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ORIGINAL PAPER

Hydroxyobtustyrene protects neuronal cells from chemical hypoxia-induced cell death

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Abstract
Hydroxyobtustyrene is a derivative of cinnamyl phenol isolated from *Dalbergia odorifera* T. Chen. The heartwood, known as 'JiangXiang', is a traditional Chinese medicine. Previous studies showed that hydroxyobtustyrene inhibited the biosynthesis of prostaglandins, which are mediators of neuronal cell death in ischemia. However, it currently remains unclear whether hydroxyobtustyrene protects neurons against ischemic stress. In the present study, we investigated the protective effects of hydroxyobtustyrene against sodium cyanide (NaCN)-induced chemical ischemia. Hippocampal neurons were cultured from the cerebral cortices of E18 Wistar rats. The effects of hydroxyobtustyrene on neuronal survival and trophic effects were estimated under lower and higher cell density conditions. After the treatment of 1 mM NaCN with or without hydroxyobtustyrene, an MTT assay, Hoechst staining, and immunocytochemistry for cyclooxygenase (COX)-2 were performed. Hydroxyobtustyrene increased cell viability under lower, but not normal density conditions. Neither the neurite number nor the length was influenced by hydroxyobtustyrene. NaCN significantly decreased viability and increased fragmentation in cell nuclei, and these changes were prevented by hydroxyobtustyrene. Moreover, NaCN increased the number of COX-2-positive neurons, and this was significantly prevented by the co-treatment with hydroxyobtustyrene. Therefore, hydroxyobtustyrene protected cultured hippocampal neurons against NaCN-induced chemical ischemia, which may be mediated by the inhibition of COX-2 production.

Keywords Ischemia · Cyclooxygenase · *Dalbergia odorifera* · Hippocampus · Apoptosis

Introduction

Cerebral ischemia is a devastating event that has become the third largest cause of mortality and the leading cause of disability worldwide [1], and results in serious impairments in central and peripheral functions. However, the treatment of cerebral ischemia remains highly unsatisfactory. A number of clinical trials on pharmacological neuroprotective strategies for cerebral ischemia have resulted in disappointment [2]. Therefore, the discovery of novel neuroprotective agents for the treatment of cerebral stroke is urgently needed.

Cyanide is a well-known neurotoxin and select neurons are particularly susceptible to its toxic effects [3]. A number of patients who have survived acute cyanide intoxication exhibit selective damage in the cortex, cerebellum, and basal ganglia [4–7]. Cyanide inhibits the respiratory chain, the utilization of oxygen, and mitochondrial functions [8, 9]. Since neurons are vulnerable to the loss of oxygen, cyanide produces neuronal death by apoptosis or necrosis [10, 11]. Cyanide treatment (chemical ischemia) is commonly used as an experimental model to study ischemia in vitro.

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin (PG) production from arachidonic acid [12]. There are two isoforms of COX (COX-1 and COX-2) in the brain [13]. COX-2 is expressed under ischemic conditions, and enhances the production of PGs and reactive oxygen

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species (ROS) as a side product [12]. Some of these products are toxic for neurons, and the inhibition of COX or its downstream signals has been reported to protect neurons in ischemia models including cyanide-induced chemical ischemia [14–18]. Therefore, COX and its downstream molecules are potential targets in the treatment of ischemia.

Dalbergia odorifera T. Chen is a medium-sized evergreen tree belonging to the Leguminosae family. Its heartwood, known as 'JiangXiang', is a traditional Chinese medicine and is widely used to dissipate blood stasis, stop bleeding, and relieve pain, ischemia, swelling, necrosis, and rheumatic pain [19]. Previous studies reported that hydroxyobtustyrene, a derivative of cinnamyl phenol extracted from *D. odorifera*, inhibited PG biosynthesis [20], and exerted antioxidant effects (unpublished data). However, it currently remains unknown whether hydroxyobtustyrene protects neurons against ischemic stress. In the present study, we investigated the protective effects of hydroxyobtustyrene against NaCN-induced chemical ischemia.

Materials and methods

Ethics

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science, and were conducted according to the guidelines of the National Institute of Health and the Japan Neuroscience Society.

