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Original Article

Lidocaine hydrochloride increased apoptosis of SH-SY5Y cells through p38 MARK mediated DNA methylation

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Abstract: With extensive application of local anesthesia (LA) recently, neuronal toxic injury and general toxicity of LA have aroused wide attention, how to prevent and treat LA induced injury has been an important subject of clinical anesthesia. As one of main injury mechanisms of LA, we speculated that p38 mitogen-activated protein kinase (p38 MARK) probably mediates DNA methylation to induce neuronal damage. In the study, 10 mM lidocaine hydrochloride treated SH-SY5Y cell was applied as the nerve cell injury model, p38 MARK was inhibited by SB203580 to verify our conjecture. Cells were divided into control, lidocaine hydrochloride (Lido) group and Lido + SB203580 group. Cell proliferation, cycle and apoptosis were analyzed by means of CCK8 and flow cytometry (FCM). DNA methylation under the different conditions was detected by ELISA. Expression levels of apoptosis and DNA methylation related gene products including Bax, Bcl-2, PCNA, DNMT1, DNMT3a, DNMT3b and MeCP2 were observed through real time PCR (RT-PCR) and western blot. Our results indicated the negative correlation between p38 MARK and cell proliferation in condition of lidocaine hydrochloride treatment. Inhibition of p38 MARK pathway reduced apoptosis rate and mitigated DNA methylation level with up-regulation of PCNA and down-regulation of Bax/Bcl-2 and DNA methylation related proteins. It was proved that lidocaine hydrochloride increased apoptosis of SH-SY5Y cells through p38 MARK mediated DNA methylation, which could provide a profile for preventing and treating LA induced neuronal toxic injury.

Keywords: Lidocaine hydrochloride, SH-SY5Y cell, p38 MARK, DNA methylation

Introduction

Regional block, with definite anesthetic effect, quick anesthesia recovery and low influence on whole body, has been one of common methods of surgical anesthesia and postoperative analgesia in recent years. The meta-analysis indicated that in comparison with general anesthesia, regional block could reduce intraoperative blood loss, decrease metabolism and oxidative stress, improve postoperative revascularization, and lower the occurrence rate of thrombosis and adverse cardiac events [1, 2]. However, local anesthetics (LA) also have their untoward effects on nervous tissues such as ultrastructure occurrence and injury to metabolism and electrophysiology. Thus, with the widespread application of regional block, toxic damage of LA to nerve cells has aroused broad attention, how to decrease and prevent such damages has become a hot area of research in recent clinical science field [3].

The toxicity of LA is related to dosage, concentration and action time. However, even in condition of clinical commonly used concentration of LA, toxic injury to nervous cells can also be induced, appearing as swelling, degeneration, nervous demyelinating, chromosome dissolution and programmed cell death [4, 5]. p38 MARK is considered as one of primary mechanisms to induce toxic injuries of LA probably through mediating DNA methylation [6-10]. Several lines of evidence proved that inhibition of p38 MARK could mitigate neuronal injury caused by bupivacaine, ropivacaine and lidocaine [8, 11, 12].

In the study, SH-SY5Y cells were applied as the nerve cell model to investigate neuronal cyto-

toxicity induced by LA-lidocaine hydrochloride. We speculated that lidocaine hydrochloride leads to cell injury probably through p38 MARK mediated DNA methylation and tried to prove it.

Materials and methods

Cell culture and treatment

SH-SY5Y cells were purchased from Shanghai Jining Shiye Co. Ltd. (Shanghai, China). Cells were cultured in compete medium mixed by Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc, New York, USA), then incubated in 5% CO_2 incubator at 37°C. Cells were randomly divided into three groups: control group, lidocaine (Lido) group and Lido + SB203580 group. Cell in Lido group were treated with 10 mM lidocaine (Suzhou Pulu Biotechnology Co. Ltd., Jiangsu, China) for 24 h. Cells in Lido + SB203580 group, except addition with 10 mM lidocaine, were additionally treated with p38 MARK inhibitor SB203580 (MedChemexpress LLC., New Jersey, USA) to inhibit p38 MARK pathway.

