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Original article

Citrus flavonoid 3,5,6,7,8,3′,4′-heptamethoxyflavone induces BDNF *via* cAMP/ERK/CREB signaling and reduces phosphodiesterase activity in C6 cells

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ABSTRACT

Background: Brain-derived neurotrophic factor (BDNF) is associated with onset of several central nervous system disorders, e.g., Parkinson's disease, Alzheimer's disease, depression, epilepsy, and chronic pain. In our previous *in vivo* studies using ischemic and depression mouse models, we revealed that citrus flavonoid 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) exerts neuroprotective effects by enhancing the expression of BDNF in astrocytes within the hippocampus. Therefore, in the present study, we examined the mechanism of BDNF induction by HMF *in vitro* using rat C6 glioma cells.
Methods: C6 glioma cells were treated with HMF (10 μM) or HMF + U0126 (10 μM), HMF + H89 (1 μM), or HMF + K252a (200 nM) for 48 h. The protein level of mature BDNF (m-BDNF), phosphorylated-ERK (p-ERK) and phosphorylated-cAMP-response element binding protein (p-CREB) were measured using western blot analysis. To clarify the mechanism of HMF for increasing m-BDNF, the inhibitory effect of phosphodiesterase 4B (PDE4B) and PDE4D, and intracellular cAMP levels were examined by ELISA.
Results: Our findings revealed that the m-BDNF-inducing activity of HMF was abolished by U0126 but not by H89 or K252a. HMF was found to phosphorylate (activate) ERK and cAMP-response element binding protein (CREB), a BDNF transcription factor. HMF inhibited PDE4B and PDE4D activity. Moreover, 10 μM HMF elevated intracellular cAMP levels in C6 cells.
Conclusions: These findings suggest that HMF might exert its neuroprotective effects by inducing m-BDNF expression in C6 cells, model cell line of astrocytes, *via* the activation of cAMP/ERK/CREB signaling and inhibiting PDE4B or PDE4D.

Introduction

3,5,6,7,8,3′,4′-Heptamethoxyflavone (HMF) is a polymethoxyflavone found in citruses. In our recent studies, we revealed that the HMF passes through the blood–brain barrier [1,2] and exerts neuroprotective effects in ischemic [3,4] and depression [2,5] mouse models by enhancing the expression of the brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of secreted proteins, in the hippocampus. Immunohistochemical analyses of the brains of these ischemic and depression mouse models showed that the main site of BDNF expression was the astrocytes. However, it has been unknown whether HMF affects astrocytes or not, and how produces BDNF. Therefore, in the present study, we attempted to elucidate the neuroprotective mechanism(s) and targets of HMF used rat C6 glioma cells (rat glial tumor cell line).

BDNF is initially synthesized as a precursor (pro-BDNF) and subsequently cleaved to generate mature (m-BDNF). Since it has been reported that the cleaved m-BDNF modulates neuronal morphology and synaptic plasticity, including long-term potentiation (LTP) in the CNS [6], the decrease of m-BDNF lead to the onset of several CNS disorders. Furthermore, m-BDNF is produced and secreted from neuron but also from astrocyte and microglia [7,8], thus promoting induction of m-BDNF in glial cells are expected as a new therapeutic target for CNS disorders such as depression [9].

Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CREB, cAMP-response element binding protein; EPAC, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; HMF, 35,6,7,8,3′,4′-heptamethoxyflavone; MAPK, mitogen-activated protein kinase; MTT, 3-45-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDE, phosphodiesterase; PKA, protein kinase A.

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Since C6 cells elicit similar response with cultured primary glial cells, it is the most appropriate cell line to examine the sensitivity of glial cells to various substances and conditions [10,11]. In addition, it has been reported that differentiated C6 cells exhibit astrocytes specific markers such as glial fibrillary acidic protein (GFAP) and S 100β protein [10,11]. Based on these knowledge, it has been considered that this cell line is the simple model of astrocytes.

