Activation of protein kinase C βII/ε-c-Jun NH_2-terminal kinase pathway and inhibition of mitogen-activated protein/extracellular signal-regulated kinase 1/2 phosphorylation in antitumor invasive activity induced by the polymethoxy flavonoid, nobiletin

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Abstract

Flavonoids from medicinal plants have been therapeutically administered for cancer therapy. We recently reported that nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) exhibits its novel antitumor invasive activities by suppressing the production of pro-matrix metalloproteinases (proMMPs) and augmenting the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) *in vivo* and *in vitro*. In the present study, intracellular target molecules associated with the actions of nobiletin against tumor invasion were identified. Nobiletin inhibited the phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase (MEK) 1/2, but not the activity of Ras or the phosphorylation of Raf. Moreover, a MEK1/2 inhibitor, U0126, mimicked nobiletin's ability to decrease the production of proMMPs-1 and 9 in human fibrosarcoma HT-1080 cells stimulated by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). In addition, neither the activity of phosphatidylinositol 3-kinase (PI3K) nor the phosphorylation of Akt was influenced by nobiletin. However, nobiletin was found to augment the phosphorylation of c-Jun NH_2-terminal kinase (JNK), a downstream signal factor of the PI3K-Akt pathway, in TPA-treated HT-1080 cells. A similar augmentation of JNK phosphorylation was observed on treatment with a PI3K inhibitor, LY-294002. Furthermore, nobiletin enhancement of TIMP-1 production in TPA-stimulated HT-1080 cells was found to be diminished by adding a JNK inhibitor, SP600125. Moreover, protein kinase C (PKC) inhibitor experiments showed that PKCβII/ε were associated with the nobiletin-mediated augmentation of JNK phosphorylation. Therefore, these results introduce novel evidence that the antitumor effects of nobiletin are finely regulated by the following intracellular mechanisms: (1) the inhibition of MEK1/2 activity is involved in the suppression of MMP expression and (2) the activation of the novel PKCβII/ε-JNK pathway is associated with the augmentation of TIMP-1 expression. [Mol Cancer Ther 2004;3(7): 839–47]

Introduction

Metastatic progression of malignant tumors requires the proteolytic degradation of extracellular matrix components in basement membranes and stromal tissues, and matrix metalloproteinases (MMPs) have been shown to play important roles in the breakdown of the extracellular matrix (1-3). Different sets of MMPs, such as gelatinase A (M_r 72,000 type IV collagenase)/MMP-2, gelatinase B (M_r 92,000 type IV collagenase)/MMP-9, interstitial collagenase/MMP-1, stromelysin-1/MMP-3, and membrane type-MMPs have coordinately participated in the breakdown of extracellular matrix components during tumor invasion (1, 4-8). Furthermore, the enzymic activity of MMPs has been found to be inhibited by tissue inhibitors of metalloproteinases (TIMPs)-1, 2, 3, and 4 (9), which in turn inhibit the invasion and metastasis of malignant tumor cells *in vivo* and *in vitro* (10-14). Thus, it is likely that the augmentation of TIMP expression exerts an interferential effect on MMP-dependent tumor invasion.

Flavonoids from medicinal plants possess pharmacologic effects for preventing tumor progression by inhibiting tumor-cell proliferation and tumor invasion (15). The prominent flavonoids, quercetin and genistein, have been shown to exert an antitumorigenic effect on malignant tumors (16-20). In addition, genistein has been reported to suppress the expression of MT1-MMP and MMP-9 in human breast carcinoma cells (19, 21). Huang et al. (20) also found that quercetin suppresses the epidermal growth factor-induced production of MMPs-2 and 9 in human squamous carcinoma A431 cells. We recently reported that nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), a major

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component in juice from *Citrus depressa*, inhibits the invasive activity of human fibrosarcoma HT-1080 cells not only by suppressing the expression of MMPs but also by augmenting TIMP-1 production (22). In addition, Minagawa et al. (23) reported that nobiletin prevents tumor-cell invasion due to a decrease of MMP-9 production in the peritoneal dissemination of human gastric carcinoma in severe combined immunodeficient mice. Furthermore, we reported a similar preventive efficacy of nobiletin for extracellular matrix breakdown due to the transcriptional suppression of MMPs-1, 3, and 9 in articular chondrocytes and synoviocytes from rabbits (24) and humans (25), respectively. Therefore, nobiletin may be a novel candidate against tumor invasion activity *in vivo* and *in vitro*.

Various pathologic events, including tumor invasiveness, are considered to result from abnormal regulation of the activation of intracellular signal molecules. The overexpression of Ras and the phosphorylation of its downstream kinase, mitogen-activated protein/extracellular signal-regulated kinase (MEK) 1/2, promote tumor invasion due to the augmentation of MMP expression (26, 27). In addition, it has been reported that a signal transduction pathway of phosphatidylinositol 3-kinase (PI3K) contributes to the stimulation of tumor invasion (28, 29). Furthermore, investigators have reported the effects of flavonoids on the expression and activation of signal transduction molecules to address their therapeutic mechanisms. For example, genistein is a well-known tyrosine kinase inhibitor that has been reported to block signal transduction pathways mediated by mitogen-activated protein kinase in human neutrophils (30) and 1-phosphatidylinositol 4-phosphate 5-kinase in human ovarian carcinoma OVCAR-5 cells (17). In addition, quercetin has been found to inhibit protein kinase C (PKC) and/or tyrosine kinase in human HL-60 leukemia cells (18), and PIK in human breast carcinoma MDA-MB-435 cells (16). Moreover, our previous study (22) suggests that PI3K and MEK may be intracellular target molecules for the therapeutic actions of nobiletin. However, it remains unclear how nobiletin regulates the expression and activation of PI3K and MEK, and whether other crucial target molecules may be involved in the nobiletin-induced suppression of MMP expression and augmentation of TIMP-1 expression.

