Resveratrol Inhibits GABA_C Receptor-Mediated Ion Currents Expressed in Xenopus Oocytes

Byung-Hwan Lee*1, Sun-Hye Choi*1, Sung-Hee Hwang1, Hyeon-Joong Kim1, Joon-Hee Lee2, and Seung-Yeol Nah1

1Department of Physiology, College of Veterinary Medicine and Bio-Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea. 2Department of Physical Therapy, Sehan University, Yeongam 526-702, Korea

Resveratrol is a phytoalexin found in grapes, red wine, and berries. Resveratrol has been known to have many beneficial health effects, such as anti-cancer, neuroprotective, anti-inflammatory, and life-prolonging effects. However, relatively little is known about the effects of resveratrol on the regulation of ligand-gated ion channels. We have previously reported that resveratrol regulates subsets of homomeric ligand-gated ion channels such as those of 5-HT3 receptors. The γ-aminobutyric acidC (GABA_C) receptor is mainly expressed in retinal bipolar cells and plays an important role in visual processing. In the present study, we examined the effects of resveratrol on the channel activity of homomeric GABA_C receptor expressed in Xenopus oocytes injected with cRNA encoding human GABA_C ρ subunits. Our data show that the application of GABA elicits an inward peak current (I_GABA) in oocytes that express the GABA_C receptor. Resveratrol treatment had no effect on oocytes injected with H2O or with GABA_C receptor cRNA. Co-treatment with resveratrol and GABA inhibited I_GABA in oocytes with GABA_C receptors. The inhibition of I_GABA by resveratrol was in a reversible and concentration-dependent manner. The IC50 of resveratrol was 28.9±2.8 μM in oocytes expressing GABA_C receptor. The inhibition of I_GABA by resveratrol was in voltage-independent and non-competitive manner. These results indicate that resveratrol might regulate GABA_C receptor expression and that this regulation might be one of the pharmacological actions of resveratrol on the nervous system.

Key Words: GABA_C receptor, Ligand-gated ion channel, Resveratrol, Xenopus oocyte

INTRODUCTION

γ-aminobutyric acid (GABA) receptors are members of the Cys-loop family with ligand-gated ion channels, and the Cys-loop family includes proteins such as 5-hydroxytryptamine 3 (5-HT3), nicotinic acetylcholine, and glycine receptors [1]. The structure of the ligand-gated ion channels is similar to that of the pentameric ion channels. In addition, the ion channels make channel pores through the transmembrane 2 (TM2) domain [1]. The GABA receptors are classified into 3 types of receptors: GABA_A, GABA_B, and GABA_C. The GABA_A and GABA_C receptors function as anion selective channels that are permeable to chloride ions, whereas GABA_B receptors are G-protein-coupled receptors [2,3]. GABA_A receptors are composed of heteropentameric anion channels composed of α, β, γ, δ, ε, θ subunits; however GABA_C receptors can form homopentameric or heteropentameric anion channels by ρ subunits alone or with α 1 and γ 2 [2-4]. The GABA_A receptor is predominantly expressed in the central nervous system [5,6], whereas GABA_C receptors are mainly expressed in retinal bipolar cells [7,8] and have a lower abundance in cerebellum [9] and hippocampus [10]. They play an important role in vision, sleep, cognition, and memory [11].

Resveratrol is a phytoalexin found in grapes, red wine, and other berries (Fig. 1A) and is also produced as an anti-fungal chemical by plants [12]. The concentration of resveratrol in red wine is as high as 0.2–5.8 mg/l [13]. Resveratrol exhibits diverse physiological and pharmacological activities, such as anti-cancer, chemopreventive, anti-viral, cardio-protective, anti-aging, anti-inflammatory, and life-prolonging effects [13-15]. Resveratrol also has neuroprotective effects, and it attenuates neurodegenerative disorders such as Alzheimer’s disease [16]. It also attenuates neuronal cell death caused by in vitro or in vivo brain hypoxia or ischemic conditions [17,18]. Although ac-

ABBREVIATIONS: GABA, γ-aminobutyric acid; Res, resveratrol; I_GABA, GABA-mediated inward current.
cumulating evidence indicates that resveratrol has diverse beneficial properties, including protective effects on the nervous systems, relatively little is known about its effects on cells, especially with respect to the regulation of receptors involved in synaptic transmission.

