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Polymethoxylated flavones potentiate the cytolytic activity of NK leukemia cell line KHYG-1 via enhanced expression of granzyme B

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ABSTRACT

Polymethoxylated flavones (PMFs) are found in the peel tissues of some citrus species. Here, we report that PMFs, such as nobiletin, potentiate the cytolytic activity of KHYG-1 natural killer (NK) leukemia cells. Nobiletin markedly enhanced the expression of granzyme B, a serine protease that plays critical roles in the cytolytic activity of NK cells. The potentiated cytolytic activity induced by nobiletin was canceled by the granzyme B inhibitor Z-AAD-CMK. Nobiletin also increased the levels of phosphorylated CREB, ERK1/2, and p38 MAPK in KHYG-1 cells, which are known to participate in NK cell function. Inhibition of an upstream kinase of ERK1/2 failed to reduce the granzyme B expression and KHYG-1 cytolytic activity. Meanwhile, inhibition of p38 MAPK attenuated both granzyme B expression and KHYG-1 cytolytic activity. These results suggest that the primary role of nobiletin in KHYG-1 cytolytic activity lies in upregulation of granzyme B expression, at least in part, mediated through p38 MAPK function.

1. Introduction

Natural killer (NK) cells are the first line of defense against tumor cells and virus-infected cells without prior antigenic stimulation. NK cells have cytolytic granules that contain cytotoxic effector molecules, and release these granules upon binding to their target cells. One of the cytotoxic effectors, perforin, forms pore-like structures to allow passage of the cytotoxic effectors and cations into the target cells [1,2]. Perforin-deficient mice show reduced resistance to the development of spontaneous lymphoma, indicating the importance of perforin in immune surveillance [3]. Another important cytotoxic effector is the serine protease granzyme B. When this enzyme is taken up into target cells, it activates caspase 3 directly or indirectly through modulation of the mitochondrial function, leading to DNA fragmentation and apoptosis [4,5]. The requirement for granzyme B in NK cell function was demonstrated by the observation that granzyme B-deficient NK cells are defective in inducing DNA fragmentation and cell death of target cells [6]. The transcriptional regulation of the human and mouse granzyme B genes has been investigated. The upstream regions of the transcriptional start sites of the human and mouse granzyme B genes contain functional binding sites for transcription factors, such as AP-1, CREB, Ikaros, and CBF [7–9]. In addition, the downstream region of the human granzyme B gene contains a functional NF-κB binding site [10].

KHYG-1 is an NK leukemia cell line established by Yagita et al. [11]. This cell line was reported to be highly cytotoxic against the NK-sensitive cell line K562 [12]. The cytolytic granules in NK cells are usually dispersed in the cytoplasm, and become polarized upon contact with target cells. However, the granules in KHYG-1 cells are constitutively polarized [13,14]. This "priming" effect of KHYG-1 cells might support its high level of cytolytic activity.

Polymethoxylated flavones (PMFs), including nobiletin, tangeretin, and sinensetin, are abundant in the peel tissues of certain types of citrus species such as *Citrus tangerine*, *Citrus reticulata*, and *Citrus depressa* [15]. PMFs are known to exhibit pharmacological effects toward various types of cells, such as pheochromocytoma cells [16], adipocytes [17], microglial cells [18], and synovial fibroblasts [19], accompanied by modulation of cytokine expression, or intracellular signaling including kinases and transcription factors. However, little is known about the effects of nobiletin on NK cell function.

We have explored compounds from natural sources that potentiate NK cell cytolytic activity using KHYG-1 cells. Toward this aim, we tested approximately 250 compounds and found that PMFs potentiate the cytolytic activity of KHYG-1 cells. In this report, we describe the effects of nobiletin on the activation of granzyme B, taking account of the already-known in vitro function of nobiletin.

