Original Article

The effects of hinokitiol on human cells revealed by a proteomic approach

Seido Ooka[1,*], Toshiyuki Sato[2], Mitsumi Arito[2], Hiromasa Nakano[1], Yukiko Takakuwa[1], Naoya Suematsu[2], Kazuki Okamoto[2], Manae Kurokawa[2], Shoichi Ozaki[1] and Tomohiro Kato[2]

[1]Division of Rheumatology and Allergy, Department of Internal Medicine, St. Marianna University School of Medicine, Kanagawa, Japan
[2]Clinical Proteomics and Molecular Medicine, St. Marianna University Graduate School of Medicine, Kanagawa, Japan

Hinokitiol, a natural substance extracted from plants like *Chamaecyparis obtusa* var. *formosana* (Taiwan Hinoki in Japanese), is known to have various functions such as anti-bacterial activities. However, the effects of hinokitiol on human cells have not been fully understood. Thereby, we here comprehensively analyzed the effects of hinokitiol on human peripheral blood mononuclear cells (PBMCs) by a proteomic approach. Specifically, human PBMCs were cultured with or without hinokitiol for 24 hours, then the protein profiles of the PBMC samples were compared by 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE). The protein spots whose intensity was altered by the stimulation with hinokitiol were subjected to protein identification by mass spectrometric analysis and the subsequent protein data base searching. As a result, we found that the intensity of 150 protein spots was significantly altered by the hinokitiol stimulation ($p<0.05$). Out of them, the intensity of 63 protein spots was increased to more than 1.5 folds or decreased to less than 1/1.5 folds by the hinokitiol stimulation. Further, we identified 22 out of the 63 proteins, which are involved in cytoskeleton structure, polypeptide synthesis, homeostasis, and apoptosis, Taken together, this study identified multiple proteins that are significantly influenced the hinokitiol in PBMCs. Our data would be of great help to use hinokitiol more effectively and safely in daily life.

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*Correspondence should be addressed to:
Seido Ooka, Division of Rheumatology and Allergy, Department of Internal Medicine, St. Marianna University School of Medicine, Sugao 2-16-1, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan. Phone: +81-44-977-8111, Fax: +81-44-977-8593, E-mail: ooka@marianna-u.ac.jp

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Introduction

Hinokitiol (Fig.1(a)) is a natural substance found in several plants such as *Chamaecyparis obtusa* var. *formosana* and *Thujopsis dolabrata* var. *hondae*. Hinokitiol has been shown to possess a variety of bioactivities. One of the well-known activities is anti-bacterial one. For example, hinokitiol shows anti-bacterial activities against *Staphylococcus aureus, Staphylococcus epidermidis*[1-4], and *Chlamydia*[5]. Because of such anti-bacterial activities, hinokitiol has been used as a food additive for more than 20 years in Japan. In addition to the anti-bacterial activities, an antiviral effect has also been reported[6]. As for the effects of hinokitiol on mammalian cells, several studies have been reported. For example, it has been reported that hinokitiol inhibited the production of TNF-α by suppressing NF-κB, which indicated anti-inflammatory activities of hinokitiol[7]. Hinokitiol-related compound was reported to inhibit DNA damage induced by hydrogen peroxide, which indicated anti-oxidant activities of hinokitiol[8]. Also, hinokitiol was reported to induce G1 arrest of melanoma cells by negatively regulating pRb and Skp2 ubiquitin ligase, which indicated anti-tumor activities of hinokitiol[9]. On the other hand, teratogenicity associated with high concentration of hinokitiol was reported in mice[10]. This may indicate harmful effects even in humans. Therefore, the activities of hinokitiol on human cells should be investigated in more detail. We thus here investigated the effects of hinokitiol on human peripheral blood mononuclear cells (PBMCs) comprehensively using proteomic approaches. By the approaches, we identified multiple proteins affected by hinokitiol.

