Osthole-Mediated Inhibition of Neurotoxicity Induced by Ropivacaine via Amplification of the Cyclic Adenosine Monophosphate Signaling Pathway

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Abstract

Background: Ropivacaine is widely used for clinical anesthesia and postoperative analgesia. However, the neurotoxicity induced by ropivacaine in a concentration- and duration-dependent manner, and it is difficult to prevent neurotoxicity. Osthole inhibits phosphodiesterase-4 activity by binding to its catalytic site to prevent cAMP hydrolysis. The aim of this present study is to explore the precise molecular mechanism of osthole-mediated inhibition of neurotoxicity induced by ropivacaine.

Methods: SH-SY5Y cell viability and apoptosis were measured in different concentration and duration. Protein concentration was determined in each signaling pathway. The molecular mechanism of osthole-mediated inhibition of ropivacaine-caused neurotoxicity was evaluated.

Results: The study demonstrated that osthole inhibits SH-SY5Y cells neurotoxicity in a duration- and concentration-dependent manner. Moreover, ropivacaine significantly increased the expression of caspase-3 by promoting the phosphorylation of p38. Osthole-induced upregulation of cAMP activated cAMP-dependent signaling pathway, sequentially leading to elevated cyclic nucleotide response element-binding protein levels, which inhibits P38-dependent signaling and decreases apoptosis of SH-SY5Y.

Conclusions: This study display the evidence confirmed the molecular mechanism by which osthole amplification of cAMP-dependent signaling pathway, and overexpression of cyclic nucleotide response element-binding protein inhibits P38-dependent signaling and decreases ropivacaine-induced SH-SY5Y apoptosis.

Keywords

ropivacaine, osthole, neurotoxicity, p38, cyclic adenosine monophosphate, apoptosis

Introduction

The local anesthetic ropivacaine is widely used for clinical anesthesia and postoperative analgesia given its reduced cardiac toxicity, and its especially useful property of inducing a separation block in sensory and motor nerves. However, epidemiological investigations have shown that nerve exposure to high concentrations and long durations of local anesthetics could result in neurotoxicity. All local anesthetics have been reported to cause neurotoxicity in a concentration- and duration-dependent manner.
The precise molecular mechanism of neurotoxicity induced by ropivacaine is unclear, inflammation, neurotrophic factors, cell apoptosis, and intracellular calcium concentration are generally related to neurotoxicity.7-10

Ropivacaine, introduced as a pure S-(-)-enantiomer, has a low degree of neurotoxicity, but high concentration of ropivacaine triggers both neurotoxicity and cardiovascular toxicity. Several previous studies indicate that neurotoxicity induced by ropivacaine including mitochondrial dynamics, mitochondrial dysfunction and cell death that are dependent on dynamic reaction protein 1 (DRP1) expression,11 and CaMKIIβ, Cav3.2, Cav3.3 expression up-regulated.12,13 Ropivacaine inhibits the tuberin/mTOR/p70S6K signaling pathway, downregulates autophagic flux, and increases neuronal damage.14 Researchers have suggested that ropivacaine activates the MAPK/p38/Fas signaling pathway to promote neurogliocyte apoptosis,15 but a consensus on the molecular mechanism of neurotoxicity induced by ropivacaine is lacking.

Phosphodiesterase-4 (PDE4) reduces intracellular cyclic adenosine monophosphate (cAMP) levels by hydrolyzing cAMP to 5'-AMP, thereby regulating the cAMP/PKA pathway and the phosphorylation of cyclic nucleotide response element-binding protein (CREB) by PKA.16,17 PDE4-selective inhibitors interact with the catalytic pocket of the enzyme and decrease cAMP hydrolysis, as a result, the cAMP levels were increased intracellularly. The major effect of higher cAMP levels is the activation of the cAMP-dependent cAMP/PKA/CREB pathway. Greater than 20 naturally occurring PDE4 isoforms, are encoded by 4 genes (PDE4A, PDE4B, PDE4C, and PDE4D), each of which has a specific pattern of expression.18 PDE4D5 could determine the direction of the pathway by regulating the levels of several signaling proteins, such as RACK1,19 β-arrestin2,20-22 and the MAPK signaling pathway, especially ERK1/223-25 and MK2.26 PDE4D5 is also a PKA27 and oxidative-stress kinase target.28