We previously noticed the abolition of *in vivo* HMF-induced BDNF expression in the mouse hippocampus by preadministration of U0126 [2]. U0126 is a selective inhibitor of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) or MEK signaling that can prevent ERK activation by phosphorylation. ERK is an activator of cAMP-response element binding protein (CREB), a BDNF transcription factor [12]. We also previously demonstrated that HMF significantly induces the phosphorylation of both ERK and CREB in cultured neurons [13]. Altogether, these findings prompted us to investigate whether BDNF expression and ERK/CREB phosphorylation by HMF in C6 cells would be inhibited by U0126.

Due to the fact that ERK/CREB can also be activated *via* a neurotrophic factor-dependent signaling pathway [12], in the present study, we also investigated whether BDNF/tropomyosin receptor kinase B (a BDNF receptor) participated in the induction of BDNF expression using K252a, an antagonist of the tyrosine protein kinase activity of the tropomyosin receptor kinase family. Since CREB is known to be activated by cAMP-dependent protein kinase (PKA) [14], we then investigated whether PKA participated in the induction of BDNF expression using H89, a selective and potent cell-permeable PKA inhibitor.

On the other hand, we previously found that HMF can inhibit phosphodiesterase 3B (PDE3B) and PDE4B [15]. Since it has been reported that the inhibition of PDE4B and/or PDE4D results in the upregulation of BDNF expression mediated by CREB phosphorylation in the hippocampus [16], we assessed whether HMF has an inhibitory effect on both PDE4B and PDE4D. Finally, we verified whether HMF was able to increase the intracellular level of cAMP in C6 cells as we showed in our previous study that HMF increases the cAMP content in mouse spleen cells [15].

Materials and methods

Chemicals

HMF was supplied by Ushio Chemix Corporation (Omaezaki, Japan). K252a and forskolin were purchased from Wako Pure Chemical Industries (Osaka, Japan). U0126, H89, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Merck (Darmstadt, Germany). Rolipram, PDE4B, and PDE4D (recombinant protein of each isoform) were purchased from BPS Bioscience (San Diego, CA, USA). To prepare stock solutions of these chemicals (100 mM HMF, 10 mM U0126, 10 mM H89, 200 μM K252a, 10 mM forskolin, and 100 mM rolipram), each substance was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

C6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (29.2 mg/mL) in a humidified incubator with 5% CO_2 at 37 °C. All cell culture materials were purchased from Thermo Fisher Scientific (Waltham, MA, USA). For incubation with HMF, U0126, H89, or K252a, less than one-thousandth of the stock solutions (<0.1% DMSO) was added to the medium.

MTT assay

C6 cells were seeded onto a 96-well plate at 3×10^3 cells/well and cultured for 24 h. To avoid the influence of FBS on the cell viability, the medium was changed to DMEM containing 0.5% bovine serum albumin (BSA; Merck). After culturing for an additional 24 h, cells were treated with different concentrations of HMF (2–100 μM) for 48 h. The control (0 μM) was treated with 0.1% DMSO. MTT was then added to each well at a final concentration of 0.5 mg/mL, and the cultures were incubated in a humidified incubator with 5% CO_2 at 37 °C for 4 h. To solubilize the deposited formazan, we added 200 μL of DMSO to each well. The absorbance of the violet solution was measured on a microplate reader at 570 nm. The proliferation states were visualized using phase-contrast function of All-in-One Fluorescence Microscope BZ-8000 (Keyence, Osaka, Japan).

