The Major Cholesterol Metabolite Cholestane-3 β ,5 α ,6 β -Triol Functions as an Endogenous Neuroprotectant

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Overstimulation of NMDA-type glutamate receptors is believed to be responsible for neuronal death of the CNS in various disorders, including cerebral and spinal cord ischemia. However, the intrinsic and physiological mechanisms of modulation of these receptors are essentially unknown. Here we report that cholestane-3 β ,5 α ,6 β -triol (triol), a major metabolite of cholesterol, is an endogenous neuroprotectant and protects against neuronal injury both *in vitro* and *in vivo* via negative modulation of NMDA receptors. Treatment of cultured neurons with triol protects against glutamate-induced neurotoxicity, and administration of triol significantly decreases neuronal injury after spinal cord ischemia in rabbits and transient focal cerebral ischemia in rats. An inducible elevation of triol is associated with ischemic preconditioning and subsequent neuroprotection in the spinal cord of rabbits. This neuroprotection is effectively abolished by preadministration of a specific inhibitor of triol synthesis. Physiological concentrations of triol attenuate $[Ca^{2+}]_i$ induced by glutamate and decrease inward NMDA-mediated currents in cultured cortical neurons and HEK-293 cells transiently transfected with NR1/NR2B NMDA receptors. Saturable binding of [³H]triol to cerebellar granule neurons and displacement of [³H]MK-801 binding to NMDA receptors by triol suggest that direct blockade of NMDA receptors may underlie the neuroprotective properties. Our findings suggest that the naturally occurring oxysterol, the major cholesterol metabolite triol, functions as an endogenous neuroprotectant *in vivo*, which may provide novel insights into understanding and developing potential therapeutics for disorders in the CNS.

Key words: calcium; cerebral ischemia; cholestane-3 β , 5 α , 6 β -triol; NMDA; spinal cord ischemia; steroid

Introduction

Steroids and oxysterols are ubiquitous lipid components of eukaryotic plasma membranes and play vital roles in regulating the properties of cell membranes in mammalian cells (Mauch et al., 2001; Steck and Lange, 2010). Physiologically, the main metabolic pathways of cholesterol involve the biosynthesis of bile acids and steroid hormones, as well as the oxidation into oxysterols, the most abundant of which is cholestane-3 β ,5 α ,6 β -triol (triol; Javitt, 1994; Payne and Hales, 2004). According to the ancient *Chinese Pharmacopoeia*, gallstones from oxen, which mainly contain cholesterol and its metabolites, have been used medicinally

to treat various CNS disorders, including epilepsy (Gu, 1955). In traditional Indian medicine, animal gallstones have also been used as drugs for at least 4000 years (Thiyagarajan and Sunderrajan, 1992). More recent clinical epidemiological studies have shown that higher total plasma cholesterol levels correlate with lower mortality and better outcomes in patients who have had a stroke (Iso et al., 1989; Dyker et al., 1997; Olsen et al., 2007). A recent epidemical research with meta-analysis also revealed that low cholesterol is a risk of all-cause mortality in Japan (Kirihara et al., 2008), including stroke.

Ischemic stroke is a leading cause of death and disability worldwide, with few if any effective therapies (Al Hasan and Murgan, 2012). Numerous pathophysiological studies have implicated excessive stimulation of glutamate receptors, changes in the expression and function of these receptors, and/or aberrant glutamatergic neurotransmission in the mechanisms of ischemic neuronal injury and death (Budd, 1998; Mori et al., 2004; Giffard and Swanson, 2005). It is also generally accepted that disturbances in intracellular calcium ($[Ca^{2+}]_i$) homeostasis contribute to ischemia-induced neuronal injury and death (Cross et al., 2010). Overstimulation of NMDA receptors is believed to be responsible for neuronal death in the CNS induced by a variety of ischemic and traumatic insults. NMDA receptor channel-mediated Ca^{2+} overload, which plays a critical role in neuronal

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excitotoxicity, has also been implicated in neuronal death after spinal cord ischemia (Kocaeli et al., 2005; Villmann and Becker, 2007). NMDA receptors are modulated by many endogenous and exogenous substances. For example, the amino acid glycine and many neurosteroids, including 24(S)-hydroxycholesterol, facilitate the activation of NMDA receptors by glutamate (Fahey et al., 1995; Paul et al., 2013). To our knowledge, no naturally occurring NMDA receptor antagonists have so far been identified or shown to be active *in vivo* as neuroprotectants.

In the current study, we demonstrate that an endogenous oxysterol metabolite of cholesterol, triol, is able to protect neurons against glutamate-induced neurotoxicity *in vitro* and ischemia-induced neuronal injury *in vivo*. Triol is also a potent NMDA receptor negative modulator that may underlie its observed neuroprotective properties. Our findings suggest that the metabolism of cholesterol to triol may serve to reduce neuronal injury and death after ischemic CNS insults.

Materials and Methods

Reagents. Triol and 6-ketocholestanol (KC) were provided by Sigma-Aldrich. The purity of triol was >99.0%, determined under 1260 HPLC (Agilent) with an 2000ES ELSD detector (Alltech).

Animals. Sprague Dawley rats (240–270 g, males) and New Zealand white rabbits (2.0–2.5 kg, males) were used. All animals were provided by the Animal Research Laboratory at Sun Yat-sen University (Guangdong, China). The experimental protocol used was approved by the Ethics Committee for Animal Experimentation.

Human blood donors. Six healthy volunteers (20–30 years old, three males) provided their blood for triol determination in plasma.

