Effects of hyperbaric factors on lidocaine-induced apoptosis in spinal neurons and the role of p38 mitogen-activated protein kinase in rats with diabetic neuropathic pain

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Abstract. The application of lidocaine can lead to nerve damage. Evidence suggests that patients with diabetic neuropathy are at a higher risk for neurotoxicity. In the present study, the successful induction of diabetic neuropathic pain (DNP) in rats via a high-sugar, high-fat diet and intraperitoneal injection of 1% streptozotocin was verified and pronounced tactile allodynia was observed. It was found that intrathecal injections of hyperbaric lidocaine produced motor blocks of longer durations in the DNP model rats than in nondiabetic rats, or in DNP model rats injected with isobaric lidocaine. Histology of the lumbar 4-5 spinal cord revealed a significant difference in neuropathology between the DNP and nondiabetic rats. Moreover, edematous neurons and TUNEL-positive cells were observed in the hyperbaric lidocaine group. It was also found that the inhibition of p38 mitogen-activated protein kinase (p38MAPK) played a neuroprotective role in response to hyperbaric lidocaine-induced apoptosis in DNP rats, which indicates that p38MAPK plays a key role in the regulation of hyperbaric lidocaine-induced apoptosis in DNP rats. These findings suggest that hyperbaric lidocaine can promote spinal cord neuronal apoptosis in rats with DNP. Furthermore, p38MAPK might play a key role in the regulation of hyperbaric lidocaine-induced apoptosis in rats with DNP.

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Introduction

The risk of neurological complications in patients with diabetes following spinal anesthesia using local anesthetics has become increasingly concerning (1) due to the increase in the prevalence of type 2 diabetes mellitus (T2DM) worldwide (2) and the increase in the use of nerve block during surgery (3). It has been reported that ~24% of patients with T2DM experience neuropathic pain (4), and spinal cord involvement may be present in diabetic neuropathy (5).

Diabetic patients frequently undergo various types of surgery under local anesthesia, and studies have shown that patients with diabetic neuropathic pain (DNP) are more sensitive than healthy controls to peripheral nerve block-induced neurotoxicity (6,7). Lidocaine is clinically used at concentrations of 1-2%, and glucose and a local anesthetic are currently often formulated into hyperbaric local anesthetics for spinal anesthesia. It may be hypothesized that diabetic patients are at risk of damage to the spinal cord following the intrathecal injection of hyperbaric lidocaine.

Physiologically, p38 mitogen-activated protein kinase (p38MAPK) is a member of the MAPK family; enzymes in this family are important regulators of eukaryotic cell regulation. p38MAPK is activated by a variety of stressors, such as cytotoxic substances, radiation, osmotic stress, inflammatory cytokines and growth factors (8). The inhibition of p38MAPK has been shown to be potentially beneficial in experimental nerve trauma, excitotoxicity (9) and growth factor withdrawal (10). The neurotoxicity of lidocaine is mediated by the specific activation of p38MAPK, and the inhibition of p38MAPK significantly attenuates toxicity (11). Similarly, the expression of p38MAPK has been manipulated in order to develop a rat diabetic sensorimotor neuropathic model, and the inhibition of p38MAPK in this model has been shown to result in the restoration of normal nerve conduction (12). However, the function of p38MAPK in the neurotoxicity caused by hyperbaric lidocaine in DNP remains unclear. The hypothesis investigated in the present study is that intrathecally administered hyperbaric lidocaine will induce spinal cord neuron apoptosis in a rat model of DNP. The secondary aim of this

study was to determine whether p38MAPK plays a key role in the regulation of hyperbaric lidocaine-induced apoptosis in the rat model of DNP.

