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Honokiol-induced neurite outgrowth promotion depends on activation of extracellular signal-regulated kinases (ERK1/2)

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Abstract

We have found that honokiol [4-allyl-2-(3-allyl-4-hydroxy-phenyl)-phenol] can promote neurite outgrowth and mobilize intracellular Ca^{2+} store in primary cultured rat cortical neurons. In this study, we examined the effects of honokiol on extracellular signal-regulated kinases (ERK1/2) and Akt, and their possible relationship to neurite outgrowth and Ca^{2+} mobilization. Honokiol-induced neurite outgrowth in the cultured rat cortical neurons was significantly reduced by PD98059, a mitogen-activated protein kinase kinase (MAPKK, MAPK/ERK kinase MEK, direct upstream of ERK1/2) inhibitor, but not by LY294002, a phosphoinositide 3-kinase (PI3K, upstream of Akt) inhibitor. Honokiol also significantly enhanced the phosphorylation of ERK1/2 in a concentration-dependent manner, whereas the effect of honokiol on Akt phosphorylation was characterized by transient enhancement in 10 min and lasting inhibition after 30 min. The phosphorylation of ERK1/2 enhanced by honokiol was inhibited by PD98059 as well as by KN93, a Ca^{2+}/calmodulin-dependent kinase II (CaMK II) inhibitor. Moreover, the products of the phosphoinositide specific phospholipase C (PLC)-derived inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) were measured after honokiol treatment. Together with our previous findings, these results suggest that the signal transduction from PLC, IP_3, Ca^{2+}, and CaMK II to ERK1/2 is involved in honokiol-induced neurite outgrowth.

Keywords: Honokiol; Cortical neuron, rat; Neurite outgrowth; ERK1/2; CaMK II

1. Introduction

Honokiol is one of the main constituents in the stem barks of *Magnolia obovata* Thunb and *Magnolia officinalis* Rhed (Fujita et al., 1973), which have been used as a traditional medicine in China and Japan for treatment of anxiety-related disorders and digestive complains. Honokiol is known to be neuroactive. Its neuropharmacological features have been recently reviewed, especially on its anxiolytic effects (Maruyama and Kuribara, 2000). In our previous paper, we reported neurotrophic effects of honokiol on neurite outgrowth promotion and neuronal survival enhancement in the cultured rat cortical neurons (Fukuyama et al., 2002). As part of our trials to clarify its neurotrophic mechanism, we reported that honokiol can mobilize intracellular Ca^{2+} in a phospholipase C (PLC)-dependent manner in the cultured cortical neurons (Zhai et al., 2003). Although controlled Ca^{2+} increase can serve as a neurotrophic signal in up-regulating neurotrophic factor gene expression in neurons (Finkbeiner, 2000; West et al., 2001) and plays essential roles in neurotrophic functional expression (Egea et al., 2001; Kang and Schuman, 2000), there remains a need to figure out other associated signals with the neurotrophic effects of honokiol. Extracellular signal-regulated kinases (ERK1/2) and Akt signal cascades are well known to regulate neuronal differentiation, neurite outgrowth, and neuronal survival (Kaplan and Miller, 2000). In the present study, we investigated the effects of honokiol on ERK1/2

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and Akt, and the relationship of ERK1/2 phosphorylation to intracellular Ca^{2+} mobilization.

2. Materials and methods

2.1. Materials

Honokiol was isolated from *M. officinalis* Rhed (>99%). The purity was confirmed by high-performance liquid chromatography (single peak) and by [^{1}H] and [^{13}C] nuclear magnetic resonance spectra and high resolution electron ionization mass spectrum. Cell culture plates and culture dishes were from Iwaki (Chiba, Japan). Neurobasal culture medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and B27 supplement were purchased from Gibco BRL (NY, USA). PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one], LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one], and KN93 [*N*-[2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl] phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt] were from Sigma (MO, USA). Human recombinant basic fibroblast growth factor (bFGF) was from Upstate Biotechnology Inc. (NY, USA). All other agents used are high grade commercially available.