Determination of triol. All plasma and tissue samples were stored at –80°C and determined as soon as possible. Determination of triol was performed as described previously (Sevanian et al., 1994; Razzazi-Fazeli et al., 2000) with modification. To avoid oxidation during sample preparation, the whole process was carefully kept away from light and oxygen, such as solvent degasification and saturation with nitrogen, solvent evaporation under a stream of nitrogen, and addition of butylated hydroxytoluene and EDTA before sample preparation.

A total of 1.0 g of plasma or 2.0 g of 50% tissue homogenate containing 0.9% NaCl was used for analysis, and 3 β ,6 β -dihydroxyl-cholestane-24-one was used for the internal standard (IS). First, plasma or tissue homogenate was vortex extracted by adding ethyl acetate. After ethyl acetate evaporated, the residue was dissolved in hexane-ethyl acetate (9:1, v/v) and loaded onto the SPE column (Sep-Pak Vac, 3 ml/500 mg; Waters). The column was then washed with 10 ml of hexane-diethyl ether (95:5, v/v), 15 ml of hexane-diethyl ether (90:10, v/v), and 10 ml of hexane-diethyl ether (80:20, v/v) in order. Finally, the column was eluted by 6 ml of acetone. Then the acetone was evaporated, and the residue was dissolved in 100 μ l of methanol. An aliquot of the aqueous layer (10 μ l) was injected into the liquid chromatography–mass spectrometry (LC–MS) system.

The LC–MS system consists of an Agilent 1200 HPLC with a 6120 quadrupole MS equipped with atmospheric pressure chemical ionization (APCI) source and operated in positive ion mode. Chromatographic separation was performed by a XBridge C18 column (250 $\times$ 4.6 mm inner diameter, 5 μ m; Waters) at 30°C using a mobile phase of water–methanol–acetonitrile (6:44:50, v/v/v) at a flow rate of 1.0 ml/min. Determination of triol was applied in the selective ion monitoring (SIM) mode with a mass-to-charge ratio (*m/z*) of 384.7–385.7 and 400.7–401.7 for IS. Linearity was assessed by triol IS peak area ratio versus the concentrations of triol. All calibration curves were corrected by subtracting the ratio of triol IS peak area in biological samples. Assay precisions and recoveries were determined and approved for all tested plasma and tissues.

Cell culture and *in vitro* experiments. Primary cultures of spinal cord neurons, cerebellar granule neurons, and cortical neurons of rats were prepared as described previously (Dichter, 1978; Hughes et al., 1993; Berman and Murray, 1996). For glutamate excitotoxicity assay, the neuronal cultures were washed three times with Mg²⁺-free Locke's buffer and then incubated with 200 μ M glutamate and 10 μ M glycine in Mg²⁺-

free Locke's buffer for 45 min. After that, the cultures were washed three times and maintained in the normal culture medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) for 24 h in the incubator. HEK-293 cells were transfected with the plasmids containing NR1/NR2B subunits using Lipofectamine 2000 as described previously (Qiu et al., 2005). The survival rate of cells exposed to various concentrations of triol was measured by MTT assay as described previously (Sun et al., 2013).

In vivo animal model of spinal cord ischemia. The animal model of spinal cord ischemia in rabbits was performed as described previously (Johnson et al., 1993; Celik et al., 2002). Rabbits were randomly assigned into four groups (*n* = 12) as follows: (1) the ischemia group (ISC), in which the infrarenal aorta was occluded for 20 min to produce spinal cord ischemic injury; (2) the triol group, in which an intravenous infusion of 8 mg/kg triol [10% of dimethylsulfoxide (DMSO) as vehicle] was administered 30 min before spinal cord ischemia; (3) the vehicle group, in which an intravenous infusion of the same volume 10% of DMSO was administered; and (4) the sham group, in which only the aorta was exposed. During the surgical operation, a 22-gauge catheter was inserted into the ear artery to measure proximal blood pressure, and another catheter was inserted into the left femoral artery to measure distal blood pressure. Blood pressure was monitored continuously by using a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Healthcare). Rectal temperature was maintained between 38°C and 39°C by a temperature-controlled heating pad during the experiments. Arterial blood was sampled at the preischemic state, 10 min after ischemia, and 10 min after reperfusion for the determination of the partial pressure of oxygen in arterial blood (PaO₂) and partial pressure of carbon dioxide in arterial blood (PaCO₂). Arterial blood gases were measured by means of the OMNI Modular System (AVL List).

Both the neurological and histopathological evaluations were assessed at 48 h after reperfusion. A modified Tarlov criteria (Johnson et al., 1993) was used: score 0, no voluntary hindlimb function; score 1, perceptible joint movement; score 2, active movement but no ability to stand; score 3, to be able to stand but not to walk; and score 4, complete normal hindlimb motor function. For the histopathological analysis, the lumbar spinal cord was removed, and coronal sections of the spinal cord (L5 segment) were cut at a thickness of 6 μ m and stained with hematoxylin and eosin (H&E).