Materials and methods

Animals and grouping. A total of 60 healthy male Sprague-Dawley rats, weighing 220-300 g and aged 2-3 months, were used in this study. The rats were provided by Nanchang University of Traditional Chinese Medicine (Nanchang, China). Rats were housed at 25±5˚C (relative humidity, 60-70%) with a 12-h light/dark cycle. This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Nanchang University of Traditional Chinese Medicine. Rats that exhibited abnormal paw withdrawal thresholds (PWTs) and damaged nerves were eliminated from the study. After 1 week of adaptive feeding, 60 remaining rats were divided into a normal control (n=10) and DNP (n=50) group. The rat model of DNP was established via the provision of a high-fat, high-carbohydrate diet and a single intraperitoneal injection of 1% streptozotocin (STZ; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) as described below. When the DNP rat model was successful established (also as described below), the DNP group was divided into an isobaric lidocaine group (IL group; n=12), a glucose group (G group; n=13), a first hyperbaric lidocaine group (HL1 group; n=12) and second hyperbaric lidocaine group (HL2 group; n=13). All rats ate and drank freely during the experimental period.

DNP rat model. After 1 week of adaptive feeding, the DNP group was provided with a high-fat, high-carbohydrate diet for 4 weeks and then injected with a single intraperitoneal 1% STZ dose of 30 mg/kg. By contrast, the control group received a normal diet and a single intraperitoneal injection of the same volume of citric acid buffer. After 3 days, blood glucose levels were measured via samples taken from the tail veins of the rats. Fasting blood glucose levels >16.65 mmol/l were considered to be indicative of the successful establishment of a T2DM model. DNP was verified via tests of the tactile sensitivity (von Frey filament) of the plantar hindpaws that began 28 days after the intravenous STZ injection. In these tests, after allowing rats to accommodate for 15 min to a wire mesh grid, a calibrated set of von Frey filaments (0.04-15 g) was applied from below to the plantar hindpaw to determine the 50% force withdrawal threshold using an iterative method.

Intrathecal catheter. All rats were anesthetized via an intraperitoneal injection of chloral hydrate (10%, 3 ml/kg). After the DNP model was successfully established, a sterile intrathecal catheter (a polyurethane catheter with an inner diameter of 5 mm was stretched to an adjustable catheter with a thick-end diameter of 0.7 mm and a thin-end diameter of 0.5 mm) was inserted into the subarachnoid space through the lumbar 3-4 intervertebral space. The twitching of the rat's tail or hind legs was taken as an indicator of success (typically at a depth of 1.5-2.0 cm), and the catheter was fixed and sutured. The insertion of the catheter into the subarachnoid space was verified by cautious aspiration of the cerebrospinal fluid (CSF). The catheter was then flushed with normal saline (10 μl) to prevent thrombus formation. Penicillin was intramuscularly post-operatively injected to prevent infection, and the rats were then individually housed for 3 days, during which time they were checked daily for spinal cord injuries. Rats that exhibited any sensory or movement disorder were excluded from the experiment.

Intrathecal drug injection. Isobaric 2% lidocaine (10 μl) was intrathecally injected in the IL group, 10% glucose (10 μl) was intrathecally injected in the G group, and hyperbaric lidocaine (10% glucose + 2% lidocaine; 10 μl) was intrathecally injected in the HL1, HL2 and control groups. All anesthetic solutions were administered manually in single bolus injections using a microsyringe at a rate of approximately 10 μl/15 sec. Each solution was administered intrathecally in a volume of 10 μl, and was followed by the administration of 10 μl saline to flush the catheter. Injections were performed once per day for 3 days in all rats. To determine whether the inhibition of p38MAPK decreased apoptosis among the spinal neurons, the HL2 group was intrathecally injected with the p38MAPK inhibitor SB203580 at a concentration of 10 μg/10 μl once per day for 5 days after the intrathecal injection of hyperbaric lidocaine, and the HL1 group was intrathecally injected with 10 μg/10 μl dimethyl sulfoxide (DMSO).