2.2. Primary culture of cortical neurons

Cerebral cortical cells were obtained from fetuses of timed-pregnant Sprague–Dawley rats (SLC, Japan). The E18 dams were killed by cervical dislocation under ether anesthesia and the fetuses were immediately removed and placed in Dulbecco's phosphate-buffered saline (PBS). The fetuses were quickly decapitated, and the brains were removed and cleaned of meninges. The cortical hemispheres were dissected out, minced with forceps, and completely dissociated into a single-cell suspension by trypsinization (first 0.25% trypsin 20 min, then DNase I 5 min). The cell suspension then was washed 2 times with Minimum Essential Medium (MEM) (15 ml, 5 min × 200 g). Isolated neurons were diluted to the indicated density according to the following requirements, and then cultured at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. All culture media were supplemented with 50 U/ml penicillin–50 μg/ml streptomycin.

2.3. Morphological assay

For neurite outgrowth assay, the neurons were cultured in 24-well plates at the density of 9000 cells/cm^2 in DMEM medium plus 10% FBS. After 24 h, medium was changed to Neurobasal medium plus 2% B27 (NB/B27) supplement and 0.5 mM glutamine containing different concentrations of test samples. After being cultured for a further 144 h, neurons were fixed and stained with anti-microtubule-associated protein 2 (MAP2) immunohistochemical method for neurite length analysis as described previously (Fukuyama et al., 2002). Briefly, well-stained neurons, which did not grow on or near glial cells and made no connection to more than two cells, were selected for measurement of their primary (longest) neurite length indicative of overall neurite outgrowth using the software LuminaVision (ver. 1.0)/MacScope (Ver. 2.6) (Mitani Corp., Fukui, Japan).

2.4. Western blotting

Western blotting was used to detect activation of ERK1/2 and Akt phosphorylation. Methodically, neurons were cultured in 35 mm dishes in DMEM with 10% FBS at the density of 5×10^5 cells/cm^2 for 24 h; then the medium was changed to serum-free DMEM containing 1% N_2 supplement. After a further 24 h, neurons were treated for indicated intervals, and then were washed once with 1 ml ice-cold PBS and lysed in 100 μl lysis buffer (115 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM dithiothreitol (DTT), 2.5 mg/ml bromophenol blue). The lysates (15 μl) containing the same total proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to immunoblotting with antibodies of anti-phosphorylated ERK1/2, anti-ERK1/2, anti-phosphorylated Akt, and anti-Akt (Molecular Signaling, USA). Detection was accomplished using the AP-conjugated secondary antibody system (GAR-AP) (Bio-Rad, USA). The non-phosphorylated forms of ERK1/2 or Akt from the same cell lysates were also immunochemically monitored to ensure the observation of phosphorylation of ERK1/2, and Akt was observed at the same protein level. Blot density, indicative for the level of

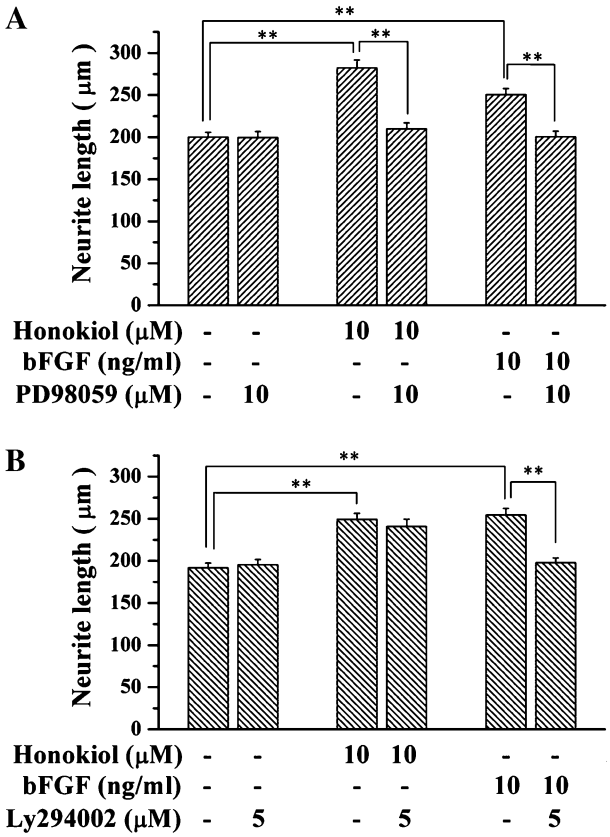

Fig. 1. Neurite outgrowth-promoting activity of honokiol is reduced by inhibition of MEK but not PI3K. Rat cortical neurons were cultured and treated for 144 h as described in the context. (A) Effect of MEK inhibitor PD98059 on honokiol and bFGF-induced neurite outgrowth; (B) effect of PI3K inhibitor LY294002 on the honokiol and bFGF-induced neurite outgrowth. Data were expressed as means ± S.E.M. (n = 80). Statistical analysis was performed with Student's t-test. $^{**}P < 0.01$. Typical one of three successfully repeated experiments was presented here.

