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Royal jelly and its unique fatty acid, 10-hydroxy-trans-2-decenoic acid, promote neurogenesis by neural stem/progenitor cells *in vitro*

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ABSTRACT

Neural stem/progenitor cells (NSCs) proliferate vigorously as neurospheres in medium containing basic fibroblast growth factor (FGF-2), but start differentiating into neurons, astrocytes or oligodendrocytes in FGF-2-free medium. An extract of royal jelly (RJ) significantly increased the percentage in the total cell population of not only neurons immunoreactive for class III β-tubulin (Tuj1) but also astrocytes immunoreactive for glial fibrillary acidic protein (GFAP), and oligodendrocytes immunoreactive for 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) generated from NSCs, but decreased that of nestin-positive NSCs. These results highlight a novel and outstanding property of the RJ, *i.e.*, that it facilitates the differentiation of all types of brain cells (neurons, astrocytes, and oligodendrocytes). On the other hand, 10-hydroxy-trans-2-decenoic acid (HDEA), an unsaturated fatty acid characteristic of RJ, increased the generation of neurons and decreased that of astrocytes from NSCs. These observations suggest that RJ contains plural components that differently influence neuronal and/or glial lineages and that HDEA is one of such components of RJ that facilitates neurogenesis by NSCs.

Neural stem cells/progenitor cells (NSCs) have self-renewal capacity and multipotent activity to differentiate into neurons, astrocytes, and oligodendrocytes during development (4, 23). Besides being present in the developing embryonic brain, NSCs also reside in the adult forebrain (4, 28), constitutively give rise to proliferating progenitor cells (21), and differentiate into neurons (17, 18), suggesting that the injured brain has the capacity for self-repair by activated NSCs. Therefore, NSCs in the mature brain are a promising target for therapy of degenerative neurological disorders including Alzheimer's and/or Parkinson's disease.

Royal jelly (RJ), which is fed to the queen honeybee, has a variety of biological activities towards various types of cells. For instance, RJ exhibits immunomodulatory properties (3, 22, 24) and inhibits the development of atoptic dermatitis-like skin lesions (26). Earlier we found that RJ had the ability to induce neurites from cultured rat pheochromocytoma PC12 cells (10), which prompted us to test the effects of RJ and its components on NSCs cultured from the central nervous system (CNS). RJ consists of proteins, sugars, lipids, vitamins, and free amino acids (2, 25), and includes various other components such as the unsaturated fatty acid 10-hydroxy-trans-2-decenoic acid (HDEA) (20). HDEA has been reported to have many pharmacological activities such as anti-tumor activity (27), collagen production-promoting activity (15), and anti-biotic activity (1), but its biological activities on CNS have remained unknown.

Therefore, in the present study we examined if HDEA could regulate cell lineages of NSCs, be-

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cause HDEA is uniquely and abundantly present in RJ (25), and docosahexaenoic acid (DHA), an unsaturated fatty acid with a 22-carbon chain, has been recently reported to promote neurogenesis by NSCs (14) (see Fig. 1). In addition we examined the influence of RJ itself on the differentiation of cultured NSCs. Our results demonstrated that RJ facilitated the generation of all types of brain cells, *i.e.*, neurons, astrocytes and oligodendrocytes and that HDEA increased neuronal, but decreased glial, generation. These results highlight a novel and outstanding property of RJ.

MATERIALS AND METHODS

RJ and fatty acid. RJ (originated from *Apis melifera*) and its unique fatty acid, HDEA, was a generous gift from Api Co. Ltd., Gifu, Japan. RJ was mixed with phosphate-buffered saline (PBS) (25% w/v) and shaken slowly overnight at 4°C. The mixture was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant (PBS-extract of RJ: PERJ) was further diluted with culture medium and used in the experiments. The dilution of RJ is expressed as that from the original product.