Behavioral testing. The effect of the lidocaine was apparent within 1 min of the injection, and the duration of the block was recorded as the time from the end of the injection to the time at which the motor response reappeared. A technician who was blinded to the animal groups performed the paw stimulation tests. The paw stimulation test was performed to assess the response of the paw to mechanical stimulus. The test was performed by stimulating the plantar surface of the hind paw with electronically controlled von Frey filaments. A positive response was defined as a quick withdrawal of the hind paw upon stimulation. When a negative response occurred, the filament of the next higher stiffness was applied. When a positive response occurred, the filament of the next lower stiffness was applied. This process was repeated until the first adjacent positive and negative reactions were identified across four consecutive measurements with stimulus intervals of 30 sec and maximum intensities of 15 g. The PWT was recorded by calculating the 50th percentile paw response threshold across the rats. The PWTs were measured prior to the induction of the diabetes model (T_1); prior to the intrathecal injections (T_2); 30 min plus 1 day after the initiation of the intrathecal injections (T_3); 30 min plus 2 days after the initiation of the intrathecal injections (T_4); 30 min plus 3 days after the initiation of the intrathecal injections (T_5); and at the end of the administrations at 5 days (T_6).

Hematoxylin and eosin (H&E) staining. At 5 days after the final intrathecal injection of lidocaine, the rats were anesthetized using intraperitoneal injections of 10% chloral hydrate (35 mg/100 g). Thoracotomy was subsequently performed to expose the heart and followed by cardiac perfusion with

0.9% saline followed by 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. Lumbar 4-5 spinal tissue was removed; sections were post-fixed in 4% paraformaldehyde for 4 h and embedded in paraffin. Transverse sections (4-μm thick) were obtained from paraffin-embedded tissue blocks. Sections were washed in xylene to remove the paraffin, rehydrated with serial dilutions of alcohol followed by a wash in PBS solution. The sections were subsequently stained with H&E. Histological images were recorded under a light microscope. The histological examinations were conducted by an investigator who was blinded to the experimental conditions.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The spinal cord was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Serial longitudinal sections (5 μm) were cut from the paraffin-embedded tissue blocks. The sections were deparaffinized in xylene, rehydrated with serial dilutions of alcohol and washed in distilled water. After treatment with 3% H_2O_2 for 10 min at room temperature, the sections were incubated with proteinase K (1:200 in TBS) for 15 min at 37˚C. TUNEL was performed using a TUNEL-based assay kit (Roche Diagnostics GmbH, Mannheim, Germany). The sections were then incubated with TdT and dUTP-digoxigenin in a humidified chamber at 37˚C for 2 h, followed by three washes in TBS. The sections were then incubated with streptavidin-biotin complex at 37˚C for 60 min, developed with 3,3'-diaminobenzidine for 10 min and counterstained with hematoxylin. Three tissue slices from each rat were observed under a microscope at x400 magnification to examine the spinal cord. Five non-overlapping fields were then randomly selected. The positive cells were counted, and an apoptotic index (AI) was calculated using the Image-Pro Plus 6.0 software analysis system and the following formula: AI (%)= number of TUNEL-positive cells/total number of cells x 100.

Western blotting. Samples from 5 rats from each group were examined via western blot analysis. The rats were intraperitoneally injected with chloral hydrate (35 mg/100 g) for anesthesia and rapidly decapitated. The lumbar spinal cord enlargement was removed, weighed, ultrasonically homogenized at low temperature, and centrifuged at 4˚C and 1,400 x *g* for 15 min. The supernatant was collected, and bicinchoninic acid was applied to determine the protein concentration in the sample following four denaturations in sample buffer at 95˚C for 5 min. Equal amounts of sample were subjected to 10% SDS polyacrylamide electrophoresis at constant voltage and then transferred to nitrocellulose membranes in skimmed milk at room temperature for 2 h. The membranes were washed in TBST (1X TBS, 0.1% Tween 20) three times for 10 min each. Rabbit monoclonal anti-p-p38MAPK antibody (dilution 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin antibody (dilution 1:5,000; Abbkine, Wuhan, China). was then added, and the samples were incubated overnight at 4˚C, rewarmed for 30 min, and washed in TBS three times for 10 min each. Alkaline phosphatase-conjugated secondary antibody (dilution 1:1,000; Shanghai Rui Qi Biological Technology, Ltd., Shanghai, China) was added, and the samples were vibrated and incubated at room temperature for 2 h. After this, they were washed in TBTS three times for 10 min each, and a BCIP/NBT alkaline phosphatase display kit (Shanghai Rui Qi Biological Technology, Ltd.) was used to reveal the reactive bands which were then analyzed by scanning with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The protein bands were normalized to internal control β-actin values to reflect the expression levels.

