Bark Constituents from Mushroom-detoxified *Rhus verniciflua* Suppress Kainic Acid-induced Neuronal Cell Death in Mouse Hippocampus

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**INTRODUCTION**

*Rhus verniciflua* Stokes, a deciduous tree in the anacardiaceae family and indigenous in East Asia, has been used as a traditional herbal medicine to treat diabetes mellitus and stomach diseases. In the present study, RVH-1 used as a traditional herbal medicine to treat diabetes mellitus and stomach diseases. In the present study, RVH-1 was isolated from *Rhus verniciflua* family and indigenous in East Asia, has been isolated from *Rhus verniciflua* var. *Stokes*, a deciduous tree in the anacardiaceae family and indigenous in East Asia, has been isolated from *Rhus verniciflua* (stigma-4-en-3-one) andRVH-2 (stigma-4-en-3,6-dione). In the present study, RVH-1 and RVH-2 were isolated from mushroom-detoxified and used as a traditional herbal medicine to treat diabetes mellitus and stomach diseases.

Urushinol, a plant allergen, has significantly restricted the medical application of *Rhus verniciflua*, although it has been reported to possess a wide variety of biological activities such as anti-inflammatory, antioxidant, and anti-cancer actions. To reduce the urushinol content while maintaining the beneficial biological activities, mushroom-mediated fermentation of *Rhus verniciflua* was carried out and this method resulted in significantly attenuated allergenicity [1]. In the present study, to examine the neuroprotective properties of mushroom-fermented stem bark from *Rhus verniciflua*, two constituents were isolated from mushroom-fermented bark and their neuroprotective properties were examined in a mouse model of kainic acid (KA)-induced excitotoxicity. KA resulted in significant apoptotic neuronal cell death in the CA3 region of mouse hippocampus. However, seven daily administrations of RVH-1 or RVH-2 prior to KA injection significantly attenuated KA-induced pyramidal neuronal cell death in the CA3 region. Furthermore, pretreatment with RVH-1 and RVH-2 also suppressed KA-induced microglial activation in the mouse hippocampus. The present study demonstrates that RVH-1 and RVH-2 isolated from *Rhus verniciflua* and detoxified using mushroom species possess neuroprotective properties against KA-induced excitotoxicity. This leads to the possibility that detoxified *Rhus verniciflua* can be a valuable asset in herbal medicine.

**Key Words:** Kainic acid, Neuroprotection, Stigma-4-en-3-one, Stigma-4-en-3,6-dione

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**Abbreviations:** RVH-1, stigma-4-en-3-one (RVH-1); RVH-2, stigma-4-en-3,6-dione; KA, kainic acid; iv, intracerebroventricular; PDGF, platelet-derived growth factor.

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sults in the death of pyramidal neurons and concurrent reactive gliosis in hippocampus [13,14].

In the present study, we examined the neuroprotective effects of RVH-1 and RVH-2, isolated from mushroom detoxified stem bark of *Rhus verniciflua*, on hippocampal CA3 neuronal damage induced by intracerebroventricular injection of KA.

**METHODS**

**Animals and reagents**

Male ICR mice weighing 23–25 g were obtained from Folas-International, Ltd. (Seoul, Korea). All of the animal experiments were conducted in accordance with the animal care guidelines of the National Institutes of Health (NIH) and Korean Academy of Medical Sciences (KAMS). Mice were housed five per cage in a room maintained at 22±2°C with an alternating 12/12 hr light/dark cycle. Food and water were available *ad libitum*. KA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). KA was prepared as a stock solution at a concentration of 1 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS, pH 7.4), and aliquots were stored at −20°C until use.

**Compounds isolated from detoxified Rhus verniciflua**

RVH-1 (stigma-4-en-3-one) and RVH-2 (stigma-4-en-3,6-dione) were isolated and identified from mushroom detoxified stem bark of *Rhus verniciflua* [1]. These compounds (Fig. 1) were orally administered using an oral zonde daily with an alternating 12/12 hr light/dark cycle. Food and water were available *ad libitum*. KA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). KA was prepared as a stock solution at a concentration of 1 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS, pH 7.4), and aliquots were stored at −20°C until use.

**Intracerebroventricular (icv) injection of KA**

The administration of KA (0.1 µg/5 µl) was performed according to the procedure established by Laursen and Belknap [15]. Briefly, KA was injected at the bregma point with a 50-µl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. Mice were sacrificed 24 hr after KA administration.