Osthole is a coumarin derivative (She Chuang Zi) that is, a widely used traditional Chinese medicine, and has many biological functions.29 Osthole displays anti-inflammatory biological functions by suppressing the activation of the NF-κB and p38/MAPK signaling pathways,30 and anti-gastric cancer activity inhibition the AKT/MAPK pathway.31 Osthole is also effective in preventing the colitis by suppressing p38/MAPK and cAMP/PKA-dependent pathways,32 and osthole modifies osteogenesis by activating the cAMP/PKA/CREB signaling pathway.33 Recently, a study demonstrated that osthole inhibited PDE4D activity by binding to the PDE4D catalytic site to prevent cAMP hydrolysis, therefore, the cAMP levels were increased and amplified cAMP/PKA-dependent signaling, eventually resulting in relaxation of airways in airway smooth muscle cells.34 Despite studies addressing several biochemical behaviors of osthole, whether osthole exerts the property for the inhibition of neurotoxicity induced by ropivacaine and its molecular mechanisms remain elusive.

In the present study, we found that SH-SY5Y cell neurotoxicity was induced by ropivacaine in a concentration- and duration-dependent manner, and that osthole inhibited neurotoxicity induced by ropivacaine in a concentration- and duration-dependent manner. Osthole inhibits PDE4D activity and decreases the hydrolysis of cAMP to 5'-AMP, thereby increasing cAMP levels and activating the cAMP/PKA/CREB pathway. CREB over expression suppresses p38/MAPK pathways and decreases SH-SY5Y cell apoptosis.

Materials and Methods

Reagents and Methods

SH-SY5Y cells with special culture medium, bovine serum albumin and all of the antibodies were purchased from Jihe Biotechnology Inc, Shanghai. The TUNEL apoptosis assay kit and total RNA extraction kit were purchased from Jingchu Biotechnology Co., Ltd, Shanghai. Masson kit and streptavidin were purchased from Jingheng Biotechnology Co., Ltd, Shanghai. Osthole with a purity greater than 98% was purchased from Mansite Biotechnology Inc, Chengdu. Ropivacaine at a concentration of 1% was purchased from AstraZeneca AB Ltd

Viability Assays for SH-SY5Y Cells

SH-SY5Y cells were cultured with 10% complete medium (minimum essential medium, MEM, supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 mg/ml)) in a humidified, 5% CO2, 37°C incubator. The medium was replaced every 2 days, and images of the passing cells were passed when they reached to 90% confluence.

The passaging cells were plated at 2 × 10^5 cells/ml in 6-well plates and 2 ml/well at 37°C with 5% CO2 for 24h, and then stimulated with six different concentration of ropivacaine, .1, .5, 1.0, 2.0, 5.0, and 10.0 mM for 8 h. Then, cells were sequentially cultured at 37°C with 5% CO2 for 7 days. Cell viability was measured using a trypan blue exclusion assay. The growth medium was replaced every 2 days, and images of SH-SY5Y cells were captured every day with an inverted microscope (Olympus, Tokyo, Japan). Following imaging, SH-SY5Y cells were trypsinized and stained with trypan blue (Mediatech, Manassas, VA, USA). A hemocytometer (Beckman, Brea, CA, USA) was used to count viable (nonstained) and nonviable (blue) cells.

Neurotoxicity Assay to Assess Osthole’s Preventative Effects

Osthole was diluted into dimethyl sulfoxide (DMSO) and mixed with culture medium to supply six different concentrations. SH-SY5Y cells were pre-treated with different concentrations of osthole for 1 h, before subsequent treatment of ropivacaine (3 mM) for 8 h, and then cells were cultured in growth medium for 7 days. Cell viability was measured using a trypan blue exclusion assay.
Flow Cytometry to Assess SH-SY5Y Cell Apoptosis

SH-SY5Y cells were cultured at $2 \times 10^5$ cells/ml in 6-well plates and 2 mL/well at 37°C with 5% CO2 for 24 h. Cells were stimulated with normal saline for 8 h and then culture with growth medium for 28d in control group, or cells were stimulated with 3.0 mM ropivacaine for 8 h and then culture with growth medium for 28d in ropivacaine group. Cells were pre-treated with 100 μM osthole for 1 h, before subsequent treatment of ropivacaine (3.0 mM) for 8 h, and then cells were cultured in growth medium for 28 days in osthole group. After 28 days, the rate of apoptosis was measured. (Figure 1.)

