Extract of *Ginkgo biloba* Leaves Attenuates Kainate-Induced Increase in Intracellular Ca\(^{2+}\) Concentration of Rat Cerebellar Granule Neurons

Aimi Kanada, Yumiko Nishimura, Jun-ya Yamaguchi, Masako Kobayashi, Kyoko Mishima, Kanna Horimoto, Kaori Kanemaru, and Yasuo Oyama*

Laboratory of Cellular Signaling, Faculty of Integrated Arts and Sciences, The University of Tokushima; Tokushima, 770–8502, Japan. Received January 25, 2005; accepted March 9, 2005; published online March 10, 2005

In order to reveal one of possible mechanisms for neuronal protective action of extract of *Ginkgo biloba* leaves (EGBL), the effect of EGBL on kainate- and KCl-induced increases in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(\text{i}\)) of rat cerebellar neurons was examined using a confocal laser microscope with appropriate fluorescent probes. EGBL at 3 \(\mu\)g/ml started to attenuate kainate-induced increase of [Ca\(^{2+}\)]\(\text{i}\) and further increase in EGBL concentration (up to 30 \(\mu\)g/ml) concentration-dependently and significantly inhibited the kainate response. The complete inhibition by EGBL was observed in some neurons when the concentration was 10—30 \(\mu\)g/ml. The kainate-induced increase in [Ca\(^{2+}\)]\(\text{i}\) was mainly due to Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channel opened by membrane depolarization via activation of kainate receptor-channel. However, the increase in [Ca\(^{2+}\)]\(\text{i}\) by KCl was not significantly affected by EGBL at concentrations where the kainate response was greatly inhibited. EGBL consisting of flavone glycosides and terpene lactones is known to be an antioxidant. Furthermore, in this study, it is shown that EGBL exerts an inhibitory action on kainate receptor (a subtype of glutamate receptor). Since some of neurodegenerative diseases are due to cell death induced by glutamate excitotoxicity and oxidative stress, EGBL may be very suitable for preventing and/or treating such diseases.

Key words *Ginkgo biloba*: glutamate; excitotoxicity; calcium; neuron; kainate

The idea that an excessive release of glutamate mediates neuronal cell death was proposed, as the excitotoxicity hypothesis, by Olney and de-Gubareff.\(^1\) Glutamate activates two types of its receptor, N-methyl-D-aspartate (NMDA) type and non-NMDA one.\(^2\) Excessive activation of glutamate receptors, resulting in increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(\text{i}\)) and generation of reactive oxygen species (ROS), is involved in neuronal disorder and degeneration.\(^3\) Both phenomena induced by glutamate are related to cell injury and death.\(^4,5\) In fact, antagonists for NMDA and non-NMDA glutamate receptors are proven to possess neuroprotective action.\(^6–8\)

Extract of *Ginkgo biloba* leaves (EGBL) is expected to treat patients with some neuronal disorders such as dementia and Alzheimer’s disease.\(^9,10\) The extract exerts protective action (or antioxidant action) on neurons suffering from oxidative stress induced by hydrogen peroxide, one of ROS, and by overload of intracellular Ca\(^{2+}\).\(^5,11,12\) EGBL and its constituents inhibit the response mediated by activation of NMDA receptor.\(^13,14\) However, there is limited information concerning the effect of EGBL on response induced by activation of non-NMDA glutamate receptor. Kainate, an agonist for non-NMDA glutamate receptor, increases [Ca\(^{2+}\)]\(\text{i}\) in neurons via activations of non-NMDA glutamate receptor and voltage-dependent Ca\(^{2+}\) channel.\(^15\) Therefore, in order to elucidate one of mechanisms for neuroprotective action of EGBL, we examined the effect of EGBL on kainate-induced increase in [Ca\(^{2+}\)]\(\text{i}\) in rat brain neurons using a laser microscope with fluorescent Ca\(^{2+}\)-indicator.