Middle cerebral artery occlusion-induced brain injury in rats. Male Sprague Dawley rats were randomly divided into seven groups (*n* = 10): a saline group, a 20% hydroxypropyl- β -cyclodextrin (HP- β -CD) group (vehicle group), and five different therapeutic time windows of triol groups, respectively. The middle cerebral artery occlusion (MCAO) model and physiological monitoring were performed as described previously (Longa et al., 1989). Briefly, the right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were ligated. A 3-0 nylon monofilament suture (Ethicon nylon suture) with a blunt tip was inserted into the internal carotid artery to a point ~17–18 mm distal to the carotid bifurcation until a mild resistance was felt, thereby occluding the origins of the anterior cerebral and middle cerebral arteries. Reperfusion was accomplished by withdrawing the suture after 120 min of ischemia. During the surgical operation, the right femoral artery was cannulated for continuous monitoring of arterial blood pressure and for sampling blood to measure PaO₂ and PaCO₂, and rectal temperature was monitored and kept between 37.0°C and 37.5°C by a temperature-controlled heating pad. The five triol groups were administered intravenously with 12 mg/kg triol at 0.5, 1, 2, 3, and 4 h after the onset of MCAO. The saline and vehicle groups were administered intravenously with the same volume of saline and vehicle as the triol groups, respectively, at 2 h after onset of MCAO.

After 24 h of reperfusion, each animal was assigned a neurological score (Longa et al., 1989): 0, no neurologic deficit; 1, failure to extend the left forepaw fully; 2, circling to the left; 3, falling to left; 4, no spontaneous walking with a depressed level of consciousness; and 5, dead.

For observation of the persistent neuroprotection of triol, one group of animals administered triol intravenously at 1 h after onset of MCAO were evaluated at day 7 after reperfusion.

After the neurological assessment, the rats were anesthetized with 10% chloral hydrate and decapitated. Brains were quickly removed

and coronally sectioned with a tissue chopper at 2 mm interval, beginning 1 mm posterior to the anterior pole, incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min, and fixed in 4.0% paraformaldehyde solution. Each brain slice was photographed and quantified using an image analysis system (Adobe Photoshop CS5). The infarct volume of each rat was corrected for swelling of the ischemic hemisphere by applying a derived formula (Swanson et al., 1990): the corrected infarct volume percentage = $100 \times (\text{contralateral hemisphere volume} - \text{non-infarct ipsilateral hemisphere volume}) / \text{contralateral hemisphere volume}$.

Ischemic preconditioning. Rabbits were randomly assigned into six groups ($n = 10$) as follows: (1) the ISC group, in which the infrarenal aorta was occluded for 20 min to produce spinal cord ischemic injury; (2) the ischemic preconditioning (IPC) group, in which 6 min of infrarenal aorta occlusion below the threshold of the ischemic injury was performed alone without the second ischemia; (3) the IPC + ISC group, in which 6 min of infrarenal aorta occlusion was followed by 30 min of reperfusion before the 20 min of infrarenal aorta occlusion; (4) the KC + IPC + ISC group, in which KC, a specific inhibitor of triol biosynthesis, was preadministered intravenously with 8 mg/kg 1 h before the IPC, followed by 30 min of reperfusion before the 20 min of infrarenal aorta occlusion; (5) the vehicle + IPC + ISC group, in which the same volume of vehicle used to dissolve KC was preadministered 1 h before the IPC, followed by 30 min of reperfusion before the 20 min of infrarenal aorta occlusion; and (6) the sham group, in which the infrarenal aorta was exposed but not occluded.

Additionally, to exclude the possibility that KC could abolish the neuroprotection of IPC through exacerbating spinal cord ischemic injury by some other mechanism rather than inhibition of triol biosynthesis, 32 rabbits were randomly assigned into four groups ($n = 8$) as follows: (1 and 2) the IPC group and the ISC group were performed the same as described above; (3) the KC + IPC group, in which KC was preadministered intravenously with 8 mg/kg 1 h before the IPC; and (4) the KC + ISC group, in which KC was preadministered intravenously with 8 mg/kg 96 min before the ISC.

During the experiments, rectal temperature was maintained between 38°C and 39°C by a temperature-controlled heating pad, blood pressure was monitored continuously, and arterial blood was sampled at the pre-ischemic state, 10 min after ischemia, and 10 min after reperfusion for the determination of PaO_2 and PaCO_2 . Both the neurological and histopathological evaluations were assessed at 48 h after reperfusion.

Measurement of $[\text{Ca}^{2+}]_i$ and patch-clamp recording. Cells were prepared and loaded with the fluorescence dye Fluo-3/AM, as described previously (Földes-Papp et al., 2003). Briefly, the cells were grown in culture medium on six-well tissue plates at 37°C. Cells were incubated for 30 min in the dark at room temperature with 5 μM membrane-permeant Fluo-3/AM and 0.02% pluronic F-127 in HBSS (in mM: 125 NaCl, 0.62 KCl, 1.8 CaCl_2 , 20 HEPES, and 6 glucose, adjusted to pH 7.4 with NaOH). The fluorescence signal was monitored and recorded by a laser scanning confocal imaging system (Qiu et al., 2005) (TCS SP2; Leica). The data analysis was processed with Origin 6.1. The change in fluorescence intensity after drug treatment as indicated was normalized with the initial intensity.

Whole-cell patch-clamp recordings were made under voltage-clamp mode at room temperature (22–25°C). Recording was performed using a Multiclamp700A amplifier and Digidata 1322 series interface (AXON

Figure 1. Physiological distribution of triol. **A**, The fundamental metabolic pathway of cholesterol. Cholesterol is transformed into cholesterol-5,6-epoxide (5,6-epoxide) via oxidation and then is hydrated into triol by ChEH. **B**, The plasma concentrations of triol in rabbits and humans. All values represent mean $\pm$ SD of six individuals. **C**, LC-MS spectra of triol in the SIM mode with m/z of 384.7–385.7 in rat blood, brain, spinal cord (S.C.), liver, and kidney. IS indicates the internal standard (3 β ,6 β -dihydroxyl-cholestan-24-one) in the SIM with m/z of 400.7–401.7 in rat liver. The APCI source and the positive ion mode were involved in the measurement. **D**, The distribution of triol in rats. All values represent mean $\pm$ SD of six rats.