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2. Materials and methods

2.1. Materials

Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone), tangeretin (4′,5,6,7,8-pentamethoxyflavone), 3,3′,4′,5,6,7,8-heptamethoxyflavone, recombinant human IL-2, U0126, SB203580, and trichostatin A (TSA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sinensetin (3′,4′,5,6,7-pentamethoxyflavone) was purchased from Funakoshi (Tokyo, Japan). KHYG-1 cells and K562 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Fetal bovine serum (FBS) was from Biological Industries (Beit-Haemek, Israel). FastStart Universal SYBR Green Master (ROX) was from Roche Applied Science (Indianapolis, IN). Antibodies against β-actin, granzyme A, granzyme B, Fas ligand, extracellular signal-regulated protein kinase (ERK) 1/2, phospho-ERK1/2 (Thr202/Tyr204), p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK (Thr180/Tyr182), CREB, phospho-CREB (Ser133), and HRP-linked anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA). The antibody against perforin was purchased from Abcam Inc. (Cambridge, MA). The antibody against granulysin was from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). The granzyme B inhibitor Z-AAD-CMK was from Merck Millipore (Billerica, MA).

2.2. Cell culture

KHYG-1 cells were cultured in RPMI-1640 supplemented with 10% FBS, 50 ng/ml human IL-2, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. K562 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate.

2.3. Cytotoxicity assay

KHYG-1 cells were cultured with each compound for specified times, collected by centrifugation, and resuspended in RPMI-1640 supplemented with 1% FBS. K562 cells cultured for 48 h were also collected by centrifugation and resuspended in RPMI-1640 supplemented with 1% FBS. The two cell types were mixed at various ratios and cocultured in round-bottom microplates in triplicate for 4 h. NK cell-mediated cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release from K562 cells using a Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Alternatively, cytotoxicity was measured in a flow cytometer (EasyCyte 6-2L; Merck Millipore) using a Guava Cell Toxicity Kit (Merck Millipore). For flow cytometric analysis, K562 cells were prelabeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and cocultured with KHYG-1 cells. Subsequently, the cells were stained with

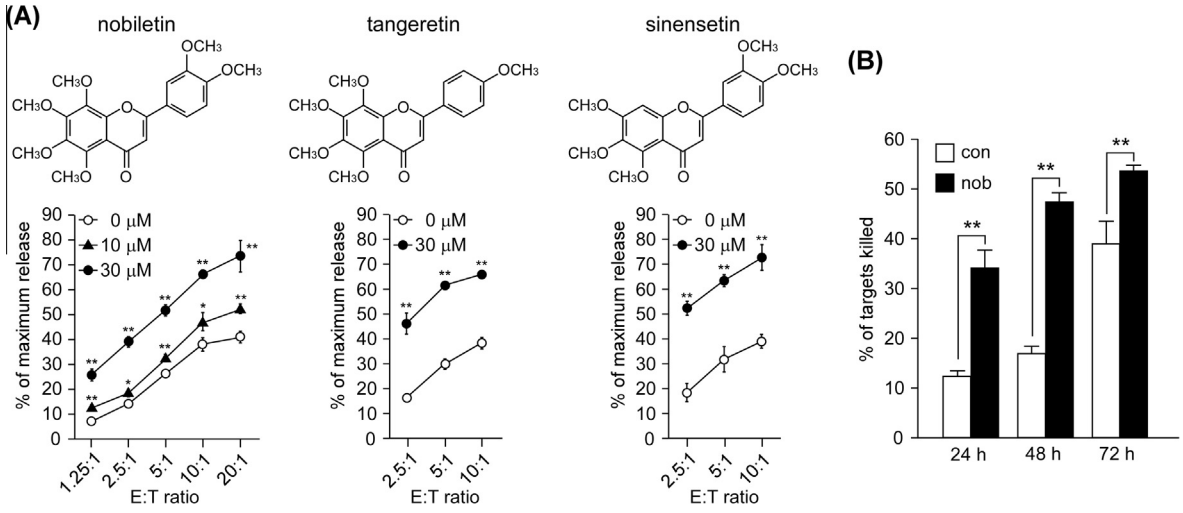

Fig. 1. Effects of PMFs on the cytolytic activity of KHYG-1 cells. (A) Lysis of K562 target cells by KHYG-1 effector cells treated with the indicated doses of PMFs for 72 h. KHYG-1 and K562 cells were washed and cocultured for 4 h at the indicated effector:target (E:T) ratios. Cytotoxicity was determined by measuring LDH release from K562 cells. (B) Lysis of K562 cells by KHYG-1 cells treated with 30 μM nobiletin (nob) for the indicated times. Cytotoxicity was measured by flow cytometry at the E:T ratio of 1:1. Values represent means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vehicle control.

