7, 8, 3’-Trihydroxyflavone Promotes Neurite Outgrowth and Protects Against Bupivacaine-Induced Neurotoxicity in Mouse Dorsal Root Ganglion Neurons

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Background: 7, 8, 3’-trihydroxyflavone (THF) is a novel pro-neuronal small molecule that acts as a TrkB agonist. In this study, we examined the effect of THF on promoting neuronal growth and protecting anesthetics-induced neurotoxicity in dorsal root ganglion (DRG) neurons in vitro.

Material/Methods: Neonatal mouse DRG neurons were cultured in vitro and treated with various concentrations of THF. The effect of THF on neuronal growth was investigated by neurite outgrowth assay and Western blot. In addition, the protective effects of THF on bupivacaine-induced neurotoxicity were investigated by apoptosis TUNEL assay, neurite outgrowth assay, and Western blot, respectively.

Results: THF promoted neurite outgrowth of DRG neurons in dose-dependent manner, with an EC₅₀ concentration of 67.4 nM. Western blot analysis showed THF activated TrkB signaling pathway by inducing TrkB phosphorylation. THF also rescued bupivacaine-induced neurotoxicity by reducing apoptosis and protecting neurite retraction in DRG neurons. Furthermore, the protection of THF in bupivacaine-injured neurotoxicity was directly associated with TrkB phosphorylation in a concentration-dependent manner in DRG neurons.

Conclusions: THF has pro-neuronal effect on DRG neurons by promoting neurite growth and protecting against bupivacaine-induced neurotoxicity, likely through TrkB activation.

MeSH Keywords: Bupivacaine • Ganglia, Spinal • Neurotoxicity Syndromes • Receptor, trkB

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Background

Recently, a group of small-molecule compounds have been identified as tyrosine receptor kinase (Trk) receptor agonists that had profound pro-neuronal effects on neurogenesis and neuronal regeneration [1–4]. These compounds hold great clinical importance for neurodegenerative diseases as they may easily penetrate blood-brain-barrier, and have great potency with half-effective concentration (EC₅₀) lower than 1 μM [2,4,5]. Among those small-molecule compounds, a new compound, 7,8,3’-trihydroxyflavone (7,8,3’-THF), was created as the derivative of 7,8-dihydroxyflavone but with better potency [1]. In the peripheral auditory system, THF was shown to rescue noise-damaged or chemically-injured auditory nerves both in vitro and in vivo [6,7]. However, the role of THF in spinal cord dorsal root ganglion (DRG) neurons has not been characterized.

Spinal cord DRG neurons are subject to local anesthetics-induced neurotoxicity [8,9], which may contribute to various types of neurologic complications in young patients with regional anesthesia [10,11]. Specifically, bupivacaine, one of the commonly used local anesthetics, is shown to induce neuronal apoptosis, growth cone collapse, and neurite retraction in DRG neurons in animal models [8,12]. Studies also showed that several of the neuronal signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatase and tensin homolog (PTEN) pathways, were closely associated with the regulation of bupivacaine-induced neurotoxicity in DRG neurons [12,13]. Member of the Trk receptors – TrkB receptor, and its ligand brain-derived neurotrophic factor (BDNF) – are both identified as tyrosine receptor kinase (Trk) receptor agonists with profound pro-neuronal effects on neurogenesis and neuronal regeneration [1–4]. These compounds hold great clinical importance for neurodegenerative diseases as they may easily penetrate blood-brain-barrier, and have great potency with half-effective concentration (EC₅₀) lower than 1 μM [2,4,5]. Among those small-molecule compounds, a new compound, 7,8,3’-trihydroxyflavone (7,8,3’-THF), was created as the derivative of 7,8-dihydroxyflavone but with better potency [1]. In the peripheral auditory system, THF was shown to rescue noise-damaged or chemically-injured auditory nerves both in vitro and in vivo [6,7]. However, the role of THF in spinal cord dorsal root ganglion (DRG) neurons has not been characterized.

In this study, we used an explant model to culture neonatal mouse DRG neurons in vitro to mimic the biological conditions of young spinal cord sensory neurons. We first investigated the pro-neuronal effect of THF on the growth of neonatal DRG neurons by examining its effect on DRG neuron neurite outgrowth. Then, we applied bupivacaine to induce anesthetics-related neurotoxicity in DRG neurons. The potential protective effect of THF and the involvement of TrkB signaling pathways were examined by apoptosis assay, neurite outgrowth assay, and Western blot assay. The results of our study may further our understanding on the possible pro-neuronal mechanisms of small-molecule TrkB agonists in spinal cord sensory neurons.