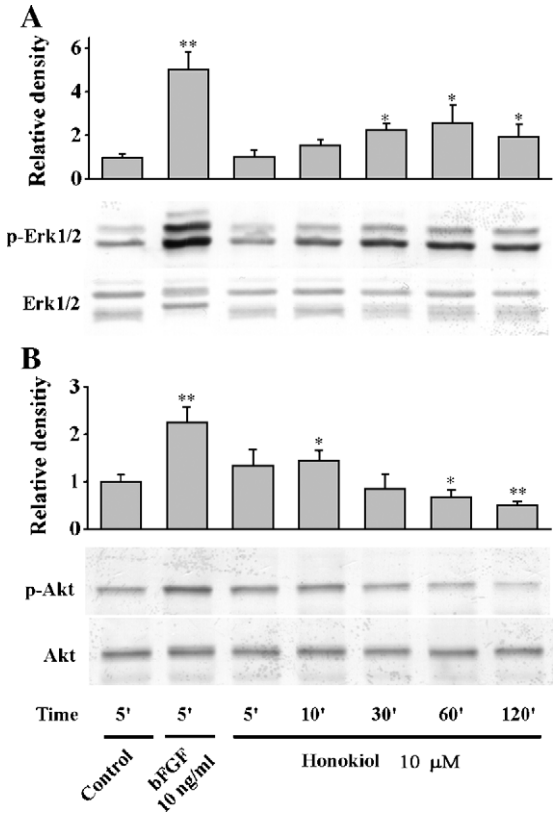

Fig. 2. Effects of honokiol on ERK1/2 and Akt phosphorylation in cultured rat cortical neurons. (A) Time course of ERK1/2 phosphorylation by treatment of honokiol over 120 min; (B) time course of Akt phosphorylation by treatment of honokiol over 120 min. 10 ng/ml bFGF was taken as positive control. There is no apparent variation of ERK1/2 or Akt phosphorylation when treated with vehicle control in parallel times (data not shown). Relative blot density was defined as phosphorylated ERK1 of treated groups to phosphorylated ERK1 of vehicle control (A) or phosphorylated Akt in treated groups to phosphorylated Akt of vehicle control (B). Data were expressed as means ± S.E.M. ($n=3$). Statistical analysis was performed with Student's t-test. $^{*}P<0.05$, $^{**}P<0.01$ vs. control.

signal proteins, was analyzed with the software NIH image (ver. 1.63). The protein remained on the gels was stained by CBB (Coomassie Brilliant Blue) and thereby equal protein amounts were shown to be loaded in each line.

2.5. Inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) measurement

Cortical neurons were cultured in 24-well plates in DMEM plus 10% FBS at the density of $2.5\times10^5/cm^2$ for 24–48 h. Then neurons were incubated with 20 μM honokiol at 37 °C for the indicated time. Reaction was terminated by the addition of ice-cold 10% perchloric acid.

For IP_3 quantification, the perchloric acid-treated samples were kept on ice for 20 min and then centrifuged at $2000\times g$ for 5 min at 4 °C. The pH of the supernatant was adjusted to 7.5 with 10 M KOH. The solution was kept on ice for 30 min and centrifuged at $2000\times g$ for 15 min at 4 °C. The concentration of IP_3 in the supernatant was determined with the IP_3 assay kit (Amersham Biosci.).

For DAG quantification, the perchloric acid-treated samples were extracted for lipids as described by Bligh and Dyer (1959) except that 0.2 M KCl–5 mM EDTA was used instead of water. The final organic phase was dried under a stream of N_2, and then the DAG in crude lipid fractions was converted to phosphatidic acid by *Escherichia coli* DAG kinase in the presence of [g-^{32}P]ATP (Preiss et al., 1986), lastly measured with the DAG assay kit (Amersham Co.).

2.6. Statistical analysis

Data analysis was performed with Originpro 7.5 (OriginLab Corp., MA, USA). Statistical significance between groups was estimated by one-way ANOVA followed by Student's t-test. $P<0.05$ was thought as significantly different.

