Effect and mechanism of hyaluronic acid on the neurotoxic injury of lidocaine

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Abstract: Hyaluronic acid (HA) is used to aid tissue repair and is a characterized inhibitor of TRPV1 channels. In this study, we investigated the effects of HA on lidocaine induced neurotoxicity and its mechanism of action. U87-MG cells with low (U87-MG-shTRPV1) or high (U87-MG-TRPV1) TRPV1 expression were studied. The control group was treated with lidocaine. The experimental group was treated with lidocaine and HA. Flow cytometry was used to assess the intracellular calcium concentration ($[Ca^{2+}]_i$) and cell apoptosis. Cell viability was detected by MTT assays. Compared to the control group, $[Ca^{2+}]_i$ of U87-MG-TRPV1 and U87-MG cells were lower at T3, T4 and T5 ($p < 0.05$), apoptosis rates of U87-MG and U87-MG-TRPV1 cells were lower ($p<0.05$), and the cell viability of U87-MG and U87-MG-TRPV1 cells were higher in the experimental group ($p<0.05$). HA reduces the toxic damage of lidocaine through blocking Ca^{2+} influx through TRPV1 channels, preventing Ca^{2+} overload, leading to nerve cell protection.

Keywords: Lidocaine, toxicity, calcium.

INTRODUCTION

The occurrence of nerve block had led to interest in the neurotoxicity of local anesthetics (Zhang *et al*., 2016). Toxicity of local anesthetics is related to drug dose and administration time (Wang *et al*., 2010; Carvalho *et al*., 2010) and often results in persistent nerve pain. An array of studies suggests that ion channels are involved in the neurotoxicity induced by local anesthetics. Wen *et al*. reported that local anesthetics increase $[Ca^{2+}]_i$ in SH-SY5Y cells leading to intracellular Ca^{2+} overload and apoptosis (Wen *et al*., 2011). Lidocaine has been shown to bind to the capsaicin site of the TRPV1 channel leading to channel activation and increased $[Ca^{2+}]_i$ (Leffler *et al*., 2008). Lu *et al*. reported that TRPV1 over expression in U87-MG cells enhances $[Ca^{2+}]_i$ and apoptosis and that TRPV1 silencing reduces lidocaine induced cytotoxicity (Lu *et al*., 2016). These studies indicate that the neurotoxicity of local anesthetics are related to TRPV1 expression and activity.

HA is a large polysaccharide composed of two disaccharide units, a D-glucuronic acid and N-acetylglucosamine. It plays an important role in cell proliferation and migration, and interacts with a variety of membrane proteins including CD44, and Toll like receptors (TLRs) (Gokce *et al*., 2017; Amann *et al*., 2017; Khanmohammadi *et al*., 2017). HA regulates cell growth, apoptosis, inflammation, and tumor occurrence. In recent years, HA has been widely used in the treatment of arthritic pain and to promote the healing of damaged tissue (Dicker *et al*., 2014). HA also interacts with TRPV1 and can reduce the channel opening frequency induced by heat stimulation or capsaicin. Experiments in isolated dorsal root neurons demonstrated that HA reduces the sensitization of TRPV1 induced by bradykinin. Subcutaneous injection of HA also alleviates the harmful response of mice to capsaicin and heat stimulation (Caires *et al*., 2015). HA differs from the competitive block of classic TRPV1 antagonists through binding to the cytoplasmic domain of TRPV1 to stabilize its closed state. HA is the major component of the nerve extracellular matrix and regulates the plasticity of neurons.

TRPV1 is widely expressed in neurons and gliocytes. It is speculated that HA protects against neurotoxicity in response to local anesthetics. In this study, TPRV1 expression in U87-MG cells was manipulated to assess its effects on $[Ca^{2+}]_i$, cell viability and apoptosis in response to lidocaine and HA treatment. The ultimate aim was to explore the effects of HA on the neurotoxicity of lidocaine and its related mechanisms.

MATERIALS AND METHODS

Cell culture

U87-MG cells were purchased from the Hanbio Company (Shanghai, China). U87-MG-shTRPV1 and U87-MG-TRPV1 cell lines were provide by Dr. Lu. Cells were cultured and maintained in RPMI 1640 medium (Gibco, Life Technologies, UK) with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37^{o}C, 5% CO2 cell culture incubator.

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Ethical approval

Subjects have given their informed consent and that the study protocol has been approved by the institute's committee on human research. Further, they should also state that animal experiments conform to institutional standards. There were only cells used in the experiment.

Cell grouping and processing

U87-MG cells, U87-MG-shTRPV1 and U87-MG-TRPV1 cells in the control group were treated 10 mmol/l lidocaine for 1 h. Cells in the experimental group were treated with 10 mmol/l lidocaine and 100 mg/l HA for 1 h.

Detection of calcium concentrations

Cells were collected at 30 min (T1), 1 h (T2), 2 h (T3), 4 h (T4) and 6 h (T5) post-treatment. Cells were centrifuged for 6 mins, the supernatants discarded and the cell concentrations adjusted to 10^6-10^7 /ml. For Ca^{2+} imaging, Fluo-3 AM (Beyotime, China) was added to a final concentration of 3-4 g/ml, and the cells were incubated for 10 min at 37°C. Cells were washed in HBS, suspended in HBSS and fluorescent intensity assessed by flow cytometry.