Material and Methods

Mouse dorsal root ganglion neuron culture

Dorsal root ganglion (DRG) neurons were prepared from postnatal 2-3-day-(P2~P3) old C57BL/6J mice (Jackson Lab, USA). Briefly, DRG clumps were quickly dissected into cold (4°C) 1X HBSS (Invitrogen, USA). They were then transferred to disociation medium with warm (37°C) Dulbecco’s modified Eagle medium (DMEM, Invitrogen, USA), 10% fetal bovine serum (FBS, Invitrogen, USA), 1 X B27 neuronal supplement (Invitrogen, USA), and 0.25% trypsin (Invitrogen, USA) for 20 min. Dissociated cells were prepared by continuously triturating DRG clumps in-and-out of 1 mL pipette tips for 10 min. They were then re-suspended in serum-free culture medium containing neurobasal medium (Invitrogen, USA), penicillin/streptomycin (PenStrep, Invitrogen, USA), and 1 X B27 neuronal supplement (Invitrogen, USA) in a tissue culture chamber with 5% CO₂ at 37°C overnight. On the second day, the floating non-neuronal cells were removed. DRG neurons were maintained in culture medium for 2–7 days, depending on the requirement of designated experiments.

Neurite outgrowth assay

Cultured DRG neurons were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) and 0.3% Triton (Sigma-Aldrich, USA) in 1X PBS (Invitrogen, USA) for 30 min, then incubated with a rabbit anti-neurofilament 2000 (NF-2000) polyclonal antibody (Santa Cruz, USA) at 37°C over night. On the second day, the culture was incubated with AlexaFluor 488 anti-rabbit secondary antibody at room temperature for 2 h. The green fluorescent images were viewed under an upright fluorescent microscope (BX51, Olympus, Japan). For each experimental condition, the averaged length of the longest 50~80 neurites were measured among at least three repeats, and then normalized to control condition.

7, 8, 3’-Trihydroxyflavone treatment assay

In DRG neuron culture, various concentrations (2, 5, 10, 20, 50, 100, 250, 500, 1000, 1500, 2000 nM) of 7, 8, 3’-Trihydroxyflavone (THF) were added for 2 days. The effect of THF on DRG neuron growth was estimated by the neurite outgrowth assay.

Western blotting assay

DRG neurons were collected from cultures and treated with a lysis buffer (50 mM Tris at pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and protease inhibitor cocktail, Millipore, USA). After checking with concentrations, proteins were separated by electrophoresis on 12% SDS-PAGE gel, and transferred to PVDF membranes. The membranes were blocked...
with 5% non-fat dry milk and 1% BSA for 2 h, followed by incubation with primary antibodies of rabbit polyclonal anti-TrkB (1:500, Novus biological, USA) and rabbit polyclonal anti-p-TrkB (1:200, Novus biological, USA) overnight at 4°C. On the second day, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000, Novus biological, USA) for 2 h at room temperature. The blots were then visualized by enhanced chemiluminescence (Pierce, USA) according to the manufacturer’s protocol.

**Bupivacaine treatment assay**

In DRG neuron culture, treatment of bupivacaine was conducted according to the method described before [12]. Briefly, 10 mM bupivacaine was added into culture for 2 h to induce substantial neuronal apoptosis [12]. The culture was washed with fresh medium 3 times (10 min / time), and maintained for another 24 h before further evaluation.

**Apoptosis assay**

DRG culture was quickly washed with PBS, and fixed by 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) for 30 min. The apoptosis of DRG neurons was examined by a terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) Apoptosis Kit (R&D Systems, USA) according to the manufacturer’s protocol. A mouse monoclonal NeuN antibody (Millipore, USA) was used to identify the DRG neurons. Fluorescent images were then examined under an upright fluorescent microscope (BX51, Olympus, Japan). Relative apoptosis was quantified as the percentage of TUNEL-positive cells among all NeuN-positive cells for each experimental condition.

**Statistical analysis**

All experiments were conducted in biological triplicates. All data are presented as mean ±SE. Statistical comparisons between means were examined by two-tailed unpaired Student’s t test on SPSS software (version 13.0, SPSS, USA). Statistically significant differences were determined as P<0.05.

**Results**

**THF promotes neurite growth and activates TrkB in DRG neurons**

We first investigated the possible pro-neuronal effect of THF on DRG neuron growth. The cultured mouse neonatal (P2–P3) DRG neurons were treated with various concentrations of THF (2, 5, 10, 20, 50, 100, 250, 500, 1000, 1500, and 2000 nM) for 2 days. Immunohistochemistry with a NF-2000 antibody was then performed. Images of green-fluorescence-positive DRG neuron neurites showed that THF significantly promoted neurite growth in a dose-dependent manner (Figure 1A). The relative lengths of DRG neuron neurites, corresponding to different THF concentrations, were compared in neurite outgrowth assay and fit with a Hill equation (Figure 1B). The EC50 concentration was determined to be 67.4 nM.

