurotoxic effects of ropivacaine: The role of the Akt signaling pathway

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Abstract. There is a growing body of evidence that suggests common complications in regional anesthesia, including transient neurological syndrome, are caused by the neurotoxicity of local anesthetics (LAs). Ropivacaine is thought to be one of the safest LAs, however, there have been several studies detailing possible neurotoxic effects. At present, the exact molecular mechanism of ropivacaine-mediated neurotoxicity is unknown. The present study was designed to explore the possible mechanisms underlying the neurotoxicity of ropivacaine. The neurotoxic effects of ropivacaine were assessed in spinal cord by TUNEL staining for apoptosis and in cultured PC12 cells by cell viability assays. Protein kinase B (Akt) activation was evaluated by immunoblotting. Ropivacaine promoted apoptosis and caused cell death in a treatment group compared with a sham-operated group. Furthermore, ropivacaine significantly diminished Akt activation. There were significantly lower Akt levels in cells exposed to ropivacaine compared with controls. The present study demonstrated ropivacaine neurotoxicity *in vivo* and *in vitro*, mediated by the Akt signaling pathway. The neurotoxicity of apoptosis with concomitant cell death, mediated by ropivacaine, may offer an explanation for its adverse effects (e.g., transient neurological syndrome).

Introduction

Local anesthetics (LAs) have been used for relieving acute, intraoperative, postoperative and chronic pain for several years. Generally LAs are thought to be safe, but the neurotoxicity of LAs remains a considerable concern in certain cases. Ropivacaine is thought to be one of the safest LAs for pain relief, however, its efficiency is low, resulting in the utilization of high concentrations of LAs to relieve pain completely (1,2). Previous studies have reported considerable neurotoxicities associated with ropivacaine, particularly at high concentrations (3,4). However, the molecular mechanisms through which LAs induce neurotoxicity remain poorly understood. Apoptosis, necrotic cell death and protein kinase B (Akt) signaling pathways may be involved (5-7).

It has been demonstrated that the activation of Akt is critical for cell anti-apoptosis (8). Survival factors suppress apoptosis by activating the serine/threonine kinase Akt, which then phosphorylates and inactivates components of the apoptotic process. Therefore, cells destined for apoptosis exhibit lower levels of Akt. Measuring Akt concentrations, under experimental conditions, may allow for an improved understanding of one of the possible mechanisms for cell death under specific conditions.

It previously has been reported (9) that high concentrations of ropivacaine result in neurotoxicity in specific cell lines. The current study was designed to determine the molecular neurotoxic mechanism of 1% ropivacaine in *in vivo* and *in vitro* models. In the present study, spinal cord *in vivo* and PC12 cell line *in vitro* models were utilized, with the aim to assess the possible neurotoxic cellular mechanistic effects of ropivacaine.

Materials and methods

Ethics and animals. The *in vivo* study was approved by the Ethics Committee for Animal Research of XiangYa Hospital in Central South University, China. Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals.

Chemicals. Commercially available ropivacaine (Naropin 10 mg/ml, Astra-Zeneca, Rueil-Malmaison, Sweden) was used and diluted with NaCl 0.9%.

Animal selection and housing. The experiment was performed using male Sprague-Dawley rats weighing 280-320 g. Rats were housed two/cage in the animal facility for one week, under 12:12 h light-dark cycle. Food and water were available at all times. Animal studies were performed following approval from the Institutional Animal Care and Use Committee in Xiangya Hospital, Central South University, China.

Surgical procedure for intrathecal catheterization. Rats were anesthetized with 10% chloral hydrate. As described

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previously (10), a sterile PE-10 tube filled with saline was inserted through the L5/L6 intervertebral space, placing the tip of the tube at the spinal lumbar enlargement level. The cannulated rats were observed for five days following intrathecal catheterization. Rats demonstrating symptoms of traumatic nerve injury or signs of neurological impairment during catheterization were excluded from further experiments.

