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Effect of *Citrus* polymethoxyfavones on neuritogenesis in neuroblastoma cells

Yoshiko Furukawa[1]*, Sono Watanabe[1], Satoshi Okuyama[1], Yoshiaki Amakura[2], Morio Yoshimura[2], Takashi Yoshida[2], Mitsunari Nakajima[1]

Abstract

We previously reported that 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF), one of the *Citrus* polymethoxyflavones (PMFs), has the ability to activate extracellular signal-regulated kinases 1/2 (ERK1/2) in cultured neurons, as well as that to rescue mice from drug-induced learning impairment. The present study showed that 1) HMF activated ERK1/2 in Neuro2a cells, a mouse neuroblastoma cell line used as a model system for studies on neuronal differentiation; 2) HMF promoted neurite outgrowth from Neuro2a cells; and 3) nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone, NBT), another PMF, acted similarly as HMF. Our findings lend further support to the notion that PMFs might be neurotrophic agents useful for the treatment of various neurodegenerative neurological disorders.

Keywords: *Neuro2a cells, neurite outgrowth, 3,5,6,7,8,3',4'-heptamethoxyflavone, polymethoxyflavone, ERK*

1. INTRODUCTION

We previously reported that 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF; Figure 1), a *Citrus* polymethoxyflavone (PMF), has the ability to activate extracellular signal-regulated kinases 1/2 (ERK1/2) in cultured neurons [1]. ERK1/2, components of the mitogen-activated protein kinase (MAPK) signaling cascade, are known to be involved in synaptic plasticity and in the development of long-term memory in the central nervous system (CNS) [2, 3]. In a previous study, we successfully demonstrated that subcutaneous injection of HMF can rescue mice from drug-induced learning impairment [1]. ERK1/2 is also known to be a critical intracellular signaling intermediate for neuronal differentiation [2]. In the rat pheochromocytoma cell line PC12 [4], neurotrophic factors such as nerve growth factor (NGF) elicit rapid phosphorylation of tyrosine residues of its receptor, TrkA, which subsequently activates (phosphorylates) signal transduction substrates including ERK1/2, sequentially inducing neurite outgrowth [5,6]. NGF-responsive PC12 cells can also extend neurites in response to cyclic AMP (cAMP) and its analogs [7]. An elevated level of cAMP within cells activates cAMP-dependent protein kinase (PKA), and this cAMP-dependent signaling can cause the neurite outgrowth *via* ERK activation [8]. Recently, much information showing that various phytochemicals have the ability to cause PC12 cells to differentiate (namely, extend neuritic processes) has been obtained [9~18]. Accumulating evidence has demonstrated that these compounds induce neurite outgrowth by an ERK-dependent process. These results prompted us to study whether HMF has the ability to induce the differentiation of neuronal cells. The well-

[1] Department of Pharmaceutical Pharmacology, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan
*Corresponding author e-mail address: ***furukawa@cc.matsuyama-u.ac.jp***
[2] Department of Pharmacognosy, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan

studied *in vitro* model systems for neuronal differentiation are not only rat PC12 cells [4] but also mouse neuroblastoma cells [19] including Neuro2a cells [20]. Unlike NGF-responsive PC12 cells, neuroblastoma cells are NGF-insensitive; and their differentiation and neurogenesis are induced by retinoic acid [21] or cAMP [22]. Although these cells have been scarcely used for investigations of the activities of phytochemicals, a recent report has shown that genipin, a natural iridoid compound, induces neuritogenesis in Neuro2a cells [23], as well as in PC12h cells [24,25] through a nitric oxide (NO)/cyclic GMP (cGMP)/cGMP-dependent protein kinase (PKG) pathway that leads to the activation of ERK. Neuro2a cells have the merit that their morphologic responses are better than those of PC12 cells [22]. Thus, using Neuro2a cells in the present study, we investigated whether HMF could induce their neuronal differentiation. Nobiletin (5,6,7,8,3,4′-hexamethoxy flavone, NBT), which has 6 methoxy groups instead of the 7 in HMF, has been reported to induce neurite extension from PC12 cells [10] by activating a cAMP/PKA/MEK/ERK/MAPK-dependent, but not TrkA-independent, pathway [11]. We thus also studied whether NBT would also have the ability to induce the differentiation of Neuro2a cells.

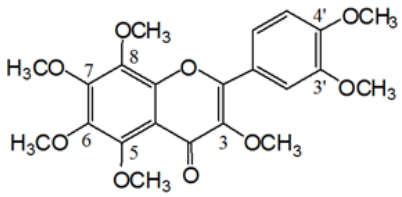

Figure 1: Chemical structure of 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF).

2. EXPERIMENTAL SECTION

2.1.Cultures of Neuro2a Cells. Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, U.S.), penicillin (100 U/mL), and streptomycin (100 μg/mL). N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP) sodium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) to yield a 100 mM stock solution. HMF was prepared from commercial orange oil (Wako, Osaka, Japan) as previously reported [1]. NBT was purchased from Wako. Both HMF and NBT were dissolved in dimethyl sulfoxide (DMSO) to yield 100 mM stock solutions. The final concentration of DMSO was 0.1 % in all cultures used in the present study.