We then investigated the molecular pathway associated with pro-neuronal effect of THF on promoting DRG neurite outgrowth. We used Western blot assay to examine the protein level of TrkB receptor, as well as phosphorylated TrkB (p-TrkB). It showed that protein levels of TrkB were unchanged by treatment of different concentrations of THF, but levels of p-TrkB were significantly unregulated with the applications of higher concentrations of THF (Figure 1C). Thus, our data strongly suggests that THF promoted DRG neuron growth by acting as a TrkB agonist.

**THF rescues bupivacaine-induced apoptosis in DRG neurons**

One of the commonly used anesthetics, bupivacaine, was shown to induce neurotoxicity in DRG neurons in a concentration-dependent manner [12]. As we showed THF had pro-neuronal effect on DRG neuron growth, we wondered whether THF could also rescue bupivacaine-induced neurotoxicity in DRG neurons.

To test this hypothesis, we maintained DRG culture for 3 to 5 days, followed by pre-treatment of THF for 24 h. We then used a high concentration of bupivacaine – 10 mM (2 h) – to induce significant neuronal apoptosis in DRG neurons. Twenty-four hours after bupivacaine treatment, a TUNEL assay was conducted to evaluate the apoptosis among DRG neurons. A NeuN neuronal-antibody was used alongside TUNEL assay to precisely identify the neuronal population in the culture. The immunohistochemical results demonstrated that while there was no THF pre-treatment (Control), most of the DRG neurons (NeuN-positive) were apoptotic (TUNEL-positive) due to bupivacaine-induced neurotoxicity (Figure 2A, left column). However, when DRG neurons were pre-treated with 50 nM or 1 µM THF, bupivacaine-induced neuronal apoptosis was markedly reduced (Figure 2A, right two columns). Quantified measurement of TUNEL assay showed that the percentage of apoptotic DRG neurons was significantly reduced, from 85.3±4.5% with no THF pre-treatment (Control), to 45.2±7.3% with 50 nM THF pre-treatment, then to 19.6±5.7% with 1 µM THF (Figure 2B, * P<0.05).

Thus, our data suggest that THF pre-treatment was effective in reducing bupivacaine-induced neuronal apoptosis in DRG neurons.
THF rescues bupivacaine-induced neurite retraction in a TrkB-dependent manner in DRG neurons

We then evaluated the protective effect of THF pre-treatment on bupivacaine-induced neurite retraction in DRG neurons. At 24 h after bupivacaine treatment, immunohistochemical results with a neurite outgrowth assay demonstrated that there was no THF pre-treatment (Control), but bupivacaine introduced significant neurite retraction in DRG neurons (Figure 3A, left column). However, when DRG culture was treated with either 50 nM or 1 µM THF, neurite retraction was markedly rescued (Figure 3A, right two columns). Quantified measurement by neurite outgrowth assay indicated that DRG neurite lengths were increased by 379±33% with 50 nM THF pre-treatment, and by 1571±79% with 1 µM THF, compared to the neurite length under control condition (Figure 3B, * P<0.05).

Figure 1. 7, 8, 3’-Trihydroxyflavone has a pro-neuronal effect on DRG neuron neurite growth. DRG neurons were extracted from neonatal (postnatal 2~3 days) mouse spinal cord and cultured in vitro. Various concentrations of 7, 8, 3’-Trihydroxyflavone (THF) were added into culture for 48 h, followed by immunohistochemistry with a neurofilament 2000 (NF2000) antibody to identify the neurite outgrowth of DRG neurons. (A) Representative fluorescent images of NF2000 immunostaining are shown for DRG neurons treated with no THF (Control), 50 nM THF, and 1 µM THF. (B) The relative neurite lengths of DRG neurons treated by various concentrations of THF were characterized by a neurite outgrowth assay and fitted with a Hill equation. The EC₅₀ was determined to be 67.4 nM. (C) Western blot analysis was carried out to evaluate the protein expressions of TrkB and phosphorylated TrkB (p-TrkB).
Figure 2. 7, 8, 3’-Trihydroxyflavone protects DRG neuron from bupivacaine-induced apoptosis. (A) DRG neurons were pre-treated without 7, 8, 3’-Trihydroxyflavone (THF) (Control), or 50 nM and 1 µM THF for 24 h. They were then treated with 10 mM bupivacaine for 2 h to induce anesthetic-induced neurotoxicity. At 24 h after bupivacaine treatment, a TUNEL immunostaining (Red) was carried out to characterize the apoptosis in DRG neurons. A mouse monoclonal NeuN antibody (Blue) was used to identify the nuclei of DRG neurons. (B) Relative apoptosis, quantified as the percentage of TUNEL-positive cells against NeuN-positive cells, was compared between conditions of no THF pre-treatment (control) and of 50 nM or 1 µM THF pre-treatments (* P<0.05).
We also evaluated whether the protection of THF on DRG neurite retraction was correlated with the activation of the TrkB signaling pathway. The result of Western blot confirmed this hypothesis by showing that, in bupivacaine-injured DRG neurons, phosphorylated TrkB (p-TrkB) was increased by THF pre-treatment in a concentration-dependent manner, whereas TrkB protein levels were unaltered (Figure 3C).

