

Neuroprotective Effect of Fucoidan against *N*-methyl-D-aspartate-Induced Excitotoxicity in Rat Hippocampus

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ABSTRACT

Fucoidan, a sulfated polyanionic polymer of L-fucose, is obtained from brown marine macroalgae. In the present study, neuroprotective effect of fucoidan against *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity in the hippocampus was investigated. The patch clamp study revealed that fucoidan significantly inhibited NMDA receptor-activated ion current in the acutely dissociated hippocampal CA1 neurons. In an organotypic hippocampal slice culture, fucoidan inhibited NMDA-induced neuronal cell death in a dose-dependent manner. The present study showed that fucoidan possesses a neuroprotective effect against NMDA-induced excitotoxicity, and that the suppressive effect of fucoidan on the NMDA-induced ion current can be suggested as being the underlying neuroprotective mechanism of fucoidan.

Key words: fucoidan, *N*-methyl-D-aspartate, hippocampus, patch clamp, slice culture

INTRODUCTION

Glutamate receptors play several important roles for various physiological, developmental, and pathological processes in the central nervous system (CNS) (Komuro and Rakic, 1993; Meldrum, 2000). The glutamate receptors are divided into the ionotropic and metabotropic receptors. The ionotropic glutamate receptors are further sub-classified

into the *N*-methyl-D-aspartate (NMDA) receptor, the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and the kainate receptor. The ionotropic glutamate receptors not only mediate normal intercellular communications but they are also involved in neuronal injury and death (Hollmann and Heinemann, 1994). Exposure to glutamate receptor agonists is known to lead to neuronal cell death in most cases, whereas the antagonists of glutamate receptor appear to offer neuroprotection (Drain et al., 1999; Stoshi et al., 2001).

Fucoidan is obtained from brown marine macroalgae, and it is a sulfated polyanionic polymer of L-fucose. Fucoidan has been reported to have va-

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rious biological activities, including inhibiting leukocyte recruitment (Preobrazhenskaya et al., 1997), aiding in the re-vascularization of ischemic tissue (Luyt et al., 2003), its anti-thrombotic activity (Collic-Jouault et al., 2003), and it also exerts anti-tumor and anti-microbial effects (Kim et al., 1997). However, there is no information on the effects of fucoidan against the glutamate-induced excitotoxicity in neurons.

In the present study, the modulatory effect of fucoidan on the ion currents activated by glutamate and its subtype agonists were studied by using the nystatin-perforated patch clamp technique under voltage-clamped conditions on acutely dissociated hippocampal CA1 neurons of rats. In addition, NMDA is a major subtype agonist of glutamate-induced excitotoxicity, the neuroprotective effect of fucoidan on the NMDA-induced neuronal cell death in the brain slices was confirmed by the organotypic hippocampal slice culture technique.

MATERIALS AND METHODS

Materials

The fucoidan used in this study was obtained from Riken Vitamin Co., Ltd. (Tokyo, Japan). Most of the drugs used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Patch clamp study

Hippocampal CA1 neurons were dissociated by a previously described method (Kim et al., 1997). In brief, Sprague-Dawley rats 10~15 days old were decapitated under anesthesia induced by Zoletil 50[®] (10 mg/kg, i.m.; Vibac Laboratories, Carros, France). The brains were removed and transverse slices (400 μ m thick) were made using a microslicer (DTK-1000, DSK, Tokyo, Japan). The slices were incubated with 1 mg/6 ml of pronase (protease XIV; Sigma Chemical Co.) for 40~80 min, and the slices were subsequently incubated with thermolysin (protease X, Sigma Chemical Co.) for 10~20 min at 32°C. The CA1 regions of the slices were identified, micropunched out and mechanically dissociated. For studying the NMDA-activated current, Mg²⁺-free standard solution with 10⁻⁶ M glycine was used. The electrical recordings were performed in the nystatin-perforated patch recording mode with

the voltage clamped at -50 mV.