**Immunohistochemistry**

All mice were sacrificed 24 hr after KA injection. Mice were transcardially perfused and post-fixed for 4 hr in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose, sectioned coronally (40 µm) on a freezing microtome, and collected in cryoprotectant for storage at −20°C. For cresyl violet staining, the sections were mounted in gelatin-coated slides and allowed to air-dry overnight. The mounted sections were submerged in 0.1% cresyl violet solution for 5 min. The sections were rinsed in 70% ethanol and dehydrated in a graded series of ethanol, immersed in xylene, mounted in Permout (Fisher Scientific, NJ, USA) and cover slipped. The free-floating immunohistochemistry of the brain of the brain sections was processed as previously described [16]. Sections that were collected from cryoprotectant were washed with PBS, pre-incubated for 30 min in 0.1 M PBS with 1% bovine serum albumin and 0.2% Triton X-100, and incubated for 2 days at 4°C with the following primary antibodies: OX-6 (1:1,000; BD Pharmingen) or NeuN (1:1,000; Santa Cruz, Santa Cruz, CA, USA). After 2 days of incubation with primary antibody, the antigens were detected with 3,3-diaminobenzidine tetrahydrochloride using Vectastain Elite ABC kits (Vector, Burlingame, CA, USA). Sections were mounted, air-dried, dehydrated through graded ethanol, cleared in histoclear, and cover slipped using Permout.

**In situ labeling of DNA fragmentation**

The analysis of cells exhibiting DNA fragmentation, which are suggestive of apoptosis as previously described [17], was performed according to the manufacturer’s instructions using terminal deoxynucleotidyl transferase with peroxide-12-UTP nick-end labeling (TUNEL) (Roche Molecular Biochemicals, Indianapolis, IN, USA). Brain sections for TUNEL staining were prepared according to the same procedure as above for immunohistochemistry. The percentage of TUNEL-positive cells was assessed by analysis of digitized images from 5 or more microscopic fields of TUNEL-stained cells from TIFF files (Adobe Photoshop).

**Statistical analysis**

Data were analyzed using Mann-Whitney’s U test and SPSS software 12K (SPSS statistics, Chicago, IL, USA) for independent samples to compare mice treated with KA and saline, and mice treated with KA and RVH-1 or RVH-2. A value of *p*<0.05 was accepted as statistically significant. Results are expressed as mean±SEM values.

**RESULTS**

**Both RVH-1 and RVH-2 attenuated KA-induced neuronal cell death**

Intracerebroventricular injection of KA resulted in extensive neuronal cell death in the CA3 region of mouse hippocampus, whereas neuronal cell death was negligible in vehicle-, RVH-1-, or RVH-2-treated mice (Fig. 2). Although both RVH-1 and RVH-2 attenuated KA-induced neuronal cell death, they did not completely prevent neuronal cell death at a RVH concentration of 10 mg/kg. RVH-2 appeared to be more protective than RVH-1. However, dose-dependent inhibition of KA-induced neuronal cell death was not observed (data not shown).
Both RVH-1 and RVH-2 attenuated KA-induced apoptotic neuronal cell death

Representative and quantitative analysis of apoptotic neuronal cell death was determined using TUNEL staining (Fig. 3). In accordance with the data from cresyl violet staining (Fig. 2), TUNEL-positive neurons in the CA3 region were observed abundantly in KA-injected mice. Mice pretreated with RVH-1 or RVH-2 showed attenuated neuronal cell damage (Fig. 3). There was no apparent difference between RVH-1 and RVH-2. The number of TUNEL-positive neurons was negligible in vehicle-treated mice (oral administration of saline as pretreatment and icv injection of PBS instead of KA), RVH-1-only-treated mice (oral administration of RVH-1 or RVH-2 for 7 days daily as pretreatment and icv injection of PBS instead of KA), and RVH-2-only-treated mice.

Protection of pyramidal neurons by RVH-1 and RVH-2 was confirmed with neuronal immunostaining in the CA3 region of hippocampus

To further elucidate the neuroprotective effects of RVH-1 and RVH-2 on pyramidal neurons in the hippocampus, immunostaining of NeuN, a neuronal marker, was carried out. In accordance with the data from cresyl violet and TUNEL staining, both RVH-1 and RVH-2 attenuated loss of pyramidal neurons in the CA3 region of mouse hippocampus (Fig. 4). Neither RVH-1 nor RVH-2 had a noticeable effect on the viability of pyramidal neurons.

Both RVH-1 and RVH-2 attenuated KA-induced microglial activation

Given the previous report that KA-induced neuronal cell death accompanies microglial activation in the hippocampus [13], the suppressive effect of RVH-1 and RVH-2 on KA-induced microglial activation was examined using immunostaining with OX-6, a microglial activation marker. The intracerebroventricular administration of KA resulted in a considerable amount of microglial activation in the hippocampus, especially in the CA3 region where neuronal cell death was most obvious (Fig. 5). However, seven daily administrations of RVH-1 and RVH-2 prior to KA markedly attenuated KA-induced microglial activation (Fig. 5).