Primary cultures. NSCs were obtained from the telencephalon of 15.5-day-old rat embryos, and cultured in proliferation medium consisting of equal volumes of Dulbecco's modified Eagle's minimum essential medium and F12 medium supplemented with insulin (25 μg/mL), apo-transferrin (100 μg/mL), progesterone (20 nM), putrescine (100 μM), selenium (30 nM), and basic fibroblast growth factor (FGF-2; 10 ng/mL), as described previously (12). Cultures were replenished with fresh proliferation medium every day, and passaged 5 days after seeding. For the differentiation experiments, the cells were plated on coverslips coated with poly-L-ornithine, and cultured in FGF-2-free proliferation medium (differentiation medium).

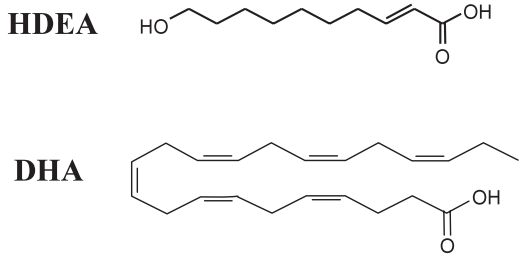

Fig. 1 Chemical structures of 10-hydroxy-trans-2-decenoic acid (HDEA) and docosahexaenoic acid (DHA).

Immunocytochemical procedures. For fluorescence immunostaining, cells were fixed for 3 min by adding an equal volume of 4% (w/v) paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) to the culture medium, and post-fixed for 10 min with the same fixative solution. After washings with PBS, the cells were treated with PBS containing 2% (w/v) skim milk for 30 min to reduce non-specific antibody binding. They were then reacted with primary antibody at 4°C overnight. The primary antibodies included anti-neuron-specific class III β-tubulin (Tuj1) mouse antibody (R & D Systems, Minneapolis, MN, USA), anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) mouse antibody (Sigma, St. Louis, MO, USA), anti-glial fibrillary acidic protein (GFAP) rabbit antibody (Dako, Copenhagen, Denmark), and anti-nestin mouse antibody (BD Biosciences Pharmingen, San Diego, CA, USA). After washings for 5 min with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit IgG donkey antibodies (1.0 μg /mL, Chemicon, Temecula, CA, USA) for 2 h at room temperature, washed with PBS, and mounted in Permafluor (Thermo Fisher; Waltham, MA, USA).

RESULTS AND DISCUSSION

Intracellular signaling of PERJ

Previously we observed that PERJ activates mitogen-activated protein kinase (MAPK) (9, 10) and protein kinase A (PKA) (Hattori *et al.*, unpublished results) in cultured rat pheochromocytoma PC12 cells by acting predominantly through adenosine A_{2A} receptors. Therefore, the effects of PERJ on extracellular signal-regulated kinase 1 or 2 (ERK1/2) and cAMP-response element-binding protein (CREB) in cultured NSCs were evaluated, because activations of ERK1/2 and CREB are checkpoints to assess the activation of the Ras/MAPK cascade and PKA pathways, respectively. PERJ at a 500-fold- or 100-fold dilution enhanced the phosphorylation of both ERK1/2 and CREB (Fig. 2), demonstrating that the PERJ was effective on NSCs at these concentrations.

Effects of PERJ on differentiation of NSCs

It is well known that NSCs proliferate vigorously as neurospheres in the presence of FGF-2, but stop proliferation and differentiate into neurons, astrocytes or oligodendrocytes when FGF-2 is withdrawn from the culture medium (4, 12, 23). Primary NSCs maintained in the proliferation medium were transferred to the differentiation medium (FGF-2-free)