Intrathecal administration of drugs. Following successful intrathecal catheterization, 72 rats were randomly divided into two groups (2x36). In group R, rats received 8 injections of 1% ropivacaine (0.12 ml/kg), at 90-min intervals, for a duration of 12 h. Ropivacaine concentration was determined by a pilot study (11). A dosing interval of 1.5 h was based on a study by Rose *et al* (12). An identical amount of saline was administered by the same method in the group NS (control group). The catheter was flushed with 10 μl of saline to account for the dead-space of the catheter. At each injection, the solution was administered over seconds and then capped with a pin. Each group was observed on days 1, 3, 5, 7, 14 and 28 (n=6 at each time point).

Spinal cord section specimen. The animals in each group were sacrificed at each time point under terminal anesthesia. Transverse sections of the spinal lumbar enlargement region (L2-L6) in each animal were removed en bloc and each was dissected into two gross sections (A and B). The transverse specimen with spinal cord L3 (sample A) was used for TUNEL staining (see below) and embedded in paraffin. The posterior section specimen (sample B) was used for immunostaining examination.

TUNEL staining for apoptosis. For the detection of apoptosis, we used a Fluorescein FragEL™ DNA fragmentation detection kit QIA39 (Calbiochem, Darmstadt, Germany) to identify apoptotic nuclei in paraffin-embedded tissue sections. TUNEL was performed on 5-μm thick transverse sections of sample A. We used fluorescence microscopy and a x40 oil lens. Fields under x40 lenses within one section were randomly selected for quantification of TUNEL-positive cells by counting. Data were expressed as average count/field/animal. Images were captured under high-power magnification (x200).

Western blot analysis. Sample B specimens from each group were homogenized in a lysis buffer containing protease inhibitors and phosphatase inhibitors. Supernatants were obtained following centrifugation at 10,000 x g (4˚C, 5 min). Protein concentration was estimated using the Bradford reagent (Sigma, St. Louis, MO, USA). The protein was mixed with laemmli sample buffer and heated at 99˚C for 5 min. For western blot analysis, an equal amount of protein (50-80 μg) was loaded into each well and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred from the gel to polyvinylidene fluoride (Millipore, Bedford, MA, USA) membranes and blocked in 5% skimmed milk prepared in 1X TBST. Membranes were incubated with the following primary antibodies overnight at 4˚C: anti-Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-phospho-Akt (Stressgene, San Diego, CA, USA) or anti-β-actin (Santa Cruz Biotechnology, Inc.; each at 1:1,000). Blots were developed using Beyo ECL plus detection system (Beyotime Institute of Biotechnology, Shanghai, China) and relative band density was measured using FluroChem FC2 System (NatureGene Corp., Medford, NJ, USA).

Cell culture and treatments. PC12 cells (ProMab Bitechnologines Inc., Hunan, China) were routinely cultured in growth medium (GM) composed of DMEM and 10% fetal bovine serum (FBS; Hyclone SH30084.03, Haikelong, China) at 37˚C and 5% CO_2. The cells were pre-seeded at appropriate densities. Ropivacaine was prepared as stocks of 1% (15 mmol/l) in GM with the pH adjusted to 7.4. The concentrations of ropivacaine used in the present study were based on dose-dependent neurotoxicity effects and on previous *in vitro* studies (13,14). The concentrations of ropivacaine were 1, 2.5, 5, 10, 15 and 25 mM. A high concentration of ropivacaine was included since it most likely causes cell death in PC12 cells. Each assay was observed at 48 h.

MTT cell counting assay. The cell toxicity of ropivacaine was assessed using the 3-(4,5-dimethyl-thiszol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Cayman Chemical Co., Ann Arbor, MI, USA). Cells were plated at 4,000 cells/well in 96-well plates and grown in GM in the absence (control group NS) or presence of ropivacaine (group R) for 48 h. MTT assay was performed using an enzyme-linked immunosorbent assay, measuring optical density values at 570 nm (630 nm calibration).