After 28 d of culture, cells were digested by adding .5 mL of trypsin to each well and then centrifuged at 1500 r/min for 5 min. The supernatant was discarded, and cells were washed with 1 mL of cold PBS in each group. Cells were centrifuged at 1500 r/min for 5 min, discarding the supernatant. Then, 300 μL Binding Buffer and 5 μL Annexin V-FITC were added in each group and incubated in the dark for 15 min. Cells were resuspended in 200 μL Binding Buffer, 5 min prior to analysis using a BD FACSCalibur flow cytometry. Then 10 μL PI was added in each group. A minimum of $1.0 \times 10^4$ cells within the gated region were analyzed.

Western Blotting

SH-SY5Y cell samples were lysed for 20 min placed on ice in RIPA lysis buffer and centrifuged at 12 000 r/min for 20 min at 4°C, and the supernatant was reserved.

Protein concentration was determined using a bicinechonicin acid (BCA) protein concentration assay kit. The protein concentration was adjusted by adding 5×loading buffer and PBS according to the concentration determination results to ensure a consistent protein concentration between different groups. After the concentration was adjusted, the samples were incubated at 95°C for 5 min.

Protein samples were electrophoretically separated using a 10% SDS polyacrylamide gel, followed by transfer to .45 μm PVDF membranes at 200 mA for 90 min. PVDF membranes were soaked in TBST (containing 5% skim milk) for 2 h at room temperature, and the PVDF membrane was subsequently soaked in primary antibody (1:1000) diluted with TBST (containing 2% BSA solution) and incubated at 4°C overnight. After washing with TBST thrice, the HRP labeled secondary antibody was diluted (1:1500) with TBST, and the PVDF membrane was soaked in the secondary antibody solution for 1 h at room temperature.

The results were visualized using an automatic chemiluminescence image analysis system (Beyotime Biotechnology Co., Ltd, Shanghai, China). Image grayscale values were analyzed using Image J software.

Assay to Determine PDE Activity in Vitro

The PDELight HTS cAMP phosphodiesterase kit (Lonza, LT07-600) was used to determine PDE activity, per operated according to the manufacturer’s instructions (Protocol B). One microliter of PDE eluate expressed in eukaryotes was incubated with osthole for 20 min in PDE buffer (10 mM Tris-HCl, pH = 7.5). Then, 2 μM of cAMP was added, and the solution was kept under incubation for 60 minutes. Then, 10 μl of the stop solution was added to the reaction to stop further phosphodiesterase activity, and 20 μl of the reconstituted AMP detection reagent was then added to the wells followed by incubate for 10 minutes at room temperature, luminescence was measured using a Varioskan Flash device (Thermo Fisher Scientific) after 10 minutes later.

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistics. Parametric data expressed as mean ± standard deviation (mean ± SD), One-way analysis of variance (one-way ANOVA) was used for comparisons between groups. Kruskal–Wallis test was conducted for analysis of normalized data. A $P$-value less than .05 was considered statistically significant.

![Figure 1](image-url) The different stimulation methods of SH-SY5Y cells in three groups.
Results
The IC50 of ropivacaine inhibits SH-SY5Y cells viability. SH-SY5Y cells were stimulated with six different concentrations (.1, .5, 1.0, 2.0, 5.0, and 10.0 mM) of ropivacaine for 8 h, then the cells were sequence cultured in growth medium. We counted the percentage of death cells in each concentration (8%, 12%, 48%, 69%, 73%, 80%, respectively) after 72 h, and calculated the mean inhibitory concentration (IC50) by probit regression analysis from twelve experiments. The IC50 of ropivacaine that inhibited SH-SY5Y cells viability was 3.22 ± 1.1 mM (Figure 2A). Ropivacaine significantly inhibited SH-SY5Y cells viability compared with the control. The neurotoxicity caused by ropivacaine was duration- and concentration-dependent (Figure 2B).

The IC50 of osthole-mediated inhibition of SH-SY5Y cell neurotoxicity
The IC50 of osthole-mediated inhibition of SH-SY5Y cell neurotoxicity induced by ropivacaine was 28.6 ± 6.2 μM and the IC80 was 95.0 ± 12.6 μM (Figure 3A). SH-SY5Y viability was significantly lower in the ropivacaine group compared with the control group, and the SH-SY5Y viability was no significantly different between the osthole group and the control group (Figure 3B). Osthole exerted its effects in a duration- and concentration-dependent manner to prevent neurotoxicity induced by ropivacaine.