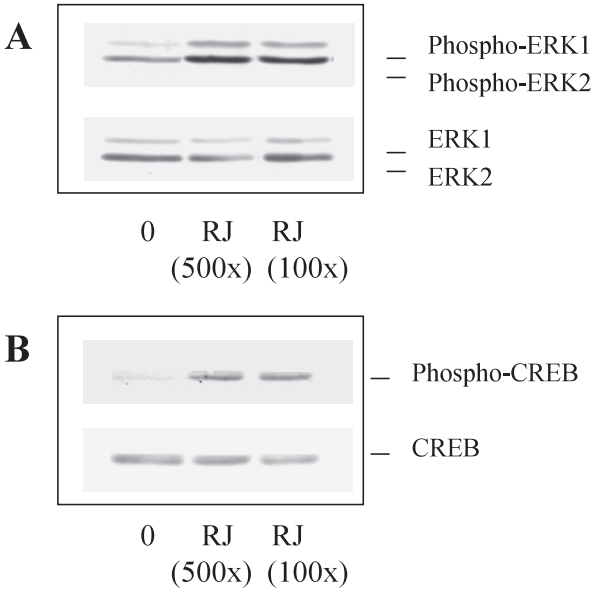

Fig. 2 RJ stimulates the phosphorylation of both ERK1/2 and CREB in cultured NSCs. NSCs growing as neurospheres were plated on poly-L-ornithine-coated 6-well plates, and cultured in the proliferation medium for 2 days. Then, the cells were incubated with 500 or 100 fold-diluted PERJ, and harvested 30 min after the administration. Cell protein (5 μg) was separated on a 10% gel of SDS-PAGE, and ERK1/2, its phosphorylated forms (A), and CREB and its phosphorylated forms (B) were detected by Western immunoblotting using the respective specific antibodies.

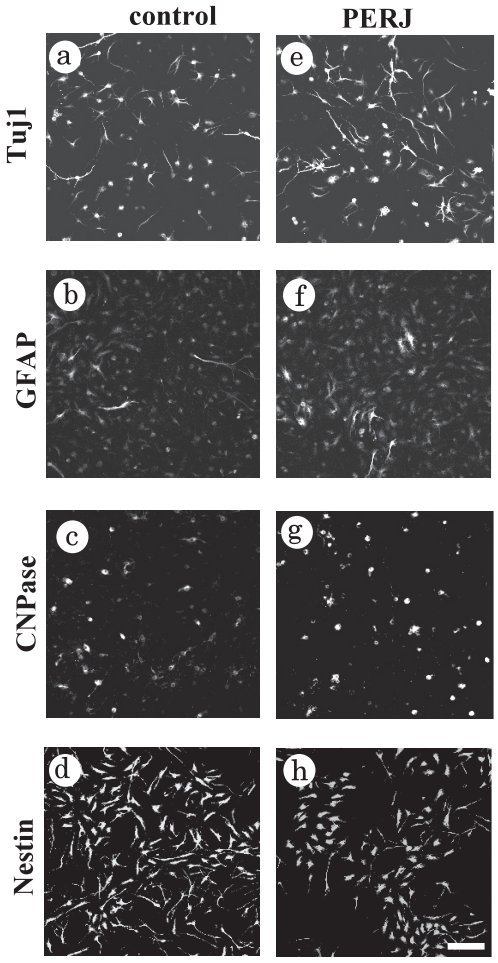

Fig. 3 Photographs of cells expressing the differentiation marker of neurons, astrocytes, oligodendrocytes or the undifferentiated NSCs before and after treatment with RJ. Dissociated cell suspensions of NSCs were plated on coverslips coated with poly-L-ornithine. The cells were maintained for 2 days in the proliferation medium, and then transferred to the differentiation medium containing vehicle (a, b, c, d) or 500 fold-diluted PERJ (e, f, g, h) and cultured for another 5 days. The fixed cells were then reacted with antibody against Tuj1 (a, e), GFAP (b, f), CNPase (c, g) or nestin (d, h) and subsequently incubated with Alexa Fluor 488-conjugated secondary antibodies. Scale bar indicates 50 μm.