Western blot analysis

C6 cells were seeded onto a 6-well plate at 1×10^5 cells/well and cultured for 24 h. The medium was then changed to DMEM containing 0.5% BSA, and the cells were cultured for an additional 24 h to avoid the influence of FBS on intracellular signaling. After 30 min of incubation with U0126 (10 μM), H-89 (1 μM), or K252a (200 nM), cells were treated with 10 μM HMF for 48 h. Cell extracts were prepared as previously described [13], and equal amounts of protein (30 μg) were analyzed by immunoblotting. The primary antibodies used were rabbit monoclonal anti-BDNF (1 : 1,000; Thermo Fisher Scientific, Waltham, MA, USA), phospho-CREB (p-CREB) (Ser133; 1 : 1,000; Cell Signaling, Danvers, MA, USA), and anti-CREB (1 : 2,500; Cell Signaling) and rabbit polyclonal anti-β-actin (1:1,000; Cell Signaling), anti-phospho-p44/42 MAPK (p-ERK1/2) (Thr202/Tyr201; 1 : 1,000; Cell Signaling), and anti-ERK1/2 (1 : 2,500; Merck). The secondary antibody was horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (Cell Signaling). Immunoreactive bands were detected by ECL Prime (GE Healthcare, Buckinghamshire, Little Chalfont, UK), and band intensities were measured with a ChemiDoc™ Touch Imaging System (Bio Rad, CA, USA). A quantitative analysis of band intensities was conducted using Image Lab™ Software (Bio Rad, CA, USA).

PDE activity assay

The PDE assay was performed using a Cyclic Nucleotide Phosphodiesterase Assay Kit (Enzo, Farmingdale, NY, USA) as previously described [15]. The reaction mixture contained 10 μg/mL PDE4B or PDE4D, 0.5 mM cAMP, 5,000 kU/mL 5′-nucleotidase, 10 mM $MgCl_2$, 0.1 mg/mL BSA, 0.05% Tween-20, and 10 mM Tris-HCl (pH = 7.4). As a PDE inhibitor, rolipram (1, 10, and 100 μM) or HMF (1, 10, and 100 μM) was added and incubated at 37 °C for 30 min. The control was treated with DMSO. The final absorbance of the green solution was measured on a microplate reader at 655 nm.

Quantification of intracellular cAMP

C6 cells were seeded onto a 96-well plate at 5×10^3 cells/well with DMEM containing 10% FBS and cultured until confluence. Cells were then incubated with rolipram (1 or 10 μM), HMF (1 or 10 μM) in the presence of forskolin (10 μM) for 15 min. Treatment with forskolin alone was denoted as control (CON) and basal condition cAMP level (untreated C6 cells) was denoted as a NONE. The intracellular amounts of cAMP were measured using a cAMP Complete ELISA Kit (Enzo) as previously described [15].

Statistical analysis

Results are expressed as the mean ± standard error of the mean. Significant differences were analyzed by one-way analysis of variance test with a Dunnett's *post-hoc* test. A p-value < 0.05 was considered statistically significant.

Results

Effects of HMF on C6 cell viability and proliferation

The optimal HMF concentration required to induce BDNF expression in C6 cells was determined by treating cells with different concentrations of HMF (2–100 μM) for 48 h. HMF treatment showed a loose, bell-shaped, dose–response curve at 5–50 μM, with maximal cell viability (115.4% ± 2.1%) at 10 μM ($p < 0.01$, Fig. 1A). At the concentration of 100 μM HMF treatment showed cytotoxicity. Consistent result of MTT assay, the proliferation of C6 cells were promoted at 10 μM HMF but were suppressed at 100 μM HMF (Fig. 1B). We also investigated the influence of HMF on morphology changes (neurite outgrowth) of C6 cells, but the any differences were not obtained between control and HMF 10μM (data not shown). Based upon these results, 10 μM HMF was used in subsequent experiments.

Effects of HMF on mature BDNF expression in C6 cells

In our previous *in vivo* studies, we demonstrated that the upregulation of BDNF expression by HMF in the mouse hippocampus was inhibited by pretreatment with U0126, suggesting that HMF induces BDNF expression *via* ERK/MAPK signaling [2]. To confirm this phenomenon *in vitro*, we investigated whether 48 h HMF treatment with or without U0126, K252a and H89 pretreatment would affect BDNF expression in C6 cells. Since m-BDNF plays crucial role for neuroprotective effect, we examined m-BDNF expression in C6 cells. As shown in Fig. 2, while m-BDNF expression in HMF-treated cells (153.0% ± 13.9%) was significantly higher ($p < 0.01$) than that in CON (0.1% DMSO, 100% ± 5.3%), U0126 pretreatment significantly suppressed ($p < 0.05$) this HMF action (69.4% ± 6.2%). In contrast, the effect of HMF was slightly suppressed but not significant by pretreatment with K252a (120.7% ± 5.8%) and H89 (129.4% ± 9.6%).