Statistical analyses. SPSS 19.0 statistical software (IBM SPSS, Armonk, NJ, USA) was used to analyze the results. Normally distributed numerical data are presented as the means plus standard deviations. Single factor analysis of variance was used to examine the differences among and between the groups. When the differences were significant, SNK-q inspections were used to compare pairs of groups. $P<0.05$ was considered to indicate a statistically significant result.

Results

Survival rate. At the end of the experiment, 43 rats survived. The numbers of surviving rats in the normal control, HL1, HL2, IL and G groups were 10, 8, 9, 8 and 8, respectively; therefore, the percentages of rats in these respective groups that were considered to be successful models were 100, 66.7, 69.2, 66.7 and 61.5%.

Behavior evaluated by PWT and motor block duration tests. No significant differences were detected in PWT between any groups at T_1. The rats in group G moved normally at all time points. In comparison with the control group, the PWTs at $T_{2\text{-}5}$ of the HL1, HL2, IL and G groups were reduced ($P<0.05$). The PWT of the HL2 group was elevated compared with that in the HL1 group at T_6 ($P<0.05$; Fig. 1A). The durations of the motor blocks in the HL1 and HL2 groups were longer compared with those in the control group, and also compared with those in the IL group at T_3, T_4 and T_5 ($P<0.05$; Fig. 1B).

H&E staining of the spinal cords. Histological examination of the spinal cords revealed no obvious nerve fiber injury in groups C, IL and G. Edema in the nerve fibers and slight widening of the nuclei of the neurons were particularly evident in group HL1. In group HL2, edema of the nerve fibers and widening of the nuclei of the neurons were rarely observed (Fig. 2).

TUNEL assay. TUNEL staining was used to examine the effects of p38MAPK inhibition on cell apoptosis in the spinal cord in a rat model of lidocaine-treated DNP. The number of TUNEL-positive cells was significantly increased compared with that in the control group following hyperbaric lidocaine injection in group HL1. The AI in group HL2 was significantly decreased following the administration of SB203580 compared with that in group HL1 ($P<0.05$). Furthermore, there was no significant difference between group HL2 and the control group (Fig. 3).

Western blotting. The effect of the p38MAPK inhibitor SB203580 on the spinal cords in group HL2 was investigated