CCK8

Cell viability of SH-CY5Y cells in each group was detected through using CCK8 kit (Shanghai Beyotime Biotechnology Co. Ltd., Shanghai, China). Cells were grouped, and seeded into 96-well plates at amount of 100 μL per well, and then incubated at 37°C in 5% CO_2 incubator for 4 h. After added with 10 μL CCK reagent to each well, cells were putted into 5% CO_2 incubator at 37°C for 1-4 h. Optical density (OD) value of each group was observed at 450 nm by a spectrophotometer (Sigma-Aldrich Co. LLC., USA).

Flow cytometry

Cells in logarithmic phase were collected and seeded into 6-well plates. Cells were digested by EDTA free trypsin (Shanghai Beyotime Biotechnology Co. Ltd., Shanghai, China), stained with Annexin V-FITC and propidium iodide (Shanghai BestBio Science Co. Ltd., Shanghai, China), and incubated in dark place for 15 min at room temperature afterwards. Cell cycle and apoptosis rate of each group was detected by EPICS XL-MCL FCM (Beckman Coulter, Inc., USA) with excitation wavelength 488 nm and emission wavelength 530 nm.

ELISA

DNA methylation level was measured by applying total DNA methylation quantitative test kit (Epigentek Group Inc., USA). Cells were divided into control, Lido, and Lido + SB203580 group. Detection was conducted according to the manufacturer's instructions. OD values were read at 420 nm on a spectrophotometer (Sigma-Aldrich Co. LLC., USA).

RT-PCR

Expression levels of Bax, Bcl-2, PCNA, DNMT1, DNMT3a, DNMT3b and MeCP2mRNA were detected by means of RT-PCR. Cells were seeded into 6-well plates at a density of 2×10^7 cells/well, divided into 3 groups: control, Lido, and Lido + SB203580 group. Total RNA was extracted with Trizol (Thermo Fisher Scientific Inc, New York, USA) according to the manufacture's instruction. Concentration of extracted RNA was read through a UV spectrophotometer (Thermo Fisher Scientific Inc, New York, USA). cDNA was synthesized by reverse transcription. β-actin was applied as the internal control to monitor the efficiency of RT-PCR. All primers in this study were designed by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China). The specific primer sequences for each gene were listed as the follows: 5' GCCAGCAAACTGGTGCTCAA 3' and 5' CCAACCACCCTGGTCTTGGA 3' for Bax (product: 126 bp); 5' CACTGGCCAGGGTCAGAGTT 3' and 5' TGGCCATAGACCCTGTCAGC 3' for Bcl-2 (product: 178 bp); 5' CAACGAGGCCTGCTGGGATA 3' and 5' TCTTCATTGCCGGCGCATTT 3' for PCNA (product: 189 bp); 5' CAGGCCTGAGAACACCCACA 3' and 5' AGTACACCTGGACGCACTCG 3' for DNMT1 (product: 161 bp); 5' GGACCGAAAGGACGGAGAGG 3' and 5' CTTGGAGATCACCGCAGGGT 3' for DNMT3a (product: 160 bp); 5' GCTCACAGGGCCCGATACTT 3' and 5' GGCGAAGAGGTGTCGGATGA 3' for DNMT3b (product: 159 bp); 5' CAGCAGCGTCTGCAAAGAGG 3' and 5' TTTGGCCTTGGCATGGAGGA 3' for MeCP2 (product: 183 bp) and 5' GCCGGGACCTGACTGACTAC 3' and 5' GTCAGGCAGCTCGTAGCTCT 3' for β-actin (product: 188 bp).