In the present study, we showed that nobiletin inhibited the phosphorylation of MEK1/2, but not the activity of Ras or the phosphorylation of Raf, in human fibrosarcoma HT-1080 cells treated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). In addition, although nobiletin was shown to neither modulate the activity of PI3K nor the phosphorylation of Akt, it was, however, found to increase the phosphorylation of c-Jun NH_2-terminal kinase (JNK), which is a downstream molecule in the PI3K-Akt pathway. Moreover, we showed that the augmented phosphorylation of JNK resulted from the nobiletin-induced activation of PKCβII/ε, which might be associated with the up-regulation of TIMP-1 and the down-regulation of proMMP-9 production by nobiletin in HT-1080 cells. Therefore, we suggest that nobiletin divergently regulates the expression of MMPs and TIMP-1 by novel mechanisms that lead to the inhibition of MEK1/2 activity and the activation of JNK dependent on the increased activity of PKCβII/ε in HT-1080 cells.

Materials and Methods

Cell Culture and Treatment

Human fibrosarcoma HT-1080 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Eagle's MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Asahi Techno Glass, Tokyo, Japan) and nonessential amino acids (Invitrogen). After reaching confluence, the cells were treated with nobiletin that was isolated from the juice of *C. depressa* ''Hayata'' (24), a PI3K inhibitor, LY-294002, a potent MEK1/2 inhibitor, U0126, a potent and selective JNK inhibitor, SP600125 (BioMol Research Laboratory, Plymouth Meeting, PA), and/or TPA (Sigma Chemical Co., St. Louis, MO) in MEM supplemented with nonessential amino acids and 0.2% lactalbumin hydrolysate (Sigma) for up to 24 hours. Otherwise, the cells were pretreated with PKC inhibitors: Gö6976 (for types α, βI, and μ PKC), Gö6983 (for types α, β, δ, and ξ PKC), or Ro-31-8425 (for types α, βI, βII, and ε PKC) (Calbiochem-Novabiochem, San Diego, CA) for 30 minutes, and then treated with fresh medium supplemented with nobiletin for 90 minutes. Furthermore, for the last 30 minutes, the combined treatment of nobiletin and TPA was carried out after adding concentrated TPA solution to the cell culture. The harvested culture media were stored at −20°C until use and the cells were subjected to preparation of the cytosol fraction.

Preparation of Cytosol Fraction

The cells were washed once with Ca^{2+}- and Mg^{2+}-free PBS [PBS(−)] and then homogenized in 10 mmol/L HEPES-KOH (pH 7.8), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1% NP40, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 μmol/L pepstatin, 10 μmol/L leupeptin, and 1 mmol/L sodium orthovanadate. After centrifugation at 5,000 × *g* at 4°C, the resultant supernatant was collected as the cytosol fraction and used for Western blotting and the analysis of kinase activities. Protein concentrations were measured by the method of Lowry et al. (31).

Western Blotting

MMPs and TIMP-1 in the harvested culture media were analyzed by Western blotting using 10% or 12.5% acrylamide gel under reducing conditions (22). Proteins separated in the gel were electrotransferred onto a nitrocellulose membrane, and the membrane was reacted with sheep anti-(human proMMP-1) or anti-(human TIMP-1) antibody (kindly provided by Dr. H. Nagase), which was then complexed with horseradish peroxidase-conjugated goat anti-(sheep IgG)IgG. Immunoreactive proMMP-1 and TIMP-1 were visualized with enhanced chemiluminescence-Western-blotting detection reagents (Amersham Bioscience, Tokyo, Japan). For the detection of phosphorylated cellular proteins, aliquots (30 μg) of cytosol protein were analyzed by Western blotting using specific rabbit antibodies against

phosphorylated MEK1/2 (Ser217/221), Raf (Ser259), Akt (Ser473), and JNK (Thr183/Tyr185), and rabbit antibodies against Raf, MEK1/2, and JNK (New England Biolaboratories, Beverly, MA) under non-reducing conditions. Immunoreactive Raf, MEK1/2, and JNK, and phosphorylated Raf, MEK1/2, Akt, and JNK were detected with enhanced chemiluminescence-Western-blotting detection reagents after being complexed with horseradish peroxidase-conjugated anti-rabbit IgG. Relative amounts of the immunoreactive proteins were quantified by densitometric scanning using an Image Analyzer LAS-1000 plus (Fuji Film, Tokyo, Japan).

Measurement of Ras Activity

Ras activity was measured using Ras Activation Assay kits (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. The collected cells were lysed in the supplied lysis buffer, and the cell lysate (500 μg) was subjected to an affinity precipitation with agarose-bound Ras binding domain of Raf-1 at 4°C for 30 minutes. GTP-bound Ras in the precipitate was analyzed by Western blotting using mouse monoclonal anti-Ras antibody. Immunoreactive GTP-bound Ras was detected with enhanced chemiluminescence-Western-blotting detection reagents after being complexed with horseradish peroxidase-conjugated anti-mouse IgG. Relative amounts of the immunoreactive proteins were quantified by densitometric scanning using an Image Analyzer LAS-1000 plus (Fuji Film).