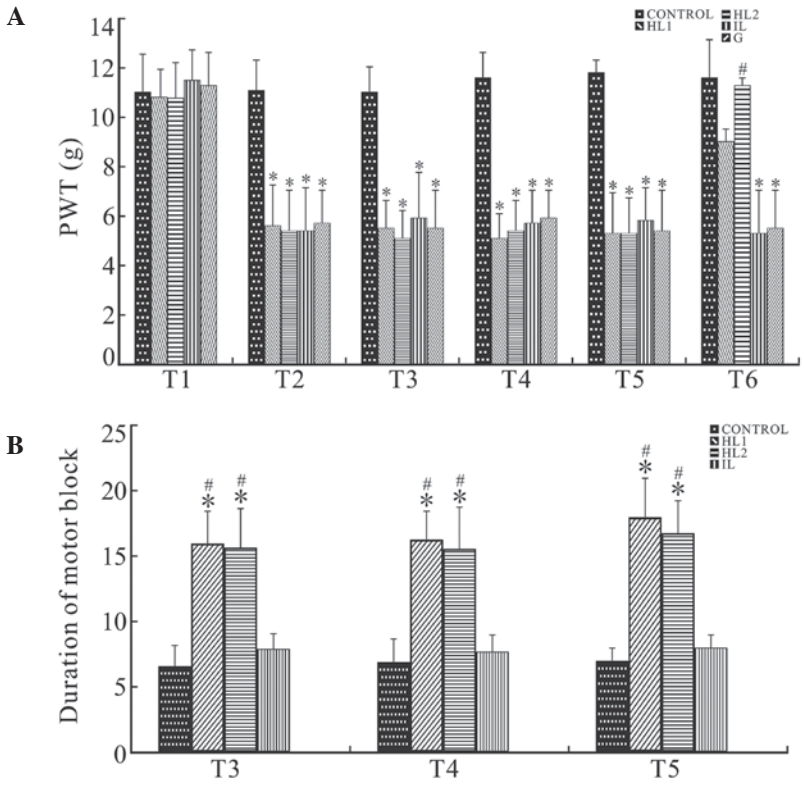

Figure 1. Rat behaviors. (A) Effects of lidocaine and lidocaine combined with glucose on PWT. The PWTs were measured prior to the induction of the diabetes model (T1); prior to the intrathecal injections (T2); 30 min plus 1 day after the initiation of the intrathecal injections (T3); 30 min plus 2 days after the initiation of the intrathecal injections (T4); 30 min plus 3 days after the initiation of the intrathecal injections (T5); and at the end of the administrations at 5 days (T6) in the control, HL1, HL2, IL and G groups. *P<0.05 vs. control; $^{\#}$P<0.05 vs. HL1. There were no significant differences in PWT between the groups at T_1. (B) Time courses of the motor blocks in the control, HL1, HL2, IL and G groups were measured from the end of the injection of the drugs to the time at which the motor responses reappeared. *P<0.05 vs. control; $^{\#}$P<0.05 vs. IL. PWT, paw withdrawal threshold; HL1, hyperbaric lidocaine group; HL2, hyperbaric lidocaine group treated with SB203580; IL, isobaric lidocaine group; G, glucose group.

