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Short Communication

Honeybee Royal Jelly and Nobiletin Stimulate CRE-Mediated Transcription in ERK-Independent and -Dependent Fashions, Respectively, in PC12D Cells

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Abstract. To prove the pharmacological actions of honeybee royal jelly (RJ) on the nervous system, we examined the effects of RJ on CRE-mediated transcription. RJ increased CRE-mediated transcription in PC12D cells. Moreover, CRE-mediated transcriptional activity by RJ was enhanced by nobiletin. U0126, a MEK inhibitor, inhibited CRE-mediated transcription by combining RJ plus nobiletin without affecting transcription by RJ alone. These results suggest that RJ stimulates CRE-mediated transcription via an ERK-independent cascade, whereas the increasing CRE-mediated transcriptional effect by nobiletin is dependent on ERK phosphorylation. Combining RJ plus nobiletin may activate effectively neuronal functions via enhancement of CRE-mediated transcription.

Keywords: honeybee royal jelly, CRE-mediated transcription, Alzheimer's disease

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is characterized by multiple cognitive deficits, including memory loss, with a devastating impact on the whole society. Consequently, a tremendous effort is being devoted to the development of drugs that prevent or delay neurodegeneration in the brains of patients with AD (1). With respect to the impaired cognition and memory in neurodegenerative disease of the Alzheimer type, much attention has been paid especially to cyclic AMP (cAMP) / cAMP-dependent protein kinase (PKA) / cAMP-response element (CRE)-binding protein (CREB)–dependent signaling linked to CRE-mediated transcription, which plays an essential role in a variety of species ranging from *Drosophila* to mammals (2). Accumulating evidence indicates that CRE-mediated transcription-mediated signaling plays a crucial role in hippocampal long-term potentiation associated with learning and memory (3).

Large numbers of natural resources have provided not only useful pharmacological tools (4 – 6) but also novel leading compounds for drug development (7). In the course of our survey of substances having the activity to potentiate CRE-mediated transcription to repair dysregu-

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lated neuronal functions from natural resources, we examined the effects of honeybee royal jelly (RJ) on CRE-mediated transcription in PC12D cells. RJ is the gelatinous substance secreted from young nurse worker bees (*Apis mellifera*) that is the sole food for the queen bee. The secreted substance is believed to be a functionally active supplement not only for the bees but also for humans. In support of this idea, RJ has been shown to contain a wide variety of biologically active compounds, including free amino acids, proteins, short chain hydroxy fatty acids, and vitamins (8). Moreover, RJ has been actually reported to be associated with many health-promoting activities (9, 10).

On the other hand, our previous study showed that nobiletin, a polymethoxylated flavone from the peel of *Citrus depressa*, stimulated CRE-dependent transcription via a cAMP/PKA/MEK/ERK/MAP-kinase signaling cascade, modulating neuronal gene expression, and thereby protected neuronal function in PC12D cells (11). Moreover, we also reported that nobiletin rescues olfactory-bulbectomized (OBX)-induced cholinergic neurodegeneration, accompanied by improvement of impaired memory in OBX mice (12).

Here we describe the evidence that RJ has the activity to enhance CRE-mediated transcription in PC12D cells. Moreover, for the first time, we find that nobiletin enhances the CRE-mediated transcriptional activity by RJ via the ERK/MAP-kinase signaling cascade.