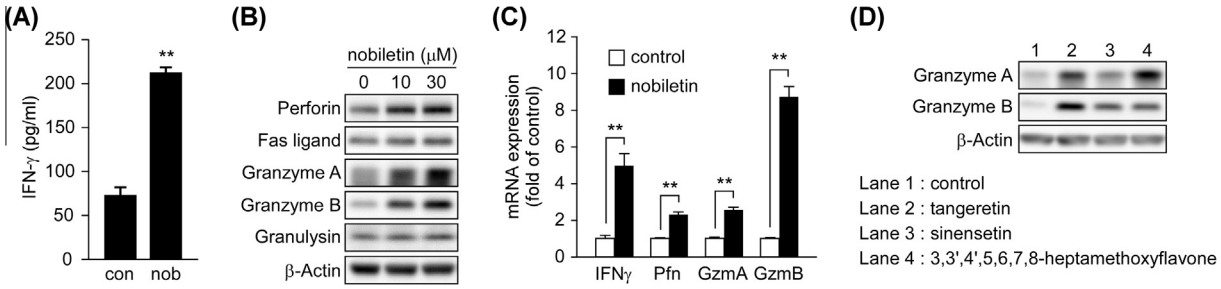

Fig. 2. Expression of IFN-γ and cytotoxic effector molecules. (A) The IFN-γ levels in culture supernatants of KHYG-1 cells treated with nobiletin (30 μM) for 24 h were measured by ELISA. (B) Protein levels of cytotoxic effectors in KHYG-1 cells treated with the indicated doses of nobiletin for 24 h. (C) Nascent mRNA levels of IFN-γ, perforin (Pfn), granzyme A (GzmA), and granzyme B (GzmB) in KHYG-1 cells. The expression of each gene was normalized to the expression of GAPDH. Relative ratios of gene expression vs. control (assigned a value of 1) are indicated. Values represent means ± SD ($n = 3$). $^{**}P < 0.01$, vs. control (con). (D) Protein levels of granzymes A and B in KHYG-1 cells treated with PMFs other than nobiletin. Cells were treated with each PMF at 30 μM for 24 h and the protein levels were determined by Western blot analysis.