Western blot

Cells were seeded in 6-well plates at a density of 2×10^7 cells/well, grouped into control, Lido, and Lido + SB203580 group. Cells were har12

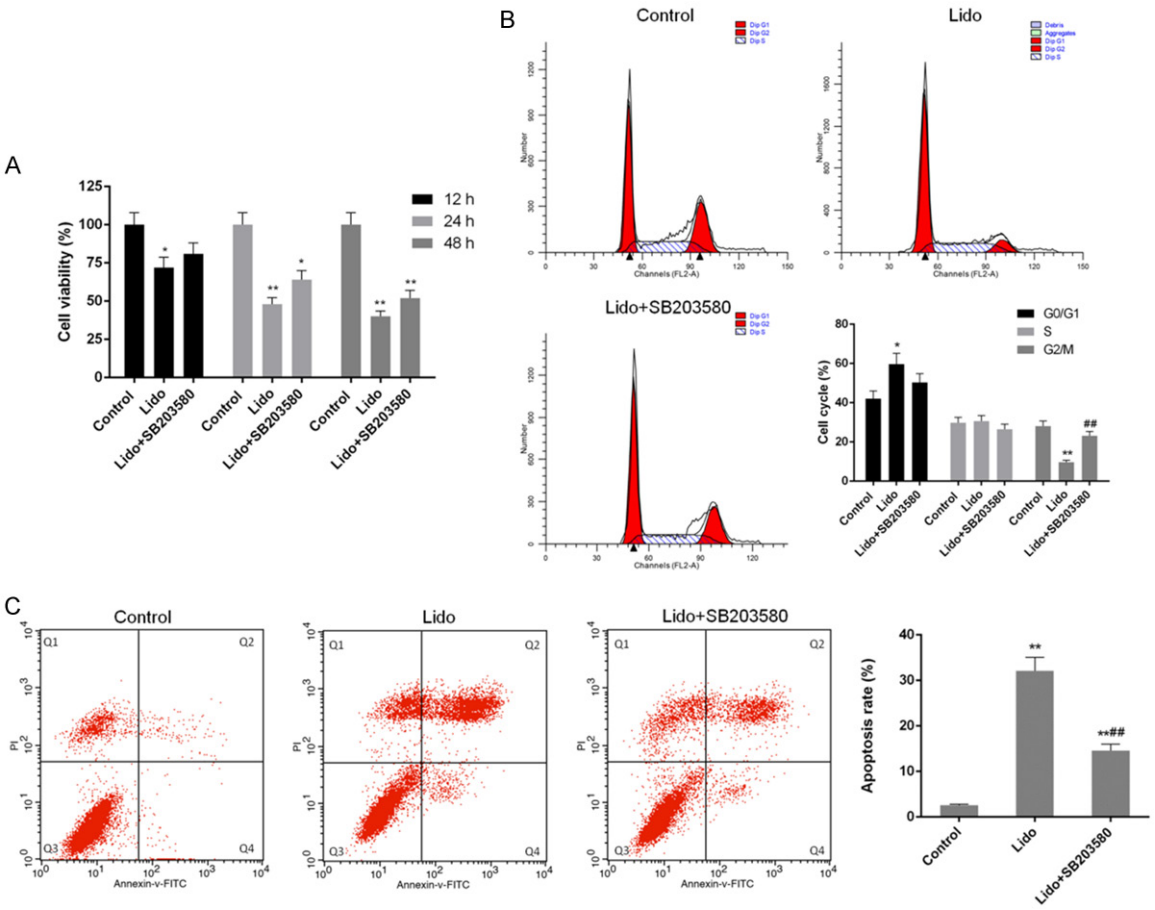

Figure 1. Observation of changes in cell viability, cell cycle and apoptosis in control, Lido (10 mM lidocaine hydrochloride for 24 h), and Lido + SB203580 (10 mM lidocaine hydrochloride for 24 h) group. A: Inhibition of p38 MARK mitigated the decrease of cell viability induced by lidocaine hydrochloride. B: Inhibition of p38 MARK substantially revert the changes of cell cycle induced by liodcaine hydrochloride to normal condition. C: Inhibition of p38 MARK reduced apoptosis rate based on lidocaine hydrochloride induced injury. Data were presented as mean ± SD, n=3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. Lido group.