Recently, we demonstrated that resveratrol regulates 5-HT3A receptor expression [19]. In the present study, we examined the effects of resveratrol on homomeric GABA\(_C\) receptor channel activity and found that resveratrol inhibits GABA\(_C\) receptor channel activity in a concentration-dependent, voltage-independent, and non-competitive manner. These results indicate that resveratrol might play a role in the regulation of GABA\(_C\) receptor channel activities.

**METHODS**

*Materials*

The cDNAs for human GABA\(_C\) receptor \(\rho 1\) subunit were purchased from Thermo Fisher Scientific Inc. (Wyman Street Waltham, MA, USA). Fig. 1A shows the chemical structure of resveratrol. Resveratrol used in this study was dissolved in dimethyl sulfoxide (DMSO) as previously reported [20] and was diluted with bath medium before use. Resveratrol was stored in the dark because it is light sensitive, and a fresh stock solution was prepared for every experiment. The final DMSO concentration was less than 0.1%. Resveratrol and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

*Oocyte preparation*

*Xenopus laevis* care and handling were performed in accordance to the guide for the Care and Use of Laboratory Animals, published by NIH, USA. Frogs underwent surgery only twice, separated by an interval of at least 3 weeks. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester for oocyte isolation. Oocytes were separated by collagenase treatment along with gentle shaking for 2 h in a CaCl\(_2\)-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Only stage 5 or 6 oocytes were collected and maintained at 18°C, with continuous gentle shaking in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 5 mM HEPES; pH 7.5) supplemented with 0.5 mM theophylline and 50 \(\mu\)g/ml gentamycin. All the solutions were changed daily. All the experiments were performed within 2–4 days following the isolation of the oocytes [21].

*Oocyte recording*

A single oocyte was placed in a small Plexiglas net chamber (0.5 ml) and was constantly superfused with ND96 medium in the presence or absence of GABA or resveratrol during recording. The microelectrodes were filled with 3 M KCl and had a resistance of 0.2–0.7 MΩ. Two-electrode voltage-clamp recordings were performed at room temperature using Oocyte Clamp (OC-725C, Warner Instrument) with Digidata 1200A. For most of the electrophysiological experiments, the oocytes were clamped at a holding potential of \(-80\) mV. For the current-voltage relationship, voltage ramps were applied from \(-100\) to \(+40\) mV for 300 ms.

*eRNA preparation of GABA\(_C\) receptor \(\rho 1\) and micro-injection*

A recombinant plasmid containing a human GABA\(_C\) receptor \(\rho 1\) cDNA insert was linearized by digestion with the appropriate restriction enzymes. The cRNAs from linearized templates were obtained by using an in vitro transcription kit (mMessage mMachine; Ambion, Austin, TX) with a T3 polymerase. The RNA was dissolved in RNase-free water at 1 \(\mu\)g/\(\mu\)l, divided into aliquots, and stored at \(-80\)°C until use. Oocytes were injected with \(\mathrm{H}_2\mathrm{O}\) or mouse glycine \(\alpha 1\) receptor cRNAs (5–10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). The injection pipette was pulled from the glass capillary tubing used for the recording electrodes, and the tip was broken to obtain an outer diameter of approximately 20 \(\mu\)m [21]. The final cRNA products were re-suspended with RNase-free water at a concentration of 1 \(\mu\)g/\(\mu\)l and stored at \(-80\)°C.