containing or lacking PERJ, and cultured for another 5 days. In the control experiment without PERJ (Fig. 4B), the percentage of neurons, astrocytes or oligodendrocytes generated from NSCs in the total cell population was comparable with that shown in our previous report (12), demonstrating that present experimental conditions were sound and the NSCs were available for the purpose. We used 500 fold-diluted PERJ for the experiment, because significant phosphorylation of ERK1/2 and/or CREB was obtained at that concentration, as shown in Fig. 2. PERJ significantly increased the percentage of not only Tuj1-positive cells (neurons) but also GFAP-positive cells (astrocytes) and CNPase-positive cells (oligodendrocytes) in the total cell population, and decreased that of nestin-positive cells (undifferentiated cells; Figs. 3, 4). It is important to clarify the mechanism underlying the actions of PERJ toward NSCs. Therefore, we examined the ratio of nestin-positive cells to total cells after treatment with PERJ in the proliferation or differentiation medium. The ratio was essentially the same in both circumstances (Fig. 4), suggesting that the PERJ facilitated cell differentiation rather than maintained cell survival. If the PERJ could allow the differentiated neurons, astrocytes or oligodendrocytes to survive more effectively, the treatment before FGF-2 removal would not affect the generation of these differentiated cells. Namely, the PERJ was likely to increase the generation of the differentiated cells and decrease the number of undifferentiated cells such as nestin-positive cells. These results highlight a novel and outstanding property of the PERJ, namely, that it facilitates the differentiation of all types of brain cells, *i.e.*,

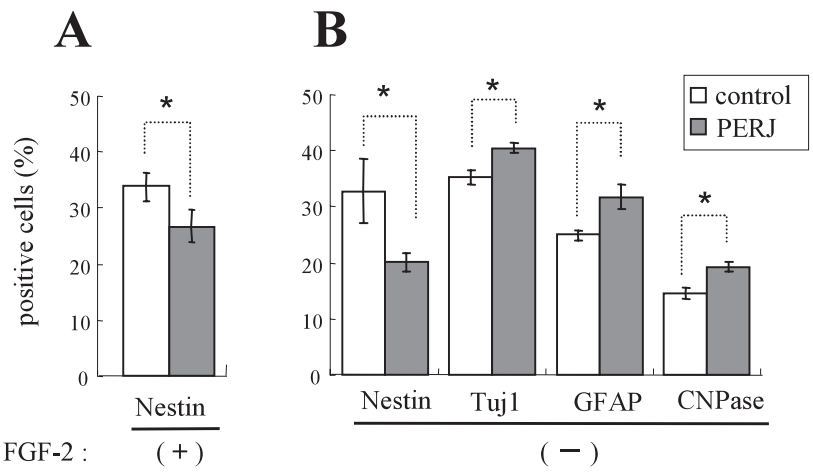

Fig. 4 RJ increases the ratio of the cells expressing neuronal, astrocytic or oligodendrocytic cell marker, and decreases the ratio of the undifferentiated cells. Dissociated cell suspensions of NSCs were plated on coverslips coated with poly-L-ornithine. A: The cells were cultured in the proliferation medium (containing 10 ng/mL FGF-2) with 500 fold-diluted PERJ or vehicle (control) for 2 days, and then cultured for another 5 days in the differentiation medium (FGF-2-free) without PERJ. B: The cells were cultured for 2 days in the proliferation medium, and then transferred to the differentiation medium containing 500 fold-diluted PERJ or vehicle (control) and cultured for another 5 days. The fixed cells were then reacted with antibody against Tuj1, GFAP, CNPase or nestin, and subsequently incubated with Alexa Fluor 488-conjugated secondary antibodies. The number of antigen-positive cells was counted, and the ratio to total cells was calculated. The values are expressed as the mean + SE (n = 4). Significance of differences from the value of PBS-treated cells was determined by Student's *t*-test for each antigen. Significance, *$p < 0.05$.

neurons, astrocytes, and oligodendrocytes, suggesting that RJ contains plural active components that influence the respective cell lineages differently.

Effects of HDEA on NSCs

We next examined the effect of HDEA on NSCs, because it is a unique fatty acid of RJ (Fig. 1). Exposure of the cells to HDEA after the removal of FGF-2 increased the percentage of Tuj1-positive cells and simultaneously decreased that of GFAP- or CNPase-positive cells (Fig. 5). This reciprocal response between neuronal and glial populations suggests that HDEA affected a neuronal lineage of the neural progenitor cells having the ability to generate both neuronal and glial cells. We previously observed that brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of neurotrophic factors, similarly affected cultured NSCs (12). Thus, HDEA supposedly committed neural progenitors to the fate of neuronal lineage similarly as BDNF.