Statistical analysis

Data were expressed as mean ± S.D. Differences among groups were evaluated through variance analysis and student's t-test. Statistical significance was defined as $P < 0.05$, significant statistical difference was defined as $P < 0.01$.

Results

Inhibition of p38 MARK pathway migrated the decrease of cell viability induced by lidocaine hydrochloride

Changes of cell viability among different groups were detected by CCK8 assay. The result showed that cell proliferation of SH-SY5Y cells was markedly decreased under the condition of lidocaine hydrochloride compared with normal group ($P < 0.05$ or $P < 0.01$). With addition of p38 MARK inhibitor SB203580, cell viability in Lido + SB203580 group was enhanced based on only lidocaine hydrochloride treated cells. All the effects were in a concentration-dependent manner (**Figure 1A**).

Cell cycle was affected by lidocaine hydrochloride treatment

Cell cycle of Lido group was evidently influenced by lidocaine hydrochloride. FCM detected that 59.69 ± 5.50% of cells stayed in the phase of G0/G1 in Lido group while 42.06 ± 4.00% of cells were in control group. The rate of cells in the phase of G2/M was substantially reduced from 28.10 ± 2.50% in control group to 9.71 ± 0.95% in Lido group. The differences were both significant ($P < 0.05$ and $P < 0.01$). Inhibition of p38 MARK pathway substantially revert the changes of cell cycle induced by lidocaine

