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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

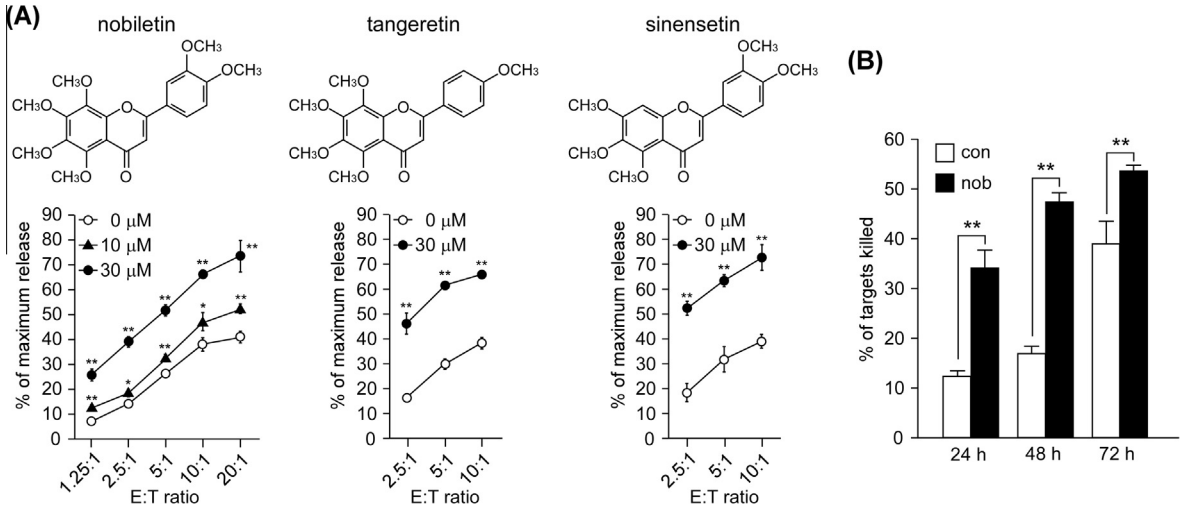

Fig. 1. Effects of PMFs on the cytolytic activity of KHYG-1 cells. (A) Lysis of K562 target cells by KHYG-1 effector cells treated with the indicated doses of PMFs for 72 h. KHYG-1 and K562 cells were washed and cocultured for 4 h at the indicated effector:target (E:T) ratios. Cytotoxicity was determined 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.

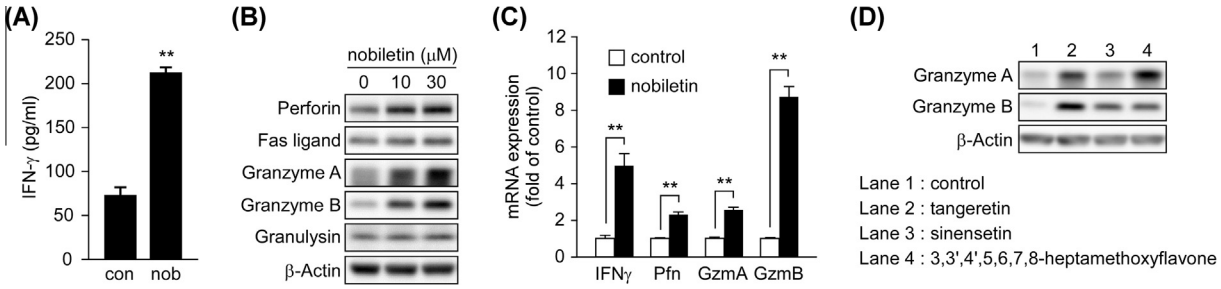

Fig. 2. Expression of IFN-γ and cytotoxic effector molecules. (A) The IFN-γ levels in culture supernatants of KHYG-1 cells treated with nobiletin (30 μM) for 24 h were measured by ELISA. (B) Protein levels of cytotoxic effectors in KHYG-1 cells treated with the indicated doses of nobiletin for 24 h. (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.