Cell culture

Hippocampal neurons were cultured from the cerebral cortices of E18 Wistar rats (Japan SLC Inc., Shizuoka, Japan) under aseptic conditions as previously described [21]. Briefly, brain tissue was cut into small pieces and transferred to a centrifuge tube containing 0.25% trypsin (Sigma) and 0.1% DNase 1 (Boehringer Mannheim, Mannheim, Germany) dissolved in DMEM (Gibco/Invitrogen, Carlsbad, CA, USA). Tissue pieces were incubated at 37 °C for 15 min with gentle agitation every 5 min and then gently triturated using a flame-narrowed Pasteur pipette. The brain tissue suspension was centrifuged at 1000 rpm for 5 min, and the resultant pellet was resuspended in feeding medium, which contained DMEM with 5% fetal bovine serum, 5% horse serum, and 1% penicillin–streptomycin solution. The cell suspension was passed through sterilized lens paper before being diluted with feeding medium, and was then seeded at 1.25×10^5 or 2.5×10^5 cells/cm^2 on 24-well plastic plates precoated with polyethylenimine (Sigma). Cells were incubated at 37 °C in a 90 and 10% mixture of atmospheric air and CO_2, respectively. Two days after plating (day 2), cells were treated for 24 h with 10 μM cytosine arabinoside (Sigma) to remove actively dividing cells. The medium was changed the next day (day 3), and subsequently twice per week.

Synthesis of hydroxyobtustyrene

Hydroxyobtustyrene (Fig. 1) was synthesized as previously reported [20]. 3-Methoxycatechol (700 mg) was reacted with 3-phenyl-2-propen-1-ol (670 mg) in 90% formic acid at 60 °C for 30 min to afford hydroxyobtustyrene (120 mg). The yield of the product was approximately 9.4%. Hydroxyobtustyrene was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C. In our preliminary experiments using cultured septal neurons, hydroxyobtustyrene increased cell viability at a concentration of 100 or 300 nM, but not 10 nM. Therefore, we used 100 nM hydroxyobtustyrene in the present study.

Analysis of neurite length

Immunocytochemistry for MAP-2 was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). On day 3 and day 6, hydroxyobtustyrene (100 nM) was added to the culture medium. Cells were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) (pH 7.5 at 4 °C) for 20 min. Fixed cells were blocked with horse normal serum and incubated at 37 °C for 2 h with a primary antibody (mouse anti-MAP-2 antibody, Sigma), followed by an incubation with the secondary antibody at 37 °C for 30 min. Signals were detected with the ABC kit and DAB kit (Vector Laboratories). Stained neurons were imaged with a microscope (IX71; Olympus, Tokyo, Japan), and the longest neurite length in each neuron was measured using the National Institutes of Health (NIH) ImageJ software.

3-[4,5-Dimethlthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

On day 3 and day 6, NaCN (1 mM) with or without hydroxyobtustyrene (100 nM) was added to the culture medium for 24 h. After the 24-h treatment, neurons were washed with PBS and then continuously cultured in normal medium. On

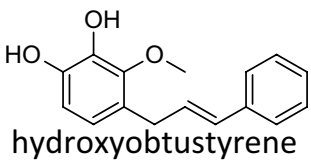

Fig. 1 Chemical structure of hydroxyobtustyrene

day 9, MTT (0.5% final concentration, Wako) was added to cultured neurons at 37 °C for 4 h. Formazan crystals were dissolved in acidic isopropanol (0.04 M HCl in isopropanol), and their absorbance was measured at 550 nm.

DNA staining

NaCN (1 mM) with or without hydroxyobtustyrene (100 nM) was added to the culture medium on day 4. After 24 h, DNA staining was performed with Hoechst 33342 (Wako) in hippocampal neurons [22]. The neurons were fixed with 4% paraformaldehyde for 20 min. After two washes with PBS, the neurons were incubated with Hoechst 33342 (5 µg/ml, Wako) for 15 min at room temperature, and then observed with a fluorescence microscope (IX71; Olympus).

COX-2 immunostaining

After the 4-h treatment with NaCN with or without hydroxyobtustyrene on day 4, the cells were fixed with 4% paraformaldehyde in 0.01 M PBS (pH 7.5) at 4 °C for 20 min. The fixed cells were blocked with blocking solution (5% non-fat milk, 1% bovine serum albumin in PBS), and were incubated at 37 °C for 2 h with a primary antibody (goat anti-COX-2 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an incubation with a secondary antibody (Alexa 488-conjugated anti-goat IgG, molecular probes) at 37 °C for 30 min. Stained neurons were imaged with a microscope (IX71; Olympus), and the stained area was measured using NIH ImageJ software.

Data analysis

Data are expressed as the mean ± SEM. The significance of differences was evaluated using a parametric one-way analysis of variance followed by Bonferroni's multiple comparison test. All statistical analyses were performed using Graphpad Prism (Graphpad Software Inc., San Diego, CA, USA). The criterion for significance was $p < 0.05$ in all statistical evaluations.