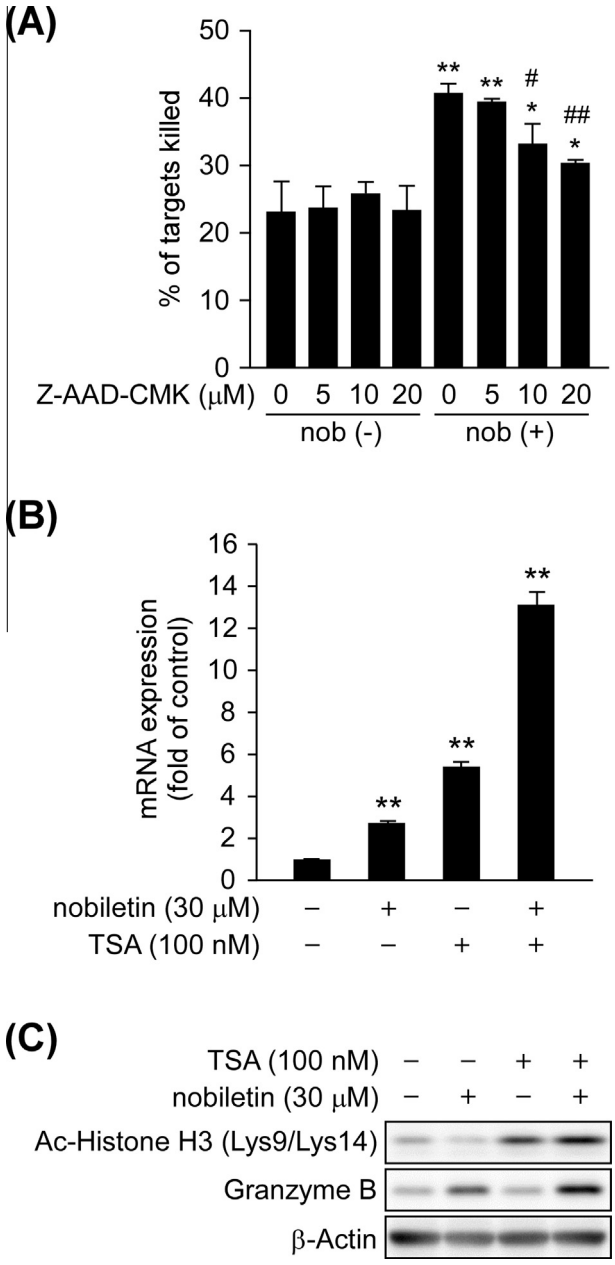

Fig. 3. Mechanism underlying granzyme B induction by nobiletin and its significance in the cytolytic activity of KHYG-1 cells. (A) Effects of the granzyme B inhibitor Z-AAD-CMK on the cytolytic activity of KHYG-1 cells. Cells treated with the indicated doses of Z-AAD-CMK were cultured with nobiletin (0 or 30 μM) for 24 h. Cytotoxicity was measured by flow cytometry at an E:T ratio of 1:1. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Z-AAD-CMK(−)nobiletin(−). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Z-AAD-CMK(−)nobiletin(+). (B) Gene expression of granzyme B, and (C) acetylation of histone H3 and protein level of granzyme B with a combination of nobiletin and TSA on KHYG-1 cells. KHYG-1 cells cultured for 24 h were treated with TSA (0 or 100 nM) and nobiletin (0 or 30 μM), and cultured for a further 24 h. Total RNA and protein were purified and subjected to quantitative real-time PCR and Western blot analysis, respectively. The expression of each gene was normalized to the expression of GAPDH. Relative ratios of gene expression vs. TSA(−)nobiletin(−) as a control (assigned a value of 1) are indicated. Values represent means ± SD ($n = 3$). $^{**}P < 0.01$, vs. TSA(−)nobiletin(−) control.

7-amino-actinomycin D (7-AAD). The CFSE(+)7-AAD(+) double-positive cells were defined as damaged K562 cells.

2.4. Cytokine measurement

The levels of IFN-γ in the culture supernatants of KHYG-1 cells were determined using a LEGEND MAX Human IFN-γ ELISA Kit (BioLegend, San Diego, CA) according to the manufacturer's protocol.

2.5. Immunoblotting

Proteins were extracted from KHYG-1 cells using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) in the presence of a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Wako Pure Chemical Industries Ltd.). Equal amounts of protein were resolved by SDS–PAGE and transferred to Immobilon-P PVDF membranes (Merck Millipore). The membranes were probed with the required primary antibodies diluted in Immuno-Enhancer Solution A (Wako Pure Chemical Industries Ltd.) for 1 h at room temperature, washed with TBS-T, and incubated with HRP-linked secondary antibodies diluted in Immuno-Enhancer Solution B (Wako Pure Chemical Industries Ltd.) for 1 h at room temperature. The immunoreactive proteins were visualized and detected using an ECL-Select Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan) and a GeneGnome-5 chemiluminescent imaging system (Syngene, Cambridge, UK).