Gelatin Zymography

Aliquots (10 μL) of the harvested culture media were subjected to SDS-PAGE with 10% acrylamide gel containing gelatin (0.6 mg/mL) (DIFCO Laboratories, Detroit, MI). The gel was washed with 50 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 10 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 0.1% Triton X-100, and then incubated in 50 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 10 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$ at 37°C. Thereafter, the gel was stained with 0.1% Coomassie Brilliant Blue R-250, and gelatinolytic activity was detected as unstained bands on a blue background.

Measurement of PI3K Activity

PI3K activity was measured by the method of Fukui and Hanafusa(32) with some modifications. Aliquots (750 μg) of cytosol protein were incubated with rabbit anti-(PI3K, p85) antibody (Upstate) for 18 hours at room temperature, and then the immunoreactive complex bound to added protein A-Sepharose (Amersham Bioscience) was incubated for 2 hours at 4°C. After centrifugation, the resultant precipitate containing PI3K was re-suspended in 75 μL of TNE buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 5 mmol/L EDTA] and then incubated with 10 μL of phosphatidylinositol (2 mg/mL) in TNE buffer and 10 μL of 100 mmol/L $MgCl_2$. Next, an enzymic reaction was started by adding 5 μL of [γ-^{32}P]ATP (5.5 kBq) (DuPont NEN, Boston, MA) and carried out for 15 minutes at 37°C. After the reaction was terminated by adding 20 μL of 1 mol/L HCl, synthesized ^{32}P-labeled phosphatidylinositol 3-phosphate was extracted with chloroform/methanol (1:1, v:v), analyzed by TLC on a silica-gel 60 F_{254}-coated column (Merck, Darmstadt, Germany) in a chloroform/methanol/H_2O/25% ammonia solution (60:47:11.3:2, v:v:v:v), and then detected by exposing the plate to X-ray film at −80°C. Relative amounts of the ^{32}P-labeled phosphatidylinositol 3-phosphate were quantified by densitometric scanning using an Image Analyzer LAS-1000 plus (Fuji Film).

Measurement of PKC Activity

PKC activity in the cytosol fraction (50 μg) was measured using a PKC Enzyme Assay System (Amersham Bioscience) and [γ-^{32}P]ATP (7.5 kBq) (DuPont NEN) according to the manufacturer's instructions.

Statistical Analysis

ANOVA was used for statistical analysis. The Fisher test was applied when multiple comparisons were done.

Results

Nobiletin Inhibits the Phosphorylation of MEK1/2

Our previous finding (22) that a MEK1/2 inhibitor, PD98059, mimics the actions of nobiletin against tumor invasion by suppressing the expression of proMMPs-1 and 9 in HT-1080 cells allows us to speculate that the Ras-Raf-MEK pathway may be inhibited by nobiletin. Therefore, we examined the effect of nobiletin on the activity of Ras and the phosphorylation of Raf and MEK in HT-1080 cells. As shown in Fig. 1A GTP-bound Ras was constitutively detected in HT-1080 cells as previously reported (33). The levels of GTP-bound Ras were slightly but not statistically increased by TPA treatment, while they were unchanged by nobiletin (64 μmol/L). In addition, the constitutive expression of phosphorylated Raf was found to decrease in TPA-stimulated HT-1080 cells, whereas there were no significant changes in Raf protein levels (Fig. 1B), as previously reported (34, 35). Furthermore, nobiletin (64 μmol/L) did not influence both levels of phosphorylated Raf and its protein in the presence or absence of TPA. On the other hand, HT-1080 cells constitutively expressed phosphorylated MEK1/2 and the phosphorylation was augmented by TPA treatment (Fig. 2A). Furthermore, the augmented levels of phosphorylated MEK1/2 were found to decrease in nobiletin (64 μmol/L)-treated cells. A similar inhibition of MEK1/2 phosphorylation was observed in HT-1080 cells treated with a MEK1/2 inhibitor, U0126 (1 μmol/L). However, neither nobiletin, U0126, nor TPA altered the constitutive levels of MEK1/2 protein (Fig. 2B), indicating that nobiletin inhibited only TPA-induced phosphorylation of MEK1/2. Moreover, U0126, as well as PD98059 (22), was found to decrease the TPA-induced production of proMMPs 1 and 9 in a dose-dependent manner (0.1 to 1 μmol/L; Fig. 3). Therefore, these results suggest that the selective inhibition of MEK1/2 phosphorylation by nobiletin results in suppressing the production of proMMPs-1 and 9.

Nobiletin Does Not Influence PI3K Activity

We reported that a PI3K inhibitor, LY-294002, mimics the nobiletin-induced suppression of proMMP-9 production and the augmentation of TIMP-1 production in