Results

Protective effects of hydroxyobtustyrene on cultured hippocampal neurons against cell death induced under low-density conditions

In order to evaluate the effects of hydroxyobtustyrene on cell viability and neurite length, MTT assays and MAP-2 staining were performed on hippocampal cultured neurons. Hydroxyobtustyrene increased cell viability under lower density conditions (1.25×10^5 cells/cm^2, Fig. 2a), but not under normal density conditions (2.5×10^5 cells/cm^2, Fig. 2b). Under lower density conditions, the number of neurites from soma (Fig. 2d) and the mean longest neurite length (Fig. 2e) were not influenced by the hydroxyobtustyrene treatment.

Protective effects of hydroxyobtustyrene on cultured hippocampal neurons against cell death induced by chemical ischemia

In order to evaluate the effects of hydroxyobtustyrene on cell viability in the case of chemical ischemia, MTT assays were performed in 1 mM NaCN-treated neurons under normal cell density conditions (Fig. 3). NaCN significantly decreased cell viability, which was prevented by hydroxyobtustyrene (Fig. 3).

In order to morphologically evaluate the protective effects of hydroxyobtustyrene, DNA was visualized using Hoechst staining (Fig. 4). After 24-h exposure to NaCN, the marked fragmentation of cell nuclei was observed. The co-treatment with hydroxyobtustyrene significantly decreased the fragmentation of nuclei. Hydroxyobtustyrene alone did not influence the morphology of nuclei or cell viability in the MTT assay.

Hydroxyobtustyrene prevented COX-2 expression induced by chemical ischemia

We investigated whether hydroxyobtustyrene decreased NaCN-induced COX-2 expression (Fig. 5). After 4-h exposure to NaCN, the size of the COX-2-positive area markedly increased. The co-treatment with hydroxyobtustyrene significantly prevented the increase in the size of the COX-2-positive area. Hydroxyobtustyrene alone did not influence the size of the COX-2-positive area.

Discussion

In the present study, we showed that hydroxyobtustyrene prevented lower density- and chemical ischemia-induced cell death in cultured hippocampal neurons. In addition, hydroxyobtustyrene prevented COX-2 expression induced by NaCN. Therefore, hydroxyobtustyrene may be a neuroprotective substance against ischemic damage, and its effects may be mediated by the inhibition of COX-2 expression.

The lower the cell density, the more difficult it is to maintain cultures of primary neurons [23]. Neuronal death under low density conditions is caused by a lack of paracrine trophic support from adjacent neurons and glia [24, 25]. Moreover, the lower the cell density, the greater the reduction in the intracellular ATP concentration needed to induce cell death following exposure to cellular stress