Effects of HMF on ERK/CREB signaling in C6 cells

We previously reported that HMF significantly induced the phosphorylation of CREB in cultured neurons [13]. CREB is a downstream target of p-ERK [17] and a BDNF transcription factor [12]. To further elucidate the mechanism of HMF action, we

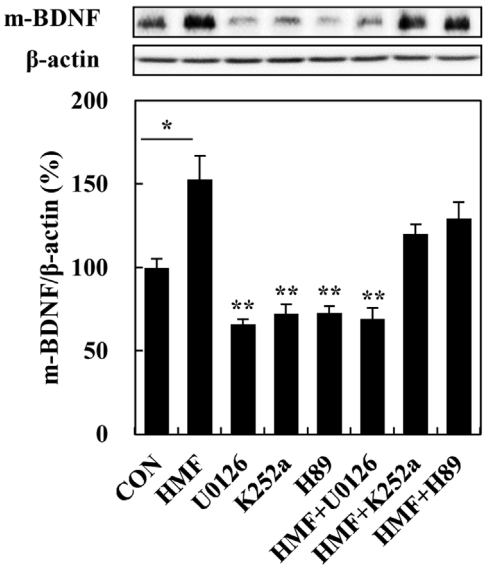

Fig. 2. Effect of HMF on mature BDNF expression in C6 cells. C6 cells were treated with 10 μM HMF for 48 h with or without 10 μM U0126, 200 nM K252a, or 1 μM H-89 pretreatment. Representative band patterns of m-BDNF and β-actin are shown at the top of the graph. Quantitative analysis of the m-BDNF/β-actin ratio was performed using Image Lab™ Software. Symbols show significant differences *vs.* HMF. The values represent the mean ± SEM ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$.

examined the influence of HMF with or without U0126 pretreatment on the expression of p-ERK and p-CREB in C6 cells. We analyzed the ratio of p-ERK2 to total ERK2 among ERK1 and ERK2, which share 83% amino acid identity, because only the ERK2 isoform has been associated with neurogenesis and cognitive function [18]. The expression of both p-ERK2 (Fig. 3A) and p-CREB (Fig. 3B) was significantly promoted by HMF treatment (154.4% ± 18.2%, $p < 0.05$; 153.8% ± 4.7%, $p < 0.01$, respectively) compared with CON (0.1% DMSO, 100% ± 7.0% and 100% ± 8.7%, respectively), and this effect was significantly inhibited (both $p < 0.01$) by pretreatment with U0126 (86.3% ± 8.1% and 94.4% ± 3.9%, respectively).

Effects of HMF on PDE4B and PDE4D activity

PDE4B and PDE4D have been reported to induce the elevation of intracellular cAMP levels, followed by induction of BDNF in the CNS [16]. Therefore, we examined whether HMF could exert an inhibitory effect on PDE4D as well as PDE4B [15]. We found that the activity of PDE4B (Fig. 4A) and PDE4D (Fig. 4B) was significantly inhibited (both $p < 0.01$) by 100 μM HMF (64.4% ± 5.0% and 52.9%

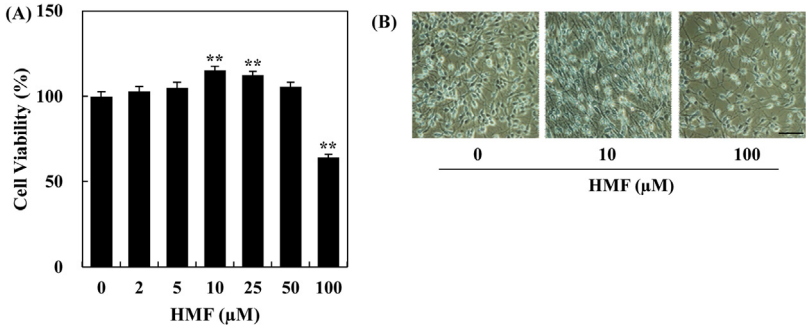

Fig. 1. Effect of HMF on C6 cell viability and proliferation. C6 cells were treated with different concentrations of HMF (2–100 μM) for 48 h (A). The state of C6 cells proliferation at 10 μM and 100 μM HMF at 48 h (B). The scale bar shows 50 μm. Cell viability is expressed as a percentage of the control (0 μM). Symbols show significant differences *vs.* control (0 μM). The values represent the mean ± SEM ($n = 5$), $^{**}p < 0.01$.