2.2.Immunoblot Analysis. The cells were seeded in 6-well plates (2×10^5 cells/well), cultured for 24 h in normal medium, and then for 24 h in low-serum (2% FBS) medium. The cells were then incubated with test compounds for 30 min. The preparation of lysates from cells and immunoblotting were performed as previously described [1]. The density ratio of pERK2 to total ERK2 in untreated cultures (none) was determined and expressed as 1 arbitrary unit. Results are presented as the mean ± SEM (n = 4 different cultures).

2.3. Assessment of Process Formation. The cells were seeded into 35-well dishes (2×10^4 cells/well). After a 24-h incubation in normal medium, the cells were then incubated for 24 ~ 72 h in the medium containing 2% FBS and the desired test compound. Neurite outgrowth was determined by manually tracing the length of the longest neurite per cell for a given 100 cells in a field (200 × magnification) under a phase-contrast microscope (Biozero, Keyence, Osaka, Japan).

3. RESULTS SECTION

3.1. Effect of HMF on ERK Activation of Neuro2a Cells. We tested at first by immunoblot analysis whether HMF had the ability to promote the phosphorylation of ERK1/2 in Neuro2a cells. As a positive control, dbcAMP, a membrane-permeable cAMP analog, was used here. When the cells were treated with 100 μM HMF for 30 min, ERK1/2 were significantly phosphorylated, as was the case with 5 mM dbcAMP (Figure 2). These observations indicate that HMF could indeed induce the phosphorylation of these ERKs in Neuro2a cells.

3.2. Effect of HMF on Neuronal Differentiation of Neuro2a Cells. Next we tested whether HMF could induce neurite extension of Neuro2a cells. At 24 h after the addition of 5 mM dbcAMP, a few short processes extended from the cell bodies. Almost similar morphological changes of the cells were observed after the treatment with 50 μM HMF (Figure 3A). At 48 h, the neurite extension induced by HMF or dbcAMP was more obvious than that seen after treatment for 24 h (Figure 3B). We next examined the dose-response relationship of the effect of HMF on neurite extension in Neuro2a cells. After the cells had been treated with HMF at the concentration of 0, 10, 25, 50 or 100 μM or with 5 mM dbcAMP for 24 ~ 48 h, the length of neurites that grew out from each cell was measured for a given 100 cells in a field observed under a phase-contrast microscope.

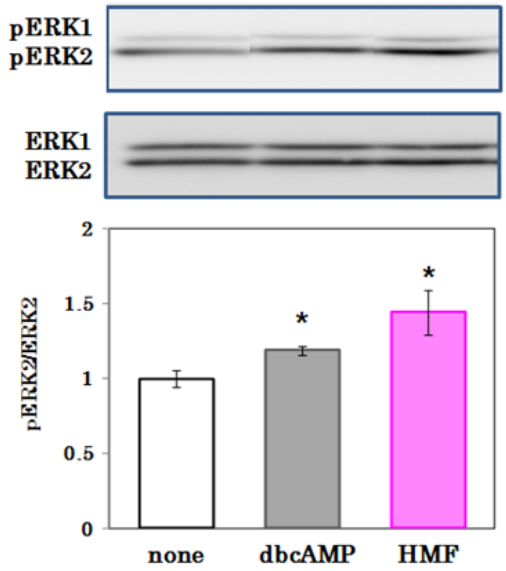

Figure 2: Effects of HMF and dibutyryl cAMP (dbcAMP) on activation of extracellular signal-regulated kinases 1/2 (ERK1/2) in Neuro2a cells. Significant difference in values between the compound-treated and non-treated cultures: * $P < 0.05$ (Student's t test).

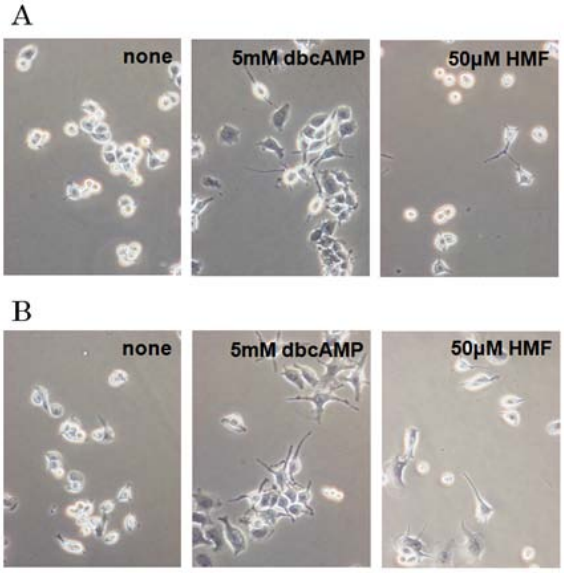

Figure 3: Effects of HMF and dbcAMP on neurite outgrowth from Neuro2a cells. Cells were treated with 50 μM HMF or 5 mM dbcAMP for 24 h (**A**) or 48 h (**B**), after which phase-contrast photomicrographs of the cells were taken.