Measurement of cell viability. Trypan blue exclusion assay was used to measure cell viability. Cells were cultured in GM in group NS or group R and plated at 20,000 cells/well in 24-well plates. Time and concentrations of ropivacaine were as described for the MTT assay. The medium was changed and images of the cells were captured daily with an inverted microscope (Olympus, Tokyo, Japan). Following image capture, cells were trypsinized and stained with trypan blue (Mediatech, Manassas, VA, USA). Viable (non-stained) and non-viable (blue) cells were counted using a hemocytometer (Beckman, Brea, CA, USA).

Cell western blot analysis. PC12 cells were grown in GM in group NS or group R for 48 h. Cells were harvested and lysed in a lysis buffer containing protease and phosphatase inhibitors. Protein quantification, SDS-PAGE and immunoblotting were performed as described previously (15), using the following primary antibodies: anti-Akt and anti-phospho-Akt (Thr308; Cell Signaling, Danvers, MA, USA; each at 1:1,000) and anti-β-actin (Abcam, Cambridge, MA, USA; 1:5,000). Blots were developed and the relative band density was measured as described for sample B specimens.

Statistical analysis. Statistical data were from multiple experiments or measurements and were presented as the mean ± SEM (n=6). The significance of differences ($P<0.05$) was evaluated by one-way ANOVA followed by the Bonferroni/Dunnett post hoc test, where appropriate. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was utilized to perform the tests.

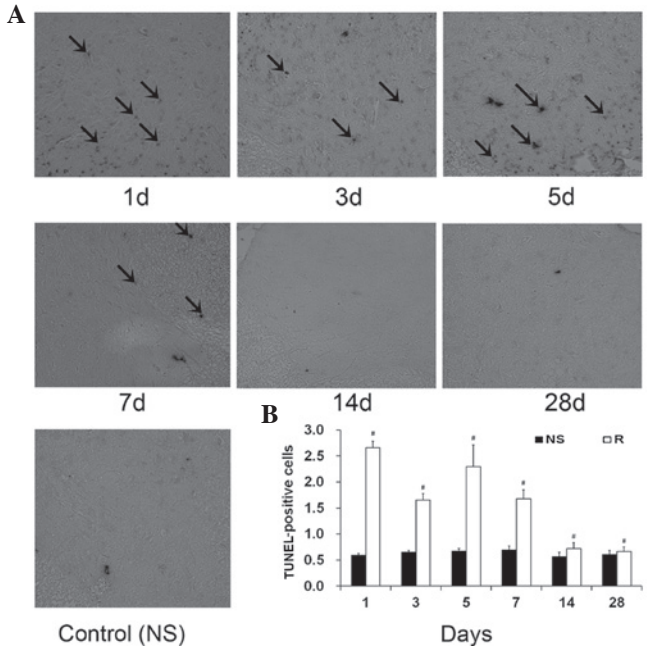

Figure 1. Effect of 1% ropivacaine on apoptosis in spinal cord. Group R, ropivacaine treatment; group NS, saline control treatment. (A) TUNEL staining was performed. Original magnification, x200; scale bar: 5 μm. (B) Analysis of apoptotic cells in spinal cord of groups R and NS at defined time points. #P<0.05 vs. group NS.

Results

Surgical procedure. No visual evidence of CSF leakage was recorded following catheter placement or during the experiments. The rats demonstrated no signs of clinical complications of the central nervous system. Postmortem examination confirmed that all catheters were sited correctly.

Measurement of apoptosis - TUNEL staining. TUNEL staining was performed to determine whether exposure to 1% ropivacaine on days 1, 3, 5, 7, 14 and 28 triggers apoptosis-mediated neurotoxicity (Fig. 1). The percentages of TUNEL-positive cells in group NS were 0.030±0.005, 0.032±0.008, 0.034±0.007 and 0.035±0.003% on days 1, 3, 5 and 7, respectively. The percentages of TUNEL-positive cells in group R were 1.33±0.26, 0.89±0.15, 1.15±0.18 and 1.34±0.21% at the same time points. The mean number of TUNEL-positive cells in sample A of group R was significantly higher than that in group NS on days 1, 3, 5 and 7 (P<0.05).