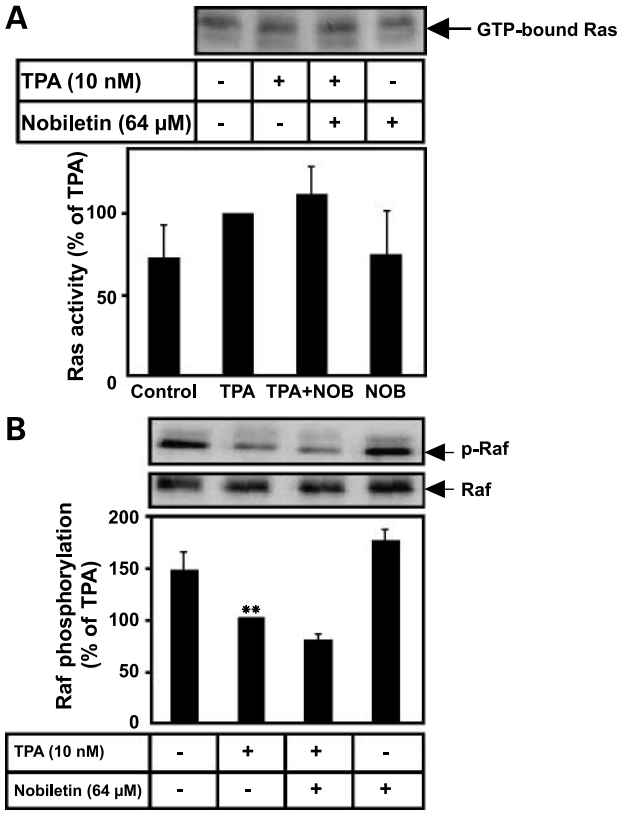

Figure 1. No effects of nobiletin on the activity of Ras and the phosphorylation of Raf in HT-1080 cells. Confluent HT-1080 cells were pretreated with nobiletin (*NOB*; 64 μmol/L) for 1 hour, and then treated with TPA (10 nmol/L) for another 30 minutes. Cytosol fractions were prepared as described in Materials and Methods and subjected to Western blotting for GTP-bound Ras (**A**), phosphorylated Raf (*p-Raf*), and Raf (**B**; *top* and *middle panels*, respectively). Three independent experiments were highly reproducible and typical data are shown. The relative amounts of GTP-bound Ras and phosphorylated Raf were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100%. *Columns*, mean of three independent experiments; *bars*, SD. **, significantly different from untreated (*Control*) cells ($P < 0.01$). *M*, mol/L.

HT-1080 cells (22). Therefore, we examined the effects of nobiletin on PI3K activity and the phosphorylation of Akt, a downstream regulator, in transducing the PI3K signal. As shown in Fig. 4A, the activity of PI3K was constitutively detected in HT-1080 cells and TPA treatment did not influence this activity. In addition, LY-294002 (50 μmol/L) was found to inhibit the PI3K activity in both the presence and absence of TPA ($P < 0.01$). Furthermore, although TPA slightly enhanced the phosphorylation of Akt ($P < 0.05$), LY-294002 decreased the levels of TPA-induced and constitutive phosphorylation of Akt in HT-1080 cells ($P < 0.05$ and 0.001, respectively; Fig. 4B). Moreover, neither PI3K activity nor the phosphorylation of Akt was modulated by nobiletin (64 μmol/L) in untreated and TPA-treated HT-1080 cells (Fig. 4). Therefore, these results suggest that both PI3K and Akt are not direct-target molecules of nobiletin for inducing its action against tumor invasion. However, because of the similar effects of

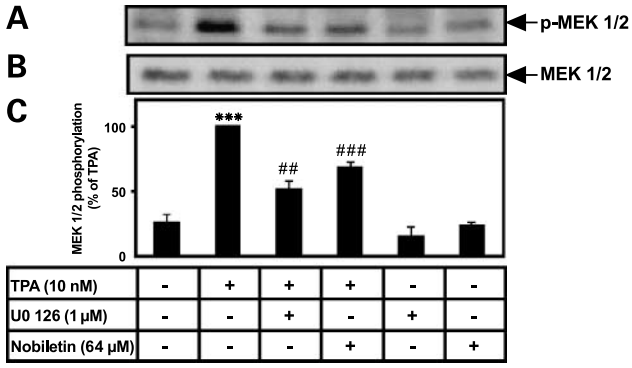

Figure 2. Inhibition of the phosphorylation of MEK1/2 by nobiletin in HT-1080 cells. Confluent HT-1080 cells were pretreated with nobiletin (64 μmol/L) or U0126 (1 μmol/L) for 1 hour, and then treated with TPA (10 nmol/L) for another 30 minutes. Cytosol fractions were prepared as described in Materials and Methods and subjected to Western blotting for phosphorylated MEK1/2 (*p-MEK1/2*) (**A**), and MEK1/2 (**B**). Three independent experiments were highly reproducible and typical data are shown. **C,** the relative amounts of phosphorylated MEK1/2 were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100%. *Columns*, mean of three independent experiments; *bars*, SD. ***, significantly different from untreated cells ($P < 0.001$). ## and ###, significantly different from TPA-treated cells ($P < 0.01$ and 0.001, respectively). *M*, mol/L.

nobiletin and LY-294002 on the regulation of proMMP-9 and TIMP-1 production (22), it seems that the antitumor action of nobiletin is associated with the regulation of signal-transduction factor(s) located downstream of the PI3K-Akt pathway.