Kawakita *et al.* (14) recently reported that docosahexaenoic acid (DHA) facilitates neuronal differentiation of NSCs in culture, and directly stimulates the neurogenesis in the dentate gyrus of the hippocampus. DHA is one of the predominant polyunsaturated fatty acids in the brain, having 22 carbons and belonging to the ω-3 fatty acid group (Fig. 1). DHA is essential for normal brain development (7), and its chronic oral administration enhances long-term memory in young and aged rats (5, 6). A reduction in DHA concentration in brain impairs the spatial learning tasks regulated by olfactory bulb, where neurogenesis occurs in adulthood (8). Thus, DHA is suggested to play crucial roles in brain development and to act to maintain brain function. It is interesting that dietary DHA was protective and ameliorated impairment of spatial cognition learning ability in amyloid-beta-infused rats (11). As suggested by this present study, HDEA may stimulate neurogenesis in the mature brain, and play roles similar to those of DHA. HDEA might have more merits to pass through blood-brain barrier, because HDEA is an unsaturated fatty acid smaller than DHA.

Neurogenesis is promoted by basic helix-loop-helix transcription factors Mash1, Math1 and/or NeuroD but suppressed by another set, Hes1 and Hes5 (13, 16, 19). Our previous report demonstrated that BDNF facilitated the levels of Mash1, Math1, and NeuroD mRNAs in cultured NSCs and resulted in substantial neuronal differentiation (12). Although the nature of the intracellular signals of HDEA that regulate the fate of NSCs is still unknown, HDEA is likely to partly mimic the effects of BDNF.

Our present results may be a clue to understand a variety of biological activities of RJ toward the

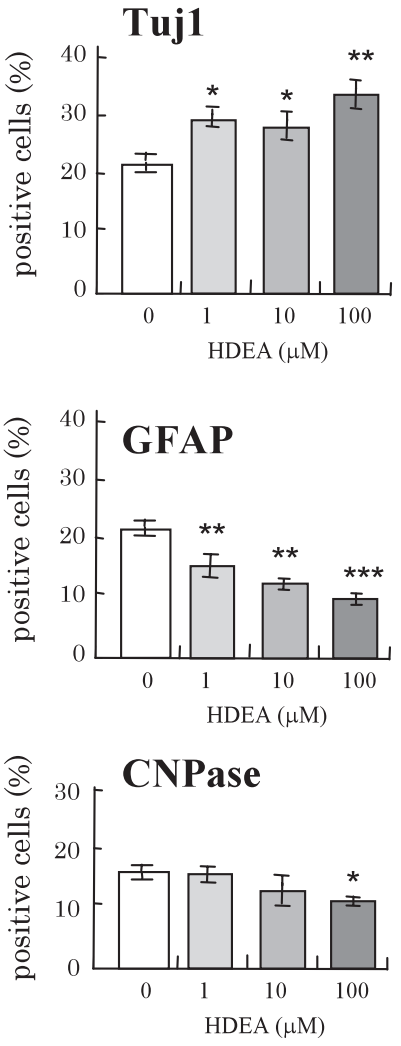

Fig. 5 HDEA stimulates neurogenesis but suppresses gliogenesis. Dissociated cell suspensions of NSCs were plated on coverslips coated with poly-L-ornithine. The cells were maintained for 2 days in the proliferation medium, and transferred to the differentiation medium containing various concentrations of HDEA and cultured for another 5 days. The fixed cells were then reacted with antibody against Tuj1, GFAP, CNPase or nestin, followed by Alexa Fluor 488-conjugated secondary antibodies. The number of antigen-positive cells was counted, and the ratio to total cells was calculated. The values are expressed as the mean ± SE (n = 4). Significance of differences from the value of the cells cultured without HDEA was determined by Tukey's test for each antigen. Significance, *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$.

CNS at the molecular level, and may provide a novel strategy for nutritional and/or clinical applications. It is urgent to examine the neurogenic activity of HDEA and RJ *in vivo*; if effective, they may serve as a tool for protection against and therapy for some particular neurological disorders.

Acknowledgements

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