Korean red ginseng excitation of paraventricular nucleus neurons via non-N-methyl-D-aspartate glutamate receptor activation in mice

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It has been reported that Korean red ginseng (KRG), a valuable and important traditional medicine, has varied effects on the central nervous system, suggesting its activities are complicated. The paraventricular nucleus (PVN) neurons of the hypothalamus have a critical role in stress responses and hormone secretions. Although the action mechanisms of KRG on various cells and systems have been reported, the direct membrane effects of KRG on PVN neurons have not been fully described. In this study, the direct membrane effects of KRG on PVN neuronal activity were investigated by using a perforated patch-clamp in ICR mice. In gramicidin perforated patch-clamp mode, KRG extract (KRGE) induced repeatable depolarization followed by hyperpolarization of PVN neurons. The KRGE-induced responses were concentration-dependent and persisted in the presence of tetrodotoxin, a voltage sensitive Na+ channel blocker. The KRGE-induced responses were suppressed by 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), a non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, but not by picrotoxin, a type A gamma-aminobutyric acid receptor antagonist. The results indicate that KRG activates non-NMDA glutamate receptors of PVN neurons in mice, suggesting that KRG may be a candidate for use in regulation of stress responses by controlling autonomic nervous system and hormone secretion.

Keywords: Korean red ginseng, paraventricular nucleus neurons, patch-clamp techniques

Introduction

Ginseng (Panax ginseng Mayer) root has been used as a traditional medicine in many Asian countries and as an alternative medicine throughout the world [1]. Korean red ginseng (KRG) is a type of ginseng that, after cultivation for 4 to 6 years, goes through cleaning, steaming, and drying processes [44]. KRG has multiple underlying pharmacologic actions [17,29]. It has been reported that KRG has several pharmacologic functions including enhancing memory [16], anti-hypertensive [15], antitumor [45], anti-diabetic [41], erectile potentiation [6], and anti-stress [17]. Ginseng has a long history in Asia and is used for its wide spectrum of stamina enhancement and coping capacity functions associated with multiple stresses such as fear, anxiety, cold, heat, and pain as well as chemical or noxious stimuli [3,10,12,17,36]. Stress is a form of response to unexpected or perilous situations [30]. Recently, several studies have demonstrated that KRG has a beneficial role in the prevention of industrial (occupational) fatigue [17], inhibiting tumor metastasis [27], immunization against human immunodeficiency virus [37], and physicochemical stresses [18]. In addition, it has been shown that ocatillol, an extract from American ginseng, induces mitral cell activity through ionotropic glutamate receptors in vitro by using a whole-cell patch-clamp [42].

The paraventricular nucleus (PVN) neurons of the hypothalamus have a critical role in integrating autonomic and endocrine functions [39]. The PVN is a complex, heterogeneous structure adjacent to the third ventricle and is composed of magnocellular and parvocellular neurons, identified by their functions [38]. The magnocellular neurons release vasopressin or oxytocin into the posterior pituitary gland and into the bloodstream [7,31],

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and they show no low-threshold spikes after hyperpolarizing pulses larger than $-100$ mV [11]. The parvocellular neurons are composed of neurosecretory and preautonomic neurons and show discrete or bursting low-threshold spikes after hyperpolarizing pulses larger than $-100$ mV [11]. The parvocellular neurosecretory neurons synthesize regulatory hormones and their axons are involved in regulating secretion from the anterior pituitary gland and in collaborating with the hypothalamo-pituitary-adrenocortical (HPA) axis [32,33]. Although many studies have reported action mechanisms of KRG on various cells and systems, the direct membrane effects of KRG on PVN neurons, which are involved in stress responses, have not been fully described. In the current study, the direct effects of a KRG extract (KRGE) on PVN neuronal activities were investigated by using a gramicidin perforated patch-clamp technique.

Materials and Methods

Experimental animals

All experiments adhered to Chonbuk National University Animal Welfare and Ethics Committee’s guidelines regarding the use and care of animals (protocol No. CBU-2014-0042). The ICR mice were housed under 12 h light/12 h dark cycles with free access to food and water.