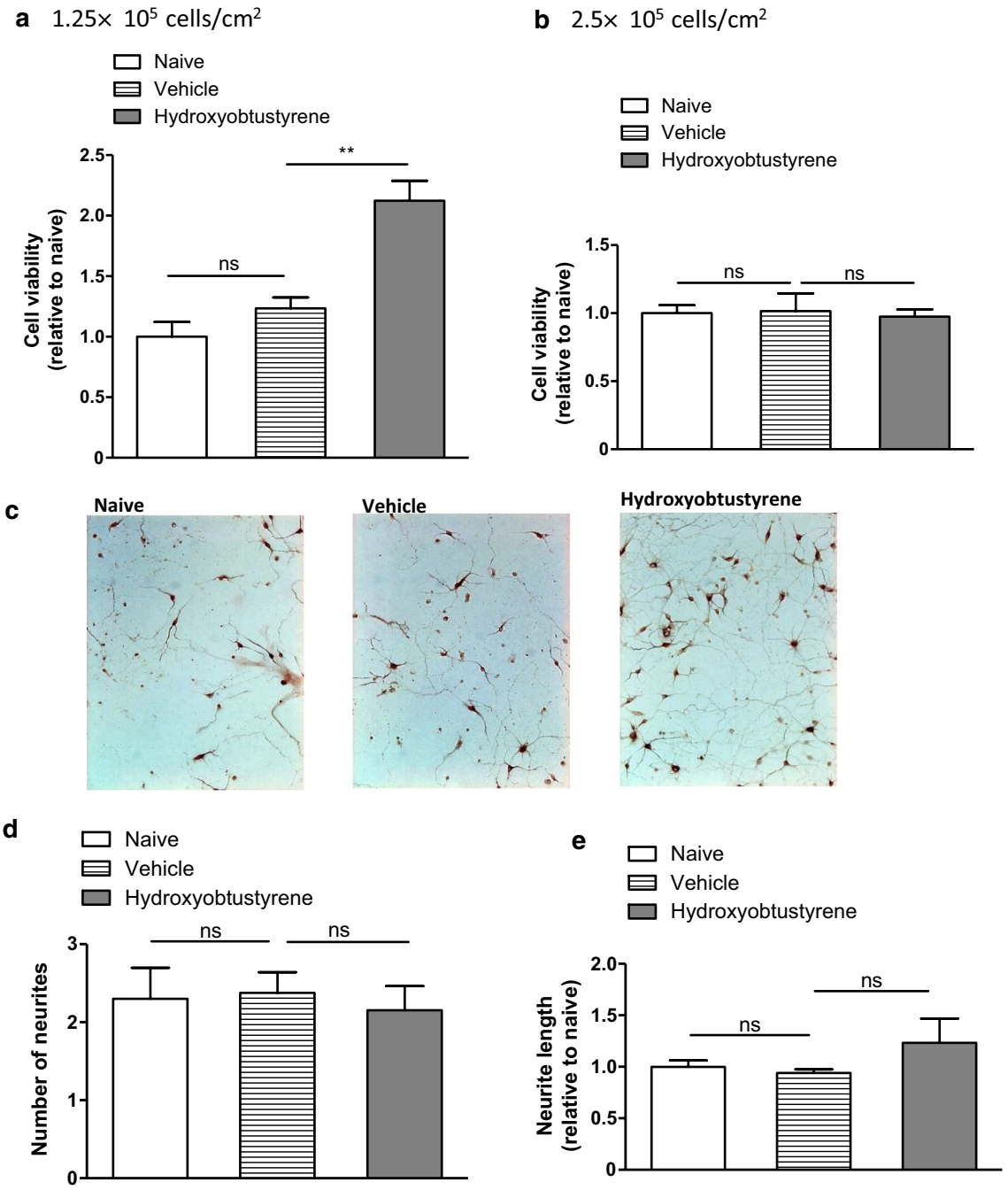

Fig. 2 Hydroxyobtustyrene increased neural cell viability in a cell density-dependent manner. Primary cultured rat neurons were exposed to each compound containing medium for 6 days. **a**, **b** Cell density-dependent neuronal viability assessed by the MTT assay ($n=4$). **c** Representative images of MAP-2-like immunoreactivities in primary cultured neurons (1.25×10^5 cells/cm^2). **d**, **e** Quantification of the number of neurites and neurite length per cell. Data are derived from three independent experiments. Neurites were detected by MAP2 immunostaining ($n=4$). Data were expressed as mean ± SEM. $^{**}p<0.01$, *ns* non-significant vs the vehicle group, one-way ANOVA followed by Bonferroni's test

[26]. Since hydroxyobtustyrene influenced cell viability under lower cell density conditions only, hydroxyobtustyrene may protect neurons from the lack of trophic support or intracellular ATP. However, hydroxyobtustyrene itself may have no neurotrophic effects, similar to brain-derived neurotrophic factor, because neurite length was not influenced by its treatment.

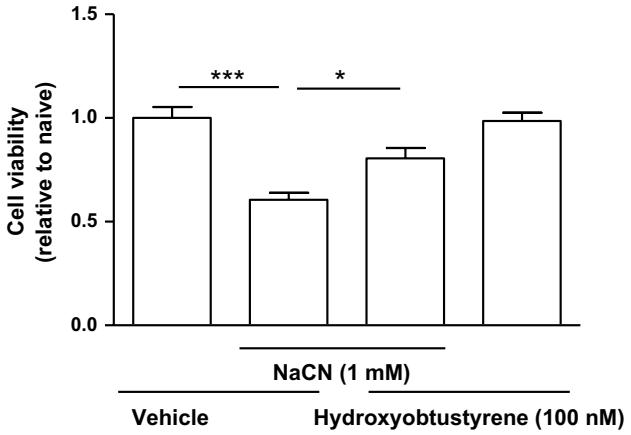

Fig. 3 Hydroxyobtustyrene rescued neuronal cell death induced by chemical ischemia. Primary cultured rat neurons were exposed to 1 mM NaCN-containing medium for 6 days with or without 100 nM hydroxyobtustyrene. Neuronal viability was assessed by the MTT assay ($n=30$). Data were expressed as mean ± SEM. ***$p<0.001$, *$p<0.05$ vs NaCN + the vehicle group, one-way ANOVA followed by Bonferroni's test