2.6. Quantitative real-time PCR

Purification of nascent RNA was performed using a Click-iT Nascent RNA Capture Kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, KHYG-1 cells were treated with nobiletin (0 or 30 μM) for 8 h, and then incubated with 5-ethynyl uridine (EU) for 16 h to label nascent RNA. Total RNA including EU-labeled RNA was isolated using a High Pure RNA Isolation Kit (Roche Applied Science). The EU-labeled RNA was then biotinylated and captured using streptavidin-conjugated beads. Purified nascent RNA was reverse-transcribed with a Primescript RT Reagent Kit (Takara Bio, Shiga, Japan) to obtain cDNA. SYBR Green-based quantitative real-time PCR was performed using an ABI 7500 Real-Time PCR System (Life Technologies) according to the manufacturer's protocol. The primer sequences used in the real-time PCR were as follows: GAPDH, 5′-CAAGGCTGTGGGCAAGGT-3′ and 5′-GGAAGGCCATGCCAGTGA-3′; granzyme A, 5′-CGTGCAATGGAGATTCTGGAA-3′ and 5′-GGAAGTGACCCCTCGGAAA-3′; granzyme B, 5′-GCCCCTACATGGCTTATCTTATGA-3′ and 5′-GAAGCCACCGCACCTCTTC-3′; IFN-γ, 5′-GAATGTCCAACGCAAAGCAA-3′ and 5′-GCTGCTGGCGACAGTTCAG-3′; perforin, 5′-TGTCGAGGCCCAGGTCAA-3′ and 5′-CCTTGGCTTCGGCAGAGAT-3′. Data analyses were performed with 7500 System SDS software version 1.3.1 (Life Technologies).

2.7. Statistical analysis

Results were expressed as means ± SD. Statistical analyses of the data were conducted using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. PMFs potentiate the cytolytic activity of KHYG-1 cells

We have explored compounds from natural sources that potentiate the cytolytic activity of the human NK leukemia cell line KHYG-1. Evaluation of cytolytic activity was performed by determination of the LDH activity derived from K562 target cells. As a result, nobiletin significantly potentiated the cytolytic activity of KHYG-1 cells in a dose-dependent manner (Fig. 1A). Two other citrus PMFs, tangeretin and sinensetin, also potentiated the cytolytic activity (Fig. 1A). The intensities of the cytolytic activity induced by these PMFs were almost equal (approximately 1.8- to 2.1-fold)

