Nobiletin, a citrus flavonoid, activates vasodilator-stimulated phosphoprotein in human platelets through non-cyclic nucleotide-related mechanisms

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Abstract. Nobiletin, a bioactive polymethoxylated flavone, has been described to possess a diversity of biological effects through its antioxidant and anti-inflammatory properties. Vasodilator-stimulated phosphoprotein (VASP) is a common substrate for cyclic AMP and cyclic GMP-regulated protein kinases [i.e., cyclic AMP-dependent protein kinase (PKA; also known as protein kinase A) and cyclic GMP-dependent protein kinase (PKG; also known as protein kinase G)] and it has been shown to be directly phosphorylated by protein kinase C (PKC). In the present study, we demonstrate that VASP is phosphorylated by nobiletin in human platelets via a non-cyclic nucleotide-related mechanism. This was confirmed by the use of inhibitors of adenylate cyclase (SQ22536) and guanylate cyclase [1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)], since they prevented VASP phosphorylation induced by nobiletin. Furthermore, this event was also not affected by specific inhibitors of PKA (H-89), PKG (KT5823) and PKC (Ro318220), representing cyclic nucleotide-dependent pathways upon nobiletin-induced VASP phosphorylation. Similarly, inhibitors of p38 mitogen-activated protein kinase (MAPK; SB203580), extracellular signal-regulated kinase 2 (ERK2; PD98059), c-Jun N-terminal kinase 1 (JNK1; SP600125), Akt (LY294002) and nuclear factor-κB (NF-κB; Bay11-7082) did not affect nobiletin-induced VASP phosphorylation. Moreover, electron spin resonance, dichlorofluorescein fluorescence and western blotting techniques revealed that nobiletin did not affect hydroxyl radicals (OH•), intracellular reactive oxygen species (ROS) and on protein carbonylation, respectively. Furthermore, the nobiletin-induced VASP phosphorylation was surprisingly reversed by the intracellular antioxidant, N-acetylcysteine (NAC), but not by the inhibitor of NADPH oxidase, diphenyleneiodonium chloride (DPI). It was surprising to observe the differential effects of nobiletin and NAC on VASP phosphorylation in human platelets, since they both have been reported to have antioxidant properties. The likely explanation for this discrepancy is that NAC may bind to allosteric sites on the receptor different from those that nobiletin binds to in human platelets. Taken together, our findings suggest that nobiletin induces VASP phosphorylation in human platelets through non-cyclic nucleotide-related mechanisms. Nevertheless, the exact mechanisms responsible for these effects need to be further confirmed in future studies.

Introduction

Nobiletin, a major flavonoid polymethoxylated flavone (5,6,7,8,3',4'-hexamethoxyflavone; PMF) found in citrus, has been found in very high concentrations in immature citrus peels (1). It can been extracted from different citrus species, such as *Citrus reticulata* Blanco (mandarin orange), *Citrus unshiu* Markovich, *Citrus depressa* (shiikuwasa) and *Citrus limon* (lemon) (2-4). Nobiletin has been reported to be an encouraging antioxidant and anti-inflammatory agent in the treatment of asthma, colitis and Alzheimer's disease (2,5,6). Nobiletin has been reported to protect PC12 cells from hydrogen peroxide (H_2O_2)-induced cytotoxicity (7), and to atttenuate ethanol-induced liver injury by augmenting the phosphorylation of AMP-activated protein kinase (AMPK) (8). This compound

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possesses potent anti-neuroinflammatory abilities by suppressing the activation of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways, as well as the translocation of nuclear factor-κB (NF-κB) and the subsequent gene expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β (9). A previous study demonstrated that nobiletin was the most potent inhibitor of neuroinflammation among 8 common tangerine flavonoids (10). Another study revealed that the administration of nobiletin protected rat brains from ischemic damage by activating the Akt/cyclic AMP response element-binding protein (CREB) signaling pathway (11).

Vasodilator-stimulated phosphoprotein (VASP) is a regulator of actin reorganization in platelets. As VASP is a common downstream target of various signaling pathways, an increasing attention to this molecule has been paid in platelet studies (12-14). Cyclic AMP-dependent protein kinase (PKA; also known as protein kinase A) is considered the main mediator of the numerous effects associated with increased cyclic AMP levels. In platelets, PKA activation has been shown to be involved in the phosphorylation of VASP (15). It is also noteworthy that cyclic AMP may cross-activate the cyclic GMP-dependent protein kinase (PKG; also known as protein kinase G) in some vascular tissues (16). Ii has also been shown that PKA and PKG are not the only kinases able to phosphorylate VASP, but that protein kinase C (PKC) may also have this ability (17). VASP phosphorylation in response to cyclic AMP/cyclic GMP in platelets correlates with fibrinogen receptor inhibition (18). In addition, platelets from VASP-knockout mice exhibit enhanced thrombin-induced platelet activation and impaired cyclic AMP-dependent inhibition (19). These studies suggest an important role of VASP in signal transduction pathways in platelets, its phosphorylation closely correlating with adenylate cyclase stimulation, platelet cyclic AMP/cyclic GMP increase, and the inhibition of platelet aggregation.