3. Results

3.1. Neurite outgrowth-promoting activity of honokiol depends on MEK activation

In a previous paper (Fukuyama et al., 2002), we demonstrated that honokiol (0.1–10 μM) promoted neurite outgrowth in the primary cultured rat cortical neurons (Fig. 1). To explore the role of ERK1/2 and Akt in the honokiol-induced neurite outgrowth, we

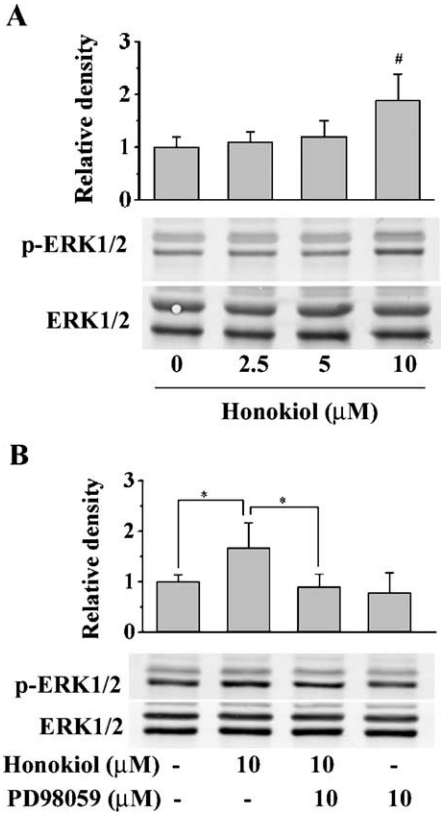

Fig. 3. Dose-dependent manner of honokiol-induced ERK1/2 phosphorylation and its inhibition by PD98059. (A) ERK1/2 phosphorylation under treatment of honokiol (60 min); (B) honokiol-induced ERK1/2 phosphorylation was decreased by PD98059. PD98059 was added to culture medium same with honokiol, and sustained for 60 min. Relative blot density was defined as phosphorylated ERK1 of treated groups to phosphorylated ERK1 of vehicle control. Data were expressed as means ± S.E.M. ($n=3$). Statistical analysis was performed with Student's t-test. $\#P<0.05$ vs. vehicle control (0 μM honokiol), $^{*}P<0.05$.

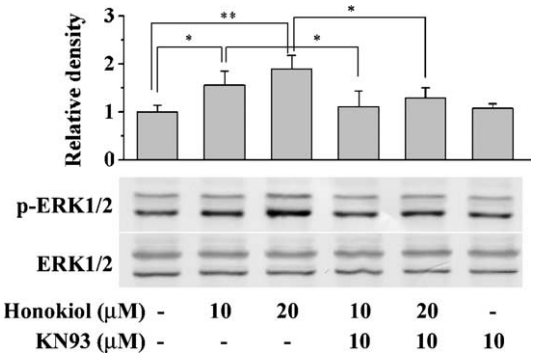

Fig. 4. Effect of CaMK II inhibition on the honokiol-induced ERK1/2 phosphorylation. KN93 was added to culture medium with honokiol, and sustained for 60 min. Relative blot density was defined as phosphorylated ERK1 of treated groups to phosphorylated ERK1 of vehicle control. Data were expressed as means ± S.E.M. ($n=3$). Statistical analysis was performed with Student's t-test. $^{*}P<0.05$, $^{**}P<0.01$.

took advantage of the availability of PD98059, a specific inhibitor of the MEK (direct upstream of ERK1/2) (Alessi et al., 1995), and LY294002, a specific inhibitor of PI3K (upstream of Akt) (Vlahos et al., 1994). 10 μM PD98059 inhibited both honokiol-induced and bFGF-induced neurite outgrowth (Fig. 1A). 5 μM LY294002 had no effect on honokiol-induced neurite outgrowth, while it inhibited bFGF-induced neurite outgrowth (Fig. 1B). It is known that ERK1/2 plays neurotrophic roles in neurite outgrowth (Shin et al., 2002). The suppression of bFGF-induced neurite outgrowth by LY294002 suggests that Akt plays a role in neurite development. Compared with bFGF as positive control, these results suggest that ERK1/2, but not Akt, may be involved in honokiol-activated signal cascades.