MATERIALS AND METHODS

Materials Extract of *Ginkgo biloba* leaves (EGBL) was obtained from Icho-Ha Sangyo Co., Ltd., Ibaraki, Japan. EGBL was initially dissolved in 50% ethanol. EGBL solution (1—30 mg/ml) was added to bathing solution to achieve appropriate concentrations (1—30 \(\mu\)g/ml). Ethanol as a solvent at 0.05% did not affect any fluorescence measurements from cells. Other chemical reagents except for fluorescent probes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise mentioned.

Cell Preparation Experimental method for dissociation of rat cerebellar neurons was similar to those described in previous studies.\(^6,16,17\) In brief, the slices of cerebellum dissected from 10- to 16-d-old Wistar strain rats were treated with 1000 protease units/ml dispase (Godo Shusei, Tokyo, Japan) in Tyrode’s solution for 50—60 min at a temperature of 32—34 °C. After enzymatic treatment, cerebellar neurons were dispersed by triturating in Tyrode’s solution. Since the majority of neurons were characterized by small size and spherical shape under microscopic observation, they were ascertained as cerebellar granule cells. These cells responded to kainate, resulting in an increase in [Ca\(^{2+}\)]\(\text{i}\).\(^15\) The cells were placed on culture dishes and incubated at 35—36 °C in Tyrode’s solution for 50—60 min before use. The application of chemicals to neurons was performed by a Y-tube technique.\(^18\)

Measurements To estimate the change in intracellular Ca\(^{2+}\) concentration of neurons with intact membranes, two fluorescent probes, fluo-3-AM (Dojindo Chemical Laboratory, Kumamoto, Japan) and propidium iodide (Molecular Probes Inc., Eugene, MO, U.S.A.), were used.\(^16,17\) Fluo-3 fluorescence is used for monitoring the change in [Ca\(^{2+}\)]\(\text{i}\).\(^19\) Propidium iodide, which is highly impermeant to intact membrane, cannot stain intact live cells. Excitation wavelength for fluo-3 and propidium was 488 nm produced by argon laser. Emissions were detected at wavelengths of 530±15 nm for fluo-3 fluorescence and 605 nm or longer for propidium fluorescence. Fluo-3 fluorescence was monitored from intact neurons that were not stained with propidium by a confocal laser microscope (Meridian Instruments Far East, Tokyo, Japan) and analyzed by a computer (Evolution V-Q

* To whom correspondence should be addressed. e-mail: oyama@ias.tokushima-u.ac.jp © 2005 Pharmaceutical Society of Japan

**Statistical Analysis** Numerical values are expressed with mean and its standard deviation. Statistical analysis was performed by Dunnett test. A $p$ value of $<0.05$ was considered significant.

**RESULTS**

**Effect of EGBL on Kainate-Induced Response of Rat Cerebellar Neurons** Kainate at 30 $\mu$m increased the intensity of fluo-3 fluorescence in some neurons (Fig. 1), but not all, and the number of cells responding to kainate was greater than those responding to glutamate or NMDA.\(^{15}\) CNQX, an antagonist for non-NMDA glutamate receptor, at 10 $\mu$m almost completely suppressed the kainate-induced augmentation of fluo-3 fluorescence.\(^{15}\)

Control intensity of fluo-3 fluorescence monitored from neurons before the application of kainate was 359±204 (control value, arbitrary unit, mean±S.D. of 25 experiments). The application of 30 $\mu$m kainate increased the intensity of fluo-3 fluorescence to 1045±222 ($n=5$) under control condition. EGBL (1—30 $\mu$/g/ml) did not significantly affect the intensity of fluo-3 fluorescence.