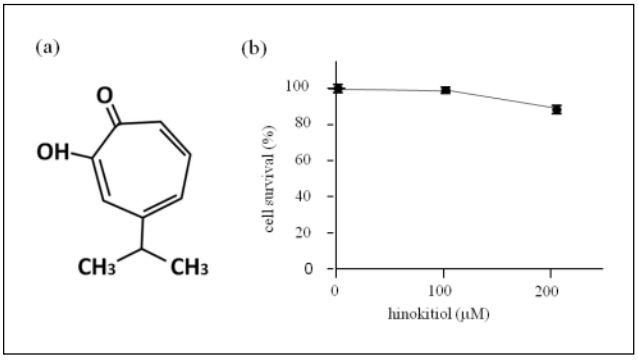

Fig.1 Effects of hinokitiol on the survival of PBMCs

2 x 10^6 PBMCs were incubated with various concentration of hinokitiol for 24 hours in each of three healthy donors, then the cell viability was measured. The cell viability without hinokitiol was defined as 100%.

Material and Methods

1)Preparation of PBMC samples, cell culture, and protein extraction

Peripheral blood samples were obtained with informed consents from 3 healthy subjects (two 29-year-old males and one 28- year-old male). PBMCs were isolated from the blood samples by gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden).

4x10^6 PBMCs in each sample were cultured in the RPMI 1640 medium with or without 100 μM of hinokitiol (Tokyo Chemical Industry, Co., Ltd, Tokyo, Japan) in a humidified atmosphere at 37℃ with 5% CO_2 for 24 h. Then, proteins were extracted from each of the PBMC samples into a cell lysis buffer (4% 3-[(3-Cholamidopropyl)dimethylammonio] propanesulfonate, 7 M urea, 2 M thiourea, 30 mM Tris-HCl (pH 8.0)). The lysates were subjected to the proteomic analysis as described below. As a preliminary experiment, PBMC samples were similarly cultured with 0-200 μM of hinokitiol, and the cell viability was measured. This study was approved by the ethics committee of St. Marianna University School of Medicine.

2)Two-dimensional electrophoresis (2DE)

The protein profiles of PBMCs stimulated by hinokitiol were compared to those of non-stimulated ones by the quantitative 2DE system of the two-dimensional differential gel electrophoresis (2D-DIGE) as described previously[11]. Specifically, a mixture of an equal amount of 6 samples (non-treated and hinokitiol-treated samples for each of the 3 subjects) was prepared as an internal control, which was further labeled with Cyanine dye 3 (Cy3; Cy Dye DIGE Saturation dye; GE Healthcare, Buckinghamshire, UK). 2.5 μg of each protein samples, similarly labeled with Cyanine dye 5 (Cy5), was mixed with 2.5 μg of the Cy3-labeled internal control sample. Then the mixture was applied onto an isoelectric focusing (IEF) gel (Immobiline Drystrip pH 3-10 NL 24 cm; GE Healthcare), by which the proteins were separated by their respective isoelectric points. Subsequently, the separated proteins were further separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To compare spot intensities among different samples, the Cy5-fluorescent intensities of the protein spots were normalized by the Cy3-fluorescent intensities of identical spots by using the Progenesis (Nonlinear Dynamics, Newcastle, UK) program, and the normalized Cy5-intensi-

ties were used for the comparison. The protein spots whose expression levels were altered by the stimulation with hinokitiol was detected quantitatively.

3)Protein identification

For identification of proteins, 2-DE gel fragments with approximately 1 mm in diameter, which corresponded to protein spots of interest by the image analysis, were recovered and washed in double-distilled water for 15 min. Then, the gel fragments were cut into small pieces and decoloured in 200 μl of decolouring solution (25 mM ammonium hydrogen carbonate, 50% acetonitrile) at room temperature for 10 min. The gel pieces were rehydrated in 10 μl of trypsin solution (50 mM ammonium hydrogen carbonate, 5 mM calcium chloride, 0.02 μg/μl trypsin) and incubated at 37℃ for 16 h for digestion of the contained proteins with the trypsin. The digested peptides were extracted from the gel pieces using trifluoroacetic acid (TFA) and acetonitrile. Specifically, the digested products were supplemented with 50 μl of 5% TFA in 50% acetonitrile solution and vortexed. After centrifugation, the supernatant was recovered. After three more cycles of this extraction, the supernatant was concentrated down to 10 μl by an evaporator. The peptide sample solution was stored at -20 ℃ until mass spectrometric analysis. The peptide mixture was desalted using a ZipTip μ-C18 prior to mass analysis. Masses of the digested peptides in the samples were determined using a MALDI TOF/TOF MS (Ultraflex, Bruker Daltonics, Bremen, Germany). The matrix for peptide analysis by MALDI TOF/TOF MS consisted of either 10 mg/ml cyano-4-hydroxycinnamic acid. The peptide and protein were identified using the Mascot software program (Matrix Science, London, UK) from the mass of degradation products of the peptides by the MS/MS analysis.