Instrument). Signals were filtered at 10 kHz. The pClamp 9.0 software system (Molecular Devices) was used for data recording and analysis. Patch pipettes were pulled from 1.2 mm outside diameter, 0.5 mm inner diameter glass pipettes (local product) using a P-97 horizontal puller (Sutter Instruments). The pipettes were filled with internal solution containing the following (in mM): 140 CsF, 1 CaCl_2 , 10 HEPES, 11 EGTA, 2 tetraethylammonium, and 4 MgCl_2 , pH 7.3, adjusted with CsOH (290–300 mOsm). The bath solution contained the following (in mM): 140 NaCl, 5.4 KCl, 20 HEPES, 10 glucose, and 2 CaCl_2 , pH 7.4, adjusted with NaOH and HCl (320–330 mOsm). Unless stated otherwise, inward NMDA-mediated currents (I_{NMDA}) were induced by local application of 200 μM NMDA with 3 μM glycine, 10 μM bicuculline, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 1 μM TTX, and 1 μM strychnine in bath solution. The maximal inward current value was measured as the peak current. The effect of triol was not tested until three stable NMDA-induced currents were achieved, after the formation of whole-cell configuration.

Binding assay. Saturation experiments were performed with cultured cerebellar granule neurons and HEK-293 cells transfected with or without NR1/NR2B receptors. Briefly, the cultured cerebellar granule neurons and HEK-293 cells were washed three times, and then different concentrations of [^3H]triol were added to the cultures to incubate for 20 min, after which the cultures were washed three times with ice-cold incubation buffer containing the following (in mM): 10 glucose, 124 NaCl, 5 KCl, 1.2 CaCl_2 , 1.2 MgCl_2 , and 15.6 Tris, pH 7.4, at an interval of 10 min. Finally, 300 μl of 10% SDS was added to lyse the neurons and

Figure 2. Neuroprotective effects of triol on glutamate-induced excitotoxicity in cultured neurons *in vitro*. **A**, Representative photomicrographs of the neuroprotective effects of triol against glutamate (Glu)-induced neurotoxicity in cultured spinal motoneurons. The control group (Control), neurons without any treatment; the glutamate group (Glu), neurons treated with 200 μ M glutamate; the MK801 + Glu and Triol + Glu groups, neurons pretreated with 10 μ M triol or 10 μ M MK-801 for 30 min before the addition of glutamate. Motoneurons were observed after being incubated with glutamate for 24 h. Scale bars, 30 μ m. **B–D**, Survival rates of spinal cord motoneurons (**B**), cortical neurons (**C**), and cerebellar granule neurons (**D**) with or without triol. The survival rates were calculated by comparison with the corresponding control cultures. All values represent mean $\pm$ SD. * p < 0.05 and ** p < 0.01 compared with the glutamate group, respectively.

HEK-293 cells, and the radioactivity of the lysate was measured by liquid scintillation 1 h later. Nonspecific binding was defined in the presence of 1 mM triol, which is 2000 times the highest concentration of [3 H]triol used in the saturation analysis.

For the competition analysis, 90 nM [3 H]MK-801, a standard radioactive ligand for NMDA receptor binding assays, was added to the cultured cerebellar granule neurons to incubate for 20 min. Then different concentrations of triol were added to cultures to incubate for 30 min to displace the [3 H]MK-801 binding. After that, the cultures were washed three times with ice-cold incubation buffer at an interval of 10 min. Finally, 300 μ l of 10% SDS was added to lyse the neurons, and the radioactivity of the lysate was measured by liquid scintillation 1 h later.

Statistic analysis. The scores of hindlimb motor function and the numbers of normal neurons were analyzed using a nonparametric method (Kruskal–Wallis test), followed by the Mann–Whitney U test. The other variables were expressed as mean $\pm$ SD and were analyzed using one-factor ANOVA, followed by a *post hoc* least significant difference test. The correlation between the neurological function Tarlov score and the number of normal neurons in the anterior spinal cord was analyzed using Spearman's rank correlation. p < 0.05 was considered to be statistically significant.

Results

Tissue distribution of triol

In preliminary experiments, we found that rabbits with hypercholesterolemia had reduced neuronal injury and neurological impairment after spinal cord ischemia compared with rabbits with normal cholesterol (our unpublished data). However, previous studies, including our own, failed to demonstrate neuroprotective effects of cholesterol itself (Olsen et al., 2007; our

unpublished observations). In many tissues, cholesterol is rapidly oxidized to cholesterol-5,6-epoxide, followed by hydration to triol by cholesterol 5,6-epoxide hydrolase (ChEH; Nashed et al., 1985; Fig. 1A). Triol has been demonstrated to be a major metabolite of cholesterol in humans (Sevanian et al., 1994; Schroepfer, 2000), and triol plasma levels are elevated in hypercholesterolemic rabbits and humans (Schroepfer, 2000; Leonarduzzi et al., 2005). We next determined triol concentrations in human and rabbit plasma by LC–MS and found plasma levels of 0.37 and 0.12 μ M in humans and rabbits, respectively (Fig. 1B). We then systematically measured the distribution of triol in various rat tissues. As shown in Figure 1D, triol levels in various rat tissues ranged from 1.07 to 0.32 μ M (spinal cord > liver > brain $\geq$ kidney > plasma).