Augmentation of JNK Phosphorylation by Nobiletin and LY-294002

Since JNK has been reported to be a downstream factor in the PI3K-Akt pathway (36, 37), we examined the regulation of JNK phosphorylation by nobiletin and LY-294002 in HT-1080 cells. Both nobiletin and LY-294002 were found to enhance the TPA-induced phosphorylation of JNK (Fig. 5A and C). However, there were no changes in the constitutive levels of phosphorylated JNK and its protein

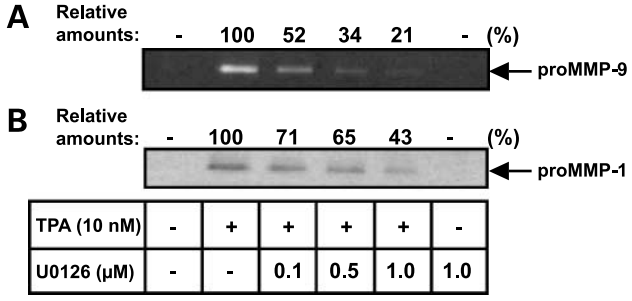

Figure 3. Inhibition of the production of proMMP-9 and proMMP-1 by the MEK1/2 inhibitor, U0126, in HT-1080 cells. Confluent HT-1080 cells were treated with TPA (10 nmol/L) and/or U0126 (0.1 to 1 μmol/L) for 24 hours and then the harvested culture media were subjected to gelatin zymography (**A**), and Western blotting for proMMP-1 (**B**). Three independent experiments were highly reproducible and typical data are shown. The relative amounts of proMMP-9 and proMMP-1 were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100%. *M*, mol/L.

under these treatments (Fig. 5B). Furthermore, the nobiletin-enhanced production of TIMP-1 was found to be diminished by a JNK inhibitor, SP600125 (20 μmol/L), in TPA-stimulated HT-1080 cells (Fig. 6). Thus, it is suggested that JNK may be an intracellular target molecule for nobiletin's antitumor invasive actions. Moreover, our finding that nobiletin did not alter PI3K activity suggests a novel pathway of nobiletin-mediated JNK phosphorylation, which may differ from the signal pathway evoked by inhibiting PI3K activity.

Involvement of PKCβII/ε in Nobiletin-Augmented JNK Phosphorylation

PKC has been reported to participate in the regulation of extracellular signal-regulated kinase (ERK) or JNK activity (38, 39). We first showed that nobiletin transiently augmented PKC activity within 10 minutes (1.4-fold, $P < 0.01$) (data not shown). In addition, to clarify whether nobiletin-augmented PKC activity may be associated with JNK phosphorylation, PKC inhibitor experiments were done. As shown in Fig. 7A, Gö6976 (100 nmol/L) (for types α, βI, and

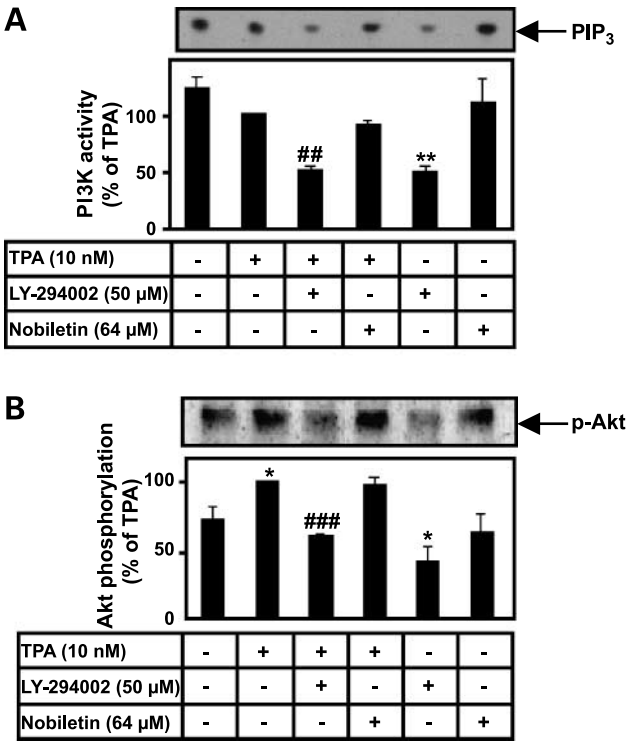

Figure 4. No effects of nobiletin on PI3K activity and Akt phosphorylation in HT-1080 cells. Confluent HT-1080 cells were pretreated with nobiletin (64 μmol/L) and LY-294002 (50 μmol/L) for 1 hour, and then treated with TPA (10 nmol/L) for another 30 minutes. Cytosol fractions were prepared as described in Materials and Methods and subjected to assay for PI3K activity (**A**), and Western blotting for phosphorylated Akt (*p-Akt*) (**B**). The relative amounts of phosphatidylinositol 3-phosphate (*PIP_3*) and phosphorylated Akt were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100% (*bottom panels* in **A** and **B**, respectively). *Columns*, mean of three independent experiments; *bars*, SD. * and **, significantly different from untreated cells ($P < 0.05$ and 0.01, respectively). ## and ###, significantly different from TPA-treated cells ($P < 0.01$ and 0.001, respectively). *M*, mol/L.