Native RJ was produced by Yamaguchi's organic beeculture (13) and thereafter immediately frozen. The frozen RJ was dried and provided as a frozen and dried powder by Japan Royal Jelly Co., Ltd., R&D center (Miyagi). The frozen and dried powder of RJ was dissolved in phosphate-buffered saline (PBS) with rotating tubes overnight to prepare 25% (w/v) solutions. The RJ solution was then centrifuged at 12,000 × *g* for 10 min and the supernatant was further sterilized by filtration (14). These sterilized solutions were stored at −20°C until use. Nobiletin was extracted and isolated as described previously (11). PC12D cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum in a 5% CO_2 incubator at 37°C (11). For transient transfection and reporter gene assay, cells were seeded in 48-well plates at a cell density of 8.0×10^4 cells per well and cultured for 24 h. Transfection and reporter gene assay were conducted as described previously (11). pCRE, firefly luciferase reporter plasmids containing CRE sequence inserted into the upstream of a TATA-like promoter region taken from herpes simplex thymidine kinase promoter, were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). A Renilla (seapansy) luciferase control vector, phRG-TK (Promega, Madison, WI, USA), was also used as an internal control to normalize difference in transfection efficiency. PC12D cells were transfected with 0.2 μg of pCRE and 0.04 μg of phRG-TK per well using LipofectAMINE 2000 (Invitrogen Corporation, Carlsbad, CA, USA). Thereafter cells were harvested to assay the activities of firefly and seapansy luciferase by using a dual luciferase assay kit (Promega). For immunoblotting, PC12D cells were seeded in 35-mm dish at a density of 1×10^6 cells/dish. After 24 h, cells were treated with RJ and the indicated compounds for 15 min. Cells were washed with PBS and lysed with lysis buffer [10 mM HEPES, 1 mM EDTA, 1% SDS, 1 × protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 0.32 μM okadaic acid, 0.1 μM calyculin A, 0.8 mM NaF, and 0.25 mM sodium orthovanadate, pH 7.4]. Lysates were heated in Laemmli sample buffer, electrophoresed on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After incubation with Tris-buffered saline (10 mM Tris-HCl and 100 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 5% skim milk for 2 h, the blot was incubated with anti-phospho-ERK or ERK antibodies (dilution 1:2000) at 4°C overnight, followed by incubation with anti-rabbit IgG antibody HRP-linked in a dilution of 1:2000 for 2 h. These antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence detection reagents (Thermo Scientific, Rockford, IL, USA). Data are presented as means ± S.E.M. Differences were compared using Student's *t*-test or one-way ANOVA followed by the Bonferroni correction for multiple comparisons. A level of $P < 0.05$ was considered to be statistically significant.

RJ appreciably stimulated CRE-dependent transcription in PC12D cells as determined by reporter gene assay. In fact, RJ concentration-dependently increased CRE-dependent transcription in PC12D cells; it stimulated CRE-dependent transcription at of 100 μg/ml with an EC_{50} value of approximately 200 μg/ml (Fig. 1). RJ had no cytotoxicity at concentrations ranging from 50 to 2,400 μg/ml (data not shown).

We next examined the effects of combining RJ plus nobiletin on stimulated CRE-dependent transcription and ERK phosphorylation in PC12D cells. As shown in Fig. 2A, CRE-mediated transcriptional activity significantly increased in the combination group [100 μg/ml RJ plus 30 μM nobiletin (25.7 ± 2.4)] compared to that in the RJ alone group [100 μg/ml RJ alone (14.6 ± 0.4)]. Next, we examined by immunoblotting the effects of RJ alone or the combination of RJ plus nobiletin on ERK phosphorylation in PC12D cells (Fig. 2B). Consistent with the results of CRE-mediated transcriptional activity de-

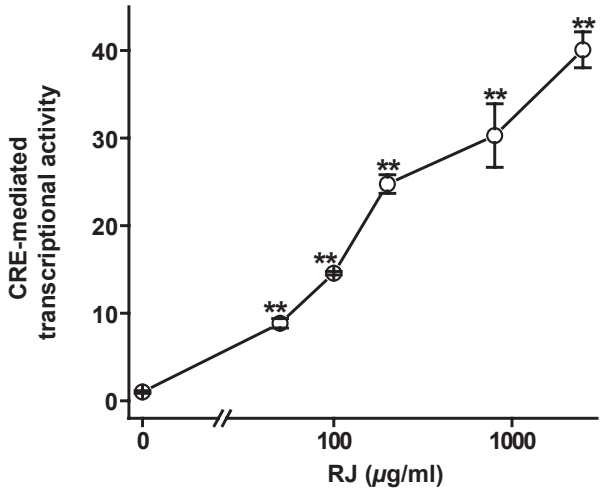

Fig. 1. Concentration-dependent stimulatory effects of RJ on CRE-dependent transcription in PC12D cells. Cells were seeded in 48-well plates at a cell density of 8.0×10^4 cells per well and cultured for 24 h. Cells were then subjected to transfection with pCRE reporter gene followed by treatment with RJ at the indicated concentrations for 5 h. Values are means ± S.E.M. (n = 4). RJ: royal jelly. $^{**}P < 0.01$ vs. vehicle control.