DISCUSSION

In the present study, we demonstrated that RVH-1 and RVH-2 isolated from mushroom-detoxified stem bark of *Rhus verniciflua*, exhibited neuroprotective activity against KA-induced excitotoxic damage in the CA3 region of mouse hippocampus. RVH-1 and RVH-2 significantly attenuated KA-induced pyramidal neuronal cell death and also sup-
Fig. 4. Representative images of neuronal protection by RVH-1 and RVH-2 against KA-induced neuronal cell death in the CA3 region of hippocampus. In order to further elucidate the neuroprotective properties of RVH-1 and RVH-2, NeuN immunoreactivity, which specifically stains pyramidal neurons in the hippocampus, was examined. Pretreatment of RVH-1 or RVH-2 attenuated KA-induced death of pyramidal neurons in the CA3 region of hippocampus. RVH-2 showed more neuronal protection against KA-induced excitotoxicity compared to RVH-1. RVH-1 and RVH-2 showed no noticeable change in neuronal viability. More than three mice were used for each group. Scale bar: 100 μm.

Fig. 5. Suppressive effects of RVH-1 and RVH-2 on KA-induced microglial activation. Given the previous report that KA-mediated neuronal death accompanies microglial activation, expression of OX-6, a microglial activation marker, was examined with immunohistochemical staining at 24 hr after KA or vehicle treatments. KA resulted in increased microglial activation (KA). However, KA-induced microglial activation was attenuated with RVH-1 or RVH-2 pretreatment. RVH-2 appears to be more suppressive than RVH-1. RVH-1 or RVH-2 showed negligible effects on microglial activation. Representative images were obtained from three independent experiments. More than three mice were used for each group. Scale bar: 100 μm.

RVH-1 and RVH-2, which belong to the stigmasterol family, were previously isolated from various natural plants [6]. Although RVH-1 and RVH-2 have been reported to inhibit platelet-derived growth factor (PDGF)-driven proliferation of hepatic stellate cells [18], biological activity of these compounds has not been extensively studied. In the meantime, it has been reported that stigmasterol exhibits anti-inflammatory effects in a primary culture model of osteoarthritic chondrocytes by inhibiting the production of pro-inflammatory mediators [4]. The authors suggested that the anti-inflammatory properties of stigmasterol might be mediated through the inhibition of the NF-kB pathway. Given the previous report that activation of the NF-kB pathway might contribute to neuronal death in a KA-induced excitotoxicity model [19], it is plausible that RVH-1 and RVH-2 might exert a protective effect through inhibition of NF-kB signaling in a KA-induced excitotoxicity model.

Excitotoxicity may be involved in a variety of pathological conditions such as stroke, traumatic brain injury, and spinal cord injury [20-22]. Furthermore, accumulating evidence suggests that excitotoxicity contributes to the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [23,24]. In accordance with a previous study [13], we found that icv administration of KA resulted in the death of neurons mainly in the CA3 region of mouse hippocampus and daily pretreatment of RVH-1 and RVH-2 for 7 day significantly reduced KA-induced death of pyramidal neurons in mouse hippocampus. A single pretreatment with these compounds showed no noticeable inhibition of KA-induced neuronal death (data not shown), suggesting that a single treatment with these compounds is not sufficient to be effective. Although these compounds exerted protective effects against KA-induced excitotoxicity, it is not certain whether the neuroprotection by RVH-1 and RVH-2 is through the suppression of the NF-kB pathway. Therefore, further studies are necessary to determine the mechanism by which these compounds protect KA-induced neuronal cell death.

KA-induced excitotoxicity also accompanies activation of microglia in the hippocampus [13]. Microglia, the major immunocompetent cells in the CNS, are believed to play an important role in inflammatory processes in the brain [25]. Concomitant microglial activation during excitotoxicity might contribute to secondary damage by producing neurotoxic factors such as proinflammatory cytokines and nitric oxide [25,26]. It has been reported that a pathogenic
amount of nitric oxide contributes to neuronal damage in many disease conditions including neurodegenerative diseases [27-29]. In the present study, RVH-1 and RVH-2 significantly attenuated KA-induced microglial activation in the mouse hippocampus. However, it is not clear whether these compounds directly suppressed the microglia or whether the microglial suppression is secondary to the neuroprotection of these compounds.

In conclusion, the present study demonstrates that RVH-1 and RVH-2 possess neuroprotective properties in an animal model of KA-induced excitotoxicity. These compounds might be potentially valuable in the treatment of brain pathologies associated with excitotoxic neuronal damage such as epilepsy, stroke, and traumatic brain injury. However, further studies are necessary to clearly understand the mechanisms by which these compounds protect neurons against excitotoxicity. The present study suggests the possibility that detoxification of *Rhus verniciflua* utilizing mushrooms could be a valuable approach to yield neuroprotective compounds from *Rhus verniciflua*.

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REFERENCES