Neuroprotective effects of triol *in vitro*

Exposure of cultured spinal cord and cortical and cerebellar granule neurons to high neurotoxic concentrations of glutamate results in a significant reduction in the number of surviving neurons (Fig. 2A). Pretreatment of neurons with triol (5–15 μ M) for 30 min before glutamate exposure increases the survival of spinal cord motoneurons, cortical neurons, and cerebellar granule neurons (Fig. 2B–D) in a concentration-dependent manner. In contrast, cholesterol itself had no neuroprotective effects at these same concentrations, and neither compound had an effect on neuronal survival in untreated neurons (data not shown).

Figure 4. The neuroprotective effects of triol treatment on brain injury induced by MCAO at 24 h and 7 d after reperfusion. **A**, Representative TTC-stained brain sections at 24 h after reperfusion. Infarction area (pale region) is significantly reduced by intravenous administration of triol (12 mg/kg) at five different therapeutic time windows, respectively, compared with that of the saline or vehicle group. **B**, Corresponding percentage of infarction volume to the contralateral hemisphere volume at 24 h after reperfusion. The therapeutic time window of triol was used at 0.5, 1, 2, 3, and 4 h after onset of MCAO. **C**, Corresponding neurological outcome of rats at 24 h after reperfusion. **D**, Representative TTC-stained brain sections at 7 d after reperfusion. Infarction area (pale region) is significantly reduced by intravenous administration of triol (12 mg/kg) at 1 h after onset of MCAO compared with that of the saline or vehicle group. **E**, Corresponding percentage of infarction volume to the contralateral hemisphere volume at 7 d after reperfusion. **F**, Corresponding neurological outcome of rats at 7 d after reperfusion. * $p < 0.01$ and ** $p < 0.01$ compared with the saline group.

of both the control and vehicle-treated groups ($p < 0.01$; Fig. 3D). In addition, physiologic variables, including blood pressure, blood gases, and rectal temperature, among these surgical operation groups had no significant differences.

Therapeutic time window of triol after transient cerebral ischemia in rats

We used the transient MCAO model in rats to test whether triol is an effective neuroprotectant and the therapeutic time window

Figure 5. Neuroprotective effects of spinal cord IPC on spinal cord injury and triol elevation in the spinal cord in rabbits. **A**, Neurological scores of animals at 48 h after reperfusion in the ISC, IPC + ISC, KC + IPC + ISC, Vehi + IPC + ISC, IPC, and Sham groups, which had an average neurological score of 0.8, 3.1, 1.5, 3.2, 4.0, and 4.0, respectively. ISC, Ischemic injury; IPC, ischemic preconditioning; Vehi, vehicle; KC, 6-ketocholestanol (a specific ChEH inhibitor of triol biosynthesis). **B**, Representative results of lumbar spinal cord sections (L5) stained with H&E. Slices were chosen from rabbits rated as having an average score. Note the darkly stained cytoplasm of dead neurons (arrowheads) compared with the fine granular cytoplasm and Nissl substance of the viable cells (arrows). Scale bars, 50 μ m. **C**, The number of normal neurons of the anterior spinal cord at 48 h after reperfusion in the ISC, IPC + ISC, KC + IPC + ISC, Vehi + IPC + ISC, IPC, and Sham groups. (Figure legend continues.)

after cerebral ischemia. As shown in Figure 4, transient MCAO in rats leads to a large infarct volume in the ipsilateral hemisphere at 24 h after reperfusion, which affects a significant portion of the frontal and parietal cortices, caudate–putamen, and diencephalon (Fig. 4A). The infarct volume induced by MCAO was $31.53 \pm 1.69\%$ for the saline group and $29.87 \pm 2.52\%$ for the vehicle-treated group (20% HP- β -CD), respectively (Fig. 4B). To explore a possible therapeutic time window of triol treatment, 12 mg/kg triol was administered to rats at 0.5, 1.0, 2.0, 3.0, and 4.0 h after onset of MCAO, respectively. Triol treatment markedly reduced the extent of the infarct volume compared with saline- or vehicle-treated animals (Fig. 4A,B) at the all time points. The infarct volumes were reduced to 7.17 ± 1.43 , 9.70 ± 1.20 , 10.86 ± 0.84 , 14.76 ± 1.25 , and $16.98 \pm 2.12\%$, respectively (vs $29.87 \pm 2.52\%$ in vehicle-treated rats). Triol treatment also improved functional neurological outcome as demonstrated by better neurologic scores in the triol-treated animals compared with those in the vehicle-treated ones ($p < 0.05$; Fig. 4C). Together, these morphological and behavioral data demonstrate that neuronal injury induced by MCAO can be markedly reduced by administration of triol even if the treatment takes place 4 h after ischemic injury. Furthermore, the neuroprotection of triol is persistent rather than temporal, simply delaying cell death, as evidenced by smaller infarct volumes and better neurologic scores at 7 d after reperfusion compared with those of vehicle-treated rats ($p < 0.01$; Fig. 4D–F). However, the physiological parameters, such as blood pressure, blood gases, and rectal temperature, among the surgical group animals had no significant changes.