Osthole attenuated SH-SY5Y cells apoptosis rate induced by ropivacaine
Flow cytometry results showed that the SH-SY5Y cells apoptosis rate was significantly decreased in the osthole group compared with the control and ropivacaine groups, and ropivacaine significantly increased the rate of apoptosis (Figures 4A-4D).

Discussion
The present study demonstrated that osthole inhibits the activity of PDE4D and amplified the cAMP/PKA-dependent signaling pathway in SH-SY5Y cells, which suppresses the
Figure 3. Concentration- and duration-dependent osthole inhibits SH-SYSY cell neurotoxicity induced by ropivacaine. (A) Sigmoid-shaped curves for the concentration-response relationship were calculated by probit regression analysis using the results from twelve experiments with six different concentrations of osthole. The IC_{50} of osthole-mediated inhibition of SH-SYSY cell neurotoxicity induced by ropivacaine was $28.6 \pm 6.2 \, \mu M$ and the IC_{80} was $95.0 \pm 12.6 \, \mu M$. (B) SH-SYSY cells were stimulated with 3.0 mM of ropivacaine (Ropivacaine and osthole group) or normal saline (control) for 8 h. The cells were sequence cultured with 100 μM (osthole) or normal saline (control and ropivacaine group) for 7 days. SH-SYSY cell viability was significantly lower in the ropivacaine group compared with the control group, and SH-SYSY cell viability was no significantly different between the osthole group and the control group (n = 3). Data are represented as the means ± SD, and were analyzed using Kruskal-Wallis test, *P < .01, **P < .001.

Figure 4. Osthole inhibits SH-SYSY cell apoptosis induced by ropivacaine. (A), (B), and (C) show the rate of apoptosis in the three groups. (D) The rate of apoptosis in ropivacaine group (38.4 ± 1.6%) was significantly increased compared with the control (32.88 ± 1.5%), and osthole group (28.6 ± 1.0%). Apoptosis was determined by flow cytometry. All data are reported as the means ± SD, and were analyzed using Kruskal-Wallis test. (n = 4), *P < .05 and **P < .01.
p-38/MAPK-dependent signaling pathway and decreases SH-SY5Y cell apoptosis induced by ropivacaine. A previous study showed that osthole binding of catalytic sites on PDE4D5 inhibits PDE activity.34

Figure 5. Osthole augments cAMP-mediated signaling and inhibits p38/MAPK signaling in SH-SY5Y cells. (A)–(C) The expression of cAMP, p-PKA, PKA, p-CREB, and CREB in the osthole group was increased compared with that in the control group, (D)–(E) The expression of p-38, p38, and caspase-3 in the osthole group was decreased compared with that in the control group. All data are presented as the means ± SD (n = 3), and were analyzed using Kruskal–Wallis test, **P < .05 and ***P < .01, ****P < .001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 6. Osthole inhibits the activity of PDE4D5. (A) Osthole inhibited the activity of PDE in a concentration-dependent manner, and 1.0 μM osthole efficiently inhibited the activity of PDE4D5 compared with the control group (n = 3). (B) Concentration-response curve of osthole inhibition of PDE4D5 activities. Sigmoid-shaped curves for the concentration-response relationship were calculated by probit regression analysis using the results from twelve experiments with six different concentrations of osthole. The IC50 of osthole inhibition of PDE4D5 activity was 6.22 ± 2.6 μM. All data are presented as the means ± SD, and were analyzed using Kruskal–Wallis test, **P < .01.

SH-SY5Y cell damage induced by ropivacaine in a concentration- and time-dependent manner,11 and the study showed that SH-SY5Y cells were exposed to different concentrations of ropivacaine for 20 minutes. Cell death was induced by
ropivacaine in a concentration-dependent manner, and the 50% cell lethality (IC50) of ropivacaine was 13.43 ± 0.61 mM. This higher concentration of ropivacaine is unsuitable for long duration clinical anesthesia. In our study, we used SH-SY5Y cells stimulated with different concentrations of ropivacaine for 8 h, the cells viability was inhibited by ropivacaine in a concentration-dependent manner. The IC50 of ropivacaine-induced SH-SY5Y neurotoxicity was 3.22 ± 1.1 mM, (Figure 2A). Then, SH-SY5Y cells were stimulated with 3.0 mM of ropivacaine for 8 h, and cell viability was inhibited by ropivacaine in a time-dependent manner, (Figure 2B) SH-SY5Y cell viability was significantly inhibited in the ropivacaine group compared with the control group.