In our previous study, we found that nobiletin (10-30 μM) inhibited collagen and arachidonic acid-induced platelet aggregation in a concentration-dependent manner through the inhibition of the activation of the phospholipase C (PLC) γ2-PKC cascade and Akt/MAPK signaling pathways, and also found that nobiletin prolonged closure time in human whole blood *ex vivo* and increased the occlusion time of thrombotic platelet plug formation in mice (20). Moreover, at a maximum concentration of 30 μM, nobiletin had no effects on the intracellular levels of cyclic AMP or cyclic GMP in human platelets, which is consistent with the recent observation by Vaiyapuri *et al* (21). Of note, our preliminary experiments revealed that nobiletin (30 μM) markedly increased VASP phosphorylation in a similar manner to prostaglandin E$_1$ (PGE$_1$) (Fig. 1A). Therefore, it is of considerable interest to understand what the novel mechanisms responsible for nobiletin-induced VASP phosphorylation, since cyclic nucleotides do not play a role in this process.

Materials and methods

Chemicals and reagents. Nobiletin (≥97%), collagen (type I), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), SQ22536 (inhibitor of adenylate cyclase), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; inhibitor of guanylate cyclase), heparin, PGE$_1$, nitroglycerin (NTG), H89 (inhibitor of PKA), KT583 (PKG inhibitor), LY294002 (Akt inhibitor), Ro318220 (PKC inhibitor), DPI (NOX inhibitor), Bay11-7082 (NF-κB inhibitor), PD98059 (ERK2 inhibitor), SB203580 (p38 MAPK inhibitor, SP600125 (JNK inhibitor) and N-acetylcysteine (NAC) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-VASP (Cat. no. GTX132176) and anti-VASP (phospho Ser157; Cat. no. GTX32362) polyclonal antibodies (pAbs) were purchased both from GeneTex, Inc. (Irvine, CA, USA). The anti-α-tubulin monoclonal antibody (mAb; Cat. no. MS-581-P0) was purchased from NeoMarkers (Fremont, CA, USA). Hybond-P polyvinylidene difluoride (PVDF) membranes, an enhanced chemiluminescence western blotting detection reagent, the horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG; Cat. no. RPN4301), and the sheep anti-mouse IgG (Cat. no. RPN4201) were all purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Nobiletin was dissolved in 0.5% dimethyl sulfoxide (DMSO) and stored at 4˚C.

Platelets. Our study was approved by the Institutional Review Board of Taipei Medical University, Taipei, Taiwan and conformed to the directives of the Helsinki Declaration. Human platelet suspensions were prepared as previously described (22). Blood was collected from 20 healthy human volunteers (who had given informed consent and had taken no medication during the 2 weeks preceding collection) and was mixed with an acid-citrate-dextrose solution. Following centrifugation at 120 x g for 37˚C, the supernatant (platelet-rich plasma) was supplemented with 0.5 μM PGE$_1$ and 6.4 IU/ml of heparin. The washed platelets were finally suspended in Tyrode's solution containing 3.5 mg/ml of bovine serum albumin (BSA). The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Immunoblotting. The washed platelets (1.2x10^9 cells/ml) were pre-incubated with 30 μM nobiletin or 0.5% DMSO for 3 min. The reaction was terminated by using 10 mM ethylenediaminetetraacetic acid (EDTA), and the platelets were immediately re-suspended in 200 μl of a lysis buffer. Samples containing 80 μg of protein were separated on a 12% acrylamide gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrotransferred onto the PVDF membranes by using a Bio-Rad semidry transfer unit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl and 0.01% Tween-20) containing 5% BSA for 1 h and probed with various primary antibodies. The membranes were incubated with the HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3,000 in TBST) for 1 h. Immunoreactive bands were detected using an enhanced chemiluminescence system. Ratios of the semi-quantitative results were obtained by scanning the reactive bands and quantifying the optical density using a video densitometer and Bio-profil Biolight software, version V2000.01 (Vilber Lourmat, Marne-la-Vallée, France).

Measurement of hydroxyl radicals (OH•) using electron spin resonance (ESR) spectrometry. ESR spectrometry was performed using a Bruker EMX ESR spectrometer (Bruker, Billerica, MA, USA) as previously described (23). The

platelet suspensions (3.6x10⁸ cells/ml) were treated with 1 μg/ml of collagen, 500 μM nobiletin or 0.5% DMSO for 3 min in a separate vial. The suspensions were incubated for 5 min, and 100 μM DMPO were added before ESR was conducted. The ESR spectrometer was operated at a power of 20 mW and 9.78 GHz, and a scan range of 100 G and a receiver gain of 5x10⁴ was applied. Moreover, a Fenton reaction solution (50 μM FeSO$_4$ + 2 mM H$_2$O$_2$) was also pre-treated with a solvent control (0.5% DMSO) or nobiletin (500 μM) for 3 min. The rate of free-radical generation is expressed in the following equation: inhibition rate = 1 - [signal height (nobiletin)/signal height (solvent control)], as previously described (23).

Measurement of intracellular reactive oxygen species (ROS) by fluorometric assay. The method, based on a fluorometric assay, was adapted to measure the intracellular ROS. Briefly, the platelet suspensions (3.6x10⁸ cells/ml) were incubated (20 min, 37˚C in the dark) in 24-well microtiter plates with 20 μM of dichlorofluorescein fluorescence (DCF) stock in ethanol. The content of each well was then transferred into a 5 ml tube for centrifugation (335 x g, 10 min, 37˚C). After removing the supernatant, the suspensions were subsequently resuspended in Hank's Balanced Salt Solution (HBSS) buffer and transferred to the wells. Following treatment with various concentrations (50, 100, and 500 μM) of nobiletin and 1 μg/ml of collagen, the generation of the fluorescent product, DCF, was followed in an automated plate fluorescence reader (Beckman Coulter, Miami, FL, USA) using an excitation wavelength of 485 nm and emission wavelength of 555 nm. The effect of nobiletin and collagen on ROS production was compared to the resting platelets set as 100% of the ROS-induced fluorescence.