Triol mediates neuroprotection after ischemic preconditioning

We next used a rabbit IPC model to explore the relationship between neuroprotection and the spinal cord concentrations of endogenous triol. The rabbits in the IPC + ISC group exhibit significantly higher neurological scores than those in the ISC group (average score of 3.1 vs 0.8, respectively, $p < 0.01$; Fig. 5A). As shown in Figure 5, B and C, the histopathological analysis further confirmed the marked neuroprotection observed after IPC. Coincidentally, the tissue levels of triol were significantly increased at 2 h after the IPC, were approximately fourfold greater than control levels at 4 h, and remained significantly elevated at 6 h after the IPC (Fig. 5G). Furthermore, after intravenous administration of triol (8 mg/kg), the concentration of triol in the spinal cord was $\sim 1.0 \mu\text{M}$ between 2 and 8 h (Fig. 5H), which is approximately fourfold of the basal concentration (0.25

μM) and equivalent to the highest endogenous triol concentration induced by IPC ($1 \mu\text{M}$, 4 h after IPC treatment).

Pretreatment of rabbits with a specific ChEH inhibitor of triol biosynthesis (Sevanian and McLeod, 1986), KC (8 mg/kg), 60 min before IPC abolished both the neuroprotection (Fig. 5A–C) and triol increase (Fig. 5G) induced by IPC. However, as shown in Figure 5D–F, pretreatment with KC did not exacerbate the relatively severe ischemic injury (20 min of spinal cord ischemia, ISC) as well as the mild ischemia (6 min of spinal cord ischemia, IPC). Together with the observed neuroprotective effects of administered triol in the rabbit spinal cord ischemia and rat MCAO models, these data suggest that the increase of endogenous triol after IPC and the neuroprotection induced by IPC are casually related to one another.

Triol blocks $[\text{Ca}^{2+}]_i$ increase induced by glutamate and I_{NMDA} in cortical neurons

Using laser scanning confocal calcium imaging (Földes-Papp et al., 2003), we found that triol reduced the $[\text{Ca}^{2+}]_i$ increase triggered by glutamate in a concentration-dependent manner (Fig. 6A,B). We further examined the effects of triol on NMDA currents in primary cultured cortical neurons. The formal recording was not started until three stable current traces were obtained. No significant NMDA current reduction was observed after recording for 7 min, in the absence of triol (Fig. 6C). In contrast, NMDA currents were time and concentration dependently decreased by $\sim 25\%$ and $\sim 60\%$ at 7 min in the presence of $1 \mu\text{M}$ (Fig. 6D,G) and $10 \mu\text{M}$ (Fig. 6E,G) triol, respectively. The “time-dependent” decrease of NMDA currents by triol is reminiscent of a use-dependent inhibition. To provide evidence supporting this hypothesis, we did not activate NMDA receptors between 2 and 7 min (5 min interval, no use) and found, under such a paradigm, that NMDA current was only decreased by 13.94% at 7 min in the presence of $10 \mu\text{M}$ triol (Fig. 6F,G), which was much less than that of the $\sim 60\%$ decrease observed in Figure 6, E and G. Obviously, this inhibition is use dependent: less NMDA receptor use produces less NMDA current inhibition.

Inhibitory effects of triol on $[\text{Ca}^{2+}]_i$ induced by NMDA in HEK-293 cells transfected with NMDA receptors

We next transfected HEK-293 cells with NR1/NR2B NMDA receptors and examined the effects of triol on $[\text{Ca}^{2+}]_i$ induced by NMDA. As shown in Figure 7A–C, application of $200 \mu\text{M}$ NMDA fails to increase $[\text{Ca}^{2+}]_i$ in wild-type HEK-293 cells (Fig. 7A), but a prominent elevation in $[\text{Ca}^{2+}]_i$ was observed in HEK-293 cells transfected with NR1/NR2B NMDA receptors (Fig. 7B). Triol significantly reduces $[\text{Ca}^{2+}]_i$ responses in a concentration-dependent manner (Fig. 7B,C). MK-801 ($10 \mu\text{M}$), used as a positive control, also reduces NMDA-induced $[\text{Ca}^{2+}]_i$ elevation. In contrast, cholesterol ($10 \mu\text{M}$) has no effect on NMDA-induced $[\text{Ca}^{2+}]_i$ increases in these same cells.

Binding of $[^3\text{H}]$ triol to primary cultured cerebellar granule neurons

To further investigate the action of triol on NMDA receptors, we studied the binding of $[^3\text{H}]$ triol to primary cultured cerebellar granule neurons. The results demonstrate specific and saturable binding of $[^3\text{H}]$ triol with an apparent K_d of $279.21 \pm 43.88 \text{ nM}$, whereas nonspecific binding was linear with increased $[^3\text{H}]$ triol concentrations (Fig. 7D,E). Finally, we studied the effects of triol on the specific binding of $[^3\text{H}]$ MK-801 to NMDA receptors expressed on cultured cerebellar granule neurons. We found that

←

(Figure legend continued.) * $p < 0.05$ and ** $p < 0.01$ compared with the ISC group, respectively. **D**, Neurological scores of animal at 48 h after reperfusion in the IPC, KC + IPC, ISC, and KC + ISC groups. The rabbits in the IPC group and KC + IPC group all displayed normal neurological function. Also, the neurological scores in the rabbits of the ISC group were not different from those of the KC + ISC group. ns, No significance. **E**, Representative results of lumbar spinal cord sections (L5) stained with H&E. Slices were chosen from rabbits rated as having an average score. Note the darkly stained cytoplasm of dead neurons (arrowheads) compared with the fine granular cytoplasm and Nissl substance of the viable cells (arrows). Scale bars, $50 \mu\text{m}$. **F**, The number of normal neurons of the anterior spinal cord at 48 h after reperfusion in the IPC, KC + IPC, ISC, and KC + ISC groups. The rabbits in the IPC group and KC + IPC group all displayed almost intact neurons. Also, the number of normal neurons in the rabbits of ISC group was not different from those of the KC + ISC group. **G**, Triol levels in the rabbit spinal cord were significantly elevated 2 h after IPC, peaked at 4 h, and kept significantly high at 6 h. However, when KC, an inhibitor of ChEH, was preadministered intravenously 1 h before IPC, triol in the spinal cord 4 h after IPC was at a relatively low level and showed no difference from the sham group. ** $p < 0.01$ and *** $p < 0.001$ compared with the sham group. **H**, Triol levels in spinal cord after intravenous administration.