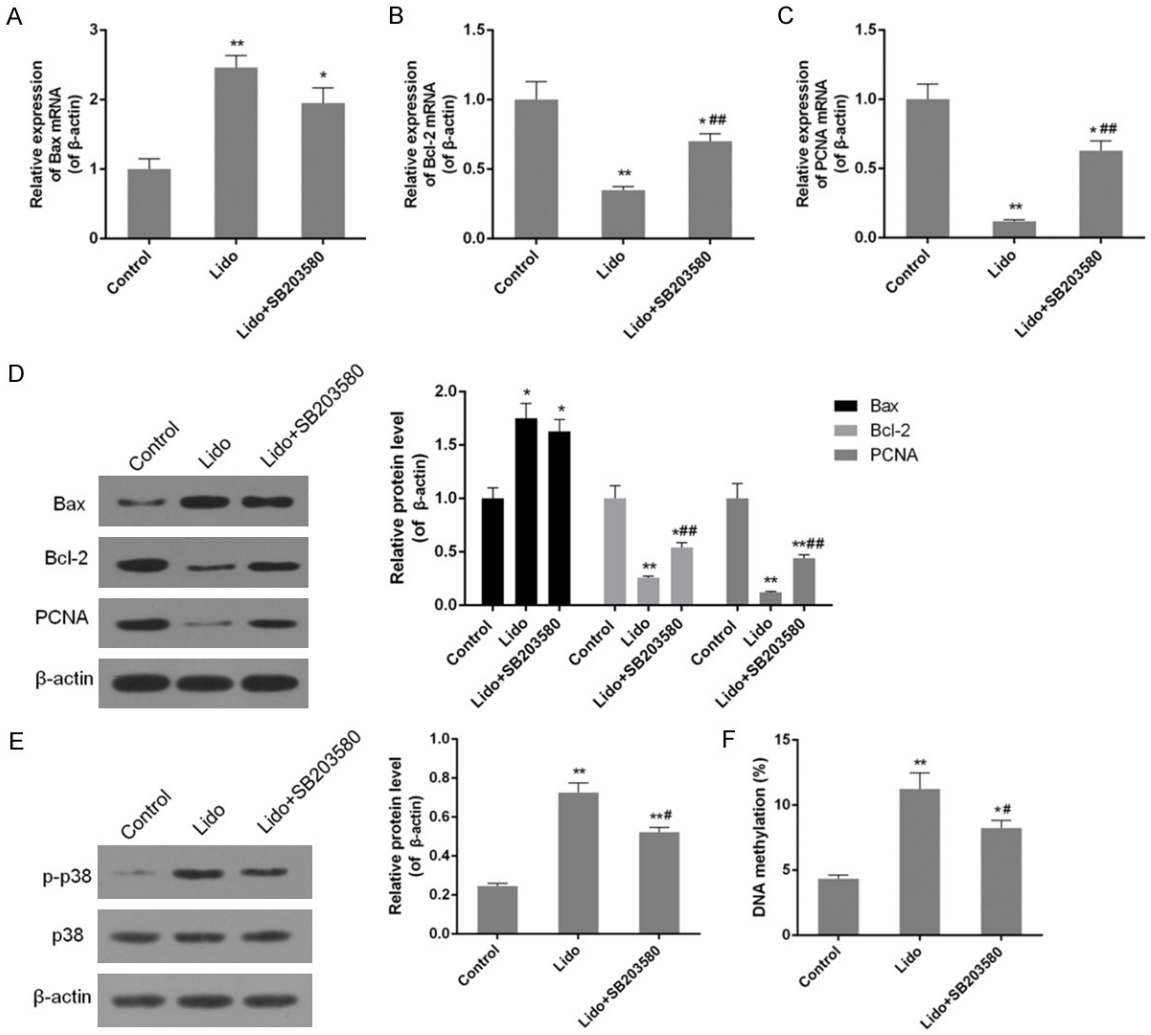

Figure 2. Expression level of apoptosis related genes including Bax, Bcl-2 and PCNA in control, Lido (10 mM lidocaine hydrochloride for 24 h), and Lido + SB203580 (10 mM lidocaine hydrochloride for 24 h) group. A: Inhibition of p38 MARK down-regulated the expression of Bax mRNA in lidocaine hydrochloride induced injury. B: Inhibition of p38 MARK up-regulated the expression of Bcl-2 mRNA in lidocaine hydrochloride induced injury. C: Inhibition of p38 MARK up-regulated the expression of PCNA mRNA in lidocaine hydrochloride induced injury. D: Inhibition of p38 MARK down-regulated the expression of Bax protein, but up-regulated the protein levels of Bcl-2 and PCNA in lidocaine hydrochloride induced injury. E: SB203580 decreased the expression ratio of p-p38 and p38 to inhibit activation of p38 MARK pathway. F: Inhibition of p38 MARK mitigated DNA methylation based on lidocaine hydrochloride induced injury. Data were presented as mean ± SD, n=3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. Lido group.

hydrochloride to normal condition. There was no significant difference between control and Lido + SB203580 group (**Figure 1B**).

Inhibition of p38 MARK pathway reduced apoptosis rate based on lidocaine hydrochloride induced injury

Cell apoptosis rate of each group was analyzed by FCM. The result indicated a dramatic rise of apoptosis rate from 2.57 ± 0.25% in control group to 32.07 ± 3.00% in Lido group ($P < 0.01$). Inhibiting p38 MARK pathway obviously decreased the apoptosis rate to 14.58 ± 1.40% based on lidocaine hydrochloride treated cells ($P < 0.01$), although the difference between Lido and SB203580 and control group was still significant ($P < 0.01$) (**Figure 1C**).

Inhibition of p38 MARK pathway down-regulated the expression of Bax but up-regulated that of Bcl-2 and PCNA based on lidocaine hydrochloride treatment

Expression level of mRNA and protein of Bax, Bcl-2 and PCNA was detected via respective

RT-PCR and western blot. The tendencies of influences in mRNA and protein level were nearly accordant. In Lido group, lidocaine hydrochloride treatment nearly doubled the expression of Bax based on control group ($P < 0.05$ or $P < 0.01$). Meanwhile, Bcl-2 and PCNA were significantly down-regulated by lidocaine hydrocholoride in comparison with normal cells ($P < 0.05$ or $P < 0.01$). The gaps of this gene expression between normal and lidocaine hydrochloride treated cells were distinctly narrowed with Inhibition of p38 MARK pathway. In Lido + SB303580 group, the expression of Bax was decreased while that of Bcl-2 and PCNA was markedly increased compared to Lido group ($P < 0.05$ or $P < 0.01$) (**Figure 2A-D**).