Detection of protein carbonyls. The expression of protein carbonyl was monitored in 2,4-dinitrophenylhydrazine (DNPH)-derivatized proteins using an OxyBlot Protein Oxidation Detection kit (Millipore, Billerica, MA, USA). Briefly, the washed platelets (1.2x10⁹ cells/ml) were incubated with collagen (1 μg/ml) and nobiletin (50-500 μM) or 0.5% DMSO for 3 min. The reaction was terminated by using EDTA, and the platelets were immediately re-suspended in 200 μl of a lysis buffer. Samples containing 30 μg of protein were separated by electrophoresis on 12% SDS-PAGE, and western blotting procedures were followed as described in immunoblotting assay. A primary rabbit monoclonal antibody (1:150) against DNP (Cat. no. S7150; Millipore) and a secondary anti-rabbit IgG antibody (1:300) were used to detect protein carbonyl.

Statistical analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations (n). Values of 'n' refer to the number of experiments, and each experiment was conducted using different blood donors. Differences between groups in the experiments were assessed using an analysis of variance (ANOVA). When this analysis indicated significant differences among group means, the groups were compared using the Student-Newman-Keuls method. A value of P<0.05 was considered to indicated a statistically significant difference. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Effects of cyclic nucleotides on nobiletin-induced VASP phosphorylation in washed human platelets. It has been demonstrated that cyclic nucleotides can induce VASP Ser157 phosphorylation in human platelets (24). Thus, in the present study, we investigated whether the nobiletin-induced VASP phosphorylation is dependent on cyclic nucleotides. The phosphorylation level of VASP in unstimulated platelets was at lower levels; however, the addition of 1 nM PGE$_1$ and 10 μM NTG and 30 μM nobiletin significantly increased this phosphorylation compared with the resting group (Fig. 1A). Moreover, VASP phosphorylation was absolutely abolished by pre-treatment with SQ22536 (100 μM), an inhibitor of adenylate cyclase or ODQ (10 μM), an inhibitor of guanylate cyclase. Moreover, pre-treatment with SQ22536 or ODQ did not significantly reverse the nobiletin (30 μM)-induced VASP phosphorylation (Fig. 1B). These results indicate that nobiletin-induced VASP phosphorylation in human platelets is not dependent on cyclic nucleotide-related pathways.

These results were further confirmed by using PKA and PKG inhibitors, since it has previously been described that VASP is a substrate of cyclic nucleotide (cyclic AMP/cyclic GMP)-dependent PKA-PKG (25), which phosphorylate it at 3 sites: Ser157, Ser239 and Thr278. These sites are phosphorylated, with differing kinetics, both *in vitro* and in intact human platelets (26). In the present study, as shown in Fig. 2A, the inhibitors of PKA (H89, 5 μM) and PKG (KT5823, 2 μM) were not essentially effective in reversing the nobiletin-induced VASP phosphorylation in platelets. These data indicate that PKA and PKG are not involved in the regulation of nobiletin-induced VASP phosphorylation.

MAPK, Akt and PKC have no essential function in nobiletin-induced VASP phosphorylation. To investigate whether MAPKs, including p38 MAPK, ERK and JNK, Akt and PKC, play a role in the stimulatory mechanisms of nobiletin on VASP phosphorylation in human platelets, we used the well-established inhibitors of p38 MAPK (SB203580, 10 μM), ERK (PD98059, 20 μM), JNK (SP600125, 10 μM), Akt (LY294002, 10 μM) and PKC (Ro318220, 2 μM) to examine their effects on VASP phosphorylation induced by nobiletin. The results revealed that all the tested inhibitors of p38 MAPK, ERK and JNK, Akt and PKC did not alter the nobiletin-induced VASP phosphorylation (Fig. 2B and C), which indicate that the MAPK, Akt and PKC signaling pathways do not play a role in nobiletin-induced of VASP phosphorylation.