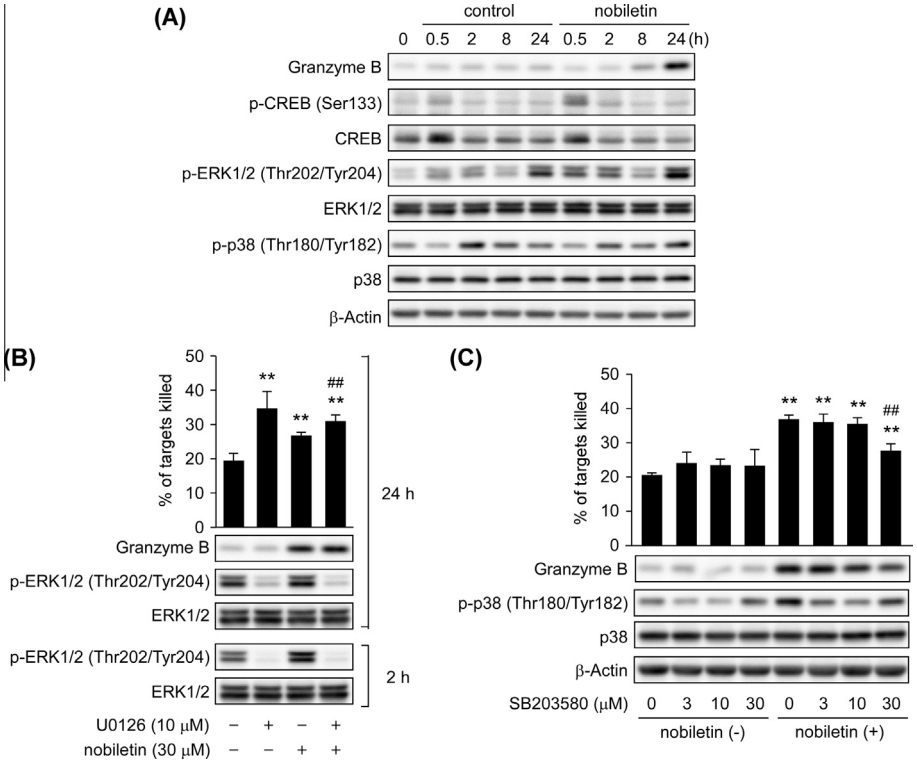

Fig. 4. Role of nobiletin in the upregulation of granzyme B expression. (A) Time-dependent changes in the expression or phosphorylation of NK cell activation-related proteins. KHYG-1 cells were treated with or without nobiletin (30 μM) for the indicated times, and total proteins were prepared and subjected to Western blot analysis. (B) Role of phosphorylated ERK1/2 in the granzyme B expression and cytolytic activity. KHYG-1 cells were treated with a combination of U0126 (30 μM) and nobiletin (30 μM). Blockade of ERK1/2 phosphorylation at 2 h was confirmed. At 24 h, the cells were recovered and subjected to a cytotoxicity assay and Western blot analysis. (C) Role of phosphorylated p38 MAPK in granzyme B expression and cytolytic activity. KHYG-1 cells were treated with the indicated doses of SB203580 and nobiletin for 24 h and subjected to a cytotoxicity assay and Western blot analysis. In (B) and (C), the cytotoxicity assay was performed using flow cytometry at an E:T ratio of 1:1. $^{**}P < 0.01$, vs. inhibitor(−)nobiletin(−). $^{\#\#}P < 0.01$, vs. inhibitor(−)nobiletin(+).

at the effector:target ratios of 5:1 and 10:1 (Fig. 1A). We also observed that a broad range of PMFs, including 3,3′,4′,5,6,7,8-heptamethoxyflavone, and other unnatural products enhanced the cytolytic activity (data not shown), suggesting that the cytotoxicity-enhancing effect on KHYG-1 cells is highly likely to be a common property of PMFs. Moreover, we confirmed that the potentiated cytolytic activity of KHYG-1 cells was already observed within 24 h of treatment with nobiletin (Fig. 1B). These results suggest that PMFs may activate an early stage of the intracellular signaling pathways leading to the cytolytic activation of KHYG-1 cells.

3.2. Nobiletin markedly increases the expression of granzyme B

Initially, we examined whether nobiletin could enhance the production of IFN-γ, which contributes to antiviral defense, in KHYG-1 cells. The cells were cultured for 24 h in the presence or absence of nobiletin and the concentrations of IFN-γ in the supernatants were quantified by ELISA. As a result, nobiletin enhanced IFN-γ production by 2.0-fold (Fig. 2A). Next, the protein levels of cytotoxic effectors were examined by Western blot analysis. As shown in Fig. 2B, nobiletin dose-dependently enhanced the expressions of perforin and granzymes A and B, but had little effect on the expressions of Fas ligand and granulysin (Fig. 2B). The effects of nobiletin on the transcriptional activation of IFN-γ, perforin, and granzymes A and B were also examined. KHYG-1 cells were cultured in the presence or absence of nobiletin for 8 h, and then treated with EU for 16 h to label nascent RNA. The results of quantitative real-time PCR analyses showed that the transcriptions of the target genes were significantly enhanced by nobiletin (Fig. 2C). In particular, the expression of granzyme B was markedly increased (8.7-fold). Similar to the finding that a broad range of PMFs enhanced the cytolytic activity of KHYG-1 cells (Fig. 1A), tangeretin, sinensetin, and 3,3′,4′,5,6,7,8-heptamethoxyflavone also increased the protein levels of granzymes A and B (Fig. 2D). While nobiletin clearly increased the protein level of granzyme A, its mRNA level was modestly increased (Fig. 2B and C). It is possible that nobiletin might increase the protein level of granzyme A through a post-transcriptional mechanism. These results suggest that the striking feature of nobiletin in KHYG-1 cells lies in its upregulation of the granzyme B gene.

3.3. Enhanced cytotoxicity induced by nobiletin is canceled by a granzyme B inhibitor

We noted the property that nobiletin markedly increased the expression of granzyme B in KHYG-1 cells. Therefore, we examined whether the upregulation of granzyme B induced by nobiletin makes a significant contribution to the increase in KHYG-1 cytolytic activity. To test this notion, cells were treated with a combination of nobiletin and the granzyme B inhibitor Z-AAD-CMK. In the presence of nobiletin, the granzyme B inhibitor dose-dependently attenuated the cytolytic activity of KHYG-1 cells (Fig. 3A), suggesting that the upregulation of granzyme B plays a primary role in nobiletin-induced cytolytic activity. In contrast, in the absence of nobiletin, the granzyme B inhibitor had little effect on the cytolytic activity of KHYG-1 cells (Fig. 3A), probably due to the low level of granzyme B in KHYG-1 cells, as described previously [12]. This raises the possibility that the gene expression of granzyme B in

