Isosteviol Sodium Protects Against Permanent Cerebral Ischemia Injury in Mice via Inhibition of NF-κB–Mediated Inflammatory and Apoptotic Responses

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Background: Isosteviol sodium (STVNa) has been reported to have neuroprotective effects against ischemia/reperfusion (I/R) injury in rats. Furthermore, recanalization treatments, including thrombolytic therapy, have several limitations. Excessive inflammation and apoptosis contribute to the pathogenesis of ischemic brain damage. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is critical to these processes and is associated with cerebral ischemia. Therefore, we studied the potential therapeutic effects and mechanisms of STVNa on permanent cerebral ischemia in mice. *Methods:* Permanent middle cerebral artery occlusion (pMCAO) was established via the suture method, followed by intravenous STVNa (7.5, 15, 30, 45, and 60 mg/kg). Neurobehavioral deficits, infarct volume, and histology were examined 24 hours after cerebral ischemia. In addition, the messenger RNA (mRNA) expression of NF-κB–related genes was detected using real-time quantitative polymerase chain reaction (qPCR). *Results:* STVNa (30 mg/kg) had significant neuroprotective effects 24 hours after pMCAO, including the reduction of the infarct volume and the improvement of the neurological severity score. Immunohistochemistry demonstrated that STVNa significantly increased the number of restored neurons and decreased the number of astrocytes. qPCR also demonstrated that the mRNA expression of inhibitor of nuclear factor kappa-B kinase-α, inhibitor of nuclear factor kappa-B kinase-β, NF-κB, inhibitor of NF-κB-α, tumor necrosis factor-α, interleukin-1 beta, Bcl2-associated X protein, and caspase-3 were significantly downregulated, whereas B-cell CLL/lymphoma 2 mRNA was upregulated with STVNa treatment compared with vehicle. *Conclusions:* These findings demonstrate a neuroprotective role of STVNa during cerebral ischemia, which may result from interactions with the NF-κB signaling pathway and the associated inflammatory and apoptotic responses. **Key Words:** Permanent cerebral ischemia—STVNa—NF-κB—inflammatory—apoptosis.

Introduction

Stroke is 1 of 3 leading causes of human death, with ischemic stroke accounting for 70% of all strokes.[1] Recanalization and neuroprotection are currently the primary treatment options for acute ischemic stroke. However, due to the narrow therapeutic time window and hemorrhagic complications associated with tissue plasminogen activator treatment,[2] this option is only used in 3%-5% of U.S. patients with ischemic stroke.[3] The goal of neuroprotective strategies is to protect the penumbra brain tissue and attenuate clinical sequelae of stroke. Although a large number of neuroprotective interventions are efficacious in small animal preclinical studies, clinical

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studies in humans have not yet demonstrated similar efficacy.[4]

Cerebral ischemia is a pathological condition characterized by an initial restriction of blood to the brain. Processes involved in cerebral ischemic damage include inflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress,[5] with particularly harmful effects of inflammation and apoptosis.[6-8] For example, numerous studies have demonstrated that the acute inflammatory response is initiated by neutrophil adherence to the ischemic endothel.[9] Other studies have demonstrated that neuronal apoptosis is commonly induced during cerebral ischemia.[10] These pathological changes have been observed during ischemic pathology in experimental animal models of stroke, as well as in humans.[6] Numerous studies investigating the underlying mechanisms of inflammation and apoptosis demonstrate a critical role of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the induction of these processes in conditions including arthritis,[11] asthma,[12] myocardial infarction,[13] and cerebral ischemia.[14-16]