NF-κB is not responsible for nobiletin-induced VASP phosphorylation in human platelets. NF-κB has been reported to function independently of gene regulation in platelets, and follwoing platelet activation, it is phosphorylated and degraded (27). NF-κB induction can be abrogated using its inhibitor, Bay11-7082 [(E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile], which is an irreversible inhibitor of IκBα phosphorylation, resulting in the downregulation of the induced NF-κB activation. Given the significance of NF-κB and platelets in atherosclerosis and inflammation, we investigated whether NF-κB plays a role in nobiletin-induced VASP phosphorylation by using its inhibitor, Bay11-7082. The data
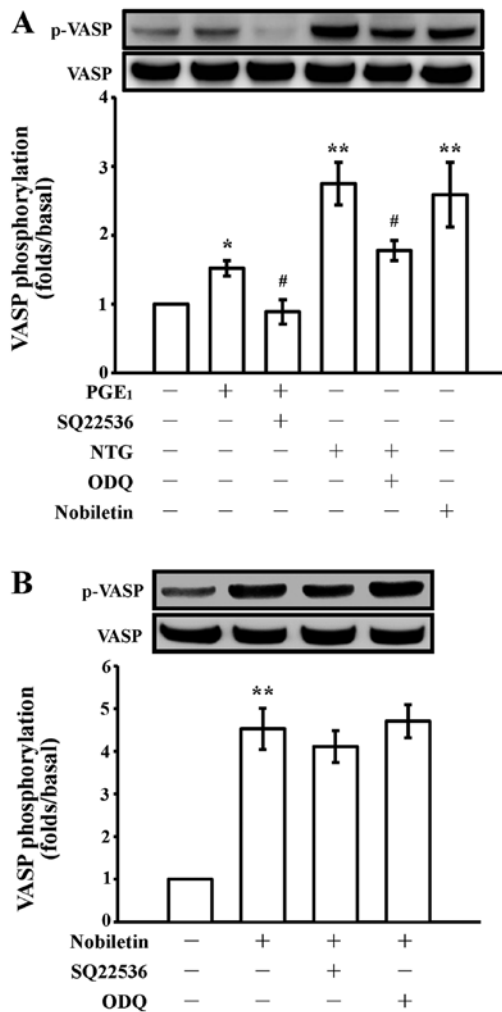
Figure 1. Effects of nobiletin and cyclic nucleotide inhibitors on vasodilator-stimulated phosphoprotein (VASP) phosphorylation in washed human platelets. (A) Human platelets (1.2×10^9 cells/ml) were pre-incubated with or without SQ22536 (100 μM) or ODQ (10 μM), followed by the addition of 1 nM prostaglandin E$_1$ (PGE$_1$), 10 μM nitroglycerin (NTG), 30 μM nobiletin, or 0.5% dimethyl sulfoxide (DMSO) (control) for 3 min; (B) human platelets were pre-incubated with SQ22536 (100 μM) or ODQ (10 μM) in the absence or presence of 30 μM nobiletin, or 0.5% DMSO (control) for 3 min. The platelets were collected, and subcellular extracts were analyzed to determine VASP phosphorylation. Values are presented as the means ± SEM (A) n=7; (B) n=4. *P<0.05, and **P<0.001, compared with the resting platelets; #P<0.05, compared with the PGE$_1$- or ODQ-treated platelets.
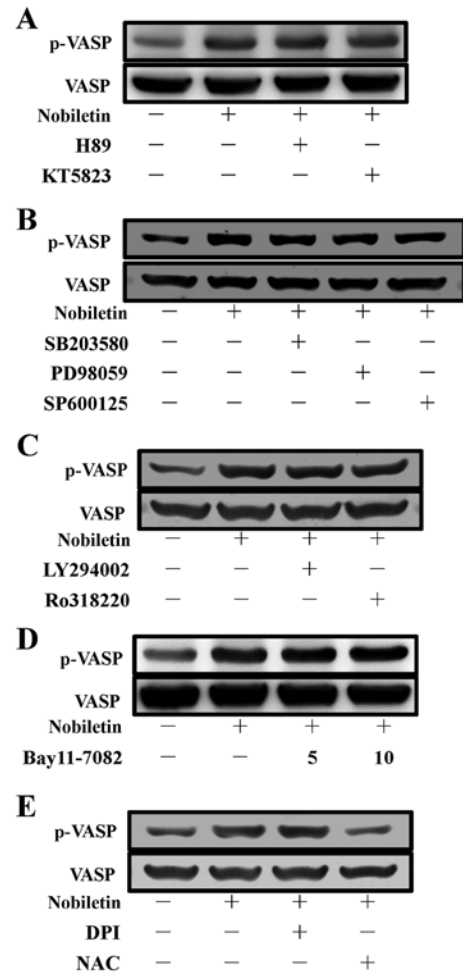

Figure 2. Effects of nobiletin on vasodilator-stimulated phosphoprotein (VASP) phosphorylation in washed human platelets. Human platelets (1.2×10^9 cells/ml) were pre-incubated with (A) 5 μM H89 [cyclic AMP-dependent protein kinase (PKA) inhibitor] or 2 μM KT5823 [cyclic GMP-dependent protein kinase (PKG) inhibitor]; (B) 10 μM SB203580 [p38 mitogen-activated protein kinase (MAPK) inhibitor], 20 μM PD98059 [extracellular signal-regulated kinase (ERK) inhibitor], or SP600125 [c-Jun N-terminal kinase (JNK) inhibitor]; (C) 10 μM LY294002 (Akt inhibitor) or 2 μM Ro318220 [protein kinase C (PKC) inhibitor]; (D) 5 and 10 μM Bay11-7082 [nuclear factor-κB (NF-κB) inhibitor] and (E) 10 μM diphenyleneiodonium chloride (DPI) and 10 mM N-acetylcysteine (NAC) for 3 min, followed by the addition of 30 μM nobiletin or 0.5% dimethyl sulfoxide (DMSO) (control). VASP phosphorylation was analyzed by immunoblotting using an anti-phospho-VASP antibody. Profiles are representative of 4 independent experiments.

indicated that Bay11-7082 had no visible effect on nobiletin-induced VASP phosphorylation (Fig. 2D), and consequently suggest that NF-κB is not involved in the process of nobiletin-induced VASP phosphorylation.

Involvement of NADPH oxidase (NOX) and ROS in nobiletin-induced VASP phosphorylation. Since it has been previously demonstrated that NOX and ROS regulate intracellular transduction pathways and many of the structural properties of NOX have been found in platelets (28), in the present study, the effects of NOX inhibitor, diphenyleneiodonium chloride (DPI), and the cell-permeable antioxidant, NAC, on nobiletin-induced VASP phosphorylation in platelets were examined. We found that DPI (10 μM) did not affect nobiletin-induced VASP phosphorylation, whereas, NAC (10 mM)

significantly antagonized this phosphorylation (Fig. 2E). These findings indicate that the stimulatory effect of nobiletin on VASP phosphorylation is also independent of NADPH oxidase, but that it is dependent on NAC-mediated inhibitory mechanisms.