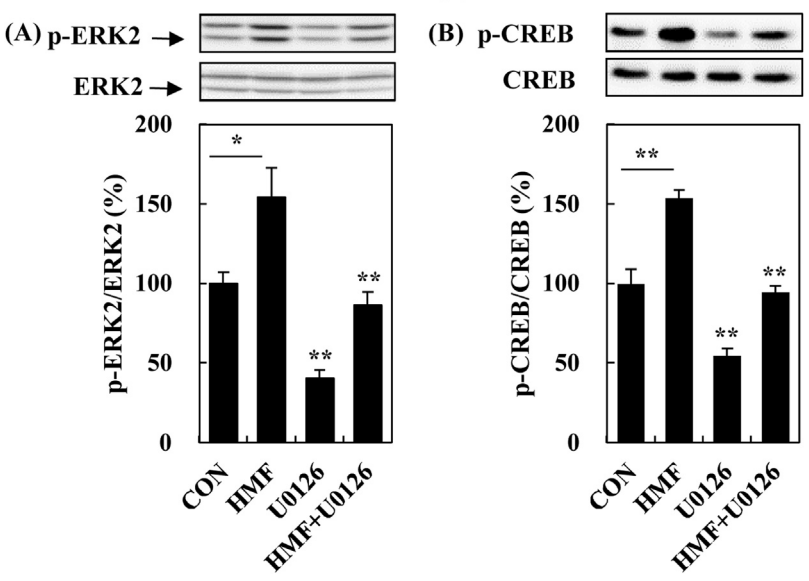

Fig. 3. Effect of HMF on p-ERK2 (A) and p-CREB (B) expression in C6 cells. Western blot analysis of p-ERK2/ERK2 ratio and p-CREB/CREB ratio in C6 cells which were treated with 10 μM HMF for 48 h with or without 10 μM U0126 pretreatment. Representative band patterns of p-ERK1/2, ERK1/2, p-CREB, and CREB are shown at the top of the graph. Quantitative analysis of p-ERK2/ERK2 and p-CREB/CREB ratios was performed using Image Lab™ Software. Symbols show significant differences *vs.* HMF. The values represent the mean ± SEM ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$.

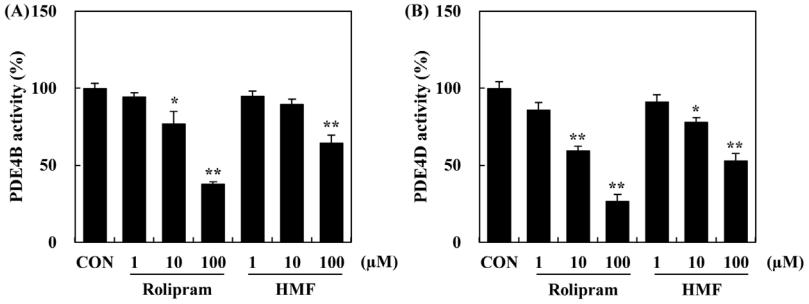

Fig. 4. Effect of HMF and rolipram on PDE4B (A) and PDE4D (B) activity. The result of inhibitory effect of rolipram (1, 10, 100 μM) and HMF (1, 10, 100 μM) on PDE4B (A) and PDE4D (B). The inhibition effect of rolipram and HMF was measured using Cyclic Nucleotide Phosphodiesterase Assay Kit. PDE activities are expressed as a percentage of the CON (DMSO treatment). Symbols show significant differences *vs.* CON. The values represent the mean ± SEM ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$.