Figure 3. 7, 8, 3'-Trihydroxyflavone protects DRG neurons from bupivacaine-induced neurite retraction. DRG neurons were pre-treated with no 7, 8, 3'-Trihydroxyflavone (THF) (Control), 50 nM or 1 µM THF for 24 h. They were then treated with 10 mM bupivacaine for 2 h to induce anesthetic-induced neurotoxicity. At 24 h after bupivacaine treatment, a neurite outgrowth assay was carried out to characterize the effect of THF on bupivacaine-induced neurite retraction. (A) Representative fluorescent images of NF2000 immunostaining are shown for DRG neurons pre-treated with no THF (Control), 50 nM THF, and 1 µM THF. (B) Relative neurite lengths were compared between DRG neurons pre-treated with no THF (Control), 50 nM THF, and 1 µM THF (* P<0.05). (C) Western blot analysis was carried out to evaluate the protein expressions of TrkB and phosphorylated TrkB (p-TrkB) in DRG neurons pre-treated with various concentrations of THF.
Thus, our data suggest that THF pre-treatment was also effective in protecting bupivacaine-induced neurite retraction in DRG neurons, very likely through activation of the TrkB signaling pathway.

**Discussion**

In the present study we discovered that, 7, 8, 3’-Trihydroxyflavone, a potent TrkB agonist small molecule, had a profound pro-neuronal effect on neuronal growth, as well as protecting against local anesthetic-induced neurotoxicity in spinal cord DRG neurons. We demonstrated that THF promoted neurite growth in neonatal DRG neuron culture in a concentration-dependent manner. The calculated EC\textsubscript{50} was calculated to be 67.4 nM, in line with other studies showing low THF EC\textsubscript{50} [2,6]. This finding was particularly encouraging as it suggests that THF can promote spinal cord neuronal development with great potency.

We also demonstrated that the THF-activated TrkB signaling pathway works by phosphorylating TrkB in DRG neurons, possibly acting as a TrkB agonist. Recently, a relative of THF – 7,8-dihydroxyflavone (DHF) – was shown to promote spinal cord motoneuron embryonic development, as well as inducing functional recovery in myotrophic lateral sclerosis [17,18]. Interestingly, DHF seemed to act through the AKT signaling pathway rather than directly on the BDNF/TrkB signaling pathway in motoneurons [17]. Therefore, it seems that complex signaling pathways may be differentially associated with THF (or DHF) in regulating spinal cord DRG neurons and motoneurons.

Studies have shown that epigenetic regulation of microRNA, as well as PTEN/AKT signaling pathways, are involved in local anesthetics-induced neurotoxicity in DRG neurons [12]. In addition, p38 mitogen-activated protein kinase (MAPK) was reported to be a critical component contributing to lidocaine-induced neurotoxicity in adult DRG neurons [9]. In the present study we demonstrated that THF had a protective effect on bupivacaine-induced neurotoxicity in DRG neurons by rescuing neuronal apoptosis and neurite retraction. Also, we showed that the TrkB signaling pathway was activated in accordance with THF treatment in bupivacaine-injured DRG neurons. Although our data suggest that THF likely acts as a survival factor through a TrkB-dependent manner in bupivacaine-injured DRG neurons, future experiments would help to elucidate the direct involvement of the BDNF/TrkB signaling pathway, as well as its association with other molecular pathways in anesthetics-induced neurotoxicity in DRG neurons.

**Conclusions**

Overall, in the present study we revealed new mechanism of 7, 8, 3’-Trihydroxyflavone as a pro-neuronal small molecule in promoting neuronal growth and protecting against bupivacaine-induced neurotoxicity in spinal cord DRG neurons. The associated signaling pathway of THF in DRG neurons is very likely through TrkB activation. These findings may help to identify novel molecular targets to be applied in future clinical settings to benefit patients with spinal cord disorders or injuries.

**Conflict of interest**

None.
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