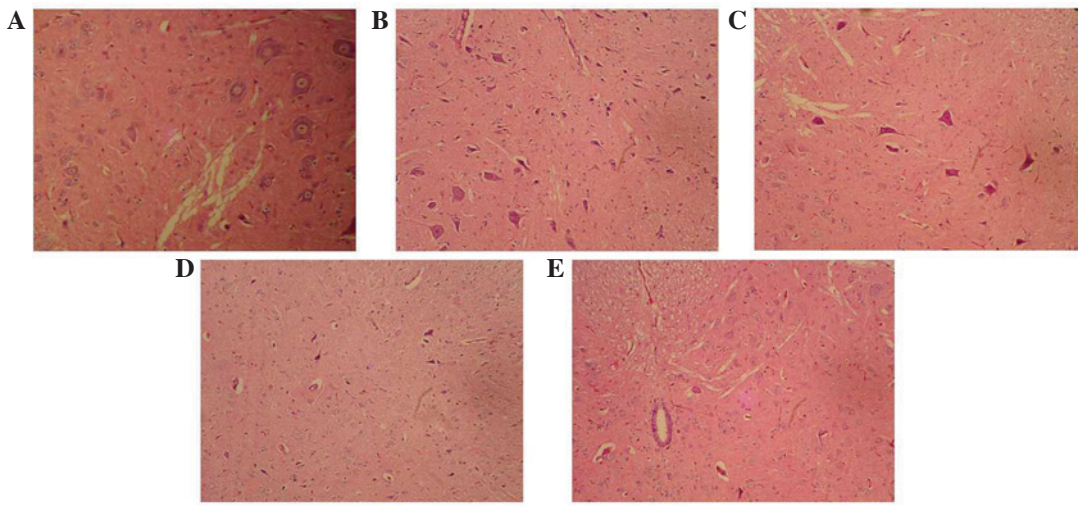

Figure 2. H&E staining of transverse sections of the spine 10 mm rostral to the conus that were obtained 4 days after the intrathecal injection of (A) hyperbaric lidocaine in the control group, (B) hyperbaric lidocaine plus dimethyl sulfoxide in the HL1 group, (C) hyperbaric lidocaine plus SB203580 in the HL2 group, (D) isobaric lidocaine in the IL group, and (E) 10% glucose in the G group. In (B), H&E stained sections of the spinal cord show edema in the nerve fibers, and slight widening of the nuclei of the neurons more than that in (C). In (C), edema of the nerve fibers and widening of the nuclei of the neurons was rarely observed. No obvious edema is visible in (A), (D) or (E). Magnification, x400.

by examining the levels of phosphorylated p38MAPK. The p-p38MAPK level was significantly decreased in group HL2 compared with that group HL1 (P<0.05), and no significant difference was observed between group HL2 and the control group. Consistent with the results of H&E staining, western blot analyses revealed that, in group HL2, p-p38MAPK expression was significantly decreased following the intrathecal injection of SB203580; hyperbaric lidocaine-induced apoptosis was