*Data analysis*

To obtain the concentration-response curve for GABA-induced current in the presence of resveratrol, the observed peak amplitudes were normalized and plotted, and then fitted to the Hill equation, described below, by using Origin software (Northampton, MA): \(\frac{y}{y_{max}}=\frac{[A]^n}{[A]^n+[IC_{50}]^n}\), where \(y\) represents percent (%) inhibition at a given concentration of resveratrol, \(y_{max}\) represents percent (%) maximal inhibition, \(IC_{50}\) is the concentration of resveratrol producing half-maximum inhibition of the control response to GABA, \([A]\) is the concentration of resveratrol, and \(n\) is the interaction coefficient. All the values are presented as the mean±S.E.M. The differences between mean values of control and that of resveratrol treatment data were analyzed using unpaired Student’s \(t\) test and one-way ANOVA test. A value of \(p<0.05\) was considered statistically significant.
**RESULTS**

**Effect of resveratrol on \(I_{\text{GABA}}\) in oocytes that express homomeric GABA\(C\) receptors**

The addition of GABA to the bathing solution induced a large inward current in oocytes injected with GABA\(C\) receptor, indicating that functional GABA\(C\) receptors were expressed by the oocytes (Fig. 1B). Resveratrol itself had no effect on oocytes with GABA\(C\) receptors at a holding potential of \(-80\) mV (Fig. 1B). Interestingly, pre-treatment of resveratrol induced a much larger inhibition of \(I_{\text{GABA}}\) than that after co-treatment (Fig. 2A and B, \(n=9\) from 3 frogs). The inhibition of \(I_{\text{GABA}}\) by resveratrol in oocytes with GABA\(C\) receptors was reversible (Fig. 2A). Thus, these results show that resveratrol may regulate GABA\(C\) receptors channel activity, although resveratrol itself had no effect on GABA\(C\) receptor channel activity.

**Concentration-dependent effect of resveratrol on \(I_{\text{GABA}}\) in oocytes with GABA\(C\) receptor**

Since pre-treatment of resveratrol enhanced the inhibition on \(I_{\text{GABA}}\) in oocytes that expressed GABA\(C\) receptor as compared to the \(I_{\text{GABA}}\) inhibition in co-treated oocytes, we examined the effects of resveratrol on \(I_{\text{GABA}}\) after the pre-treatment of resveratrol with GABA. In concentration-response experiments, the pre-treatment of resveratrol with GABA inhibited \(I_{\text{GABA}}\) in a concentration-dependent manner in oocytes with GABA\(C\) receptor (Fig. 2C). The IC\(_{50}\) of \(I_{\text{GABA}}\) was \(28.9\pm2.8\) \(\mu\)M for oocytes that expressed GABA\(C\) receptors \((n=6\) from 3 frogs) (Fig. 2D). Since the pre-application of resveratrol showed a stronger decrease of \(I_{\text{GABA}}\) than that observed for the co-application of resveratrol, we examined the time-dependent effects of resveratrol pre-application. As shown in Fig. 3A, the pre-application of resveratrol enhanced \(I_{\text{GABA}}\) inhibition and was time-dependent, while the time-dependent effects of resveratrol were almost saturated at 30 sec pre-application (Fig. 3B).

**Current-voltage relationship and voltage-independent inhibition of \(I_{\text{GABA}}\) in oocytes that express GABA\(C\) receptors mediated by resveratrol**

As shown in Fig. 4, the current-voltage relationship induced by GABA with voltage steps from \(-100\) to +40 mV showed a slight rectification at positive potentials in oocytes with GABA\(C\) receptors. The reversal potential of GABA\(C\) receptors was \(V_R=\sim-25.3\pm1.8\) mV, (mean\(\pm\)S.E.M., \(n=6\) from 3 frogs). Pre-treatment with resveratrol and GABA did not modify the potential reversal of GABA\(C\) receptor with a reduction of \(I_{\text{GABA}}\) \((n=6\) from 3 frogs). The inhibitory effect of resveratrol on \(I_{\text{GABA}}\) in oocytes that express GABA\(C\) receptors was independent of the membrane holding potential (Fig. 4B). Thus, resveratrol inhibited \(I_{\text{GABA}}\) by 75.0\% \((79.6\pm5.1, 79.6\pm3.1, 76.3\pm2.7, \text{and} 79.1\pm4.2\% \text{at} \sim-120, -90, -60, \text{and} -30 \text{mV membrane holding potential in oocytes with GABA}_C\text{ receptor, respectively (Fig. 4B, n=9~12, from 3 frog batches).}$$
Fig. 4. Current-voltage relationship and voltage-independent inhibition by Res. (A) Current-voltage relationships of $I_{GABA}$ inhibition by Res in GABAC receptor-expressing oocytes. Representative current-voltage relationships were obtained using voltage ramps of $-100$ to $+40$ mV for 300 ms at a holding potential of $-80$ mV. Voltage steps were applied before and after application of 2 μM GABA in the absence or presence of 100 μM Res. (B) Voltage-independent inhibition of $I_{GABA}$ in the GABAC receptors by Res. Inset: the values were obtained from the receptors in the presence or absence of 100 μM Res at the indicated membrane holding potentials.

