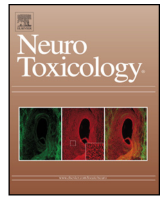
NeuroToxicology 50 (2015) 157–169

Contents lists available at ScienceDirect

NeuroToxicology



Hesperidin inhibits glutamate release and exerts neuroprotection against excitotoxicity induced by kainic acid in the hippocampus of rats

Chia Ying Chang [a,e], Tzu Yu Lin [a,f], Cheng Wei Lu [a,f], Shu Kuei Huang [a], Ying Chou Wang [d], Shang Shing Peter Chou [e], Su Jane Wang [b,c,*]

[a] Department of Anesthesiology, Far-Eastern Memorial Hospital, Pan-Chiao District, New Taipei City 22060, Taiwan
[b] Graduate Institute of Basic Medicine, Fu Jen Catholic University, No. 510, Zhongzheng Rd., Xinzhuang Dist., New Taipei City 24205, Taiwan
[c] School of Medicine, Fu Jen Catholic University, No. 510, Zhongzheng Rd., Xinzhuang Dist., New Taipei City 24205, Taiwan
[d] Department of Clinical Psychology, Fu Jen Catholic University, No. 510, Zhongzheng Rd., Xinzhuang Dist., New Taipei City 24205, Taiwan
[e] Department of Chemistry, Fu Jen Catholic University, No. 510, Zhongzheng Rd., Xinzhuang Dist., New Taipei City 24205, Taiwan
[f] Department of Mechanical Engineering, Yuan Ze University, Taoyuan 320, Taiwan

ARTICLE INFO

Article history:
Received 15 May 2015
Received in revised form 28 August 2015
Accepted 31 August 2015
Available online 2 September 2015

Keywords:
Hesperidin
Glutamate release
sEPSCs
Kainic acid
Neuroprotection
Hippocampus

ABSTRACT

The citrus flavonoid hesperidin exerts neuroprotective effects and could cross the blood–brain barrier. Given the involvement of glutamate neurotoxicity in the pathogenesis of neurodegenerative disorders, this study was conducted to evaluate the potential role of hesperidin in glutamate release and glutamate neurotoxicity in the hippocampus of rats. In rat hippocampal nerve terminals (synaptosomes), hesperidin inhibited the release of glutamate and elevation of cytosolic free Ca^{2+} concentration evoked by 4-aminopyridine (4-AP), but did not alter 4-AP-mediated depolarization. The inhibitory effect of hesperidin on evoked glutamate release was prevented by chelating the extracellular Ca^{2+} ions and blocking the activity of Cav2.2 (N-type) and Cav2.1 (P/Q-type) channels or protein kinase C. In hippocampal slice preparations, whole-cell patch clamp experiments showed that hesperidin reduced the frequency of spontaneous excitatory postsynaptic currents without affecting their amplitude, indicating the involvement of a presynaptic mechanism. In addition, intraperitoneal (i.p.) injection of kainic acid (KA, 15 mg/kg) elevated the extracellular glutamate levels and caused considerable neuronal loss in the hippocampal CA3 area. These KA-induced alterations were attenuated by pretreatment with hesperidin (10 or 50 mg/kg, i.p.) before administering the KA. These results demonstrate that hesperidin inhibits evoked glutamate release in vitro and attenuates in vivo KA-induced neuronal death in the hippocampus. Our findings indicate that hesperidin may be a promising candidate for preventing or treating glutamate excitotoxicity related brain disorders such as neurodegenerative diseases.
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1. Introduction

Population aging has increased the prevalence of neurodegenerative diseases worldwide. Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) are characterized by progressive behavioral changes including cognitive and motor abnormalities (Hague et al., 2005). These disorders are a major cause of disability and death in elderly people, and current treatment strategies are ineffective. Although the underlying mechanism in neurodegenerative diseases is unclear, excitotoxicity is considered one of the constitutive components of the disease pathogenesis (Dong et al., 2009; Lau and Tymianski, 2010; Mehta et al., 2013). Excitotoxicity is caused by excessive release of amino acids such as glutamate, a crucial excitatory neurotransmitter in the central nervous system (CNS) of mammals. Under normal conditions, glutamate acts through ionotropic and metabotropic glutamate receptors to regulate many neurological processes including cognition, learning, and memory (Meldrum, 2000; Riedel et al., 2003). However, excessive glutamate release causes the overstimulation of glutamate receptors, which leads to an overload of intracellular Ca, generation of free radicals, and subsequent

* Corresponding author at: <org>Graduate Institute of Basic Medicine, Fu Jen Catholic University</org>, <addL>No. 510, Zhongzheng Rd., Xinzhuang Dist.</addL>, New Taipei City <postalC>24205</postalC>, Taiwan.
Tel.: +886 2 29053465; fax: +886 2 29052096.
E-mail address: med0003@mail.fju.edu.tw (S.J. Wang).

http://dx.doi.org/10.1016/j.neuro.2015.08.014
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neuronal cell death (Sattler and Tymianski, 2001; Lau and Tymianski, 2010). Therefore, attenuating glutamate-induced excitotoxicity might be an effective therapeutic approach for neurodegenerative diseases. If one compound inhibits glutamate release, it would be a suitable candidate in the treatment of neurodegenerative diseases.