Inhibition of p38 MARK pathway reduced lidocaine hydrochloride induced phosphorylation of p38

Protein level of p-p38 and p38 was detected by western blot. The ratio of p-p38/p38 was dramatically increased by lidocaine hydrochloride treatment from 0.25 ± 0.01 in control group to 0.73 ± 0.05 in Lido group ($P < 0.01$). Inhibiting p38 MARK pathway by SB203580 down-regulated phosphorylation of p38, resulting in the decrease of p-p38/p38 to 0.523 ± 0.024% compared to lidocaine hydrochloride treated cells ($P < 0.05$) (**Figure 2E**).

Inhibition of p38 MARK pathway mitigated DNA methylation induced by lidocaine hydrochloride treatment

Changes in DNA methylation among different groups were detected by the means of ELISA. Treatment of lidocaine hydrochloride induced remarkable DNA methylation to 11.23 ± 1.25% based on 4.33 ± 0.31% under the normal condition. The difference between control group and Lido group was significant ($P < 0.01$). As p38 MARK pathway of SH-SY5Y cells were inhibited by SB203580, lidocaine hydrochloride induced DNA methylation was obviously migrated to 8.24 ± 0.58% ($P < 0.05$) (**Figure 2F**).

Inhibition of p38 MARK pathway down-regulated the expression of DNA methylation related genes based on lidocaine hydrochloride treatment

Expression level of mRNA and protein of DNA methylation related genes including DNMT1, DNMT3a, DNMT3b and MeCP2 was measured via respective RT-PCR and western blot. The tendencies of influences in mRNA and protein level were nearly accordant. In Lido group, the expression level of these four gene products was twice over that of control group ($P < 0.01$). Our results observed that inhibition of p38-MARK pathway down-regulated the expression of these DNA methylation related genes compared with lidocaine hydrochloride treated cells. In our study, despite of DNMT3b protein, the differences in the level of these gene products were significant between Lido group and Lido + SB203580 group ($P < 0.05$ or $P < 0.01$) (**Figure 3A-D**).

Discussion

SH-SY5Y cell line, a subline of SK-N-SH, possesses the characteristic of stem cells. As its terminal differentiated cell is similar to nerve cell in the aspect of biochemistry, ultrastructure, morphology and electrophysiology and suitable for long-term *in vitro* culture, SH-SY5Y cells are commonly used as cell model to research on the functions of nerve cell [13-15].

In the study, with treatment of 10 mM lidocaine hydrochloride for 24 h, viability of SH-SY5Y cells were markedly reduced in a time-dependent manner, and more cells were detected to stay in the phase of G0/G1 during mitosis. Moreover, cell apoptosis rate of SH-SY5Y cells under the condition of lidocaine hydrochloride was dramatically increased by nine times in the study. By the means of western blot, we detected increasing expression of phosphorylated p38 MARK and rising level of p-p38/p38, which meant the activation of p38 MARK pathway. The result of ELISA showed that DNA global methylation level was much higher in lidocaine hydrochloride induced neuronal injury in comparison with normal cells.

Apoptosis is one of cell injury mechanisms induced by lidocaine hydrochloride. In condition of lidocaine hydrochloride treatment, we found the ratio of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 was substantially increased, meanwhile, the expression of PCNA which is essential for cell replication was down-regulated, leading to higher apoptosis rate than normal cells. Additionally, lack of p38 MARK in lidocaine hydrochloride induced injury reduced