The patch pipettes were prepared from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PB-7, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with internal pipette solution and the reference electrode was 6~8 M Ω . Electrical stimulation, current recordings and filtration of the currents (at 2.9 kHz) were obtained using an EPC-7 patch-clamp amplifier (List-Electronic, Darmstadt, Germany). The current and voltage values were monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan).

Slice culture

Organotypic hippocampal slice cultures were prepared by a previously described method (Lee et al., 2003). The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. The hippocami of Sprague-Dawley rats (postnatal day 7) were isolated and then cut transversely at a thickness of 350 μ m with a McILWAIN tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). The slices were placed on Millicell-CM inserts in 6-well plates that contained 1 ml of culturing medium composed of 50% Minimum essential medium, 25% Hank's balanced salts solution and 25% horse serum. The slices were cultured for 14 days at 36°C in a 5% CO₂ incubator with a total change of medium being done every third day.

To examine whether the NMDA-induced neuronal damage happens in a dose-dependent manner, the cultures were divided into 5 groups: the control group, the 10⁻⁶ M NMDA-treated group, the 10⁻⁵ M NMDA-treated group, the 10⁻⁴ M NMDA-treated group, and the 10⁻³ M NMDA-treated group. To investigate the protective effect of the fucoidan against the NMDA-induced neuronal damage, the cultures were divided into 6 groups: the control group, the 10⁻⁴ M NMDA-treated group, the 10⁻⁴ M NMDA with 50 μ g/ml fucoidan-treated group, the 10⁻⁴ M NMDA with 100 μ g/ml fucoidan-treated group, the 10⁻⁴ M NMDA with 300 μ g/ml fucoidan-treated group, and the 10⁻⁴ M NMDA with 500 μ g/ml fucoidan-treated group.

Propidium iodide (PI, 5 μ g/ml) was added into the

cultures and the PI stained images were captured using an inverted fluorescence microscope with an attached digital CCD camera (Axiovert S100, Zeiss, Göttingen, Germany). The observed areas were measured by using the density slice function of NIH Image analysis software (version 1.65), and the percentage of the neuronal death was then calculated.

Data analysis

The data are presented as the mean±standard error of the mean (SEM). Statistical analysis was made using one-way ANOVA and Dunn's multiple comparisons, and the differences were considered significantly for $p < 0.05$.

RESULTS

Effects of fucoidan on glutamate, NMDA, AMPA, and kainate-induced ion currents in hippocampal CA1 neurons

In the patch-clamp experiment, the inward currents for almost all of the CA1 neurons tested were recorded following the application of 10^{-5} M glutamate, 10^{-4} M NMDA, 10^{-5} M AMPA, and 10^{-5} M kainate. Application of 50 μ g/ml, 100 μ g/ml, 300 μ g/ml, and 500 μ g/ml of fucoidan alone to the CA1 neurons did not elicit any ion currents.

Glutamate at a concentration of 10^{-5} M was applied every 2 min, and the magnitude of the resulting current was used as the control current. Application of glutamate along with fucoidan at concentrations of 50 μ g/ml, 100 μ g/ml, 300 μ g/ml, and 500 μ g/ml slightly decreased the amplitude of the glutamate-induced ion current to $99.5 \pm 1.29\%$ ($n=10$, $p < 0.05$), $95.4 \pm 1.18\%$ ($n=9$, $p < 0.05$), $91.0 \pm 1.34\%$ ($n=6$, $p < 0.05$), and $88.0 \pm 2.51\%$ ($n=6$, $p < 0.05$) of the control value, respectively.

NMDA at a concentration of 10^{-4} M was applied every 2 min, and the magnitude of the resulting current was used as the control current. The NMDA-induced ion current was decreased significantly by the application of fucoidan at the concentrations of 50 μ g/ml, 100 μ g/ml, 300 μ g/ml, and 500 μ g/ml to $95.0 \pm 1.50\%$ ($n=17$, $p < 0.05$), $86.8 \pm 1.65\%$ ($n=15$, $p < 0.05$), $82.2 \pm 2.01\%$ ($n=15$, $p < 0.05$), and $78.0 \pm 1.96\%$ ($n=16$, $p < 0.05$) of the control value, respectively.