Preparation of brain slices

Immature (8- to 18-day-old) male ICR mice were rapidly decapitated and the head placed in iced artificial cerebrospinal fluid (ACSF) of the following composition: NaCl 126 mM, KCl 2.5 mM, CaCl$_2$ 2.4 mM, MgCl$_2$ 1.2 mM, D-glucose 11 mM, NaH$_2$PO$_4$ 1.4 mM, and NaHCO$_3$ 25 mM (pH = 7.4 and bubbled with O$_2$ 95% and CO$_2$ 5% between 10:00 and 12:00). The forebrain was blocked and glued to the chilled stage of a vibratome (Microme, Germany). Coronal slices (150–180 μm thick) including the PVN (bregma $-0.58$ to $-1.22$ mm) were cut and kept in the oxygenated ACSF for 1 h before use at room temperature.

Electrophysiological recording and data analysis

For electrophysiological recording, a brain slice was transferred to a recording chamber and bathed with ACSF at a rate of 3 to 5 mL/min under continuous bubbling with O$_2$ (95%) and CO$_2$ (5%). The slices were fixed by a U-shaped platinum wire with single nylon strands and viewed through a microscope (BX51WI; Olympus, Japan). Glass pipettes of borosilicate capillary tubing (PG52151-4; WPI, USA) were made by using a puller (P-97; Sutter Instruments, USA). For perforated patch-clamp recording, a pipette solution containing (KCl 130 mM, NaCl 5 mM, CaCl$_2$ 0.4 mM, MgCl$_2$ 1 mM, HEPES 10 mM, and EGTA 1.1 mM [pH = 7.3 with KOH]) was passed through a 0.22 μm filter. Gramicidin (Sigma, USA) was dissolved in dimethyl sulfoxide to a concentration of 2 to 5 mg/mL and then diluted 1,000 times to a final concentration of 2 to 5 μg/mL. Access resistance was initially monitored and experiments were started when access resistance was stabilized at 50 to 90 MΩ. Stabilization typically took 15 to 20 min after gigaseal and, usually, resting was attained at under $-45$ mV. Membrane rupture was detected by a sudden overshoot of action potentials, after which the data were excluded. The membrane potential was sampled by using a Digidata 1322A digitizer (Axon Instruments, USA). Data acquisition and analysis were performed by using a Clampex clamp set (ver 9.0; Axon Instruments).

Compounds and statistics

The KRGE was kindly provided by the Korea Ginseng Corporation (Korea). The obtained KRGE was reported to contain major ginsenosides including Rb1, Rb2, Re, Rf, Rg1, Rg2, Rg3, and other minor ginsenosides (Korea Ginseng Corporation). Most test compounds were first dissolved in ACSF and then diluted by ACSF. CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione) and tetrodotoxin (TTX) were purchased from Sigma and Tocris Bioscience (UK), respectively. Data are expressed as mean ± SEM values. One-way ANOVA

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![Fig. 1](https://example.com/f1.png)

**Fig. 1.** Concentration-dependent Korean red ginseng extract (KRGE) responses on paraventricular nucleus (PVN) neurons. (A) A representative trace from a PVN neuron showing concentration-dependent responses following application of KRGE (0.3, 1.0, and 3.0 mg/mL). Cumulative graphs show the mean membrane depolarization (B) and after-hyperpolarization (C) values after treatment by KRGE (0.3, 1.0, and 3.0 mg/mL). *p < 0.05.
or a paired t-test was used for comparing more than three groups and two groups, respectively. When the test’s p value was less than 0.05, it was considered to indicate a significant difference.

Results

KRGE-induced membrane potential changes are concentration-dependent

To determine whether KRGE can directly affect PVN neuronal activities, we applied KRGE in different concentrations. A low concentration of KRGE, less than 0.3 mg/mL, showed no notable changes in PVN neuronal activity. Thus, we applied KRGE ranging from 0.3 to 3.0 mg/mL. The KRGE-induced membrane potential changes gradually increased in proportion to the concentration (panel A in Fig. 1; 0.3–3.0 mg/mL). The mean membrane depolarizations by KRGE treatment concentration were 1.59 ± 0.78 mV (0.3 mg/mL, n = 7), 3.70 ± 1.33 mV (1.0 mg/mL, n = 7), and 9.45 ± 2.20 mV (3.0 mg/mL, n = 7), respectively (panel B in Fig. 1; p < 0.05, one-way ANOVA). The mean after-hyperpolarization values (i.e., depolarization followed by hyperpolarization) by KRGE concentration were −1.07 ± 0.66 mV (0.3 mg/mL), −1.91 ± 0.96 mV (1.0 mg/mL), and −4.53 ± 1.96 mV (3.0 mg/mL), respectively (panel C in Fig. 1; p > 0.05, one-way ANOVA). As the concentration of KRGE increased, after-hyperpolarization also tended to increase, but there was no a statistically significant difference.