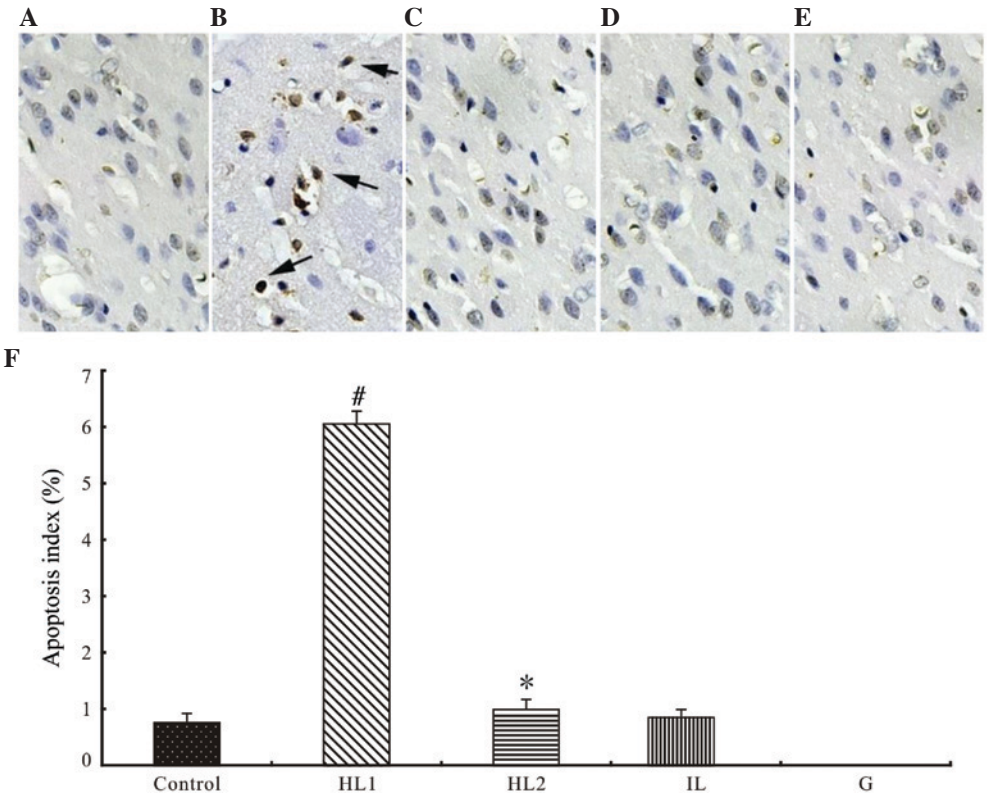

Figure 3. TUNEL-labeled cells in the spinal cord. Representative sections showing TUNEL-positive cells (arrows) in the injury areas from the (A) control, (B) HL1, (C) HL2, (D) IL and (E) G groups. (F) Apoptosis indices for all groups. The number of TUNEL-positive cells was significantly increased in the HL1 group compared with the control group. The numbers of TUNEL-labeled cells were not significantly different among the control, HL2, IL and G groups. *P<0.05 vs. HL1; #P<0.05 vs. control. HL1, hyperbaric lidocaine group; HL2, hyperbaric lidocaine group treated with SB203580; IL, isobaric lidocaine group; G, glucose group.

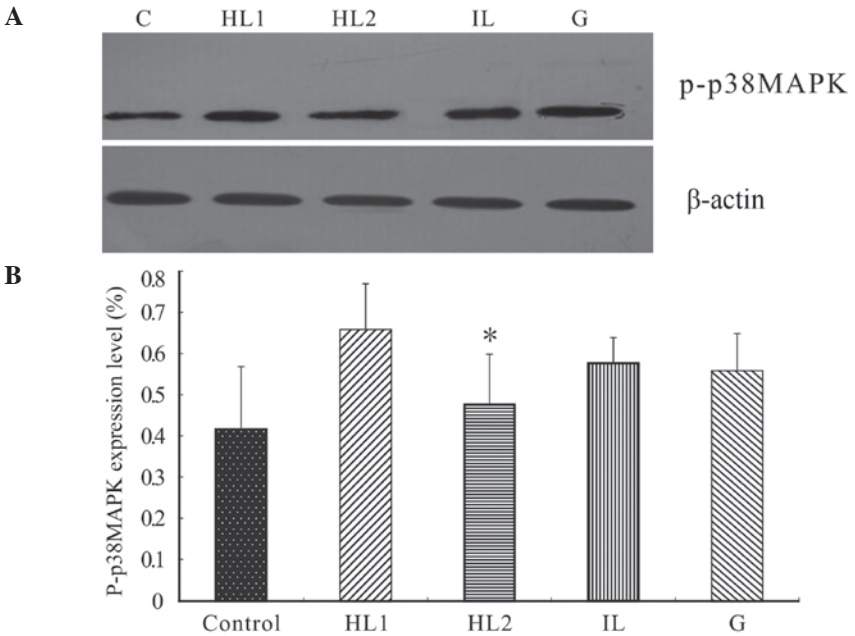

Figure 4. p-p38MAPK protein expression in the spinal cord. (A) Representative western blots showing the expression of p-p38MAPK in the spinal cord. (B) Quantification of the expression of p-p38MAPK normalized to β-actin. *P<0.05 vs. HL1. HL1, hyperbaric lidocaine group; HL2, hyperbaric lidocaine group treated with SB203580; IL, isobaric lidocaine group; G, glucose group.

also reduced in this group. These findings suggest that the intrathecal injection of SB203580 might elicit a neuroprotective effect against hyperbaric lidocaine-induced apoptosis in a rat model of DNP (Fig. 5).

Discussion

According to a method described in the literature (13), the established rat model of T2DM that is induced with a

high-sugar, high-fat diet and a low-dose intraperitoneal injection of STZ and results in insulin resistance and relatively insufficient insulin secretion . This pathogenesis of this model is similar to that of human T2DM, the modeling method is mature, and this model is currently the most commonly used method of establishing an animal model of T2DM. The fasting plasma glucose levels of groups HL, IL and G in the present study were >16.65 mmol/l, and steady, 'self-healing' phenomena did not appear. Rats were used for the induction of T2DM in the present study because diabetic neuropathy can be readily confirmed and followed in this model due to prominent tactile allodynia in the plantar hindpaw, which persists for months (14). The use of an improved lumbar catheterization method that reduces postoperative complications, such as paralysis and death, in rats was conducive to the placement and stable maintenance of the homemade catheters for long-term tests (15).