Effect of nobiletin on •OH and ROS formation. The antioxidant properties of plants or their active principles, mainly their radical scavenging activities, have been intensively investigated over the years (29-31). In this study as shown in Fig. 2E, NAC, a cell-permeable antioxidant, significantly reversed nobiletin-induced VASP phosphorylation. Thus, we examined whether the process of nobiletin-induced VASP phosphorylation is dependent on persuading •OH radicals or ROS formation. ESR spectrometry and the fluorescence-based
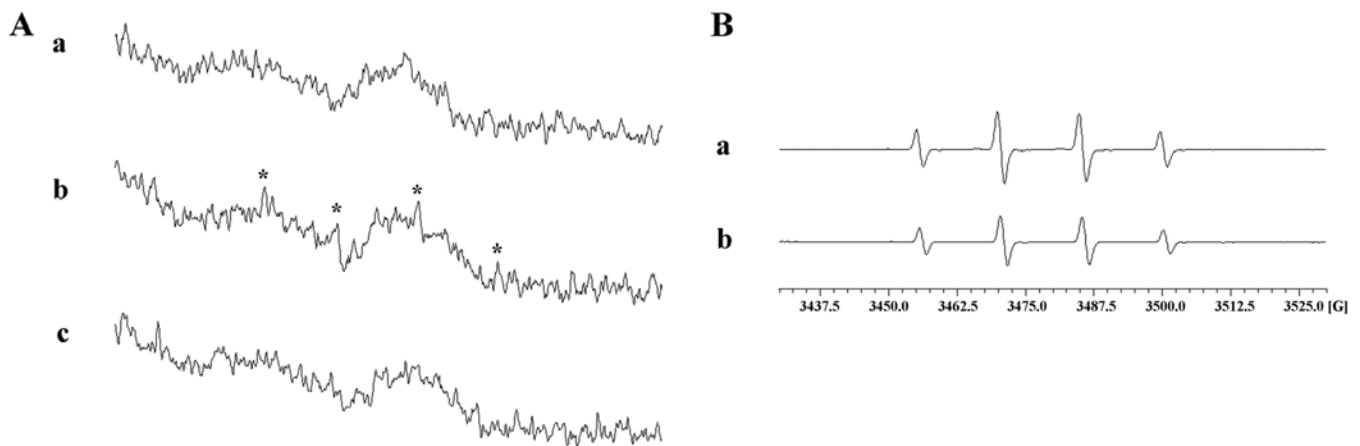
Figure 3. Effect of nobiletin on hydroxyl radical (•OH) formation by electron spin resonance (ESR) spectrometry. (A) Human platelet suspensions (3.6×10^8 cells/ml) were treated with (a) 0.5% dimethyl sulfoxide (DMSO), (b) 1 μg/ml of collagen or (c) 500 μM nobiletin for 3 min to induce •OH formation. The suspensions were incubated for 5 min, and 100 μM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were added before the analysis of ESR; (B) a Fenton reaction solution (50 μM FeSO$_4$ + 2 mM H$_2$O$_2$) was pretreated with (a) solvent control (0.5% DMSO) or (b) nobiletin (500 μM) for 3 min to induce •OH formation, and the ESR spectrometry was conducted. (A and B) Profiles are representative of 4 independent experiments; (A-b) asterisk (*) indicates the formation of •OH.

method using DCF as a fluorescent probe revealed that nobiletin, even at a higher concentration of 500 μM, did not significantly stimulate •OH radical signals, as that stimulated by either collagen (1 μg/ml; positive control) (Fig. 3A) in washed human platelets or by Fenton reaction (Fig. 3B). Furthermore, our results also demonstrated that nobiletin (50 and 100 μM) did not reduce the fluorescence intensity of DCF even at the highest concentration of 500 μM (Fig. 4A), which clearly indicated that nobiletin does not have a stimulatory effect on •OH radicals as well as on intracellular ROS.

Effects of nobiletin on protein carbonyls. At present, much attention is focused on the protein carbonyl content as the most widely used marker of the oxidative modification of proteins in correlation with a variety of pathologies (32). Since we noted that nobiletin did not affect •OH radicals or ROS production, this event was further confirmed by detecting carbonylated proteins in nobiletin (50-500 μM)-treated platelets, which was compared with collagen (positive control)-induced protein carbonylation. When the platelets were activated by 1 μg/ml collagen, protein carbonylation was markedly increased compared to the resting platelets (Fig. 4B). This augmentation is due to the oxidative stress generated by the cells themselves when activated by the exogenous exposure of the platelets to collagen. Furthermore, treatment with nobiletin at various concentrations (50-500 μM) did not significantly induce or reduce protein carbonylation, which was evidenced by observing the unlettered intensity of the carbonylated bands, as compared to the collagen-stimulated cells (Fig. 4B). Moreover, pre-treatment with NAC (10 mM) markedly reversed the nobiletin-induced VASP phosphorylation; however, treatment with NAC alone did not significantly induce VASP phosphorylation (Fig. 5A).