± 4.5%, respectively) compared with the CON (100% ± 3.2% and 100% ± 4.3%, respectively). Additionally, 10 μM HMF effectively inhibited ($p < 0.05$) PDE4D activity (77.8% ± 3.1%) but not PDE4B. The PDE4B and PDE4D-inhibitory ability of HMF was slightly weaker than that of rolipram, a nonselective PDE4 inhibitor (59.4% ± 2.8% at 10 μM, $p < 0.05$; 26.7 ± 4.2% at 100 μM, $p < 0.01$).

Effects of HMF on intracellular cAMP levels in C6 cells

Since the inhibition of PDE4 induces an elevation of intracellular cAMP in the CNS followed by activation of ERK/CREB signaling [16,19–21], we investigated whether HMF can raise intracellular cAMP levels in C6 cells. The cAMP level of basal condition C6 cells (NONE; 1.3 ± 0.7 pmol/mL) was elevated by forskolin (CON), which activates adenylate cyclase, (5.9 ± 0.4 pmol/mL, $p < 0.001$). When C6 cells were treated with HMF in the presence of forskolin, intracellular cAMP levels were significantly elevated ($p < 0.01$) by 10 μM HMF (30.9 ± 4.3 pmol/mL) but not 1 μM compared with the CON. Similar to HMF treatment, 1 μM and 10 μM rolipram treatment showed significant elevation ($p < 0.01$) of intracellular cAMP levels (30.8 ± 1.3 pmol/mL and 35.8 ± 2.9 pmol/mL, respectively) compared with the CON (Fig. 5).

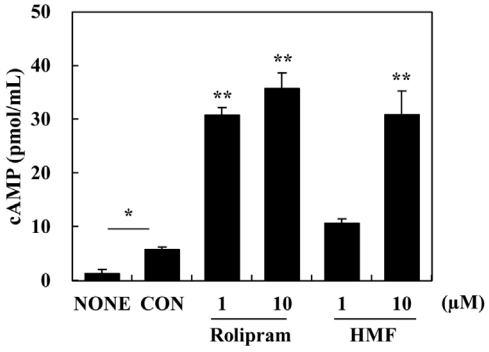

Fig. 5. Effect of HMF and rolipram on intracellular cAMP levels in C6 cells. C6 cells were treated with HMF (1, 10 μM) or rolipram (1, 10 μM) in the presence of 10 μM forskolin for 15 min. Intracellular cAMP was measured by using cAMP complete ELISA kit. Symbols show significant differences *vs.* CON. The values represent the mean ± SEM ($n = 4$), $^{*}p < 0.05$, $^{**}p < 0.01$; basal condition = NONE, forskolin alone = CON.

Discussion

We previously reported that HMF has neuroprotective effects by inducing BDNF expression in astrocytes in the CNS and has a possibility of ameliorating some CNS diseases, such as memory impairment after global cerebral ischemia and depression [2,4], but its mechanism remains unclear. In the present study, using C6 cells, we showed that HMF increases m-BDNF expression *via* activation of cAMP/ERK/CREB signaling and inhibition of PDE activity.

Increasing evidence has indicated that glial cells, especially astrocytes, are crucial in the control of brain homeostasis and function [7,8]. In particular, astrocytes play an important role as mediators of flavonoid action in the brain [22]. The results of our previous studies may support this idea because HMF exerts neuroprotective effects in the brains of mice displaying neurological disorders by enhancing the expression of BDNF in hippocampal astrocytes [2–5].

BDNF produced from neuron and glial cells exist as pro- and fully processed forms (m-BDNF) in the CNS and is a well-studied key factor in brain development, including neuronal cell survival, differentiation, migration, dendritic arborization, synaptogenesis, and plasticity [7,8,23,24]. Thus, we thought the significant increase in C6 cell viability after 10 μM HMF exposure for 48 h (Fig. 1) may be the result of increased m-BDNF expression and subsequent action induced by HMF. Consistent with our expectation, m-BDNF expression in C6 cells was significantly elevated by 10 μM HMF, and the effect was suppressed by U0126, but not by H89 and K252a (Fig. 2). These results suggesting that activating MAPK cascade is important for HMF-induced m-BDNF expression, and tropomyosin receptor B (BDNF receptor) and PKA shows lower contribution in the HMF-induced m-BDNF expression. We also revealed that 10 μM HMF phosphorylated (activates) ERK and CREB, which are upstream regulators of BDNF production in the CNS [20], but the effect was inhibited by U0126 (Fig. 3). Taken together, the effect of HMF on increasing of m-BDNF expression in C6 cells is needed activation of MAPK cascade and it indicates the possibility that HMF act on astrocytes.