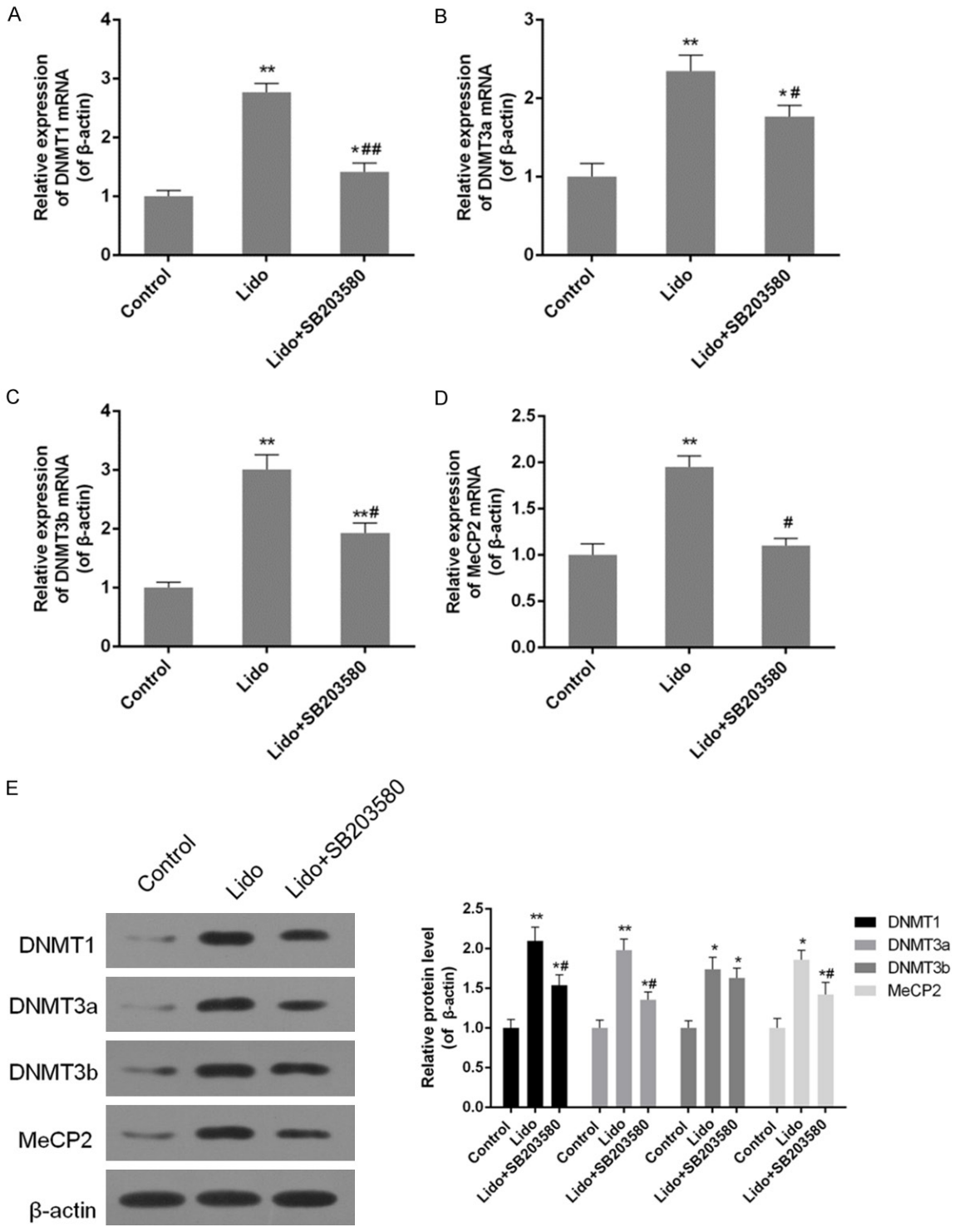

Figure 3. Expression level of DNA methylation related genes including DNMT1, DNMT3a, DNMT3b and MeCP2 in control, Lido (10 mM lidocaine hydrochloride for 24 h), and Lido + SB203580 (10 mM lidocaine hydrochloride for 24 h) group. A: Inhibition of p38 MARK down-regulated the expression of DNMT1 mRNA in lidocaine hydrochloride induced injury. B: Inhibition of p38 MARK down-regulated the expression of DNMT3a mRNA in lidocaine hydrochloride induced injury. C: Inhibition of p38 MARK down-regulated the expression of DNMT3b mRNA in lidocaine hydrochloride induced injury. D: Inhibition of p38 MARK down-regulated the expression of MeCP2 mRNA in lidocaine hydrochloride induced injury. E: Inhibition of p38 MARK down-regulated the protein levels of DNMT1, DNMT3a, DNMT3b and MeCP2 in lidocaine hydrochloride induced injury. Data were presented as mean ± SD, n=3, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. Lido group.

the ratio of Bax and Bcl-2 and increased the expression of PCNA.