Ropivacaine and the Akt pathway. Akt activation was assessed by immunoblotting with an anti-activated Akt antibody (pAkt) in sample B, as described in Fig. 2A. Ropivacaine significantly suppressed Akt activation (Fig. 2A, lanes 1-6). Multiple experiments (n=6, Fig. 2B) demonstrated that the expression levels of pAkt in group R were significantly less than those in group NS at all time points (P<0.05). Total Akt levels were also examined by immunoblotting using an anti-total Akt antibody. We observed no significant difference of total Akt proteins in group R compared with group NS (Fig. 2C; P>0.05). This correlated with the degree of apoptosis under the same conditions (compare Fig. 1B with Fig. 2A). Akt was activated in group NS cells (Fig. 3A, lane 1). By contrast, ropivacaine suppressed Akt activation (Fig. 3A, lane 2). Ropivacaine was associated with greater apoptosis in spinal cord and suppressed Akt activation.

Measurement of cell death - MTT assay. An MTT assay was performed to investigate the molecular mechanism(s) underlying this *in vitro* toxicity. To evaluate the toxic effects of ropivacaine, we performed cell counts using the culture model system PC12 (16,17). As shown in Fig. 4 by MTT assay, group NS cells grew vigorously and reached minimum confluence at 48 h; the cell survival rate was 99%. By contrast, the cell density in the presence of ropivacaine significantly declined; the cell survival rate was 46.1±7.3%. Notably, group NS cells grew to a significantly higher density than those of group R (P<0.05). These data demonstrate that ropivacaine leads to cell death.

Apoptosis and necrosis have been suggested to be two of the mechanisms by which LAs induce cell death (18). In the present study, we used trypan blue exclusion to assess the viability of PC12 cells. As shown in Fig. 5A, dead cells appear stained due to nuclear condensation. Viable cells were not stained. PC12 cells were grown in the absence or presence of ropivacaine. As already observed in Fig. 4, the total cell number in group R was significantly less than that of group NS (Fig. 5B). In group NS, dead cells (stained apoptotic and necrotic) were rare (2±1%). In group R, the percentage of stained cells was 79.2±13.4% at 48 h. Cell death percentages were calculated by normalizing non-viable cells to total cells and statistical results were exhibited (Fig. 5C). PC12 cells treated with ropivacaine had a had greater number of stained death identifiers.

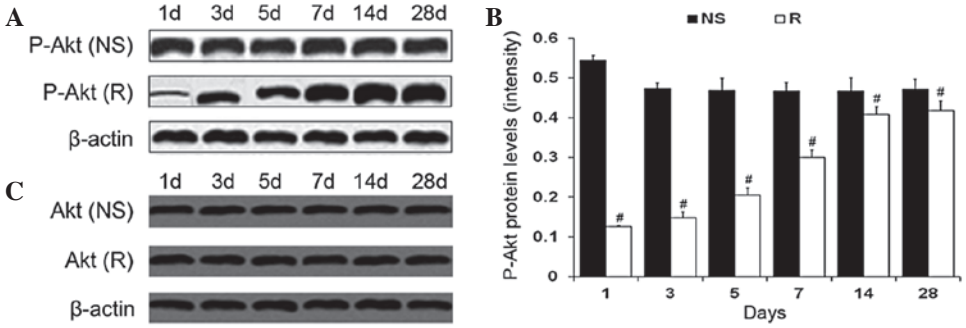

Figure 2. Effects of 1% ropivacaine on Akt activation. (A) Immunoblotting with anti-pAkt antibodies and (C) anti-total Akt antibodies. (B) Densitometric analysis of anti-pAkt signals from six independent experiments, #P<0.05.

