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Inhibitory gene expression of the Cav3.1 T-type calcium channel to improve neuronal injury induced by lidocaine hydrochloride

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2-(4-Amidinophenyl)−6-indolecarbamidine dihydrochloride (PubChem CID: 160166
Propidium iodide (PubChem CID: 104981)

ABSTRACT

Cav3.1 is a low-voltage-activated (LVA) calcium channel that plays a key role in regulating intracellular calcium ion levels. In this study, we observed the effects of lidocaine hydrochloride on the pshRNA-CACNA1G-SH-SY5Y cells that silenced Cav3.1 mRNA by RNA interference, and investigated the roles of p38 MAPK in these effects. We constructed the pNC-puro-CACNA1G-SH-SY5Y cells and pshRNA-CACNA1G –SH-SY5Y cells by the RNA interference. All the cells were cultured with or without 10 mM lidocaine hydrochloride for 24 h. The cell morphology, cell viability, Cav3.1 and p38 protein expression, cell apoptosis rate and intracellular calcium ion concentration were detected. We found that all cells treated with 10 mM lidocaine hydrochloride for 24 h showed cellular rounding, axonal regression, and cellular floating. Compared with the cells in SH-SY5Y+Lido group and NC+Lido group, those in the RNAi+Lido group showed similar changes, but of smaller magnitude. Additionally, following lidocaine hydrochloride all cells displayed increased Cav3.1 and p38 MAPK protein, apoptosis rate, and intracellular calcium ion levels; however,these changes in the RNAi+Lido group were less pronounced than in the SH-SY5Y+Lido and NC+Lido groups. The cell viability decreased following lidocaine hydrochloride treatment, but viability of the cells in the RNAi+Lido group was higher than in the SH-SY5Y+Lido and NC+Lido groups. The results showed that Cav3.1 may be involved in neuronal injury induced by lidocaine hydrochloride and that p38 MAPK phosphorylation was reduced upon Cav3.1 gene silencing.

1. Introduction

Local anesthesia, with reduced systemic effects and a rapid recovery has been widely used in the clinic. However, the potential neurotoxicity of local anesthetics is also a concern (Auroy et al., 2002; Yamashita et al., 2003; Umbrain et al., 2009). Accumulating evidence indicates that the neurotoxicity of local anesthetics is related to intracellular calcium overload (Kasaba et al., 2006, 2007). For example, 0.1% lidocaine or 0.025% bupivacaine can induce minimal changes in the cytoplasmic calcium levels in neurons of the ND7 cell-line; however, 0.5–5% lidocaine or 0.125–0.625% bupivacaine can induce increased cytoplasmic calcium levels in a concentration and exposure period-dependent manner. The increase in cytoplasmic calcium levels that was induced by lidocaine or bupivacaine was dependent on: 1) increased Ca2+influx through the plasma membrane and 2) failure of the endoplasmic reticulum to uptake the elevated cytoplasmic calcium (Johnson et al., 2002). Tetracaine can also increase intracellular calcium in growth cones (Saito et al., 2004).

Voltage-gated Ca2+ channels (VGCCs) regulate intracellular Ca2+ concentrations with changes in the electrical excitability of neurons, and play an important role in a variety of neuronal physiological processes. VGCCs are divided into high-voltage-activated (HVA) Ca2+ channels and low-voltage-activated (LVA) calcium channels. LVAs are also referred to as T-type calcium channels, and are composed of three subunits Cav3.1, Cav3.2 and Cav3.3 (Mcrory et al., 2001). T-type Ca2+ channels are activated by subthreshold membrane depolarization that is close to the resting membrane potential of the cell and produces elevated intracellular Ca2+ levels. Moreover, T-type calcium channels show a window current, resulting from the persistent Ca2+ influx through the open T-type calcium channels at the resting state (Feltz, 2006). The T-type calcium channels are very important factors in the regulation of intracellular Ca2+ concentrations (Cueni et al., 2009). Therefore, it is highly likely that the neurotoxic

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Table 1
The sequences of Cav3.1 shRNA, negative control, Cav3.1 and β-actin primers.

Primer name		Sequence
NC	up	5′-GATCCCCGCCAGCTTAGCACTGACTCTTCAAGAGAGAGTCAGTGCTAAGCTGGCTTTTTA-3′
	down	5′-AGCTTAAAAAGCCAGCTTAGCACTGACTCTCTCTTGAA GAGTCAGTGCTAAGCTGGC GGG-3′
shRNA (RNAi)	up	5′-GATCCCCCGCTTACCAACGCCCTAGAAATTTCAAGAGAATTTCTAGGGCGTTGGTAAGCGTTTTTA-3′
	down	5′-AGCTTAAAAACGCTTACCAACGCCCTAGAAATTCTCTTGAAATTTCTAGGGCGTTGGTAAGCGGGG-3′
$Ca_v3.1$	up	5′-GCCATCTTCCAGGTCATCAC-3′
	down	5′-ACCAGGCACAGGTTGATCAT-3′
β-actin	up	5′-TGGCACCCAGCACAATGAA-3′
	down	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

effect of local anesthetics is related to an increase in the intracellular Ca2+ concentration that is mediated by the T-type calcium channels.