The activation of p38 MARK is regarded as one of important mechanisms of LA induced neuronal injury. Previous study discovered that SB203580, the inhibitor of p38 MARK, reduced the neurotoxic injury which was caused by bupivacaine and ropivacaine [8]. Lirk et al. found that inhibition of activating p38 MARK was able to decrease lidocaine induced neurotoxic injury while inhibiting the activation of Caspase could not relieve [11]. Haller et al. indicated that lidocaine induced neurotoxic injury of dorsal root ganglion was relevant to p38 MARK, and its effect was in a time-dependent manner [12]. In our study, we observed decreasing expression level of p-p38/p38, which proved the inhibiting effect of SB203580 on activation of p38 MARK pathway. Cells in Lido + SB-203580 group which were treated with lidocaine hydrochloride as well as p38 MARK inhibitor were detected to have improved cell viability and proliferation and lower cell apoptosis rate and DNA methylation level, compared to only lidocaine hydrochloride treated cells.

Epigenetic phenomenon refers to heritable changes of gene expression with unaltered DNA sequences [16]. DNA methylation, one of the earliest discovered and the most characteristic genome epigenetic modifications, is a key contributor to stable state of genetic expression, and may exists in all higher living organisms [17]. In the process of DNA methylation, methyl in its donor S-adenosyl-L-methionine (SAM) is transferred into specific basic group with the catalyst of DNA methyltransferases (DNMTs) [18]. In eukaryotic cell of higher organisms, the object of methylated modification is cytosine in cytosine-phosphate-guanine (CpG) sequence, however, 60% promoters of human gene are closely related to CpG island [19-21]. So far, there are three confirmative DNMTs in mammal cells including maintenance methyltransferase DNMT1 and methyltransferase DNMT-3a and DNMT3b. During DNA replication, DNMT1 acts on hemimethylated CpGs, and duplicates methylation status of fundamental chain to its subchain [22, 23]. DNMT3a and DNMT3b create new methylated status for unmethylated CpGs [24]. Methy-CpG binding protein 2 (MeCP2) is able to connect with methylated CpG islands and combine with inhibiting compounds of chromosome transcription to inhibit transcription of promoters. Phosphoylation of MeCP2 releases transcription inhibiting compound from methylated genes to let transcription factor to connect with promoter so as to activate transcription [25, 26]. Our study detected obvious mitigation of DNA methylation and apoptosis in p38 MARK inhibited SH-SY5Y cells compared to normal SH-SY5Y cells under the condition of lidocaine hydrochloride treatment. By the means of RT-PCR and western blot, we found the expression levels of DNMT1, DNMT3a, DNMT3b and MeCP2 were positively correlated with DNA methylation level. In lidocaine hydrochloride treated cells, these gene products were markedly up-regulated. However, when p38 MARK pathway was inhibited by SB-203580, they were decreasingly expressed, resulting in comparatively low level of DNA methylation.

Conclusion

Our results indicated the negative correlation between p38 MARK and cell proliferation in condition of lidocaine hydrochloride treatment. Inhibition of p38 MARK pathway reduced apoptosis rate and mitigated DNA methylation level with up-regulation of PCNA and down-regulation of Bax/Bcl-2 and DNA methylation related proteins. It was proved that lidocaine hydrochloride increased apoptosis of SH-SY5Y cells through p38 MARK mediated DNA methylation, which could provide a profile for preventing and treating LA induced neuronal toxic injury.

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Disclosure of conflict of interest

None.

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