Fig. 5. Concentration-dependent effects of GABA on Res-mediated inhibition of $I_{GABA}$. (A) The representative traces were obtained from the GABAC receptor-expressing oocytes. $I_{GABA}$ expression shown in the upper and lower panels was elicited at a holding potential of $-80$ mV by GABA at concentrations of 1 μM and 30 μM GABA respectively. (B) Concentration-response relationship of GABA with GABAC receptors treated with GABA (0.3∼30 μM) alone or with GABA plus pre-application of 30 μM or 100 μM Res. The $I_{GABA}$ of oocytes expressing the GABAC receptors was measured using the indicated concentration of GABA in the absence (□) or presence of 30 μM (○) or 100 μM (△). Res. Oocytes were exposed to GABA alone or to GABA with Res. Oocytes were voltage-clamped at a holding potential of $-80$ mV. Each point represents mean±S.E.M. (n=9∼12/group).

Noncompetitive inhibition of GABAC receptors by resveratrol

To further study the mechanism by which resveratrol inhibits $I_{GABA}$ in oocytes with GABAC receptors, we analyzed the effect of 100 μM resveratrol on $I_{GABA}$ evoked by various GABA concentrations on oocytes with GABAC receptors (Fig. 5). Pre-application of 30 or 100 μM resveratrol with different concentrations of GABA did not shift the dose-response curve of GABA to the right (ED50, from 2.2±0.3 to 2.4±0.1 and 3.1±0.2 μM and Hill coefficient, from 1.76 to 1.87 and 1.55) in oocytes expressing GABAC receptors, indicating that resveratrol regulates GABAC receptor channel activity in a non-competitive manner (n=9∼12 from 3 frogs; Fig. 5).

DISCUSSION

In the present study, we demonstrated that (1) co- or pre-treatment with resveratrol and GABA inhibited $I_{GABA}$ expression in human GABAC receptor-expressing oocytes in a reversible and concentration-dependent manner. (2) $I_{GABA}$ inhibition caused by resveratrol occurred in a non-competitive and voltage-independent manner in GABAC receptor-expressing oocytes, indicating that resveratrol could be associated with the inhibitory regulator of $I_{GABA}$ in GABAC receptor-expressing oocytes (Fig. 4 and 5).

However, our data was insufficient for elucidating the mechanisms for resveratrol inhibition of $I_{GABA}$ in GABAC receptor-expressing oocytes. The possibility that resveratrol may act as an open channel blocker of GABAC receptors seems unlikely because the inhibitory effect of resveratrol on $I_{GABA}$ in oocytes expressing GABAC receptors was not voltage-dependent (Fig. 4). It is known that open channel blockers such as local anesthetics or hexamethonium are strongly voltage dependent, due to the charge that they carry in the transmembrane electrical field [22-24].

Another possibility may be that resveratrol is a competitive inhibitor of GABAC receptors and inhibits the receptors by interacting with the receptor-binding site(s). Competition experiment data showed that the presence of resveratrol did not change the concentration of GABA in oocytes that express GABAC receptors without changing the Hill coefficient (Fig. 5). Thus, the non-competitive modulation of GABAC receptor channel activity of resveratrol shows that resveratrol might have other binding site(s) or interaction site(s), such as those of a non-competitive inhibitor, on the GABAC receptors.