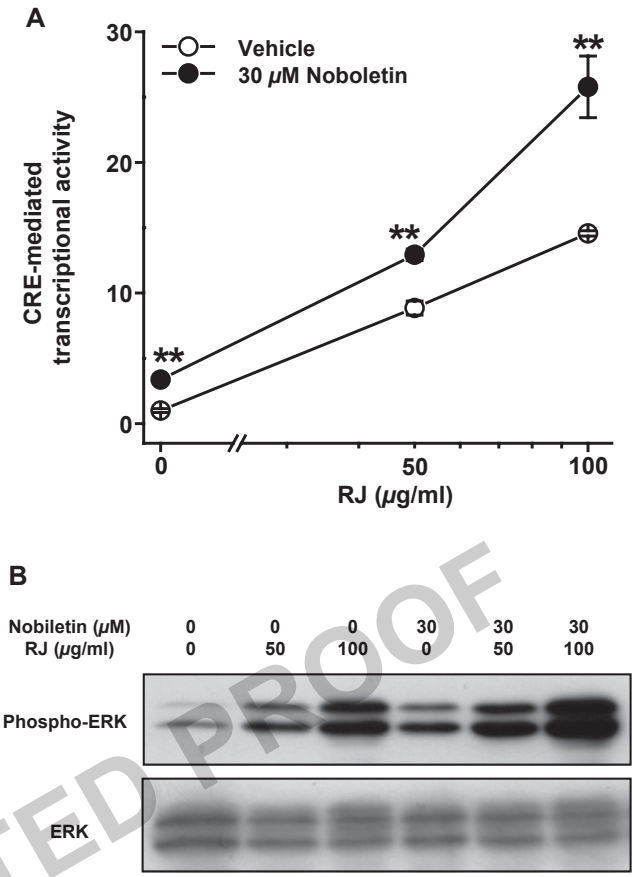

Fig. 2. Effects of combining RJ plus nobiletin on CRE-dependent transcription (A) and ERK phosphorylation (B) in PC12D cells. A: Effects of combining RJ plus nobiletin on CRE-dependent transcription. Cells were seeded in 48-well plates at a cell density of 8.0×10^4 cells per well and cultured for 24 h. Cells were then subjected to transfection with pCRE reporter genes followed by treatment with RJ at the indicated concentrations in the presence (closed circle) or absence (open circle) of 30 μM nobiletin for 5 h. RJ: royal jelly. Values are means ± S.E.M. (n = 4). $^{**}P < 0.01$ vs. RJ alone. B: Effects of combining RJ plus nobiletin on ERK phosphorylation. PC12D cells were treated with several concentrations of RJ and nobiletin for 15 min. The phosphorylation of ERK was assessed by immunoblotting. The samples were fractionated on a 10% polyacrylamide gel, followed by immunoblotting with anti-phospho-ERK antibody. ERK was used as the internal control for protein loading and transfer efficiency.

scribed above, RJ induced ERK phosphorylation in a concentration-dependent manner and nobiletin enhanced ERK phosphorylation by RJ in PC12D cells.