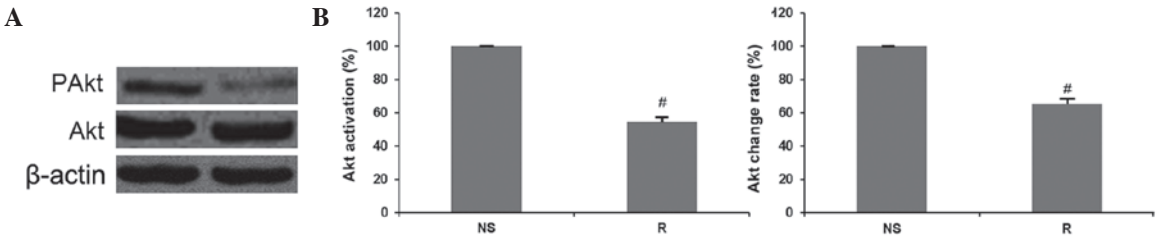

Figure 3. Effect of ropivacaine on Akt activation in PC12. Group R, ropivacaine treatment; group NS, saline control treatment. (A) Immunoblotting with anti-pAkt, anti-total Akt, and anti-β-actin antibodies, respectively. (B) Immunoblotting analysis of anti-pAkt signals (normalized to total Akt levels). Data are relative Akt activation levels and total Akt change with control set as 100%, #P<0.05.

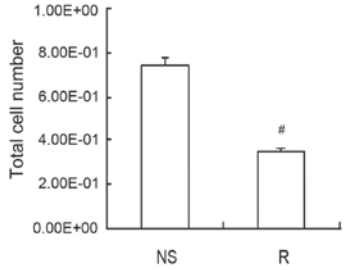

Figure 4. PC12 cells were exposed to ropivacaine for 48 h and then subjected to MTT assays. Group R, ropivacaine treatment; group NS, saline control treatment. #P<0.05 vs. group NS.

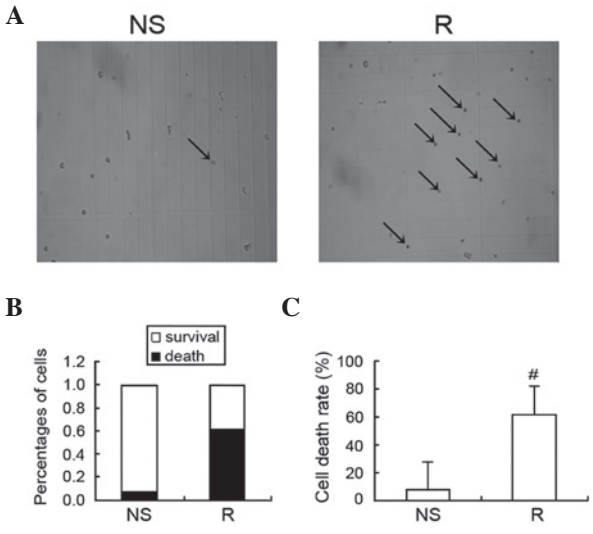

Figure 5. Toxicity of ropivacaine *in vitro*. Group R, ropivacaine treatment; group NS, saline control treatment. (A) Trypan blue assay. (B) Percentages of viable and dead cells from total cells. (C) Cell death rate (%) was normalized to the group NS. #P<0.05 vs. group NS.