Finally, the third possibility may be that resveratrol has binding sites that enable the regulation of GABAC receptor. In previous reports, we have demonstrated that the regulatory effects of resveratrol on homomeric 5-HT3 receptor...
channel activities were attenuated or abolished by site-directed mutations of amino acid residues of pre-transmembrane domain of 5-HT_{3} receptor [19]. On the basis of data from this study and that of the previous reports, we speculate that resveratrol achieves its effects through direct interactions with GABA_{C} receptors. Further studies are required to identify resveratrol binding site(s) on the GABA_{C} receptors.

Previous studies have shown that the effects of resveratrol on nervous system might be mediated by ligand-gated ion channels. For example, resveratrol-mediated neuroprotection against brain ischemia is inhibited by N-methyl-D-aspartate (NMDA) receptor antagonist [25]. Resveratrol also attenuates kainite-induced epilepsy [26]. In addition, resveratrol suppresses catecholamine secretion by inhibiting the ß3 ß4 nictinic acetylcholine receptor in adrenal medullary cells [27]. In addition, resveratrol potentiates 5-HT_{3}A receptor channel activity [19]. Thus, although resveratrol is known as a neuroprotective agent, the mechanisms of resveratrol-mediated regulation of receptor or ion channel activities at the cellular level are poorly understood.

GABA_{C} receptor is expressed in retina, thalamus, hippocampus, pituitary gland, and gut [7-10,28-30]. Its role may include visual processing, regulation of sleep-waking rhythms, pain perception, memory, learning, regulation of hormones, and neuroendocrine gastrointestinal secretion. Thus, although GABA_{C} receptor channel activity might be closely related with the regulation of visual processing and other brain functions, we currently do not understand the inhibition of GABA_{C}-receptor-mediated ion currents by resveratrol and its association with GABA_{C} receptor-related functions in nervous system. Further studies are required to determine how in vitro resveratrol-mediated inhibition of I_{GABA} is linked to GABA_{C} receptor-related in vivo pharmacology in the nervous system.

In summary, we found that resveratrol, an active ingredient found in grapes, inhibits the GABA_{C} receptor-mediated ion currents by interacting with sites that are distinct from the GABA-binding site(s), and that these results further indicate that the resveratrol-mediated GABA_{C} receptor regulation might be the cellular basis for its effects on the nervous system.

ACKNOWLEDGEMENTS

This paper was supported by the SMART Research Professor Program of Konkuk University.