5,6,7,8,3′,4′-Hexamethoxyflavone (nobiletin) is another citrus polymethoxyflavone that has been shown to activate cAMP/PKA/ERK/CREB signaling associated with learning and memory in cultured neurons [20,21]. In line with this, the present results suggest that the neuroprotective effects of HMF were mediated by the cAMP/ERK/CREB signaling pathway, but not the cAMP/PKA/ERK/CREB pathway used by nobiletin (Figs. 2 and 3). In eukaryotic cells, the effects of cAMP are known to be mediated by two intracellular cAMP receptors: classical PKA and exchange protein activated by cAMP (EPAC) [25]. We predicted that the effect of HMF might be mediated by EPAC signaling, although we have not yet examined this possibility in detail. There are two EPAC isoforms: EPAC1 is highly expressed in embryonic nerve cells, whereas EPAC2 is highly expressed in the amygdala and hippocampus of human brains [24,26]. Since EPAC2 has been reported to activate CREB expression *via* the activation of ERK in the CNS [26], it is possible that the cAMP/EPAC2/ERK/CREB pathway is involved in HMF-induced neuroprotection.

PDEs are known to be important regulators of various physiological processes mediated by cAMP or cGMP. Several lines of evidence support an important role for PDE4 (cAMP-specific) in the CNS among the 11 PDE family members [27]. In fact, the nonselective PDE4 inhibitor rolipram exerts antidepressant-like [28–30] and cognition-enhancing [31,32] effects *via* cAMP signaling [16,33,34]. Among the four members of the PDE4 family, PDE4A, PDE4B, and PDE4D, but not PDE4C, are expressed in the CNS [32]. Since the inhibition of PDE4B and PDE4D activity has been reported to be involved in antineuropsychiatric diseases, such as depression [28–30], antidepressant-like effects of HMF *in vivo* [2] might have been exerted by the inhibition of PDE4B or PDE4D. Our recent study related to the immune function of HMF revealed that this polymethoxyflavone can inhibit PDE3B and PDE4B [15]. In the present study, we showed that 100 μM HMF inhibits PDE4D and PDE4B but 10 μM HMF inhibited only PDE4D (Fig. 4). These results indicate that PDE4D is more sensitive to HMF than PDE4B, thereby elevating the level of intracellular cAMP in C6 cells (Fig. 5). However, it is yet unclear how HMF activates the ERK/CREB signaling pathway *via* elevated cAMP. Therefore, further studies are needed to confirm whether EPAC2 or other intracellular signal transmitters are involved in the effect of HMF.

In the present study, we revealed that HMF inhibits PDE4B and PDE4D activity, increasing intracellular cAMP levels and subsequently activating ERK and CREB in C6 cells. These results suggest that the neuroprotective effects of HMF-induced BDNF upregulation in the mouse hippocampus might be mediated by the inhibition of PDE4B and PDE4D followed by activation of cAMP/ERK/CREB signaling in astrocytes. The present study provides valuable evidence supporting the use of HMF as a neuroprotective phytochemical. However, it has not been clear whether HMF exerts BDNF-inducing effect in depression or ischemic condition of primary cultured astrocytes, in addition, produced m-BDNF from astrocytes by HMF treatment affect other cells such as microglia and neuron. Therefore, further studies are needed to examine the effect of HMF on primary culture astrocytes from ischemic or depression mouse model and the influence of HMF to other glial cells or neuron.

Conflicts of interest

The authors have no conflicts of interest to declare.

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