AMPA at a concentration of 10^{-5} M was applied every 2 min, and the magnitude of the resulting current was used as the control current. The magnitude of the AMPA-induced ion currents was also decreased slightly by the application of fucoidan at concentrations of 50 μ g/ml, 100 μ g/ml, 300 μ g/ml, and 500 μ g/ml to $100.0 \pm 0.54\%$ ($n=13$, $p < 0.05$), $96.6 \pm 0.53\%$ ($n=9$, $p < 0.05$), $94.9 \pm 1.29\%$ ($n=9$, $p < 0.05$), and $90.9 \pm 1.50\%$ ($n=9$, $p < 0.05$) of the control value, respectively.

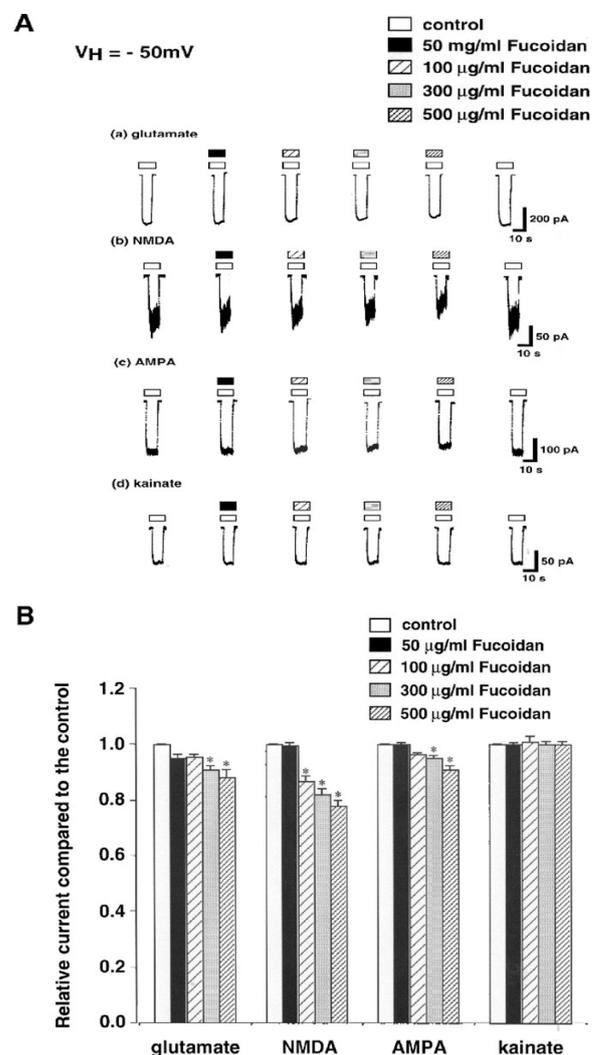


Fig. 1. Effects of fucoidan on the ion currents activated by glutamate and the other agonists of the glutamate subtype receptors in the acutely dissociated hippocampal CA1 neurons. (A) Representative ion currents. (B) Relative ion currents. The magnitudes of the inward currents elicited by 10^{-5} M glutamate, 10^{-4} M *N*-methyl-D-aspartate (NMDA), 10^{-5} M α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 10^{-5} M kainite were used as the control values. *represents $p < 0.05$ compared to the control current.