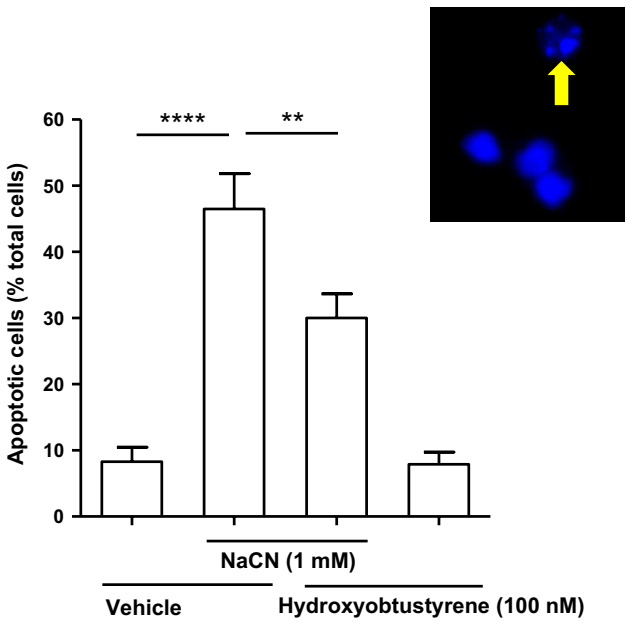

Fig. 4 Hydroxyobtustyrene prevented NaCN-induced apoptosis in primary cultured neurons. Primary cultured rat neurons (2.5×10^5 cells/cm^2) were exposed to 1 mM NaCN-containing medium for 24 h with or without 100 nM hydroxyobtustyrene. Apoptotic cells were detected by Hoechst 33342 staining. Data were expressed as mean ± SEM. ($n=5$) ****$p<0.0001$, **$p<0.01$ vs the vehicle group, one-way ANOVA followed by Bonferroni's test. Inset: a representative image of an apoptotic cell indicated by the yellow arrow. The apoptotic cell shows a shrunken and condensed morphology

Since ischemia is also reported to decrease the intracellular ATP, which leads the sequence of the pathophysiological events [33], we hypothesized that hydroxyobtustyrene may protect neurons from ischemic damage. We next investigated the effects of hydroxyobtustyrene on chemical ischemia induced by NaCN, and found neuroprotective effects in the MTT assay. The active mitochondrial dehydrogenases of living cells may cleave MTT to produce formazan, the amount of which directly correlates with the number of metabolically active cells [27]. However, the results of the MTT assay are influenced by endocytosis and the endocytic processing of its reagents. Therefore, we morphologically evaluated the protective effects of hydroxyobtustyrene by Hoechst staining, and found that hydroxyobtustyrene decreased the DNA fragmentation of neurons. These protective effects correlated with changes in the decrease in COX-2 positive neurons. In the brain, cyanide markedly increased COX-2 expression and PG production in neurons, glia and endothelial cells. In addition, the inhibition of COX or its downstream signals (e.g., prostaglandin E2 EP3 receptor) has been reported to protect neurons in ischemia models [14, 16–18, 28]. Although the detailed mechanisms have not yet been identified, EP3 receptor activation is thought to enhance neuronal cell death via the Rho kinase pathway [18]. Our results that hydroxyobtustyrene decreased COX-2 expression in the present study agree with a previous report demonstrating that hydroxyobtustyrene decreased PG production [20]. Therefore, hydroxyobtustyrene may inhibit downstream signals of COX-2 by inhibiting its expression, which may, in turn, protect neurons against ischemic damage. JiangXiang is used to treat pain and ischemia [19], in which hydroxyobtustyrene may partially play a role. It is necessary to demonstrate how hydroxyobtustyrene inhibits chemical ischemia-induced COX-2 expression.

Prolonged dosing with a selective COX-2 inhibitor is associated with an increased risk of cardiovascular events (i.e., myocardial infraction and stroke) [29, 30]. This increase in the risk of cardiovascular events is assumed to be induced by a prostacyclin I_2-thromboxane A_2 imbalance that leads to a prothrombotic state [31, 32]. Further studies are needed in order to investigate the influence of hydroxyobtustyrene on COX-1 expression and the product balance of these mediators.

In conclusion, we demonstrated that hydroxyobtustyrene exerted neuroprotective effects under lower cell density and ischemic conditions. Its derivatives may be new candidates for the treatment of ischemia and other inflammatory diseases.