Figure 6. Inhibitory effect of triol on the $[Ca^{2+}]_i$ increase induced by glutamate (Glu) and I_{NMDA} in cortical neurons. **A**, Representative results of live fluorescence intensity from cortical neurons exposed to 200 μM glutamate, pretreated with triol (numbers in parentheses indicate concentration, in micromolar). **B**, Summary of the inhibitory effects of triol on $[Ca^{2+}]_i$ induced by glutamate. The data were expressed as $(F - F_0)/F_0$ (F and F_0 indicate the fluorescence value after and before exposure to glutamate). Data represent mean $\pm$ SD ($n = 20$). * $p < 0.05$ and ** $p < 0.01$ compared with glutamate alone. **C**, **D**, Green and red current traces showing time-dependent changes of the NMDA-gated currents in the absence or presence of 1 μM triol, respectively. NMDA (200 μM) was used to induce NMDA currents. **E**, **F**, Black and blue traces showing the inhibition of NMDA currents by 10 μM triol without or with 5 min interval, respectively. **G**, Summary data showing the concentration-dependent and use-dependent inhibition of NMDA-gated currents by triol (two-way ANOVA, followed by Bonferroni's *post hoc* tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

triol readily displaced [3H]MK-801 binding (Fig. 7F) in a concentration-dependent manner. Furthermore, we found that 100 μM NMDA could significantly increase the maximum binding of [3H]triol in HEK-293 cells transfected with NR1/NR2B receptors, but [3H]triol did not bind to the wild-type HEK-293 cells (Fig. 7G).

Discussion

In the present study, we found that triol, a major metabolite of cholesterol that is present in many tissues, including the brain and spinal cord, has neuroprotective properties when studied *in vitro* and *in vivo*. Although triol was once considered a toxic metabolite of cholesterol, after prolonged exposure of endothe-

Figure 7. Inhibitory effects of triol on calcium influx induced by NMDA in HEK-293 cells transfected with NMDA receptor and binding assays of [³H]triol and [³H]MK-801. **A**, NMDA (200 μM) did not induce an increase in [Ca²⁺]_i in wild-type HEK-293 cells. **B**, Representative recordings of NMDA-evoked Ca²⁺ influx after addition of triol, cholesterol (Chol, 10 μM), and MK-801 (10 μM) for 15 min in HEK-293 cells transiently transfected with NR1/NR2B receptors (numbers in parentheses are concentration, in micromolar). **C**, Summary data indicate that triol dose dependently prevents NMDA-induced increase of [Ca²⁺]_i in HEK-293 cells transfected with NR1/NR2B receptors. All values are presented as mean ± SD (*n* = 20). **p* < 0.05 and ***p* < 0.01 compared with the NMDA group. **D**, Saturation binding assay of [³H]triol to primary cultured rat cerebellar granule neurons. The experiment repeated three times show similar results. **E**, Scatchard analysis for [³H]triol binding to primary cultured rat cerebellar granule neurons. **F**, Competitive effect of triol on [³H]MK-801 binding to NMDA receptors. **G**, Saturation binding assay of [³H]triol to HEK-293 cells transfected with NMDA receptors. All values are presented as mean ± SD of three separate experiments.

lial cells to high concentrations (Matthias et al., 1987), we found no evidence that lower physiologic concentrations were toxic to cultured neurons. In fact, we observed neuroprotective properties of triol in primary cultured neurons, including cortical neurons, cerebellar granular neurons, and neurons from the anterior spinal cord, when these cells were subsequently exposed to excitotoxic concentrations of glutamate. We also tested triol in a spinal cord ischemia model in rabbits in which ischemic injury induced by infrarenal aortic occlusion results in a highly reproducible deficit in neurological function attributable to loss of spinal cord motoneurons. Parenteral administration of triol before spinal cord ischemic injury markedly inhibited both the behavioral deficits and loss of motoneurons observed after ischemic injury. Although there exists a rather limited “time window” for neuroprotective agents after ischemic injury, we observed that triol can significantly protect against ischemic injury when administered as long as 4 h after MCAO. We also found that the neuroprotection of triol is persistent rather than simply delaying cell death, as evidenced by a potent neuroprotective effect of triol on MACO animals 7 d after reperfusion.

Does triol play a physiological role as an endogenous neuroprotectant? The presence in the CNS of abundant sources of cholesterol and the presence of its auto-oxidation product cholesterol-5,6-epoxide, as well as a biosynthetic pathway for additional hydration via CheH (Nashed et al., 1985) and relatively high tissue levels of triol present in a variety of species support a potential physiological or pathophysiological role.