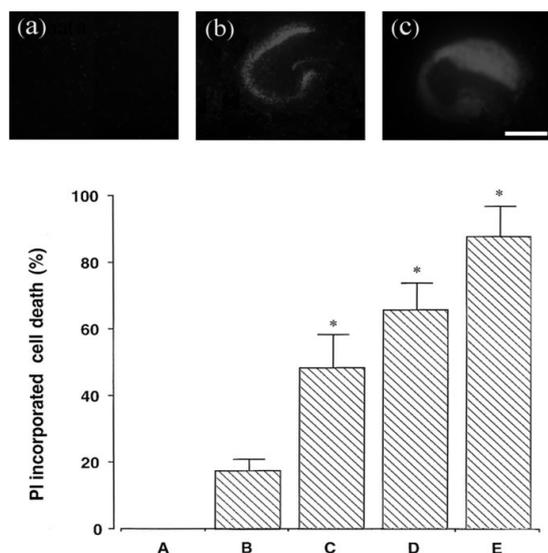


Fig. 2. The cell death induced in a dose-dependent manner by *N*-methyl-D-aspartate (NMDA) on the hippocampal CA1 area after 24 h of exposure. Upper: Representative PI fluorescence images. (a) Control group, (b) 10^{-6} M NMDA-treated group, (c) 10^{-4} M NMDA-treated group. Lower: Area diagrams of the PI incorporated cell death. (A) Control group, (B) 10^{-6} M NMDA-treated group, (C) 10^{-5} M NMDA-treated group, (D) 10^{-4} M NMDA-treated group, (E) 10^{-3} M NMDA-treated group. The scale bar represents 1 mm. *represents $p < 0.05$ compared to the control group.

Kainate at a concentration of 10^{-5} M was applied every 2 min, and the magnitude of the resulting current was used as the control current. The magnitude of the kainate-induced ion current was not affected by the application of fucoidan at concentrations of $50 \mu\text{g/ml}$, $100 \mu\text{g/ml}$, $300 \mu\text{g/ml}$, and $500 \mu\text{g/ml}$ to $100.1 \pm 0.68\%$ ($n=9$, $p < 0.05$), $101.3 \pm 1.46\%$ ($n=7$, $p < 0.05$), $100.1 \pm 0.88\%$ ($n=7$, $p < 0.05$), and $100.2 \pm 0.83\%$ ($n=6$, $p < 0.05$) of the control value, respectively (Fig. 1).

Effect of fucoidan on NMDA-induced excitotoxicity in hippocampal slice culture

To determine whether fucoidan has a neuroprotective activity on the NMDA-induced neuronal death, a hippocampal slice culture system was used. The representative PI incorporated fluorescence images of the NMDA-induced neuronal cell death captured at 24 h are shown in the upper panels of Fig. 2 and Fig. 3. The cultured hippocampal slices exposed to NMDA increased their PI incorporated fluorescence in a dose-dependent manner (Fig. 2). Treatment with fucoidan on the cultured hippocam-

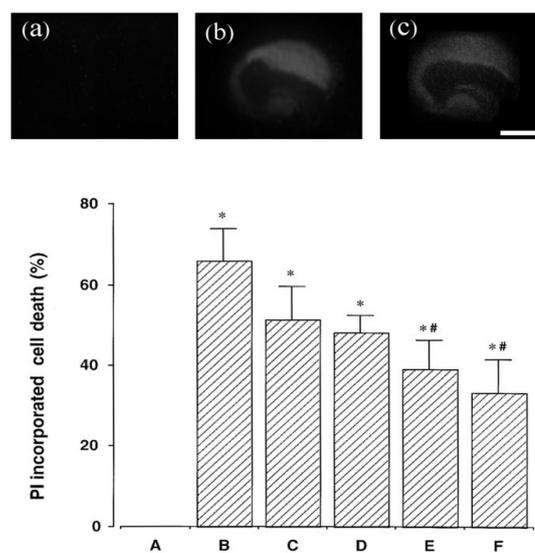


Fig. 3. Neuroprotective effect of fucoidan against the *N*-methyl-D-aspartate (NMDA)-induced cell death on the hippocampal CA1 area after 24 h of exposure. Upper: Representative propidium iodide (PI) fluorescence images. (a) Control group, (b) 10^{-4} M NMDA-treated group, (c) 10^{-4} M NMDA with $500 \mu\text{g/ml}$ fucoidan-treated group. Lower: Area diagrams of the PI incorporated cell death. (A) Control group, (B) 10^{-4} M NMDA-treated group, (C) 10^{-4} M NMDA with $50 \mu\text{g/ml}$ fucoidan-treated group, (D) 10^{-4} M NMDA with $100 \mu\text{g/ml}$ fucoidan-treated group, (E) 10^{-4} M NMDA with $300 \mu\text{g/ml}$ fucoidan-treated group, (F) 10^{-4} M NMDA with $500 \mu\text{g/ml}$ fucoidan-treated group. The scale bar represents 1 mm. *represents $p < 0.05$ compared to the control group. #represents $p < 0.05$ compared to the 10^{-4} M NMDA-treated group.