KHYG-1 cells is suppressed by an epigenetic event, such as histone deacetylation, and that nobiletin can affect this modification. Therefore, KHYG-1 cells were treated with a combination of nobiletin and the histone deacetylase inhibitor TSA. The results of quantitative real-time PCR indicated that the expression of granzyme B was solely enhanced by TSA, and additively enhanced in the presence of nobiletin (Fig. 3B). However, the results of a Western blot analysis indicated that TSA itself failed to increase the protein level of granzyme B (Fig. 3C). The cause of this inconsistency need to be addressed in future studies. Importantly, nobiletin itself failed to modulate the histone acetylation (Fig. 3C). These results suggest that a primary role of nobiletin in KHYG-1 cytolytic activity lies in the upregulation of granzyme B with the exception of changes in histone acetylation.

3.4. Nobiletin activates CREB, ERK1/2, and p38 MAPK in KHYG-1 cells

The time-course analysis indicated that the expression of granzyme B in KHYG-1 cells was scarcely changed in the absence of nobiletin (Fig. 4A). However, in the presence of nobiletin, the increase in granzyme B was detectable within 8 h and then further enhanced (Fig. 4A). Because nobiletin has been reported to induce the phosphorylation of CREB [16,17], which is also involved in expression of the human granzyme B gene [7], we examined the effect of nobiletin on CREB phosphorylation in KHYG-1 cells. Nobiletin increased the level of phosphorylated CREB at 0.5 h (Fig. 4A), raising the possibility that CREB activated by nobiletin contributes to the transcription of granzyme B. Previous reports have demonstrated that the phosphorylation of ERK1/2 and p38 MAPK might be regulated by nobiletin [16,17]. In the activation of NK cells, these MAPKs were also reported to participate in spontaneous and antibody-dependent cytotoxicity by regulating the mobility and exocytosis of granules [13,14,20–22]. Thus, we examined the effects of nobiletin on the phosphorylation of ERK1/2 and p38 MAPK in KHYG-1 cells. The phosphorylated form of ERK1/2, which was increased within 0.5 h and again at 24 h, was augmented by nobiletin (Fig. 4A and B). In the absence of nobiletin the p38 MAPK phosphorylation peaked at 2 h, while in the presence of nobiletin the phosphorylation increased again at 24 h (Fig. 4A and C). Next, we examined the significance of the enhanced phosphorylation of ERK1/2 and p38 MAPK for granzyme B expression and the cytolytic activity of KHYG-1 cells. An inhibitor of ERK activation, U0126, actually blocked the phosphorylation of ERK1/2 at both 2 and 24 h, even in the presence of nobiletin (Fig. 4B). However, inhibition of ERK1/2 phosphorylation had no effect on granzyme B expression, and rather increased the cytolytic activity of KHYG-1 cells at 24 h (Fig. 4B). It is possible that the increase in the active phosphorylated form of ERK1/2 was irrelevant to the granzyme B expression and cytolytic activity, at least in KHYG-1 cells prior to the receptor stimulation. The reason why U0126 increased the cytolytic activity of KHYG-1 cells remains unclear. In the absence of nobiletin, the p38 MAPK inhibitor SB203580 had no effects on the granzyme B expression and cytolytic activity of KHYG-1 cells (Fig. 4C). Because SB203580 does not inhibit upstream kinases of p38 MAPK, phosphorylation of p38 MAPK was not decreased by a high dose of this inhibitor (Fig. 4C). In the presence of nobiletin, it was notable that SB203580 dose-dependently decreased the granzyme B expression and cytolytic activity of KHYG-1 cells (Fig. 4C). These results suggest that p38 MAPK participates in the cytolytic activity by regulating granzyme B expression, prior to stimulation from activating receptors.

Collectively, nobiletin could potentiate the cytolytic activity of KHYG-1 cells. The primary role of nobiletin in the activation of KHYG-1 cells is marked induction of the granzyme B gene, at least in part, mediated through p38 MAPK function.

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