Ischemic preconditioning has been used to elucidate endogenous neuroprotective mechanisms (Dirnagl and Meisel, 2008). In this experimental paradigm, subtoxic ischemic insult results in subsequent neuroprotection to an otherwise toxic ischemic injury. A number of cellular mechanisms have been proposed to mediate the neuroprotective state after the processes of preconditioning (Clarkson, 2007; Zhao et al., 2013; Stetler et al., 2014). However, the actual functional effector molecules are still essentially unknown. In the present study, markedly improved neurological and histopathological outcomes were observed after ischemic preconditioning in the rabbit spinal cord as reported by a number of laboratories (Yu et al., 2006; Huang et al., 2007; Yang et al., 2008; Fang et al., 2013). Using this experimental neuroprotective paradigm, we observed increased triol concentrations in the spinal cord up to 4 h after preconditioning. This inducible increase of spinal cord triol levels was blocked by pretreatment with a specific inhibitor of triol synthesis, which also blocked the neuroprotection induced by preconditioning. These observations, combined with our *in vitro* and *in vivo* data, strongly suggest that triol may function as an endogenous neuroprotectant, possibly induced by subtoxic ischemic injury or preconditioning.

However, we found a dose difference of triol between physiological concentrations and the ones used in experiments *in vitro* and *in vivo*. From the point of view of the current methodology, the dose differences may be associated with the determination of endogenous triol. The brain or spinal cord concentrations of triol tested in our study may only represent the average ones of triol in a tissue homogenate, which may not truly reflect the concentrations in a specific region or a specific subcellular localization, such as the nerve terminal, in which triol may accumulate to reach a higher concentration to execute its neuroprotection.

Another potential factor responsible for the dose differences may also be related to the high concentrations of excitotoxins used. In the *in vitro* study, 200 μM glutamate was used to ensure the induction of a massive neuronal death. Under this condition, a relatively high dose of triol should be needed to reach its notable neuroprotective effect. A similar phenomenon has been reported

in studies of other endogenous neuroprotective agents in the literature. For example, taurine, a well established endogenous modulator, exerts its neuroprotection in rat primary cultured neurons in a high concentration of 25 mM for antagonizing 250 μM glutamate (Chen et al., 2001; Wu et al., 2005), whereas its measured endogenous concentrations are $\sim 2\text{--}9$ mM in rat brain (Porcellati, 1963; Palkovits et al., 1986). The main consideration for using relatively high doses (8 mg/kg) of triol in *in vivo* experiments is aimed to obtain a maximal neuroprotection effect under the severe pathological condition of spinal cord ischemia, because only one single dose was administered to the animals. In addition, triol binds with plasma proteins as high as $>90\%$ of the amount administered, which may limit the concentrations of the drug in the action sites. Conversely, it is notable that the concentrations of triol in spinal cord after exogenous administration with 8 mg/kg of triol at multiple time points are comparable with those induced by IPC. In both situations, triol displays definite neuroprotections. However, we believe that the exact reasons for the dose differences deserve additional investigation.

Intracellular calcium overload is generally accepted to be the main mechanism underlying neuronal toxicity (Szydlowska and Tymianski, 2010; Piccolini et al., 2013). Elevation of $[\text{Ca}^{2+}]_i$ may activate Ca^{2+} -dependent proteases, break down critical proteins or enzymes, and lead to neuronal death (Xiong et al., 2004). Molecular neuroscience has further revealed that glutamate/NMDA receptors consist of ligand-gated ion channels, by which they control Ca^{2+} influx (Evans et al., 2012; Guo et al., 2013). Glutamate-induced $[\text{Ca}^{2+}]_i$ elevation is mainly through NMDA receptor channels (Murphy and Miller, 1988). In the current study, we demonstrate that triol markedly attenuates glutamate-induced $[\text{Ca}^{2+}]_i$ increases in a concentration-dependent manner, which may account for the observed neuroprotective effects of triol in both *in vitro* and *in vivo* models that implicate NMDA receptor-mediated $[\text{Ca}^{2+}]_i$ overload. Furthermore, we observe a significant use-dependent inhibition of NMDA current by triol starting from 1 μM , which is a concentration equivalent to the basal triol level (1.07 μM) in spinal cord in rats and an elevated triol level (0.96 μM) at 4 h after IPC in rabbits.

The findings from radioactive triol binding assays demonstrate a specific binding site for triol in neurons. Furthermore, we provide evidence of direct interplay between triol and NMDA receptors with radioactive MK-801. MK-801 is widely used as a use-dependent or open channel blocker for NMDA receptors. When an NMDA receptor channel is in an open status (in use), MK-801 enters into and binds at the inside of the channels, resulting in an inhibition (Huettner and Bean, 1988; Rodríguez-Moreno et al., 2011). The displacement of MK-801 binding by triol suggests modulation on the open channel site in which MK-801 binds, implying that triol may function as an endogenous NMDA receptor open channel blocker, which is consistent with the patch-clamp findings that triol use dependently inhibit NMDA current. This observation was further supported by the fact that the $[\text{H}^3]$ triol maximum binding with NMDA receptors is significantly stimulated in the presence of 100 μM NMDA. The recombinant NMDA receptor expressed in HEK-293 cells is a more stringent molecular model and may provide a useful transgenic cellular model to clarify the effects of any potential modulators on NMDA receptors. It was reported that the functional NMDA receptor is a heteromeric protein and requires coassembly of the NR1 (which binds glycine) subunit with at least one NR2 (which binds glutamate) subunit (Paoletti and Neyton, 2007). Lines of evidence show the predominant extrasynaptic NMDA subunit NR2B acts as a central mediator for stroke injury

(Lai et al., 2011) and may represent a promising therapeutic target for stroke intervention. In our experiment, we investigate the effects of triol on NMDA receptors with a focus on NR1/NR2B subtype. These data further confirm the role of triol as a negative modulator of NMDA receptors. Additionally, it would be interesting to investigate the selectivity of triol on other NMDA subunits in the future.

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