pal slices reversed the neuronal cell death induced by the NMDA (10^{-4} M), and this resulted in a dose-dependent decrease of PI incorporated fluorescence in the CA1 areas (Fig. 3).

DISCUSSION

Fucoidan, a sulfated polysaccharide extracted from brown algae, has a wide range of biological activities, including anti-inflammatory, anti-viral, and anti-tumor activities (Matsubara et al., 2005; Yang et al., 2006; Hayashi et al., 2008). Fucoidan is known to exert immunostimulating and maturing effects on dendritic cells via a pathway involving at least nuclear factor-kappa B (Kim and Joo, 2008). Fucoidan treatment also exhibited significantly more rapid improvement of motor function and better memory retention following intracerebral hemorrhage in rats (Del Bigio, 1999).

Glutamate excitotoxicity may be involved in the pathophysiology of a large number of brain dis-

orders (Muir and Lees, 1995; Drain et al., 1999; Ishikawa et al., 1999; Meldrum, 2000). When we consider that glutamate-mediated neuronal apoptosis plays a pivotal role in neurological disorders (Sastry and Rao, 2000), the searching for therapeutic agents that can inhibit glutamate release or that selectively block the NMDA receptors may well provide extensive clinical benefits (Muir and Lees, 1995). The most widely accepted hypothesis for how NMDA receptor antagonists salvage the viability of neurons states that they inhibit the nitric oxide (NO) production that is induced by calcium's entry through the NMDA receptors following the release of glutamate from the presynaptic neurons. Ca^{2+} -dependent nitric oxide synthase (NOS) is induced by high concentrations of intracellular Ca^{2+} , and the NOS-induced NO and superoxide generation has injurious effects on the neurons (Ishikawa et al., 1999; Lee et al., 2005). It has been reported that fucoidan attenuates the hypoxia-ischemia-induced neural damage (Uhm et al., 2003).

In the present results, an application of fucoidan on the dissociated hippocampal CA1 neurons suppressed the NMDA-induced ion current in a dose-dependent manner. The glutamate- and AMPA-induced ion currents were slightly reduced by an application of fucoidan. It was also noted that fucoidan exerted no significant effect on the kainate-induced ion currents (Fig. 1).

We adapted the hippocampal slice culture technique to confirm the NMDA-induced excitotoxicity. Organotypic brain slice cultures have been extensively used to study neuronal cell death in a variety of in vitro experimental models such as ischemia, excitatory amino acid neurotoxicity, and traumatic damage (Lee et al., 2003). The brain slice culture technique has several advantages such as pharmacological accessibility, tissue specific cell connections, and the preservation of local neuronal circuits in the brain slices along with their proper patterns of innervation.

In the present results, a treatment of NMDA onto the hippocampal slice cultures for 24 h induced neuronal cell death in the hippocampal CA1 area (Fig. 2). In the subsequent experiment, we treated 10^{-4} M NMDA with various dosages of fucoidan onto the hippocampal slice cultures to examine the relative neuroprotective effect of different fucoidan

concentrations against the NMDA-induced neuronal cell death. Fucoidan displayed neuroprotective effect on the NMDA-induced excitotoxicity in a dose-dependent manner (Fig. 3).

These results from the patch-clamp study and the hippocampal slice cultures have shown that the suppressive effect of fucoidan on the NMDA-induced ion current can be suggested as the underlying neuroprotective mechanism of fucoidan against glutamate-induced excitotoxicity. Our results show that fucoidan is a possible therapeutic agent for glutamate-related CNS disorders, such as stroke.

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