Discussion

It has been considered that increased cyclic AMP/cyclic GMP in platelets activates cyclic AMP-dependent PKA and cyclic GMP-dependent PKG, and thus regulates the platelet

activation response by phosphorylating intracellular protein substrates, such as VASP. Therefore, VASP phosphorylation may act as a negative regulator of platelet activation. In this study, we demonstrated that cyclic nucleotide-related pathways were not responsible for nobiletin-induced VASP phosphorylation in washed human platelets. Considering the fact that platelets are anuclear cells, the projected hypothesis was thus confirmed by using various well established cyclic nucleotide-related inhibitors, such as adenylate cyclase inhibitor (SQ22536), guanylate cyclase inhibitor (ODQ), PKA inhibitor (H89) and PKG inhibitor (KT5823) instead of using their respective siRNAs. Our results indicated that nobiletin-induced VASP phosphorylation was not affected by these inhibitors. In addition, it was also demonstrated that this process was independent of NF-κB, MAPKs, Akt, PLCγ2/PKC, NOX and ROS, since the respective inhibitors of these molecules did not affect VASP phosphorylation induced by nobiletin. Hence, it was important to determine the actual mechanisms underlying the phosphorylation of VASP by nobiletin.

VASP is a critical protein that plays a role in regulating adhesive events that are involved in platelet aggregation (15,33). Cyclic nucleotides activate cyclic nucleotide-dependent kinases which inhibit all steps of this cascade and simultaneously phosphorylate a number of proteins, including VASP. Moreover, considering the results of a previous study that suggested that VASP plays an important role in the signaling of cyclic GMP- and cyclic AMP-dependent kinases (34), it was surprising that in this study, nobiletin stimulated VASP phosphorylation in washed human platelets without increasing the cyclic nucleotide levels. This process was examined by observing whether the adenylate cyclase inhibitor, SQ22536, and the guanylate cyclase inhibitor, ODQ, modulate nobiletin-induced VASP phosphorylation in platelets. The results confirmed that neither SQ22536 nor ODQ affected nobiletin induced VASP phosphorylation as shown in Fig. 1B. A likely explanation for this unexpected finding is that the absence of cyclic nucleotide function in VASP phosphorylation may be substituted by other members of proteins.
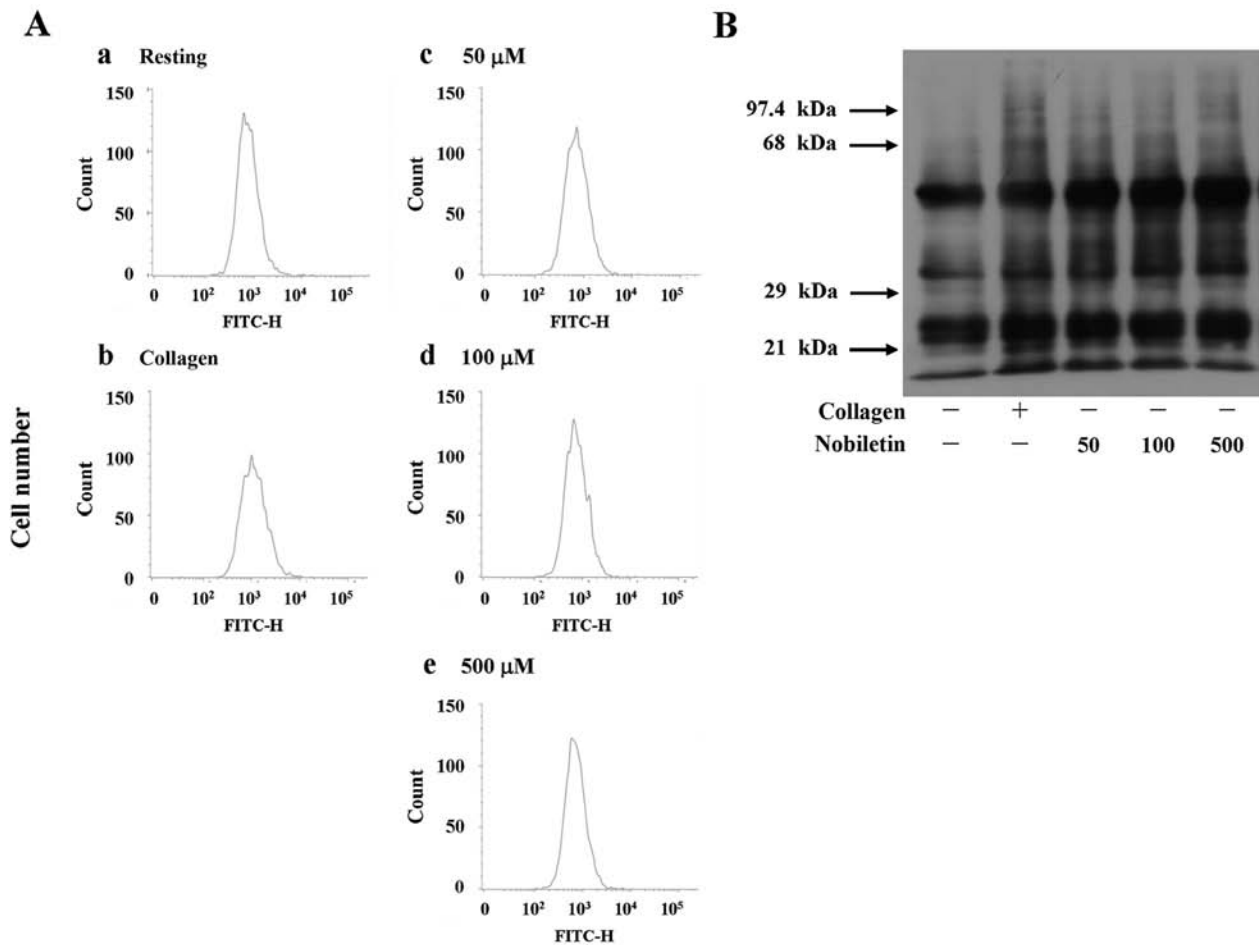
Figure 4. Effects of nobiletin on intracellular reactive oxygen species (ROS) formation and protein carbonyl expression in human platelets. (A) Platelet suspensions (3.6×10^8 cells/ml) were incubated in 24-well microtiter plates with 20 μM of dichlorofluorescein fluorescence (DCF) stock in ethanol. The content of each well was then transferred into a 5 ml tube for centrifugation (335 x g, 10 min, 37˚C). After removing the supernatant, suspensions were subsequently re-suspended in Hank's Balanced Salt Solution (HBSS) buffer and transferred to the wells. Following treatment with various concentrations (50, 100 and 500 μM) of nobiletin and 1 μg/ml of collagen, the generation of the fluorescent product DCF was followed in an automated plate fluorescence reader using an excitation wavelength of 485 nm and emission wavelength of 555 nm; (B) the expression of protein carbonyl was monitored in 2,4-dinitrophenylhydrazine (DNPH)-derivatized proteins using an OxyBlot Protein Oxidation Detection kit. (A and B) Profiles are representative of 4 independent experiments.