REFERENCES

1. Jensen M, Schousbøe A, Ahring PK. Charge selectivity of the Cys-loop family of ligand-gated ion channels. J Neurochem. 2005;92:217-225.
2. Bormann J. The 'ABC' of GABA receptors. Trends Pharmacol Sci. 2000;21:16-19.
3. Chebib M, Johnston GA. GABA-activated ligand-gated ion channels: medicinal chemistry and molecular biology. J Med Chem. 2000;43:1427-1447.
4. Milligan CJ, Buckley NJ, Garret M, Deuchars J, Deuchars SA. Evidence for inhibition mediated by coassembly of GABA_{A} and GABA_{C} receptor subunits in native central neurons. J Neurosci. 2004;24:7241-7250.
5. Bloom FE, Iversen LL. Localizing 3H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature. 1971;231:628-630.
6. McCabe RT, Wamsley JK. Autoradiographic localization of subcomponents of the macromolecular GABA receptor complex. Life Sci. 1986;39:1937-1945.
7. Wissle H, Koulou P, Brandstätter JH, Fletcher EL, Becker CM. Glycine and GABA receptors in the mammalian retina. Vision Res. 1998;38:1411-1430.
8. McCall MA, Lukasiewicz PD, Gregg RG, Peachey NS. Elimination of the rho1 subunit abolishes GABA_{C}(C) receptor expression and alters visual processing in the mouse retina. J Neurosci. 2002;22:4163-4174.
9. Drew CA, Johnston GA, Weatherby RP. Picuculline-insensitive GABA receptors: studies on the binding of (-)-baclofen to rat arrebellar membranes. Neurosci Lett. 1984;52:317-321.
10. Strata F, Cherubini E. Transient expression of a novel type of GABA response in rat CA3 hippocampal neurons during development. J Physiol. 1994;480:493-503.
11. Johnston GA, Chebib M, Hanrahan JR, Mewett KN. GABA_{C}(C) receptors as drug targets. Curr Drug Targets CNS Neural Disord. 2003;2:260-268.
12. Langkase P, Pryce RJ. A new class of phytoalexins from grapevines. Experientia. 1973;33:151-152.
13. Dudley J, Das S, Mulhejseee S, Das DK. Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose. J Nair Biochem. 2009;20:443-452.
14. Pervazi S. Resveratrol: from grapevines to mammalian biology. FASEB J. 2003;17:1985-1985.
15. Valenzano DR, Terzilasi E, Genade T, Cattaneo A, Donenici L, Cellerino A. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol. 2006;16:296-300.
16. Chen J, Zhou Y, Mueller-Steiner S, Chen LF, Kwon H, Yi S, Mucke L, Gan L. SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. J Biol Chem. 2005;280:40364-40374.
17. West T, Atzeva M, Holtzman DM. Pomegranate polyphenols and resveratrol protect the neontal brain against hypoxic-ischemic injury. Dev Neurosci. 2007;29:363-372.
18. Raval AP, Dave KR, Perez-Pinzon MA. Resveratrol mimics ischemic preconditioning in the brain. J Cereb Blood Flow Metab. 2006;26:1141-1147.
19. Lee BH, Hwang SH, Choi SH, Shin TJ, Kang J, Lee SM, Nah SY. Resveratrol enhances 5-hydroxytryptamine type 3A receptor-mediated currents: the role of arginine 222 residue in pre-transmembrane domain 1. Biol Pharm Bull. 2011;34:523-527.
20. Chan MM. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochem Pharmacol. 2002;63:99-104.
21. Lee BH, Shin TJ, Hwang SH, Choi SH, Kang J, Kim HJ, Park CW, Lee SH, Nah SY. Inhibitory effects of quercetin on muscle-type of nicotinic acetylcholine receptor-mediated ion currents expressed in Xenopus oocytes. Korean J Physiol Pharmacol. 2011;15:195-201.
22. Sine SM, Taylor P. Local anesthetics and histrionotoxins are allosteric inhibitors of the acetylcholine receptor. Studies of clonal muscle cells. J Biol Chem. 1982;257:8106-8104.
23. Heidmann T, Oswald RE, Changeux JP. Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from torpedo marmorata. Biochemistry. 1980;19:3112-3127.
24. Arias HR. Luminal and non-luminal non-competitive inhibitor binding sites on the nicotinic acetylcholine receptor. Mol Membr Biol. 1996;13:1-17.
25. Saleh MC, Connell BJ, Saleh TM. Resveratrol preconditioning induces cellular stress proteins and is mediated via NMDA and estrogen receptors. Neuroscience. 2010;166:445-454.
26. Wu Z, Xu Q, Zhang L, Kong D, Ma R, Wang L. Protective effect of resveratrol against kainate-induced temporal lobe epilepsy in rats. Neurochem Res. 2009;34:1390-1400.
27. Shinohara Y, Toyohira Y, Ueno S, Liu M, Tsutsui M, Yanagihara N. Effects of resveratrol, a grape polyphenol, on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. *Biochem Pharmacol.* 2007;74:1608-1618.

28. Boue-Grabot E, Taupignon A, Tramu G, Garret M. Molecular and electrophysiological evidence for a GABAC receptor in thyrotropin-secreting cells. *Endocrinology.* 2000;141:1627-1632.

29. Jansen A, Hoepfner M, Herzig KH, Riecken EO, Scherübl H. GABA(C) receptors in neuroendocrine gut cells: a new GABA-binding site in the gut. *Pflugers Arch.* 2000;441:294-300.

30. Chebib M. GABAC receptor ion channels. *Clin Exp Pharmacol Physiol.* 2004;31:800-804.