To determine whether the KRGE-induced responses would be desensitized by repeated applications, we applied 3.0 mg/mL KRGE successively (panel A in Fig. 2). As shown in panel B in Fig. 2, the relative membrane depolarization value in the 2nd application of KRGE was 1.28 ± 0.19 times that in the 1st application (1st 8.25 ± 1.96 mV vs 2nd 10.74 ± 2.70 mV, p > 0.05, n = 11, paired t-test). As shown in panel C in Fig. 2, the relative membrane after-hyperpolarization value in the 2nd application of KRGE was 1.34 ± 0.35 times that in the 1st application (1st −9.33 ± 2.28 mV vs 2nd −9.87 ± 1.88 mV, p > 0.05, n = 7, paired t-test). Neither changes were significantly different.

KRGE acts on PVN neurons directly

To test whether the short-lived and reproducible membrane potential changes resulting from KRGE treatment are induced
Effects of KRG on PVN neurons

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Fig. 4. Korean red ginseng extract (KRGE)-mediated membrane potential changes are mediated by activation of non-N-methyl-D-aspartate (NMDA) glutamate receptors on paraventricular nucleus (PVN) neurons. (A) A representative trace from a PVN neuron showing depolarization and after-hyperpolarization following application of 3.0 mg/mL KRGE alone and KRGE (3.0 mg/mL) with CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione), a non-NMDA glutamate receptor blocker. Mean relative membrane depolarization (B) and after-hyperpolarization (C) by KRGE (3.0 mg/mL) alone and by KRGE (3.0 mg/mL) with CNQX. *p < 0.05.

KRGE can activate non-N-methyl-D-aspartate glutamate receptors on PVN neurons

To determine which receptors are involved in the KRGE-mediated actions on PVN neurons, we applied KRGE in the presence of CNQX, a non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist. As shown in panel A in Fig. 4, the KRGE-induced depolarization (8.83 ± 1.45 mV, n = 5) and after-hyperpolarization (−3.9 ± 0.78 mV, n = 5) values decreased to 1.91 ± 1.17 mV and −0.16 ± 0.16 mV in the presence of CNQX, respectively. As shown in panels B and C in Fig. 4, the relative values of membrane depolarization and after-hyperpolarization in the presence of CNQX were 0.27 ± 0.17 and 0.06 ± 0.06 compared to those from the application of KRGE alone, respectively (p < 0.05, n = 5, paired t-test). Moreover, KRGE-mediated membrane depolarization was unaffected by the application of AP5 (20 μM), an NMDA receptor antagonist (data not shown). These results indicate that KRGE-mediated actions in PVN neurons are involved in the activation of non-NMDA glutamate receptors.

To determine whether the KRGE-induced membrane potential changes could be mediated by type A gamma-aminobutyric acid (GABA_A) receptor activation in the PVN neurons, KRGE-mediated actions on PVN neurons, we applied KRGE in the presence of CNQX, a non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist. As shown in panel A in Fig. 4, the KRGE-induced depolarization (8.83 ± 1.45 mV, n = 5) and after-hyperpolarization (−3.9 ± 0.78 mV, n = 5) values decreased to 1.91 ± 1.17 mV and −0.16 ± 0.16 mV in the presence of CNQX, respectively. As shown in panels B and C in Fig. 4, the relative values of membrane depolarization and after-hyperpolarization in the presence of CNQX were 0.27 ± 0.17 and 0.06 ± 0.06 compared to those from the application of KRGE alone, respectively (p < 0.05, n = 5, paired t-test). Moreover, KRGE-mediated membrane depolarization was unaffected by the application of AP5 (20 μM), an NMDA receptor antagonist (data not shown). These results indicate that KRGE-mediated actions in PVN neurons are involved in the activation of non-NMDA glutamate receptors.