3.2. Honokiol promotes ERK1/2 phosphorylation

To further confirm the roles of ERK1/2 and Akt in the neurotrophic mechanism of honokiol, the phosphorylations of ERK1/2 and Akt were investigated using the Western blotting method (Figs. 2, 3 and 4). While ERK1/2 phosphorylation was activated by 10 ng/ml bFGF at 5 min in primary rat neuronal cultures, under the treatment with 10 μM honokiol, ERK1/2 phosphorylation was enhanced in 10 min, and reached a plateau at about 60 min, and then sustained at least for 120 min in the observed period (Fig. 2A). On the other hand, honokiol (10 μM) increased the phosphorylation of Akt in 10 min and then turned to a fall after 30 min (Fig. 2B). Concentration-dependent studies were conducted with the 60 min treatment of honokiol for ERK1/2 phosphorylation, and below 5 μM honokiol was found to have no significant effects (Fig. 3A), whereas the increases of ERK1/2 were observed in a dose-dependent manner with 10 μM and 20 μM honokiol (Fig. 4). These enhancing effects of honokiol on ERK1/2 phosphorylation were reduced by PD98059 (Fig. 3B), a specific MEK inhibitor, suggesting that it was mediated by MEK.

3.3. Honokiol-induced ERK1/2 phosphorylation depends on CaMK II activation

As shown in Fig. 4, honokiol-enhanced ERK1/2 phosphorylation was also inhibited by addition of 10 μM KN93, a specific CaMK II inhibitor, while KN93 itself had no apparent effects on the baseline level of ERK1/2 phosphorylation (Fig. 4).

3.4. Honokiol promotes IP_3 and DAG formation

In a previous paper (Zhai et al., 2003), we presented that honokiol can mobilize intracellular Ca^{2+} in a PLC-dependent manner. As a complement of that study, we measured the formations of IP_3 and DAG, which are produced after PLC activation, by treatment with honokiol in the primary neuronal culture. Honokiol promoted both IP_3 and DAG productions, reaching their peaks in 20 s (Fig. 5). This result strongly suggests that Ca^{2+} release induced by honokiol is due to the activation of PLC.

4. Discussion

In the present study, honokiol was found to enhance ERK1/2 phosphorylation. In neurons and neuron-like cells, ERK1/2 is phosphorylated by MEK and its phosphorylated forms transfer signals into nuclei, controlling many cellular differentiation and proliferation. Injection of constitutively active ERKs into PC12 cells can directly induce neurite outgrowth (Fukuda et al., 1995; Robinson et al., 1998). Furthermore, ERK1/2 phosphorylation is accompanying

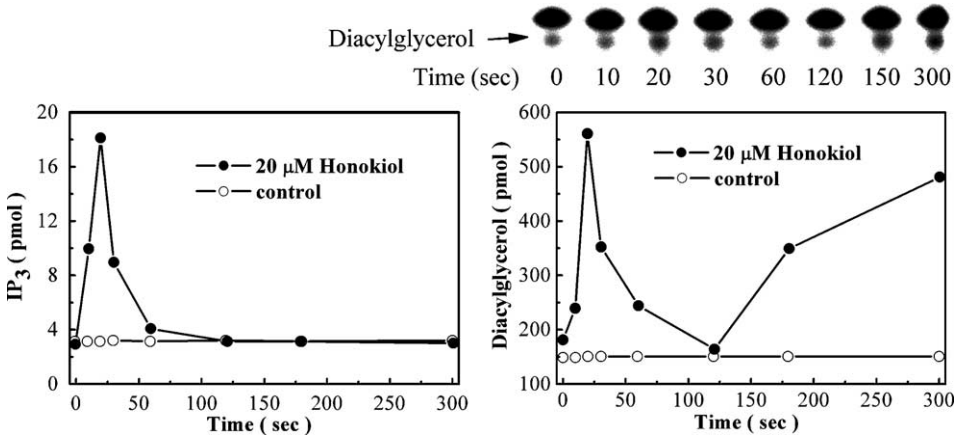

Fig. 5. Effect of honokiol on the IP_3 and DAG productions in the cultured cortical neurons. A typical result from five experiments is shown. Right-up inset is one picture from DAG separation on thin layer chromatography.