An increasing number of studies have suggested that dietary intake or supplementation of natural products can prevent or delay the onset of neurodegenerative disorders (Hwang et al., 2012; Solanki et al., 2015). Hesperidin, a flavonoid that is abundant in citrus fruits such as lemons and oranges, possesses antioxidant, anticarcinogenic, antihypertensive, antiviral, and antiinflammatory properties (Garg et al., 2001; Crozier et al., 2009; Parhiz et al., 2015). In addition, hesperidin can cross the blood–brain barrier (Youdim et al., 2003) and protect the neurons against various types of insult associated with neurodegenerative diseases including AD, PD, and HD (Menze et al., 2012; Antunes et al., 2014; Roohbakhsh et al., 2014; Wang et al., 2014). Therefore, hesperidin is a critical candidate for neurodegenerative intervention; however, the underlying mechanisms in this neuroprotective role remain unclear.

Based on the aforementioned evidence, this study investigated the following: (1) whether hesperidin inhibits glutamate release in rat hippocampal nerve terminals (synaptosomes); (2) which signaling pathway is involved in hesperidin-inhibited glutamate release; (3) whether hesperidin inhibits spontaneous excitatory postsynaptic currents in rat hippocampal slices; and (4) whether hesperidin exerts neuroprotective effects in an in vivo rat model of neurodegeneration induced by the intraperitoneal (i.p.) injection of kainic acid (KA), a glutamate analog. The findings of this study may clarify the neuroprotective role and underlying mechanism of hesperidin in inhibiting glutamate release.

2. Materials and methods

2.1. Chemicals

4-Aminopyridine (4-AP), bafilomycin A1, ω-conotoxin MVIIC (ω-CgTX MVIIC), dantrolene, 7-chloro-5-(2-chloropheny)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), dantrolene, D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), bicuculline, bisindolylmaleimide I (GF109203X), Go6976, rottlerin, and 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were purchased from Tocris Cookson (Bristol, UK). 3′,3′,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], and fura-2-acetoxymethyl ester (Fura-2-AM) were purchased from Invitrogen (Carlsbad, CA, USA). Hesperidin (>98%), ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), tetrodotoxin (TTX), kainic acid (KA) and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hesperidin, $DiSC_3(5)$, and Fura-2 were dissolved in 0.1% dimethylsulfoxide (DMSO).

2.2. Animals

Adult male Sprague-Dawley rats (150–200 g) and 8–23-day-old male Sprague-Dawley rats were purchased from BioLASCO (Taiwan Co., Ltd, Taipei, Taiwan). Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50–70%) under a regular 12 h light–dark cycle (light-off at 7 pm). Food and water were freely available. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the Far-Eastern Memorial Hospital (FEMH – 2014 – D – 001), in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use a minimum number of animals necessary to produce reliable results.

2.3. Preparation of synaptosomes

Synaptosomes, from the hippocampus of adult rats ($n = 22$), were prepared as described previously (Dunkley et al., 1986; Nicholls and Sihra, 1986). In brief, the rats were sacrificed by decapitation and the hippocampus was rapidly dissected, homogenized in a medium containing 320 mM sucrose, pH 7.4. The final synaptosomal pellet was resuspended in Hepes-buffered medium (HBM) containing (mM): 140 NaCl, 5 KCl, 5 $NaHCO_3$, 1 $MgCl_2{\cdot}6H_2O$, 1.2 Na_2HPO_4, 10 glucose, 10 HEPES (pH 7.4). Protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in a final wash to obtain synaptosomal pellets with 0.5 mg protein. Synaptosomal pellets were stored on ice and used within 4–6 h.

2.4. Glutamate release assay

Glutamate release was assayed by on-line fluorometry (Nicholls and Sihra, 1986). Pelleted synaptosomes were resuspended in HBM containing 16 μM bovine serum albumin (BSA). A 1 ml aliquot was transferred to a stirred cuvette containing $NADP^+$ (2 mM), glutamate dehydrogenase (50 units/ml), and $CaCl_2$ (1 mM). The fluorescence resulting from NADPH formation was followed in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) at excitation and emission wavelengths of 340 and 460 nm, respectively. 4-AP (1 mM), or KCl (15 mM) was added to stimulate glutamate release and hesperidin was added 10 min before depolarization. Data were accumulated at 2 s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence response was used to calculate the released glutamate as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Release traces are shifted vertically to align the point of depolarization as zero release. Release values quoted in the text and depicted in bar graphs represent the levels of glutamate cumulatively released after 5 min of depolarization, and are expressed as nmol/mg/5 min. Cumulative data were analyzed using Lotus 1-2-3 (IBM, White Plains, NY) and MicroCal Origin (Origin Lab Corp., Northampton, MA).

2.5. Synaptosomal cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) measurements

Synaptosomes (0.5 mg/ml) were preincubated in HBM containing 5 μM fura-2-AM, 0.1 mM $CaCl_2$, and 16 μM BSA for 30 min at 37 °C in a stirred test tube. After fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 1 min at 10,000 × g. The synaptosomal pellets were resuspended in HBM containing BSA, and placed in a Perkin-Elmer LS-55 spectrofluorometer at 37 °C with stirring in the presence of 1 mM $CaCl_2$. The synaptosomes were incubated for 10 min in the presence of hesperidin (50 μM) prior to being depolarized with 4-AP (1 mM). Fura-2-Ca fluorescence was determined at excitation wavelengths of 340 and 380 nm (emission wavelength, 505 nm), and data were accumulated at 2 s intervals. $[Ca^{2+}]_C$ (nM) was calculated using calibration procedures (Sihra et al., 1992) and equations (Grynkiewicz et al., 1985) described previously. Cumulative data were analyzed using Lotus 1-2-3.

2.6. Synaptosomal plasma membrane potential

The plasma membrane potential was determined using a membrane-potential-sensitive dye, $DiSC_3(5)$. $DiSC_3(5)$ is a positive charged carbocyanine that accumulates into the polarized synaptosomes which are negatively charged on the inside, where at high concentrations, the dye molecules will stack and the

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