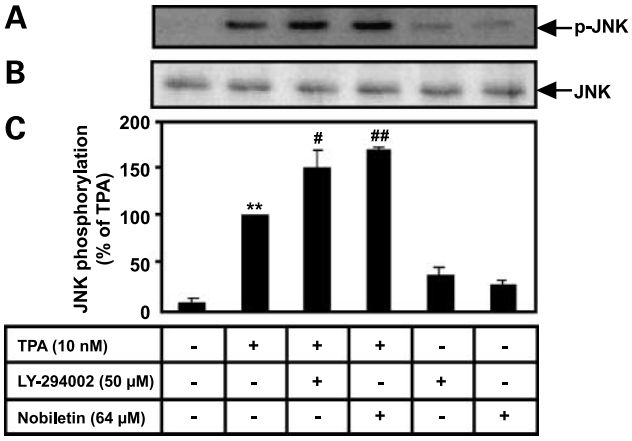

Figure 5. Augmentation of JNK phosphorylation by nobiletin and LY-294002 in HT-1080 cells. Confluent HT-1080 cells were pretreated with nobiletin (64 μmol/L) or LY-294002 (50 μmol/L) for 1 hour, and then treated with TPA (10 nmol/L) for another 30 minutes. Cytosol fractions were prepared and then subjected to Western blotting for phosphorylated JNK (*p-JNK*) (**A**) and JNK (**B**), as described in Materials and Methods. Three independent experiments were highly reproducible and typical data are shown. The relative amounts of phosphorylated JNK were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100% (**C**). *Columns*, mean of three independent experiments; *bars*, SD. **, significantly different from untreated cells ($P < 0.01$). # and ##, significantly different from TPA-treated cells ($P < 0.05$ and 0.01, respectively). *M*, mol/L.

μ PKC) did not inhibit either TPA-induced or nobiletin-enhanced phosphorylation of JNK in HT-1080 cells. In addition, although Gö6983 (100 nmol/L) (for types α, β, δ, and ξ PKC) inhibited TPA-induced JNK phosphorylation, nobiletin was adequate to augment the phosphorylation of JNK even in the presence of the inhibitor (Fig. 7B). However, nobiletin no longer augmented JNK phosphorylation in the presence of Ro-31-8425 (100 nmol/L) (for types α, βI, βII, and ε PKC), which also caused the inhibition of TPA-induced JNK phosphorylation in HT-1080 cells (Fig. 7C). Furthermore, there were no significant changes in the levels of phosphorylated JNK in HT-1080 cells treated with Ro-31-8425 alone, nobiletin alone, or Ro-31-8425 plus nobiletin. Therefore, these results provide novel evidence that nobiletin may activate PKCβII/ε and the augmented PKC activity sequentially results in an increase of JNK phosphorylation in TPA-stimulated HT-1080 cells.

Discussion

Activation of Ras has been reported to promote the proliferation and invasion of tumor cells *in vivo* and *in vitro* (40-42). In addition, Ras regulates tumoral functions by transmitting malignant signals to downstream molecules, such as Raf and MEK1/2 (43). Therefore, it is likely that the signal transduction pathway of Ras-Raf-MEK plays an important role in promoting tumor malignancy and is a therapeutic target for preventing cancer development, and tumor invasion and metastasis. Nobiletin has been found to inhibit tumor promotion, invasion, and metastasis *in vivo*

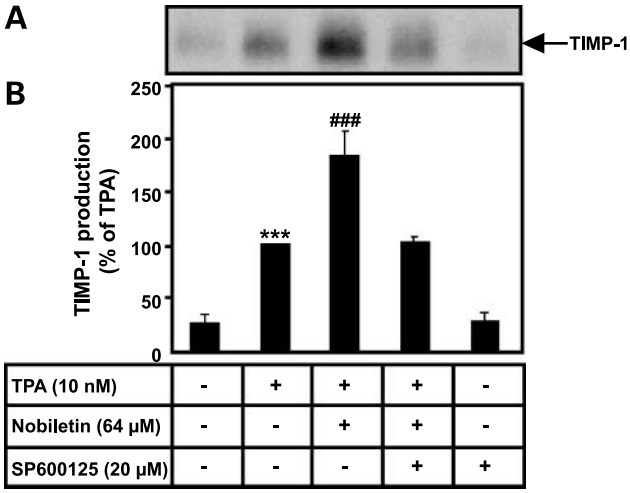

Figure 6. Suppression of nobiletin-enhanced production of TIMP-1 by JNK inhibitor, SP600125, in TPA-stimulated HT-1080 cells. Confluent HT-1080 cells were pretreated with SP600125 (20 μmol/L) for 30 minutes, and then treated with nobiletin (64 μmol/L), SP600125 (20 μmol/L), and/or TPA (10 nmol/L) for another 12 hours. The harvested culture media were subjected to Western blotting for TIMP-1 (**A**), as described in Materials and Methods. Three independent experiments were highly reproducible and typical data are shown. **B,** the relative amounts of TIMP-1 were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100%. *Columns*, mean of three independent experiments; *bars*, SD. ***, significantly different from untreated cells ($P < 0.001$). ###, significantly different from TPA-treated cells ($P < 0.001$). *M*, mol/L.

and *in vitro* (23, 44). We also reported that an MEK inhibitor, PD98059, mimics the nobiletin-induced suppression of proMMPs-1 and 9 in HT-1080 cells (22). In the present study, there were no significant changes in levels of GTP-bound Ras and phosphorylated Raf between untreated and nobiletin-treated HT-1080 cells. However, the phosphorylation of MEK1/2 was suppressed by nobiletin. A similar suppression of proMMP production and MEK1/2 phosphorylation was observed with another MEK1/2 inhibitor, U0126 in HT-1080 cells. Therefore, we propose a novel mechanism of action of nobiletin against tumor invasion: the inhibition of MEK1/2 phosphorylation leads to the suppression of MMP production in tumor cells.