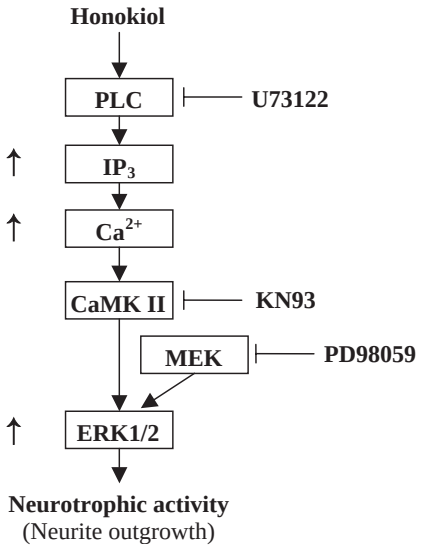

Fig. 6. A suggested event order induced in the mechanism of honokiol. IP_3 production, cytoplasmic free Ca^{2+} increase, and ERK1/2 phosphorylation were identified as effects of honokiol. The involvement of PLC, CaMK II, and MEK was shown by using their specific inhibitor, U73122, KN93, and PD98059, respectively.

neurite outgrowth enhancement by numerous neurotrophic protein factors such as basic fibroblast growth factor, *N*-cadherin, laminin (Perron and Bixby, 1999), nerve growth factor (Fukuda et al., 1995), brain-derived growth factor (Encinas et al., 1999), and some small molecules, such as α-phenyl-*N*-*tert*-butylnitron (Tsuji et al., 2001), valproic acid (Yuan et al., 2001), genipin (Yamazaki et al., 2001), cAMP (Frodin et al., 1994), lactacystin (Hashimoto et al., 2000), picrosides (Li et al., 2002), manganese (Walowitz and Roth, 1999) and so on. These findings indicate that ERK1/2 plays central roles in neurite outgrowth. MEK inhibitor PD98059 inhibited honokiol-induced neurite outgrowth and ERK1/2 phosphorylation. These results showed that MEK activation is essential for honokiol-induced neurite outgrowth, and also suggested that ERK1/2 phosphorylation plays a positive role in neurite outgrowth. The enhancement of ERK1/2 phosphorylation by honokiol was also observed in the HL-60 human leukemia cells. In this model, honokiol promoted 1,25-dihydroxyvitamin D3 and retinoic acid-induced cellular differentiation. Considering that neurite outgrowth belongs to neuronal differentiation, it can be rationalized that honokiol-induced neurite outgrowth depends on activation of ERK1/2.

Next, we tested whether honokiol-induced ERK1/2 phosphorylation was associated with other cellular events. Here we focused on relationship between Ca^{2+} mobilization and ERK1/2 phosphorylation. First of all, the measurement of IP_3 and DAG productions under treatment with honokiol confirmed our previous results that honokiol mobilizes intracellular Ca^{2+} store by PLC. Ca^{2+}–Calmodulin–CaMKs–ERKs signal cascade exists in several neuronal cells (Agell et al., 2002), and regulates important physiological roles including the neurite outgrowth (Borodinsky et al., 2002). The regulation of ERK1/2 phosphorylation by CaMK II has been found in rat cortical neurons (Choe and Wang, 2002). In the present study, honokiol-induced ERK1/2 phosphorylation was suppressed by a CaMK II inhibitor, KN93, suggesting the presence of a link between honokiol-induced Ca^{2+} mobilization and ERK1/2 phosphorylation. ERK1/2 phosphorylation is one subsequent molecular event of Ca^{2+} mobilization. Taking our previous results (Zhai et al., 2003) into consideration, honokiol-induced neurite outgrowth can be proposed as shown in Fig. 6. That is, honokiol activates PLC and then produces IP_3, which mobilizes intracellular Ca^{2+} store, and subsequently the increase of Ca^{2+} in neuronal cells activates CaMK II, which presumably enhances ERK1/2 phosphorylation via MEK.

PI3K and Akt cascade is also one of honokiol's targets. Honokiol causes a transient enhancement and subsequent lasting inhibition of Akt phosphorylation. However, PI3K inhibitor LY294002 had no effect on honokiol-induced neurite outgrowth. The role of honokiol-induced Akt phosphorylation in neurite outgrowth is inconclusive. Since Akt is well known to play an important role in neuronal survival, there remains a need to clarify the cellular outcomes of dynamic Akt phosphorylation and its relationship to Ca^{2+} mobilization and ERK1/2 phosphorylation in a continuous subject about neuroprotective mechanism of honokiol.

In conclusion, the present studies suggest that the cascade of PLC→IP_3→Ca^{2+}→CaMK II→(MEK)→ERK1/2 is involved in the neurotrophic mechanism of honokiol. These results will bring out new insights for understanding other neuroactive effects of honokiol. Our further studies are now under way to clarify how honokiol can activate PLC and what molecules in neurons are associated with honokiol-induced neurotrophic activities.

Acknowledgments

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