Some previous results demonstrated that osthole inhibited cell neurotoxicity in a concentration- and time-dependent manner. Our results confirmed that osthole prevented SH-SY5Y neurotoxicity induced by ropivacaine in the same manner. The IC50 of osthole inhibited SH-SY5Y cell neurotoxicity induced by ropivacaine was 28.6 ± 6.2 μM and the IC80 was 95.0 ± 12.6 μM (Figure 3A), and then we used 100 μM of osthole to prevent the SH-SY5Y neurotoxicity in a time-dependent manner. SH-SY5Y viability was significantly higher in the osthole group compared with that in the ropivacaine group (Figure 3B).

The cAMP levels determined the cAMP signaling output response by increasing cAMP synthesis or hydrolysis, and the PDE-mediated cAMP signaling output was amplified when the cAMP levels increased. Ropivacaine inhibits Gαs-coupled receptors in a stereoselective and noncompetitive manner, which decrease the cAMP levels. However, osthole inhibits PDE4D activity by binding to the PDE4D catalytic site, and increases the cAMP levels, the present data suggest that osthole significantly inhibits PDE4D activity (Figure 6A), and in a concentration-dependent manner (Figure 6B).

Caspase-3 is an important indicator of apoptosis when assessing the neurotoxicity of various therapies. Caspase-3 is responsible for cell apoptosis in vivo, and down regulating the expression of caspase-3 decreases the rate of apoptosis. SH-SY5Y cells were divided into three groups, and cultured with different methods, respectively (Figure 1). Our results indicated that caspase-3 protein expression levels were downregulated in the osthole-treated group, and the rate of apoptosis was significantly decreased in the osthole group compared with the control and ropivacaine groups (Figures 4A-4D). Furthermore, we investigated the cAMP/PKA signaling pathway. The protein expression levels were increased in the osthole-treated group, and a higher level of CREB suppressed the phosphorylation level of p-38, which decreased the rate of apoptosis cells induced by ropivacaine in SH-SY5Y cells.

The clinical concentration of ropivacaine can cause neurotoxicity, however, the molecular mechanism remains unclear. In our present study, both the cAMP/PKA and p-38/MAPK signaling pathways were investigated in

![Figure 7. A proposed molecular mechanism of osthole inhibition of neurotoxicity induced by ropivacaine. Ropivacaine inhibits Gαs-coupled receptors and decreases the cAMP levels. Osthole inhibits PDE4D activity to increase cAMP levels and amplify the cAMP/PKA signaling pathway, sequentially leading to elevated CREB levels, CREB overexpression inhibits p-P38/MAPK signaling and decreases apoptosis of SH-SY5Y cells induced by ropivacaine.](image-url)
osthole-treated SH-SY5Y cells. PKA is a major downstream target of cAMP. The cAMP/PKA signaling pathway plays important roles in regulating CREB by increasing P-CREB phosphorylation levels. This study showed that the molecular mechanism of neurotoxicity induced by ropivacaine involved the p-38/MAPK-dependent signaling pathway activated by ropivacaine and resulted in the promotion of neurogliocyte apoptosis. Our western blot results showed that osthole suppressed cAMP/PKA protein expression and increased cAMP, Pka, and CREB protein expression (Figures 5A-5C). CREB overexpression suppressed the p38/MAPK-dependent signaling pathway, and p38 and caspase-3 protein expression decreased (Figures 5D-5E). Therefore, SH-SY5Y cell apoptosis was decreased. The study also demonstrated that cynomorium songaricum Rupr (a traditional Chinese medicine) exhibited potential therapeutic effect on neuroprotection of ovarioctomized rats, and its effect was possibly exerted by p-CREB/CAMP/PKA signaling output to suppress p38/MAPK signaling and decrease SH-SY5Y cell apoptosis. Therefore, SH-SY5Y cell apoptosis was noted. This study provides novel biological evidence for osthole inhibition of neurotoxicity induced by ropivacaine.

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Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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