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was applied in the presence of picrotoxin (PIC), a GABA<sub>A</sub> receptor antagonist. As shown in panel A in Fig. 5, the KRGE-induced responses (depolarization 9.13 ± 2.06 mV, n = 4; after-hyperpolarization −6.88 ± 3.88 mV, n = 3) were not affected by PIC (depolarization 7.79 ± 3.57 mV, n = 4; after-hyperpolarization −5.70 ± 1.85 mV, n = 3). As shown in panels B and C in Fig. 5, the relative values of membrane depolarization and after-hyperpolarization with PIC were 0.73 ± 0.30 (n = 4) and 1.17 ± 0.38 (n = 3) times those from application of KRGE alone, respectively (p > 0.05, paired t-test), indicating no significant effect by PIC application.

**Discussion**

In the present study, we showed that KRG can activate non-NMDA glutamate receptors of the PVN neurons directly rather than via an action potential-mediated mechanism. Previous studies using whole-cell patch-clamp recordings showed that KRG activates GABA<sub>A</sub> and/or non-NMDA glutamate receptors on the preoptic gonadotropin-releasing hormone neurons [4] and on the medullary dorsal horn neurons [43] in mice. In the current study, the recordings were based on a gramicidin perforated patch-clamp, which can maintain the intracellular chloride intact and maintain cell virginity [19]. Based on the results of this study, the KRGE-induced membrane potential changes were due to direct effects on non-NMDA receptors, not by the activation of GABA<sub>A</sub> receptors.

Currently, more than 30 ginsenosides, including Rb1−2, Re, Rd, Rg1, Re, Rf, etc., have been isolated and reported to have pharmacological activity [10]. In addition, a number of studies have demonstrated that NMDA receptor-mediated Ca<sup>2+</sup> and Na<sup>+</sup> entries are suppressed by Rg<sub>3</sub> application [5,23-25]. Some studies have shown that KRGE-induced responses were mediated by the activation of the AMPA receptor, not by NMDA receptor activation [4,43]. Furthermore, it was shown that a low dose of decichine (a non-protein amino acid in roots of Panax notoginseng) contributes to reducing bleeding time by increasing the number of platelets via AMPA receptor activation [13]. The lack of AMPA receptors has contributed to enhancing the activity of the PVN neurons in hypertension rats [26]. Consequently, the effect of KRGE through AMPA receptor demonstrates that KRGE may be beneficial treatment for hypertension. Recently, it has been reported that gintonin is an important ginseng extract that can activate NMDA receptors to induce long-term potentiation via lysophosphatidic acid receptor (LPAR) mediation [34], and it can activate AMPA and NMDA receptors for synaptic enhancement by LPAR in the hippocampus [28]. In addition, gintonin can induce the release of epinephrine and norepinephrine through LPAR to increase the plasma glucose level, which may enhance physical stamina in mice [22]. The PVN neurons integrate and regulate the HPA axis to control catecholamine release [9]. Thus, among the many ingredients of KRG, gintonin may be a good candidate for further study into KRGE’s direct effects on PVN neuronal activities against stress and into determining the action mechanism involved.

In this study, KRGE induced membrane after-hyperpolarization in most cases in gramicidin perforated patch-clamp mode. Several studies have shown that after-hyperpolarization can be generated by activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels [20,21,35]. The AMPA receptor is one of non-NMDA glutamate receptors in the central nervous system, and it can be permeable to cations including Ca<sup>2+</sup> [14]. It has also been reported that Ca<sup>2+</sup>-activated K<sup>+</sup> channels are expressed in PVN neurons and have critical roles in control cardiac and renal functions [2,8]. In the neural network, AMPA is also involved in activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels, which are associated with intracellular calcium kinetics [40]. The after-hyperpolarization in the PVN neurons can be explained by an intracellular calcium increase through KRGE-induced AMPA receptor activation followed by activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels.

Since repeatable and concentration-dependent KRGE effects were not influenced by the presence of TTX, which blocks action potential-dependent transmission, we concluded that the KRGE-induced membrane potential changes were due to direct effects on the PVN neuron rather than via any action potential-mediated transmission. Direct effects of KRGE have also been reported on the gonadotropin-releasing hormone [4] and medullary dorsal horn neurons [43].

In conclusion, KRGE has excitatory effects on PVN neurons via non-NMDA glutamate receptors suggesting that KRG may be a potential target for applications regulating physiological and psychological stresses via its action on PVN neurons. However, further studies are needed to isolate and identify the compounds involved in the KRGE-mediated action mechanisms on PVN neurons.

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**Conflict of Interest**

The authors declare no conflicts of interest.

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