The p38 MAP kinase, belonging to the mitogen-activated protein (MAP) kinase family, plays a vital role in intracellular signal transduction of exogenous stimuli, and its activation is associated with the toxicity induced by local anesthetics. The agent SB203580 is one of the p38 MAPK inhibitors, which can reduce the neurotoxic injury induced by bupivacaine and ropivacaine in vitro (Lirk et al., 2008). Lirk et al. (2007) found that inhibition of p38 MAPK activation (phosphorylated p38, p-p38) can relieve neuronal-axonal damage induced by lidocaine. Those studies indicated that p38 MAPK may be involved in the local anesthetic-mediated neurotoxic injury (Haller et al., 2006).

Our previous study found that NNC 55-0396 dihydrochloride, which is a highly selective T-type calcium channel blocker, can clearly improve the injury of SH-SY5Y cells induced by bupivacaine (Wen et al., 2013b). However, three subtypes T-type calcium channels, namely Cav3.1, Cav3.2 and Cav3.3, were identified in SH-SY5Y cells (Wen et al., 2011). In addition, NNC 55-0396 dihydrochloride had no selectivity to those three subtypes.

In this study, we adopted RNA interference to inhibit Cav3.1 gene expression in the SH-SY5Y cell-line, observe changes in cell morphology, protein expression of Cav3.1 and p-p38 MAPK, cell viability, rate of cellular apoptosis and cytosolic Ca2+. Additionally, we tested the hypothesis that inhibition of Cav3.1 expression could improve SH-SY5Y cell injury that was induced by lidocaine hydrochloride.

2. Materials and methods

2.1. Materials

The SH-SY5Y cell-line was from the Shanghai Institute for Biological Sciences. psPAX2, pMD2.G lentivirus packaging system, 293FT packaging cells, pSUPERretro-puro plasmid were from Laura Biotech Company (Guangzhou, China). Plasmid maxiprep kits and DNA purification kits were from Tiangen Biotech Company (Beijing, China). DNA polymerase, DNA ligase and PrimeSTAR HS DNA polymerase were from Takara Company (Dalian, China). Anti–Cav3.1, anti-p-p38 or anti-β-actin antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Restriction enzyme Bgl II and Hind III were from NEB company (NEB, USA). Lidocaine hydrochloride was from Sigma company, USA. Other reagents used in this study were as follows: DMEM/F12 medium and fetal bovine serum (Gibco, USA), 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT, Beyotime, China) and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Beyotime, China). All reagents were from commercial suppliers and were of standard biochemical quality.

2.2. Cell culture

Cryopreserved SH-SY5Y cells were thawed in thermostated shaker at 37 °C. The nutrient solution of the SH-SY5Y cells was composed of DMEM/F12 medium with 15% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The SH-SY5Y cells were cultured in a humidified 5% CO2 incubator at 37 °C. The nutrient solution was replaced every second day.

2.3. Construction of pshRNA-CACNA1G-SH-SY5Y cells

According to our previous method that construct pshRNA-CACNA1G-SH-SY5Y cells to silence the Cav3.1 gene (Wen et al., 2013a). In brief, we designed one interference sequence and one negative control sequence using tools available on the internet. We also designed human Cav3.1 and β-actin sets of primers (Table 1). These sequences were synthesized by Shanghai Sangon Company (Shanghai, China). The interference sequences or negative control sequences were diluted to 1 μg/μl. The mixture (consisting in the passenger and guide strand − 5 μl of each – and ddH2O 15 μl) was denatured at 95 °C for 5 min and cooled slowly to anneal. The reaction system for the enzymatic digestion of pSUPERretro-puro was as follows: 4 μl NEB 10 × buffer, 1 μl Bgl II (10U/μl), 1 μl Hind III (10U/μl), 20 μl pSUPERretro-puro (0.1 μg/μl) and 14 μl ddH2O. After annealing and enzyme cutting, the synthetic constructs were combined with the digested pSUPERretro-puro plasmid to construct pNC-puro (negative control-NC) and pshRNA-puro-CACNA1G- RNAi (RNAi). The enzymatic digestion was at 37 °C for 2 h to digest and 65 °C for 10 min to inactivate. The reaction system for inserting the DNA double strands (after annealing) into the pSUPERretro-puro plasmid (after enzyme cutting) was as follows: 5 μl products of enzyme cutting, 4.5 μl products of the DNA double strands after annealing, 2 μl 10 × buffer and 1 μl T4 DNA ligase. The joint reaction system was kept at 16 °C for 24 h. The ratio of transfection complex for pNC-puro (NC) or pshRNA-puro–CACNA1G-RNAi: pSPAX2: pMD2.G was 4:3:1 (Wiznerowicz and Trono, 2003; Wei et al., 2010). 6 h after the mixture preparation at room temperature, 293T cells were cultured with this mixture for 36–48 h. Liquid supernatant of the culture was collected 3–4 times, added to the SH-SY5Y cells culture solution and incubated at 37 °C for 3 h. Then we applied new liquid supernatant for transfer, and the entire operation was repeated 2 times. The products were pNC-puro-CACNA1G-SH-SY5Y and pshRNA-CACNA1G-SH–SY5Y, respectively. Cav3.1 mRNA and protein expression were detected to assess silencing effectiveness.".

2.4. Lidocaine exposure

SH-SY5Y cells, pNC-puro-CACNA1G-SH-SY5Y (NC) and pshRNA - CACNA1G –SH-SY5Y (RNAi) were cultured with or without lidocaine hydrochloride for 24 h. Lidocaine hydrochloride in the form of a powder was dissolved in medium with serum, and the final concentrations of lidocaine hydrochloride were 10 mM. The