PI3K activation has been reported to contribute to the avoidance of apoptosis (45) and the abnormal stimulation of tumor-cell migration (28, 29), suggesting that PI3K-Akt signaling may play significant roles in the progression of tumor proliferation and metastasis. It has also been reported that flavonoids, such as quercetin and luteolin, inhibit PI3K activity (46, 47). In addition, quercetin and luteolin reportedly suppress the production of proMMP-2 and proMMP-9 in human epidermoid carcinoma A431 cells (20) and human vascular endothelial cells (48). In contrast, we showed here that nobiletin did not influence the activity of PI3K and the phosphorylation of Akt, whereas it does inhibit the expression of proMMPs-1 and 9 in HT-1080 cells (22). The discrepancy of the regulation of PI3K activity among nobiletin, quercetin, and luteolin may be explained by a previous study of Agullo et al. (47) which suggests that 3′ OH and 4′ OH in the B ring of flavones or flavonols are requisite for the inhibition of PI3K activity, and their absence may account for the lack of inhibitory activity of nobiletin against PI3K. Nonetheless, we strongly suggest that the suppressive mechanism of MMP expression by nobiletin differs from that of other flavonoids. Myung-Jin et al. (49) reported that the overexpression of phosphatase and the tensin homolog detected on chromosome 10 (PTEN) negatively regulates the PI3K-Akt pathway and thereby causes the inhibition of tumor invasion due to the suppression of MMP-9 production in U87MG glioblastoma cells. Conversely, it seems that PI3K-Akt signaling promotes tumor invasiveness along with the augmentation of MMP-9 production. In the present study, we showed that the inhibition of PI3K activity and Akt phosphorylation by LY-294002 led to the suppression of proMMP-9 production and the augmentation of TIMP-1 expression (22). These observations are similar to those for nobiletin except that the latter has no effect on PI3K and Akt. Thus, these results allow us to speculate that nobiletin may regulate the activity of signal mediator(s) downstream of the PI3K-Akt pathway, which is (are) associated with the divergent regulation of proMMP-9 and TIMP-1 production.

As far as downstream mediator(s) of the PI3K-Akt pathway is (are) concerned, JNK has been identified as a signal molecule linked to the PI3K-Akt pathway (50) and has been found to participate in tumor metastasis and tumorigenesis (51, 52), suggesting the possibility that JNK may become a target molecule for nobiletin activity against tumor invasion. The present study showed that nobiletin augmented the phosphorylation of JNK in TPA-stimulated HT-1080 cells. In addition, the inhibition of the PI3K-Akt pathway by LY-294002 caused a similar increase in JNK phosphorylation. Furthermore, SP600125 was found to diminish the nobiletin-enhanced production of TIMP-1 in TPA-treated cells, suggesting that the anti-invasive effect of nobiletin is dependent on an increase in the activity of JNK in HT-1080 cells. Therefore, the augmentation of JNK phosphorylation may act as a switch for exerting the antitumor actions of nobiletin with the divergent regulation of proMMP-9 and TIMP-1 expression. Moreover, taken together with a previous report of Levresse et al. (37), it is suggested that JNK activity may be negatively regulated by the PI3K-Akt pathway in HT-1080 cells.

Three JNK isoforms (JNK1, JNK2, and JNK3) have been identified and their cellular expression as well as their contribution to tumor progression differs for the different molecules (53-56). Recent reports using knockout mice for the *JNK1* or *JNK2* gene (57, 58) suggest that JNK1 negatively regulates and JNK2 positively regulates tumorigenesis. In the present study, although we have not identified the JNK isoform associated with the nobiletin actions, our findings that nobiletin induces antitumor proliferation due to G_0-G_1 arrest[3] and

[3] T. Sato, Y. Miyata, L. Koike, M. Yano, and A. Ito, unpublished data.

anti-invasive activity (22, 23) suggest the possibility that nobiletin may cause the phosphorylation of JNK1, the signal of which closely leads to the prevention of tumor malignancy.

In the present study, we provide novel evidence that: (1) nobiletin-activated PKC and (2) nobiletin-induced JNK phosphorylation are sequentially mediated by the activation of PKCβII/ε in HT-1080 cells. Therefore, we suggest that PKCβII/ε in addition to MEK1/2 may become target molecules for the therapeutic actions of nobiletin. Furthermore, our finding that nobiletin alone did not influence the phosphorylation of JNK allows us to speculate that additional signals may be requisite for making connections between PKCβII/ε and JNK, which could be activated by TPA. Moreover, Brändlin et al. (39) reported cross-talk among PKC, JNK, and MEK1/2 molecules, suggesting that PKCη-mediated PKCμ activation leads to an inhibition of JNK activity and to an increase in the activity of mitogen-activated protein kinase. It remains unclear whether nobiletin may directly regulate the activities of PKCβII/ε and MEK1/2, and whether nobiletin-augmented PKCβII/ε activation may be involved in interfering with the phosphorylation of MEK1/2. Further experiments will be required to clarify the regulation of PKCβII/ε, JNK, and MEK1/2 activities and their cross-talk mechanism that is characteristic of nobiletin's antitumor effect.