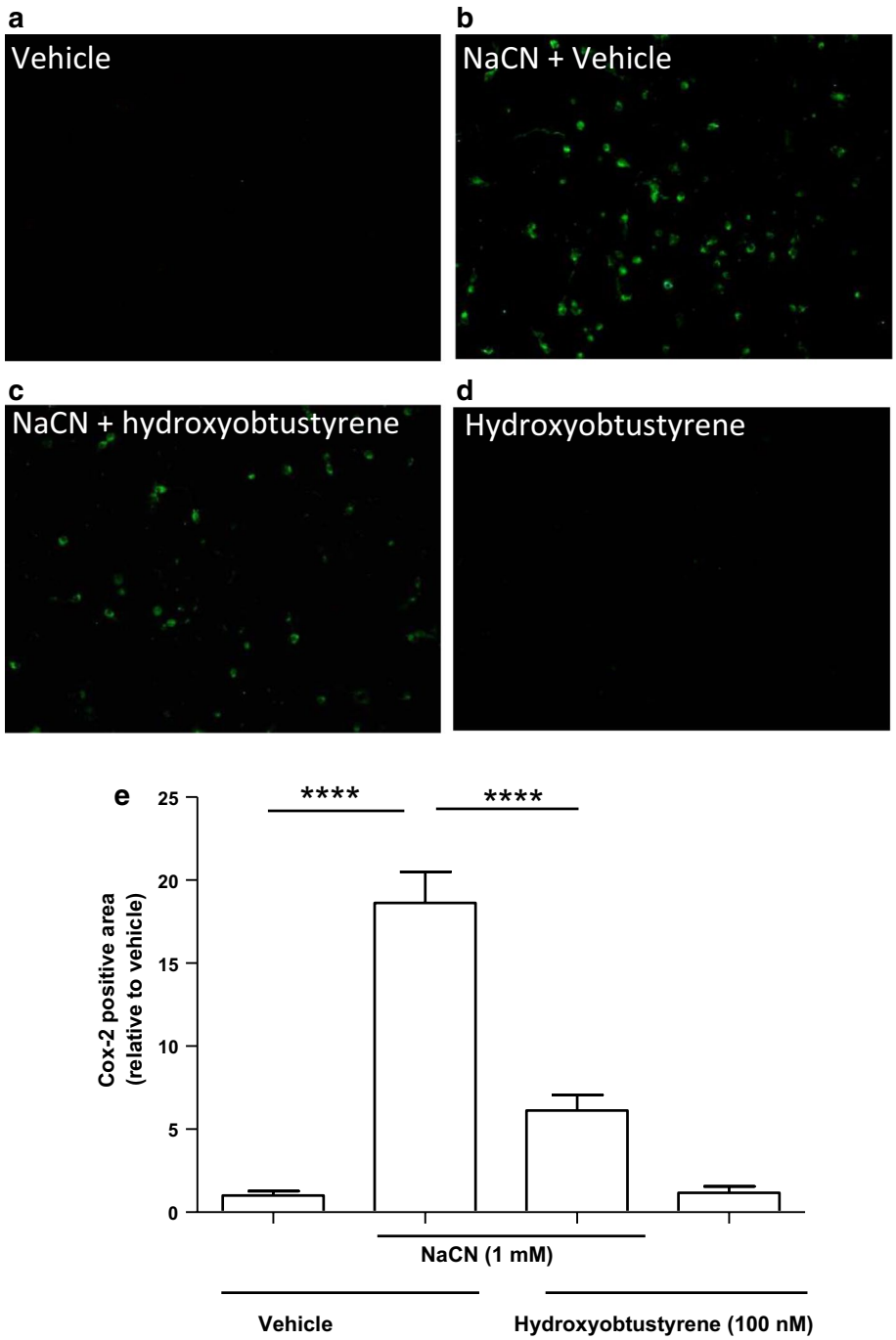

Fig. 5 Hydroxyobtustyrene decreased NaCN-induced COX-2-positive cells in primary cultured neurons. Primary cultured rat neurons (2.5×10^5 cells/cm^2) were exposed to 1 mM NaCN-containing medium for 4 h with or without 100 nM hydroxyobtustyrene. **a**–**d** Representative images of COX-2-positive cells. **e** Quantification of the number of COX-2-positive cells. Data were expressed as mean ± SEM. ($n = 5$) ****$p < 0.0001$ vs NaCN + the vehicle group, one-way ANOVA followed by Bonferroni's test

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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