To evaluate the mechanism of CRE-dependent transcription by RJ alone or the combination of RJ plus nobiletin, we examined the effects of MEK- and PKA-inhibitors on CRE-mediated transcription and ERK phosphorylation by RJ or RJ plus nobiletin. ERK phosphorylation by RJ or RJ plus nobiletin was prevented by 10 μM U0126 (a MEK inhibitor) (Fig. 3A). Interestingly, for the reporter gene assay, U0126 had no effect on CRE-mediated transcription by RJ alone, whereas CRE-mediated transcription by RJ plus nobiletin was inhibited by U0126 (Fig. 3B). U0126 alone did not affect CRE-mediated transcription (data not shown), suggesting that the decreasing the CRE-mediated transcriptional effect by U0126 depended on the inhibitory effect of ERK phosphorylation by nobiletin. These results suggest that RJ stimulates CRE-mediated transcription via an ERK cascade–independent mechanism and that increasing the CRE-mediated transcriptional effect by nobiletin was dependent on ERK phosphorylation. H89 (PKA inhibitor) at the concentration of 10 μM had no effects on ERK phosphorylation and CRE-mediated transcription by RJ, while both the effects of ERK phosphorylation and CRE-mediated transcription by combining RJ plus nobiletin were inhibited slightly but not significantly by H89 (Fig. 3: A, B).

In our previous research, 4′-demethylnobiletin, a major metabolite of nobiletin, stimulates ERK phosphorylation and enhances CRE-mediated transcription by activating a PKA/MEK/ERK pathway in cultured hippocampal neurons. Both of the effects were inhibited by U0126 and H89 (15). In our present study, both U0126 and H89 did not affect the CRE-mediated transcriptional activity by RJ, although RJ induced ERK phosphorylation. It is thought that RJ contains a wide variety of active compounds and that these active compounds enhance CRE-mediated transcription synergistically. Currently, the detailed mechanism on stimulation of CRE-dependent

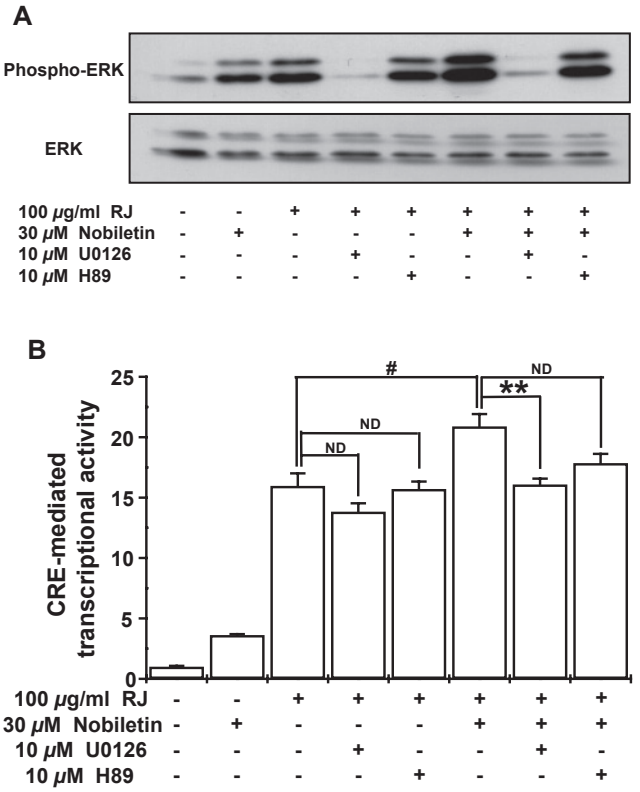

Fig. 3. Effects of protein kinase inhibitors on ERK phosphorylation (A) and CRE-dependent transcription (B) by combining RJ plus nobiletin in PC12D cells. A: Effects of protein kinase inhibitors on ERK phosphorylation. PC12D cells were treated with 100 μg/ml RJ, 30 μM nobiletin, and the protein kinase inhibitors for 15 min. The phosphorylation of ERK was assessed by immunoblotting. The samples were fractionated on a 10% polyacrylamide gel, followed by immunoblotting with anti-phospho-ERK antibody. ERK was used as the internal control for protein loading and transfer efficiency. B: Effects of protein kinase inhibitors on CRE-dependent transcription. Cells were seeded in 48-well plates at a cell density of 8.0×10^4 cells per well and cultured for 24 h. Cells were then subjected to transfection with pCRE reporter genes followed by treatment with 100 μg/ml RJ, 30 μM nobiletin, and the inhibitors for 5 h. Values are means ± S.E.M. (n = 4). $^{\#}P < 0.05$ and $^{**}P < 0.01$ vs. 100 μg/ml RJ plus 30 μM nobiletin–treatment cells. ND: No differences. RJ: royal jelly.

transcription by RJ and the isolation of active compounds are under investigation.