Stevioside, a natural sweetener isolated from the herb *Stevia rebaudiana*, has been used for decades in many countries as a food sweetener. Previous studies have shown that stevioside can reduce blood sugar and blood pressure,[17,18] which may be beneficial during cerebral ischemia. Isosteviol is obtained by the acid hydrolysis of stevioside, a process which maintains the desirable pharmacological activities of stevioside. A previous study reported that pretreatment with isosteviol inhibits NF-κB expression, thus reducing inflammation and apoptosis in a rat model of stroke.[19] However, isosteviol is a beyerene diterpene and is therefore not suitable as an aqueous injection to treat ischemic diseases because it has poor solubility and low bioavailability.[20] An injectable formulation of isosteviol sodium salt, STVNa, possesses much greater solubility and bioavailability; thus, STVNa has the potential to be widely applied as an emergency treatment.[21]

We previously demonstrated that STVNa effectively reduces the infarct volume and improves neurological deficits in ischemia/reperfusion (I/R) injury in rats.[22] However, the detailed mechanisms underlying these protective effects of STVNa have not been fully elucidated. The present study further examined the neuroprotective effects of STVNa against permanent cerebral ischemia in mice, including investigating the possible mechanisms of these effects.

Materials and Methods

Animals

Male C57BL/6 mice (7-8 weeks of age), weighing 20-25 g, were purchased from the Animal Research Centre of Guangzhou University of Chinese Medicine (Guangzhou, China). The mice were housed in a temperature-controlled environment (25°C ± 2°C), with a 12-hour light/dark cycle and free access to food and water. All efforts were made to minimize animal suffering and reduce the number of animals used. The experimental studies were approved by the Institutional Animal Care and Use Committee of Guangdong Pharmaceutical University.

Permanent Middle Cerebral Artery Occlusion

The permanent middle cerebral artery occlusion (pMCAO) model was based on the method of Longa et al,[23] via the neck arteries, as previously described. Male C57BL/6 mice were anesthetized with 4% isoflurane (Ruiwode, Shenzhen, China). Under an operating microscope, the right common carotid artery (CCA), and external and internal carotid arteries were surgically exposed through a neck incision. A 6-0 silicon-coated nylon filament was introduced into the CCA and advanced into the internal carotid artery until the tip reached the origin of the middle cerebral artery (MCA), which was detected by a mild increase in resistance. Sham-operated mice received the same experimental surgery without a filament being inserted into the MCA. The occlusion was maintained for 24 hours. The neck incision was then closed, and the mice were allowed to recover.

Laser Doppler flowmetry (PeriFlux System 5000; Perimed AB, Stockholm, Sweden) was used to measure cerebral blood flow (CBF) both before and after MCA occlusion. A drop in ipsilateral CBF below 30% of the baseline was considered sufficient for the induction of focal cerebral ischemia. Body temperatures were monitored using a rectal thermometer and maintained within normal limits (36.5°C-37.5°C) using a heating pad and a heating lamp. Heart rate, arterial oxygen saturation, and breathing rate were measured using a MouseOx Plus device (Starr Life Sciences, Inc., Oakmont, PA, USA). After surgery, mice were allowed free access to food and water.

STVNa Administration

STVNa was obtained from the Chemical Development Laboratories of Key Biological Pharmaceutical Company (Dongguan, China). Edaravone was purchased from Simcere Pharmaceutical Co., Ltd. (Nanjing, China). Drugs were administered by intravenous injections immediately following the induction of ischemia via pMCAO.

In the dose-response experiment, mice were assigned to 1 of the following 8 groups: sham (n = 8), vehicle (n = 8), STVNa (7.5, 15, 30, 45, and 60 mg/kg, n = 8 per group), and edaravone (3 mg/kg, n = 8). Dose volumes were maintained at approximately .12 mL. A single 30-mg/kg dose of STVNa was used for the histopathology and quantitative polymerase chain reaction (qPCR) experiments. In addition, the STVNa therapeutic window was investigated at a single dose of 30 mg/kg. In the pMCAO experiments, STVNa was administered at 0, 2, 4, 6 or 8 hours after pMCAO ($n_s = 8$); vehicle was administered at the time of pMCAO (n = 8). Mice were randomly assigned to groups prior to pMCAO.