Flow cytometry

In control and experimental groups, cells were collected at 12 h post-treatment. After centrifugation, culture media was discarded and cells were washed in buffer solution. Cell concentrations were adjusted to 10^6 /ml and 5 µL Annexin V and 5µl PI (Beyotime, China) were added at room temperature in the dark for 10 mins. Apoptotic cells were assessed by flow cytometry.

Cell viability assays

Cells at a density of 5×10^4 /ml were plated into 96-well culture plates in 100µl of media at 37°C, 5% CO_2 for 2 d. Cells were drug treated and 20µl of 5mg/mL MTT reagent was added to each well post-drug treatment (Beyotime, China) for 3 h. Supernatants were discarded and 150µl of DMSO was added to dissolve the MTT reagent. Absorbances were read at 570 nm.

STATISTICAL ANALYSIS

SPSS16.0 statistical software was used for analysis, and measurement data were expressed as the mean ± standard deviation. A paired *t* test was used for comparisons between the two groups, and a single factor analysis of variance was used to compare multiple groups. *P*-values < 0.05 were considered significant.

RESULTS

Changes of $[Ca^{2+}]_i$

As shown in fig.1, $[Ca^{2+}]_i$ in U87-MG cells varied over time, peaking at T3, and declining at T4 in the control group. At T3, T4 and T5, the $[Ca^{2+}]_i$ was higher than U87-MG-shTRPV1 cells ($p<0.05$). The $[Ca^{2+}]_i$ of U87-MG-TRPV1 cells peaked at T3, and was higher than U87-MG cells and U87-MG-shTRPV1 cells at T3, T4 and T5 ($p< 0.05$).

In the experimental group, the $[Ca^{2+}]_i$ in U87-MG cells varied over time, peaking at T3. At T3, T4 and T5, $[Ca^{2+}]_i$ in U87-MG cells was higher than U87-MG-shTRPV1 cells ($p < 0.05$). The $[Ca^{2+}]_i$ of U87-MG-TRPV1 cells peaked at T3, and showed no significant differences at T3, T4 and T5, compared to U87-MG cells ($p>0.05$).

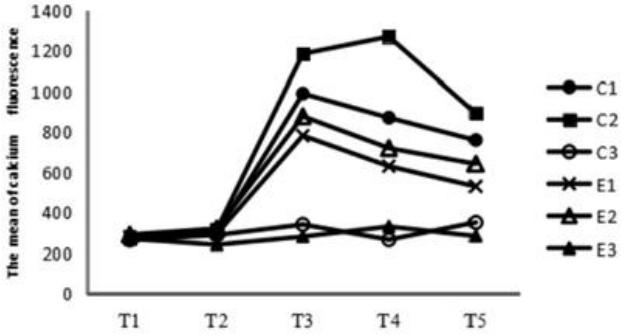

Fig. 1: *$[Ca^{2+}]_i$ assessment.* C1: U87-MG control group, C2: U87-MG-TRPV1 control group, C3: U87-MG-shTRPV1 control group, E1:U87-MG experimental group, E2: U87-MG-TRPV1 experimental group; E3: U87-MG-shTRPV1 experimental group.

There were no significant differences in the $[Ca^{2+}]_i$ between experimental and control groups at T1 and T2 ($p > 0.05$). Compared to the control group, the $[Ca^{2+}]_i$ of U87-MG and U87-MG-TRPV1 cells in the experimental group were lower at T3, T4 and T5 ($p<0.05$). There were no significant differences in the $[Ca^{2+}]_i$ of U87-MG-shTRPV1 cells any time point between the experimental and control groups ($p>0.05$).

Cell apoptosis measurements

As shown in the table 1, the rates of apoptosis in U87-MG-TRPV1 cells were higher than those of U87-MG cells ($p<0.05$), and apoptotic rates of U87-MG-shTRPV1 cells were lower than those of U87-MG and U87-MG-TRPV1 cells in the control group ($p<0.05$).

In the experimental group, there were no significant differences in apoptotic rates between U87-MG-TRPV1 and U87-MG cells ($p>0.05$). Apoptosis in U87-MG-shTRPV1 cells was lower than those of U87-MG cells and U87-MG-There was no significant difference in the apoptosis rate of U87-MG-shTRPV1 cells between the experimental group and the control group ($p>0.05$). U87-MG-TRPV1 cells ($p<0.05$).

Compared to the control group, the apoptosis rates of U87-MG and U87-MG-TRPV1 cells in the experimental group were significantly lower ($p<0.05$).