As shown in Figure 4A, HMF treatment for 24 h induced neurite extension in a dose-dependent manner. Namely, the induction of neurite extension was observed at 25 μM, which gradually strengthened up to at least 100 μM (red bars). The degree of neurite outgrowth from cells by the treatment with HMF at the concentration of 50 or 100 μM was comparable to that obtained with 5 mM dbcAMP (shaded bar). The effect of HMF at concentrations over 100 μM could not be investigated, because HMF was not fully dissolved in the culture medium at these higher concentrations.

By 48 h, the neurites had further extended at each concentration of HMF (red bars of Figure 4B). These results indicate that HMF induced neurite outgrowth in a dose and time-dependent manner.

3.3. Effect of NBT on Neuronal Differentiation of Neuro2a Cells. The effect of NBT on PC12 cells was extensively studied earlier [10,11], but its effect on Neuro2a cells was not. We therefore examined the effects of NBT on neurite outgrowth from Neuro2a cells. When Neuro2a cells were

incubated with various concentrations of NBT (25, 50 or 100 μM) for 24 h or 48 h, NBT, having 6 methoxy groups, induced neurite extension from the cells (blue bars of Figure 4A and 4B) to an extent almost comparable to that obtained by HMF with 7 methoxy groups (red bars). These results show that NBT could induce neurite extension from neuroblastoma Neuro2a cells as well as from pheochromocytoma PC12 cells and that there was no correlation between the number of methoxy residues and the neurite-extending action of PMF toward Neuro2a cells.

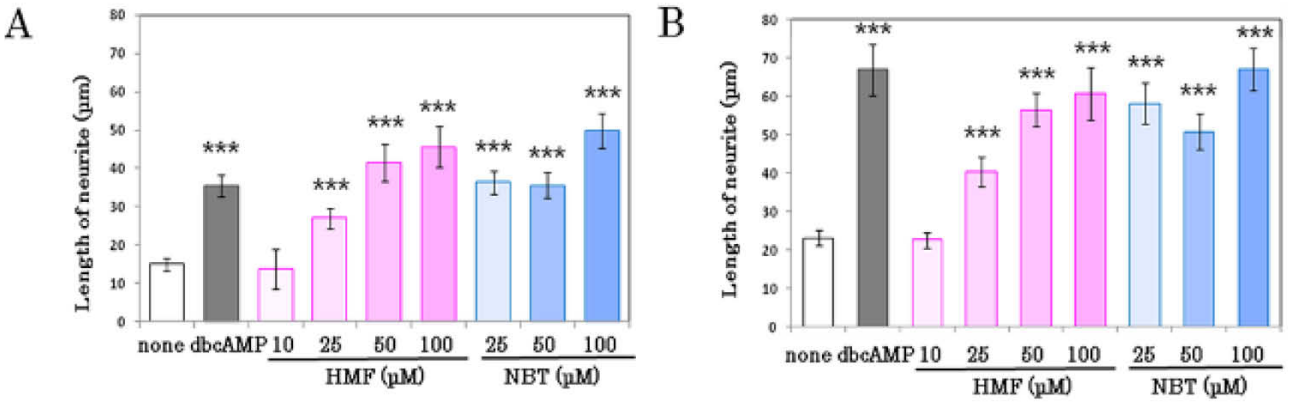

Figure 4: Effects of HMF and NBT on neurite outgrowth from Neuro2a cells. Cells were treated with various concentrations (10, 25, 50 or 100 μM) of HMF, NBT (25, 50 or 100 μM) or 5 mM dbcAMP for 24 h (**A**) or 48 h (**B**). The average radial distance of the longest neurite, measured from the neurite tip to the soma, of 100 cells was determined. Significant difference in values between the compound-treated and non-treated cells (none): $^{**}P < 0.01$, $^{***}P < 0.01$ (Student's *t* test).

NBT has been shown to have a neuroprotective effect in the CNS; i.e., it has the ability to rescue rodents from Aβ-induced impairment of learning ability [26, 27], bulbectomy-induced cholinergic neurodegeneration [10,28], and ischemia-induced learning and memory deficits [29]. We previously showed that the administration of HMF to mice treated with the NMDA receptor antagonist MK-801 restored the MK-801-induced deterioration of spatial learning performance in the Morris water-maze task [1]. NBT has been shown to have a neuritogenic activity in PC 12 cells [10,11]. In this study, we confirmed the neuritogenic activity of both HMF and NBT in Neuro2a cells. Another interesting observation we made is that HMF enhanced brain-derived neurotrophic factor (BDNF) production and neurogenesis in the hippocampus following cerebral global ischemia in mice [our unpublished data]. All these findings strongly suggest that *Citrus* PMFs, including HMF and NBT, might be useful neurotrophic agents for treating patients with various neurodegenerative neurological disorders.

4. CONCLUSIONS

This study demonstrates that HMF from *Citrus* plants had the ability to activate ERK1/2 in Neuro2a cells as well as that to induce neurite outgrowth from these cells. NBT also could facilitate the neurite outgrowth. These results suggest that PMFs might be beneficial therapeutic neurotrophic agents for treating neurodegenerative neurological disorders.

5. ACKNOWLEDGMENTS

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