Discussion

Ropivacaine is available for spinal or intrathecal use and its use in clinical practice is common. The active duration of ropivacaine is 1.6-6 h and is considered a long-acting LA (19). Ropivacaine has numerous advantages for epidural analgesia compared with traditional medicines, including bupivacaine (20-22). However, *in vitro* and *in vivo* studies suggest that ropivacaine exhibits neurotoxicity (11,14). In a previous study, we reported that following 6- or 48-h exposure during continuous spinal anesthesia, ropivacaine induced mild neuronal injury to the spinal cord and nerve roots (11). However, detailed molecular mechanisms, particularly the signaling pathways, by which the LAs exert neurotoxicity remain to be explored. The aim of the present study was to uncover the possible signaling pathways by which ropivacaine causes neurotoxicity. Numerous neurotoxicity studies have been conducted under various conditions, with exposure times ranging between 10 min and 48 h. In this experiment, we used a rat model of repeated intrathecal administration of 1% ropivacaine for 12 h and examined the long-term (for 28 days) effects on lumbar enlargement spinal cord apoptosis following drug administration. The dosing interval, of 1.5 h, was based on a study by Rose *et al* (12). We utilized the PC12 cell line and examined the toxic effects of 1% ropivacaine. Analysis of dose- and time-dependent effects in PC12 cells was based on a study conducted by Lirk *et al* (14). The present study focused on Akt, due to its critical signaling mode within cells under physiological and pathological cell survival mechanisms (8). In previous studies, Yuan *et al* suggested signaling pathways mediated by Akt are correlated to neuronal survival (23). In addition, Nakajima *et al* reported that persistent activation of Akt is important in neuronal cells (24).

In the present study, we demonstrated that ropivacaine exposure *in vivo* caused significant lumbar enlargement spinal cord apoptosis on days 1, 3, 5 and 7. In addition, we demonstrated that ropivacaine significantly inhibits cell growth and caused cell death following 48 h exposure in PC12 cells. Apoptosis is an essential process for cell integrity in development and survival. It is also triggered by physiological and non-physiological stimulation or insult, leading to pathological conditions. Although apoptosis is considered a major mechanism of LA-induced cell death, particularly in the case of neurotoxicity, LAs also induce necrotic cell death (25,26). Our results indicate that apoptosis and cell death occur when ropivacaine is administrated intrathecally and exposed to PC12 cells. Our data suggest that therapeutic concentrations of the LA ropivacaine may be sufficient to cause local anesthetic-induced long term neurotoxicity. We also identified that Akt activation was decreased in the presence of intrathecal ropivacaine and in cultured PC12 cells exposed to ropivacaine. Inhibition of the Akt pathway may explain the resultant apoptosis upon exposure to ropivacaine. An inverse correlation between the levels of Akt activation and degree of apoptosis was noted in the current study. This supports the hypothesis that LAs, in particular ropivacaine, induce apoptosis via regulation of the Akt pathway. In the first

part of this study, we assessed Akt activation by immunoblotting in repeated intrathecal ropivacaine. In the present study, we assessed Akt activation by immunoblotting with an anti-activated Akt antibody (pAkt) in PC12 cells (Fig. 2). When we examined total Akt levels by immunoblotting with an anti-total Akt antibody, we observed a significantly decreased level of total Akt proteins in group R cells compared with group NS (Fig. 3A). Compared with group NS, group R decreased Akt activity to 44.7±6.8%. Total Akt was also decreased in group R to 33.2±8.5% compared with group NS. This largely correlated with the degree of dead cells under the same conditions (compare Fig. 3B with Fig. 4 or Fig. 5B/5C). Taken together, our data suggest that ropivacaine caused PC12 cell death, at least in part, via the inhibition of Akt activation. However, we cannot completely rule out other signaling pathway(s) as significant contributors to LA-induced toxicity. p38 MAPK and c-Jun N-terminal kinase (JNK) pathways are known to be involved in ropivacaine-induced neurotoxicity (11,27). These findings raise the possibility that certain, if not all, of these pathways may modulate specific LA-induced neurotoxicity independently or collectively. Further investigation is required to address these issues. Limited clinical studies have suggested that ropivacaine may lead to transient neurological syndromes (28,29). The present study provides direct supportive evidence that *in vitro* and *in vivo* ropivacaine exposure results in neurotoxicity. The neurotoxicity of ropivacaine demonstrated in this study contributes to an emerging body of data supporting the detrimental neurotoxic effects of ropivacaine when used as an intrathecal anesthetic.

In conclusion, ropivacaine induces neurotoxicity *in vivo* and *in vitro,* and the Akt pathway is involved in the neurotoxicity of ropivacaine.

Acknowledgements

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