In conclusion, we found that RJ had the ability to stimulate CRE-mediated transcription via an ERK phosphorylation–independent mechanism in PC12D cells. It is of interest that the combined use of RJ plus nobiletin is more beneficial than RJ alone on ERK phosphorylation and CRE-mediated transcription.

References

1 Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 2002;297:353–356.

2 Gong B, Vitolo OV, Trinchese F, Liu S, Shelanski M, Arancio O. Persistent improvement in synaptic and cognitive functions in an Alzheimer mouse model after rolipram treatment. J Clin Invest. 2004;114:1624–1634.

3 Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron. 1996;16:973–982.

4 Fujiwara H, Yamakuni T, Ueno M, Ishizuka M, Shinkawa T, Ohizumi Y, et al. IC101 induces apoptosis by Akt dephosphorylation via an inhibition of heat shock protein 90-ATP binding activity accompanied by preventing the interaction with Akt in L1210 cells. J Pharmacol Exp Ther. 2004;310:1288–1295.

5 Fujiwara H, Saito SY, Hitotsuyanagi Y, Takeya K, Ohizumi Y. RA-VII, a cyclic depsipeptide, changes the conformational structure of actin to cause G_2 arrest by the inhibition of cytokinesis. Cancer Lett. 2004;209:223–229.

6 Ohizumi Y. Application of physiologically active substances isolated from natural resources to pharmacological studies. Jpn J Pharmacol. 1997;73:263–289.

7 Liu J. FK506 and cyclosporin: molecular probes for studying intracellular signal transduction. Trends Pharmacol Sci. 1993;14:182–188.

8 Hove SR, Dimick PS, Benton AW. Composition of freshly harvested and commercial royal jelly. J Apic Res. 1985;24:52–61.

9 Tokunaga KH, Yoshida C, Suzuki KM, Maruyama H, Futamura Y, Mishima S, et al. Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. Biol Pharm Bull. 2004;27:189–192.

10 Miyata T. Pharmacological basis of traditional medicines and health supplements as curatives. J Pharmacol Sci. 2007;103:127–131.

11 Nagase H, Omae N, Omori A, Nakagawasai O, Tadano T, Ohizumi Y, et al. Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities. Biochem Biophys Res Commun. 2005;337:1330–1336.

12 Nakajima A, Yamakuni T, Haraguchi M, Tadano T, Mimaki Y, Ohizumi Y, et al. Nobiletin, a citrus flavonoid that improves memory impairment, rescues bulbectomy-induced cholinergic neurodegeneration in mice. J Pharmacol Sci. 2007;105:122–126.

13 Yamaguchi K. Innovation of natural beekeeping and quality control of bee products for promising apiculture. 1. Current problems of beekeeping and recurrence of natural bee-culture for resolution of the problems. J Yunnan Agric Univ. 2004;19:322–329.

14 Hattori N, Nomoto H, Mishima S, Inagaki S, Goto M, Furukawa S, et al. Identification of AMP N1-oxide in royal jelly as a component neurotrophic toward cultured rat pheochromocytoma PC12 cells. Biosci Biotechnol Biochem. 2006;70:897–906.

15 Al Rahim M, Nakajima A, Saigusa D, Tetsu N, Ohizumi Y, Yamakuni T, et al. 4′-Demethylnobiletin, a bioactive metabolite of nobiletin enhancing PKA/ERK/CREB signaling, rescues learning impairment associated with NMDA receptor antagonism via stimulation of the ERK cascade. Biochemistry. 2009;48:7713–7721.