It is well-established that PKA is an essential negative modulator of platelet function through the inhibition of multiple biochemical events which all converge in the inhibition of platelet function. It is also noteworthy that cyclic AMP may cross-activate PKG in some vascular tissues. PKG has been identified as the intracellular mediator of the effects of cyclic GMP-elevating agents, such as nitric oxide or natriuretic peptides (35). Therefore, we wished to confirm whether nobiletin-induced VASP phosphorylation is dependent on cyclic AMP/cyclic GMP and is linked to PKA/PKG activity. In order to examine this hypothesis, the PKA inhibitor, H89, and the PKG inhibitor, KT5823, were added to the nobiletin pre-treated platelets. KT5823, an *in vitro* inhibitor of PKG, has been extensively used to demonstrate or rule out an involvement of PKG in signaling processes, and the PKA inhibitor, H89, is a chemical compound which inhibits PKA in a competitive manner (36). In this study, we found that the elevation of nobiletin-induced VASP phosphorylation in platelets was not modulated by either H89 or KT5823, and thus, it appears that VASP phosphorylation stimulated by nobiletin may be independent of cyclic AMP/PKA and cyclic GMP/PKG pathways.

A previous study suggested that PKA and PKG are not the only kinases able to phosphorylate VASP, but that PKC may also have this ability (17). Chitaley *et al* (17) reported that, in cultured rat aortic smooth muscle cells, VASP becomes phosphorylated in a PKG- and PKA-independent manner. They established that VASP is also a substrate of PKC and provide evidence for the involvement of a classical PKC isoform in VASP phosphorylation (17). Moreover, in our previous study, we reported that Akt acts as an upstream regulator of PKC (37), and this pathway was found to be responsible for phosphorylation of VASP in activated platelets (38). Hence, it is interesting to speculate whether PKC and Akt play a crucial role in regulating nobiletin-induced phosphorylation of VASP in platelets. Our results clearly demonstrated that this phosphorylation was independent of the PKC and Akt pathways, since their specific inhibitors, Ro318220 (PKC) and LY294004 (Akt), did not alter the phosphorylation status of VASP, as its level was similar in nobiletin alone-treated platelets (Fig. 2C).

MAPKs control cell proliferation, differentiation, mitosis, survival and apoptosis. Among these MAPKs, p38 MAPK, JNK1 and ERK2 have been reported to exist in platelets and are
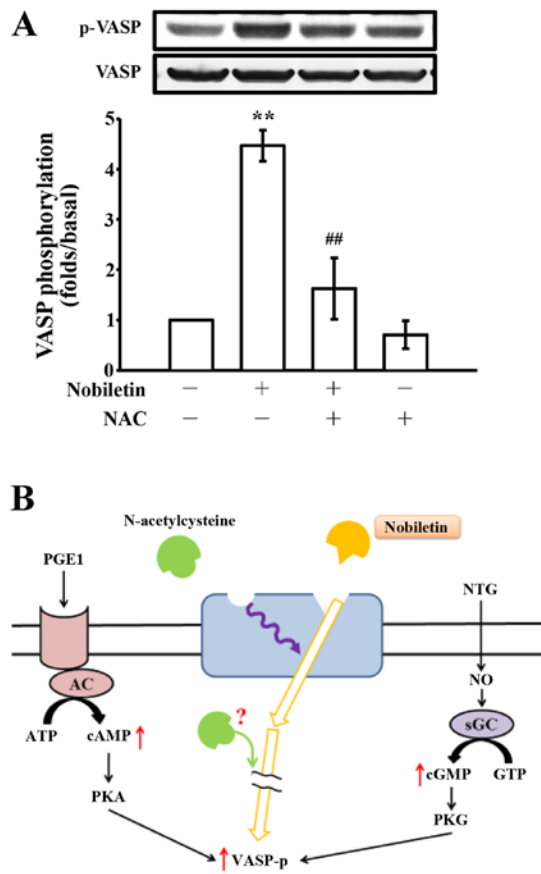
Figure 5. Effects of nobiletin and N-acetylcysteine (NAC) on vasodilator-stimulated phosphoprotein (VASP) phosphorylation in human platelets. (A) Human platelets (1.2×10^9 cells/ml) were pre-incubated with solvent control [0.5% dimethyl sulfoxide (DMSO)], with or without NAC (10 mM) and 30 μM nobiletin for 3 min. VASP phosphorylation was then analyzed by immunoblotting using an anti-phospho-VASP antibody. Values are presented as the means ± SEM (n=4). [**]P<0.001, compared with the resting platelets; [##]P<0.05, compared with the nobiletin-treated platelets; (B) schematic diagram illustrating the hypothesis of the participation of NAC and nobiletin on VASP phosphorylation in human platelets. VASP phosphorylation is induced by prostaglandin E_1 (PGE$_1$) and nitroglycerin (NTG) via stimulating cyclic AMP and cyclic GMP-regulated protein kinases [i.e., cyclic AMP-dependent protein kinase (PKA) and cyclic GMP-dependent protein kinase (PKG)], respectively. NAC interrupts nobiletin-triggered VASP phosphorylation may through binding to the allosteric sites on the receptor distinct from that nobiletin binds; or NAC directly interrupts intracellular signaling cascades which are responsible for nobiletin-triggered VASP phosphorylation through an unidentified mechanism. AC, adenylyl cyclase; NO, nitric oxide; sGC, soluble guanylate cyclase.