Tumor development and invasion are regulated by various stimuli, such as tumor-derived soluble factors and cell-cell or cell-extracellular matrix interaction *in vivo*, which activate different sets of intracellular signal pathways, including those of MEK1/2, PKC, and JNK (28, 29, 36, 59). In addition, TPA has been reported to stimulate various tumor cells to augment MMP production, which may refer to the tumorigenic properties of malignant cancers (1, 2). Furthermore, Keller et al. (60) reported that the invasive activity of HT-1080 cells is augmented by phorbol-ester treatment. In the present study, we used TPA to evaluate the intracellular mechanisms of tumor invasion and its prevention by nobiletin. We also showed that the TPA-induced activation of MEK1/2, PKC, and JNK and their regulation by nobiletin resulted in the sequential regulation of MMP and TIMP-1 expression. However, there was no alteration in the activity of these signal mediators in HT-1080 cells treated with nobiletin alone. Therefore, we suggest that nobiletin may be selectively effective against malignant tumors with elevated levels of MEK1/2 and PKC activities, and augmented expression of MMPs. Moreover, the nobiletin-mediated

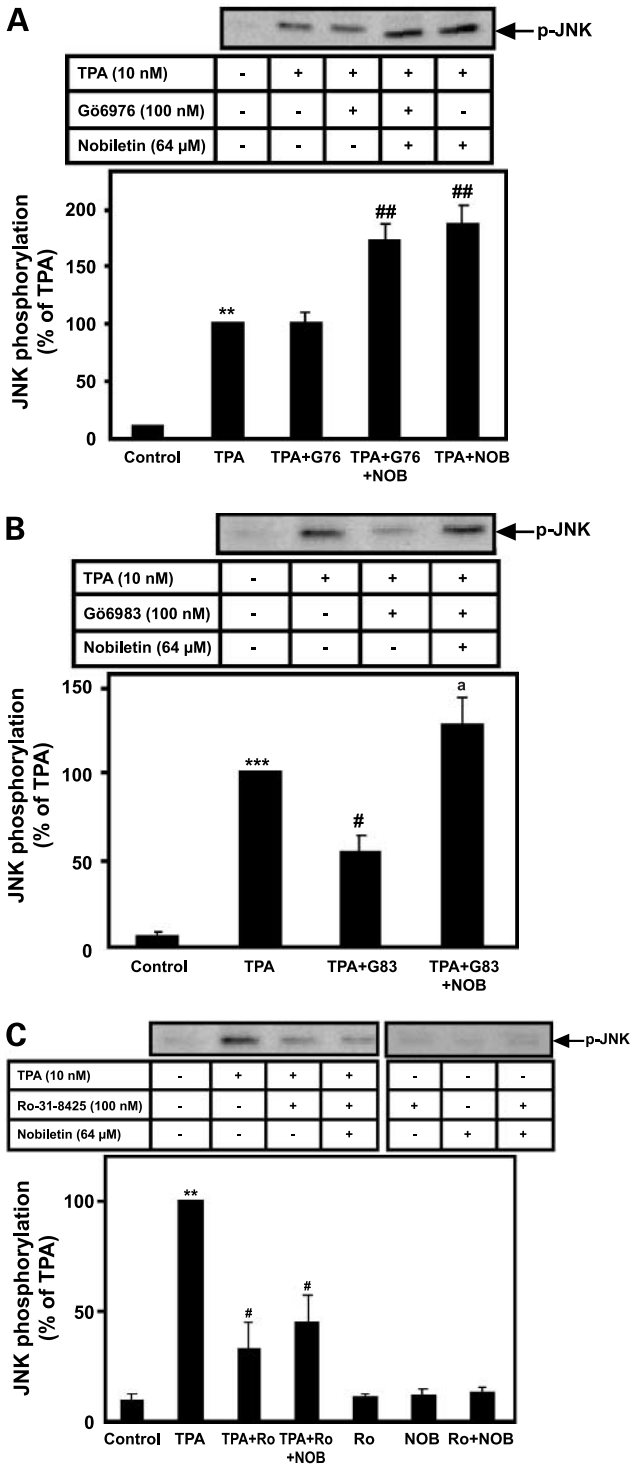

Figure 7. Involvement of PKCβII and/or PKCε in nobiletin-mediated JNK phosphorylation in HT-1080 cells. Confluent HT-1080 cells were pretreated with Gö6976 (*G76*; 100 nmol/L) (**A**), Gö6983 (*G83*; 100 nmol/L) (**B**), and Ro-31-8425 (*Ro*; 100 nmol/L) (**C**), for 30 minutes and with fresh medium containing nobiletin (*NOB*; 64 μmol/L) for another 1 hour, and then treated with TPA (10 nmol/L) for 30 minutes. Cytosol fractions were prepared and then subjected to Western blotting for phosphorylated JNK (*p-JNK*). Three independent experiments were highly reproducible and typical data are shown. The relative amounts of phosphorylated JNK were quantified by densitometric scanning, and expressed by taking TPA-treated cells as 100%. *Columns*, mean of three independent experiments; *bars*, SD. ** and ***, significantly different from untreated cells (*Control*) ($P < 0.01$ and 0.001, respectively). # and ##, significantly different from TPA-treated cells ($P < 0.05$ and 0.01, respectively). *a*, significantly different from TPA plus Gö6983 (*G83*)-treated cells ($P < 0.05$). *M*, mol/L.

inhibition of MEK1/2 and the activation of the PKCβII/ε-JNK pathway may at least partly reflect the *in vitro* and *in vivo* mechanisms of nobiletin's activity against tumor invasion (22, 23).

In conclusion, we suggest the following novel therapeutic mechanism of nobiletin's influence on tumor invasion, in that (1) nobiletin inhibits the phosphorylation of MEK1/2, which may result in the predominant suppression of proMMP production, and (2) the nobiletin-mediated activation of PKCβII/ε leads to the augmented phosphorylation of JNK, which may augment TIMP-1 production concomitant with the suppressed production of proMMP-9. Finally, these findings will provide novel approaches for the development of drugs and clinical strategies targeting intracellular signal mediators to prevent tumor invasion and metastasis.

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