Increasing evidence has shown that the neurotoxicity of lidocaine is greater than that of other commonly used local anesthetics (16). Clinically, a combination of lidocaine and 10% glucose is dispensed as hyperbaric lidocaine to hasten, improve or prolong neuronal blockade. The first finding of the present study was that the intrathecal administration of hyperbaric lidocaine resulted in a longer duration of nerve block in group HL than in groups IL, G and C. Echevarria *et al* (17) studied the mechanisms by which the duration of spinal block is increased in relation to increases in numerous CSF components, such as glucose, in diabetic patients. In the diabetic rats, the glucose level was increased, which would reduce the volume and slowed blood flow velocity in the CSF, and so might have increased the time of contact between lidocaine and the spinal cord. Therefore, hyperbaric lidocaine exhibited increased block duration. There might also be neurophysiological mechanisms that cause the spinal cords of diabetic rats to be more sensitive to local anesthetic block, which could be similar to the mechanisms of the increased duration of sciatic nerve block in STZ-diabetic rats (6).

Furthermore, in the present study, it was found that intrathecal hyperbaric lidocaine led to apoptosis of the spinal neurons in the rats with DNP when examined 5 days later. Takenami *et al* (18) reported that the intrathecal administration of 7.5% lidocaine causes lesions in the posterior roots and that 10% lidocaine also damages the posterior columns when examined 5 days after administration. Kroin *et al* (19) also reported that the intrathecal administration of isobaric lidocaine is safe in diabetic neuropathic rats at the same dose and time and that a volume of 10 μl was sufficient to spread the drug to all lumbar-sacral levels (20). In the present study, an increase in the PWT of group HL1 at T_6 was observed, which indicated that neuronal apoptosis was most evident at T_6. Compared with group IL, greater pathological changes spinal cord injury elicited in group HL1, and this observation was also confirmed by the finding that hyperbaric lidocaine promoted apoptosis of the spinal cord neurons of the rats with diabetic neuropathy. Compared with group C, the spinal cords of group HL1 exhibited less severe pathological changes, which indicated that the neurons of diabetic rats are more susceptible to apoptosis than those of normal rats. Group IL exhibited less damage and almost no disease, and these results are consistent with those of a study of diabetic rats conducted by Kroin *et al* (19). In Group G, 10% glucose did not cause apoptosis of the spinal cord neurons or motor block. The study by Hashimoto *et al* (21) found that the subarachnoid infusion of 5% lidocaine caused persistent pain, but infusions of 10% glucose and 0.9% sodium did not. The results from group G are consistent with those reported by Hashimoto *et al*. It could be considered clinically relevant that the 10% concentration of glucose did not cause neuronal apoptosis; therefore, the apoptosis of the neurons in the spinal cord is considered to result directly from the local anesthetic.

The results of the current study indicate that the mechanism by which hyperbaric lidocaine promotes apoptosis in spinal cord neurons in diabetic neuropathic rats is associated with key regulatory protein in the p38MAPK signaling pathway. In the present study, the number of apoptotic cells (as assessed with TUNEL labeling) was increased in group HL1, and the number of apoptotic cells in group HL2 (to which the p38MAPK inhibitor was administered) was significantly reduced. Lirk *et al* (22) reported that the activation of p38MAPK is a pathogenic pathway that is common to both local anesthetic-induced neuropathy and diabetic neuropathy and indicated that co-incubation with lidocaine and SB203580 reduces neurotoxicity in diabetic neurons from Zucker diabetic fatty rats *in vitro* (23). Further studies are necessary to verify the direct phosphorylation of p38MAPK.

The present study indicates that the apoptosis induced by the intrathecal administration of hyperbaric lidocaine is mediated by the activation of p38MAPK. The inhibition of p38MAPK prevents the apoptosis of spinal cord neurons. These findings provide evidence that supports the use of p38MAPK inhibitors as potential new therapeutic agents for the prevention of local anesthetic toxicity and diabetic neuropathy.

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