Table 1: Comparison of apoptosis rates (%)

	Control group	Experimental group
U87-MG	14.7 ± 1.6^{c}	9.6 ± 1.5^{NS}
U87-MG-TRPV1	21.7 ± 0.8^{b}	10.3 ± 0.7^{NS}
U87-MG-shTRPV1	6.6 ± 1.3^{a}	6.2 ± 1.0^{a}

Table 2: Comparison of cell viability (%)

	Control group	Experimental group
U87-MG	62.3 ± 4.6^{c}	81.9 ± 4.0^{NS}
U87-MG-TRPV1	47.7 ± 3.8^{b}	80.8 ± 5.1^{NS}
U87-MG-shTRPV1	93.2 ± 5.5^{a}	94.0 ± 3.7^{a}

Data are expressed as the mean ± SEM; n=6; Values with different superscripts in the same row represent significant differences ($p<0.05$), NS represents no-significant differences.

Cell viability measurements

In the control group, the viability of U87-MG-TRPV1 cells was lower than that of U87-MG cells ($P<0.05$, table 2). The viability of U87-MG-shTRPV1 cells was higher than U87-MG and U87-MG-TRPV1 cells ($p<0.05$). In the experimental group, the viability of U87-MG-TRPV1 and U87-MG cells did not significantly differ ($p>0.05$). U87-MG-shTRPV1 cell viability was higher in U87-MG compared to U87-MG-TRPV1 cells ($p<0.05$). Compared to control cells, the viability of U87-MG and U87-MG-TRPV1 cells in the experimental group were higher ($p>0.05$). There was no significant differences in the viability of U87-MG-shTRPV1 cells between the experimental and control group ($p>0.05$).

DISCUSSION

TRPV1 is a ligand gated non-selective cation channel, originally thought to be expressed in the dorsal root ganglion and trigeminal ganglion neurons. Studies have since revealed its expression in many neurons, peripheral non neural tissues, sensory neurons and afferent nerve fibers. TRPV1 is activated by capsaicin, inflammatory media and harmful thermal stimulation through capsaicin receptors (Bao *et al*., 2018; Cao *et al*., 2017; Hwang *et al*., 2017). TRPV1 channel activation enhances Ca^{2+} influx leading to an array of physiological functions and pathological mechanisms, including pain (Payrits *et al*., 2017). Leffler *et al*. (Leffler *et al*., 2008) confirmed that lidocaine activated TRPV1 by binding to its capsaicin binding site, resulting in increased Ca^{2+} influx and excitatory amino acid release in spinal dorsal root ganglion cells. Intracellular Ca^{2+} overload is an important during nerve cell apoptosis induced by local anesthetics. Intracellular Ca^{2+} overload is induced by intracellular Ca^{2+} release (Ca^{2+} induced Ca^{2+} release, CICR) mechanisms, and Ca^{2+} influx (Endo, 2009). In this study we assessed the effects of HA on $[Ca^{2+}]_i$ and apoptotic induction. We show that TRPV1 silencing reduces the increase in $[Ca^{2+}]_i$ and apoptosis induced by lidocaine, suggesting that lidocaine causes Ca^{2+} overload by activating TRPV1.

HA is a prevalent polysaccharide in the human body with good biocompatibility, degradation and low antigenicity, and is as such widely used in biomedicine. Previous studies suggest that HA regulates cell proliferation, migration, differentiation and cell matrix secretion through CD44. As the major component of the extracellular matrix, HA wraps around neurons and regulates their plasticity. TRPV1 is widely expressed in neurons and glial cells and regulates a variety of biological functions. Studies have reported that HA interacts with TRPV1 channels, decreasing the opening frequency caused by hot stimuli and capsaicin, leading to a stable reduction in Ca^{2+} influx. Studies have shown that HA can reduce TRPV1 channel sensitization of isolated dorsal root neurons induced by bradykinin, alleviating its harmful responses (Caires *et al*., 2015). Extra cellular HA binds to cell surface expressed TRPV1 to reduce its excitability. In this study, HA treatment inhibited the increase in $[Ca^{2+}]_i$ and subsequent apoptosis in cells with high TRPV1 expression, suggesting HA has protective effects on the neurotoxic injury caused by lidocaine.

We demonstrated that after 1 h of lidocaine treatment, the $[Ca^{2+}]_i$ in U87-MG cells peaked at 4 h and decreased at 6 h. TRPV1 silencing inhibited the rise in $[Ca^{2+}]_i$ induced by lidocaine. High TRPV1 expression did not increase the peak $[Ca^{2+}]_i$, but $[Ca^{2+}]_i$ was higher than low TRPV1 expressing cells. Low TRPV1 expression also reduced lidocaine induced apoptosis, whilst high TRPV1 expression significantly decreased cell viability and apoptosis. This was consistent with previous studies (Lu *et al*., 2016). In high TRPV1 expressing cells, HA inhibited the increase in $[Ca^{2+}]_i$ and delayed apoptosis. HA had no significant effect on the $[Ca^{2+}]_i$ in cells with low TRPV1 expression but protected high TRPV1 cells from lidocaine induced cell damage. Studies by Wen *et al*. (Wen *et al*., 2011), used longer lidocaine treatment times and lower drug concentrations (74 mmol/L lidocaine for 10 mins). These conditions differ from those used in the clinic.

CONCLUSION

Taken together, this study suggests that HA protects against lidocaine induced neurotoxicity. These effects are

mediated through TRPV inhibition by reducing $[Ca^{2+}]_i$. These results provide a basis for the prevention and control of the neurotoxicity of local anesthetics.

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