activated by various stimuli (39). ERK2 and JNK1 phosphorylation stimulated by collagen has been proven to be involved in platelet aggregation (40). In addition, p38 MAPK activation has also been shown in collagen-induced platelet activation and secretion, which was restored by p38 MAPK inhibitors (41). However, our findings demonstrated that the phosphorylation of VASP-stimulated by nobiletin was not modulated by the p38 MAPK inhibitor SB203580, ERK inhibitor, PD98059, and also by the JNK inhibitor, SP600125, indicating that MAPK signaling pathways do not account for the stimulatory effects of nobiletin on VASP phosphorylation. In addition, NF-κB induction can be abrogated using its inhibitor, Bay11-7082, which is an irreversible inhibitor of IκBα phosphorylation, resulting in the downregulation of the cytokine-induced NF-κB activation (42). A previous study indicated that Bay11-7082

potently inhibited collagen- and thrombin-induced platelet aggregation, and it induced VASP phosphorylation through cyclic AMP elevation and PKA activation (43). Following this, it was important to determine whether Bay11-7082 can modulate nobiletin-induced phosphorylation of VASP, and the data showed that this inhibitor did not account for the increased phosphorylation of VASP in response to nobiletin, indicating that NF-κB is also not involved in this process.

ROS derived from platelet activation may augment platelet reactivity during *in vivo* thrombus formation. Our previous study suggested that free radical species act as secondary messengers that increase cytosolic Ca^{2+} during the initial phase of platelet activation processes, and PKC is involved in receptor-mediated free radical production in platelets (44). That study also showed H$_2$O$_2$ derived from platelets is converted into •OH, and platelet aggregation can be inhibited by •OH scavengers (44). A previous study found that platelet-induced ROS production was inhibited by the radical scavenger, NAC, and the NOX inhibitor, DPI (45). We found that NAC potently reversed the stimulatory effects of nobiletin on VASP phosphorylation, but DPI did not alter this phosphorylation, which indicated that NADPH is also not involved in this process.

Protein carbonyl groups serve as a biomarker of oxidative stress, since protein oxidation typically results in increased carbonyl contents. It has been reported that H$_2$O$_2$ produces a concentration-dependent increase in the carbonylation of platelet proteins, and it can be quenched by antioxidant catalase, suggesting that carbonylation is induced by the oxidative stress generated by activated platelets (32). Several ROS, including •OH, oxidize amino acid residues in proteins to form products with carbonyl groups that can be measured after reaction with DNPH. In this study, the protein carbonyl concentrations in platelets treated with collagen, as well as with nobiletin correlated with changes in the intensity of DNPH reactive protein bands on immunoblots. In addition, an active role of ROS in VASP phosphorylation stimulated by nobiletin was monitored by DCF fluorescence as a marker for the intracellular generation of radicals. From these experiments, it was found that nobiletin-induced VASP phosphorylation is independent of ROS formation. Moreover, it has been demonstrated that NAC decreases human platelet aggregation and increases the intracellular levels of cyclic GMP by interplaying with endogenous NO (46), which is constitutively synthesized by platelets (47) and inhibits aggregation by activating the soluble guanylate cyclase (48). In this study, as shown in Fig. 2E, NAC alone did not trigger VASP phosphorylation as nobiletin did. The possible explanations for this finding may be due to the following reasons: first, NAC may bind to allosteric sites on the receptor distinct from that nobiletin binds in the human platelets to block the signals of nobiletin-triggered VASP phosphorylation; second, since NAC is a cell-permeable antioxidant, it may interrupt the intracellular signaling cascade which is responsible for VASP phosphorylation through an unidentified mechanism (Fig. 5B).

In conclusion, our previous study suggested that nobiletin represents a potential therapeutic agent for the prevention or treatment of thromboembolic disorders. In the present study, and to the best of our knowledge, we found for the first time that nobiletin stimulates VASP phosphorylation via a novel non-cyclic nucleotide-dependent mechanisms in washed human platelets. In addition, an unexpected result was

observed in that NAC reversed the nobiletin-induced VASP phosphorylation. However, the exact inhibitory mechanisms of action of NAC warrant further investigation.

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