As shown in Fig. 1, 30 $\mu$/g/ml EGBL greatly suppressed the kainate response at 15 min after the start of EGBL treatment. In the presence of EGBL, the kainate-induced increases in the intensity were 1074±248 ($n=5$) for in the case of 1 $\mu$/g/ml EGBL, 814±345 ($n=5$) for 3 $\mu$/g/ml EGBL, 486±160 ($n=5$) for 10 $\mu$/g/ml, and 484±232 ($n=4$) for 30 $\mu$/g/ml, respectively. Thus, EGBL at 3 $\mu$/g/ml or more concentration-dependently attenuated the kainate-induced augmentation of fluo-3 fluorescence, suggesting that EGBL possesses an inhibitory action on the kainate response. Results are normalized and summarized in Fig. 2.

**Possible Site of Action of EGBL** In the response to kainate in rat cerebellar neurons, there are two steps to increase [Ca\(^{2+}\)], activation of kainate receptor leading to membrane depolarization and then activation of voltage-dependent Ca\(^{2+}\) channel by the depolarization.\(^{15}\) Nicardipine at 30 $\mu$m completely suppressed the augmentation of fluo-3 fluorescence by 100 mM KCl and decreased the number of cells responding to kainate with increasing the intensity of fluo-3 fluorescence.\(^{15}\) Therefore, it is important to see whether EGBL suppresses kainate receptor, voltage-dependent Ca\(^{2+}\) channel, or both.

Control intensity of fluo-3 fluorescence was 405±188 ($n=20$). Under control condition, 30 $\mu$m kainate increased the intensity of fluo-3 fluorescence to 1728±342 ($n=5$) while it was 2125±248 ($n=5$) in the case of 50 mM KCl. However, in presence of 30 $\mu$/g/ml EGBL, respective increases were 732±352 ($n=5$) for 30 $\mu$m kainate and 1929±449 ($n=4$) for 50 mM KCl. Thus, EGBL greatly suppressed the kainate response, but not the response induced by 50 mM KCl. Results are normalized and summarized in Fig. 3.

As described above, it is suggested that EGBL suppresses kainate receptor rather than voltage-dependent Ca\(^{2+}\) channel (Fig. 3). To seek the site for inhibitory action of EGBL on kainate receptor, the effect of increasing kainate concentra-
tion on the EGBL-induced inhibition was examined.

Control intensity of fluo-3 fluorescence was 639 ± 375 (n = 29). Kainate at 30 μM and 100 μM increased the intensity of fluo-3 fluorescence to 1785 ± 301 (n = 7) and 2372 ± 127 (n = 8), respectively. However, in presence of 30 μg/ml EGBL (15 min preincubation), the increases were 891 ± 370 (n = 6) for 30 μM kainate and 2001 ± 187 (n = 7) for 100 μM kainate. Thus, it is suggested that EGBL competes for receptor site with kainate. Results are normalized and summarized in Fig. 4. It is noted that the preincubation of neurons with EGBL is required to exert the inhibitory action on kainate receptor.

DISCUSSION

EGBL is used to treat or prevent some neurodegenerative diseases as well as stroke although the weight of evidence regarding its effect is not sufficient to define clinical practice. We have shown that EGBL protects neurons suffering from oxidative stress induced by hydrogen peroxide and decreases oxidative metabolism in resting and Ca2+–loaded neurons. Oxidative stress is also induced by abnormally increasing [Ca2+]i in neurons. Therefore, if EGBL prevents increasing [Ca2+]i of neurons by excessive activation of glutamate receptor, it would contribute to the neuroprotective action of EGBL. In this study, EGBL at concentrations of 3 μg/ml or more decreased the increase in [Ca2+]i induced by kainate via an activation of non-NMDA receptor (Fig. 2). At concentrations of 10—30 μg/ml, EGBL completely suppressed the increase in [Ca2+]i induced by kainate in some neurons (Fig. 2). Thus, EGBL may exert neuroprotective action via suppression of increase in [Ca2+]i by activation of non-NMDA glutamate receptor.

Kainate-induced increase in [Ca2+]i of neurons is mediated via activations of both non-NMDA receptor and voltage-dependent Ca2+ channel (Fig. 3). Although voltage-de-

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