4) Statistical analysis

Difference of the protein spot intensity in 2D-DIGE results was evaluated by Student's *t*-test.

Result

1)The stimulation of human PBMCs by hinokitiol and detection of proteins altered by the stimulation of hinokitiol by 2D-DIGE

We first estimated the dose effect of hinokitiol on the survival of human cells cultured for 24 h using PBMCs. As a result, 100 μM of hinokitiol did not influence the survival of PBMCs substantially (98.0%, Fig.1(b)). We thereby selected 100 μM for the stimulation of PBMCs by the following 2D-DIGE investigation. To investigate the effect of hinokitiol on human PBMCs comprehensively, we compared the total protein profiles between hinokitiol-stimulated and non-treated PBMCs by 2D-DIGE. Specifically, $4x10^6$ PBMCs were incubated with or without 100 μM of hinokitiol in each of three healthy donors. Then the extracted proteins were separated by 2DE, by which the protein profile of each of the 6 PBMC samples in total was obtained as shown in Fig. 2. In this analysis, each of the samples labeled with Cy5 and the internal control sample of the mixture of the 6 samples labeled with Cy3 were simultaneously separated by the same gel. The intensity of the protein spots detected by Cy5 in each sample was normalized by the intensity of the identical protein spots

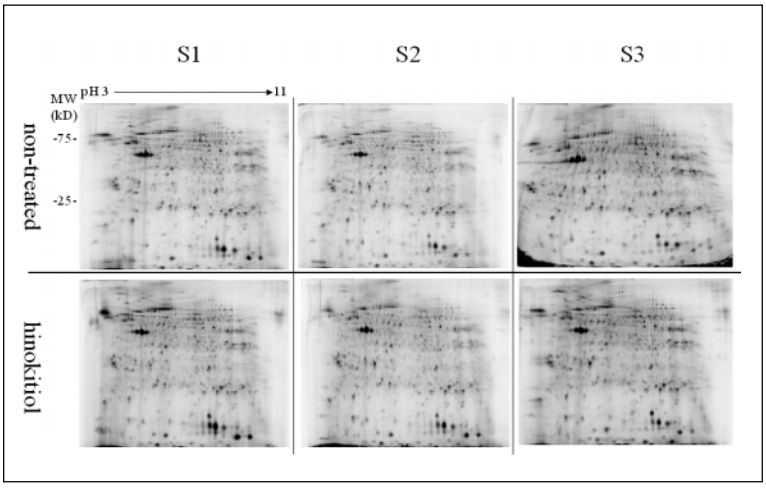

Fig.2 The protein profiles of PBMCs stimulated with hinokitiol or non-treated

$4x10^6$ PBMCs were incubated with or without 100 μM of hinokitiol in each of three healthy donors (S1-3). Each of the 6 PBMC samples in total was labeled with Cy5 was mixed with the standard samples (a mixture of the 6 samples) labeled with Cy3, and then was electrophoresed 2-dimesionally. The protein profiles of the 6 samples obtained by Cy5 detection are shown. The intensity of the protein spots detected by the Cy5 image in each sample was normalized by the intensity of the identical protein spots detected by the Cy3 image in the standard sample. Then, the normalized intensity of the protein spots was compared between the hinokitiol-stimulated and non-treated samples. MW, molecular weight; pI, isoelectric point.

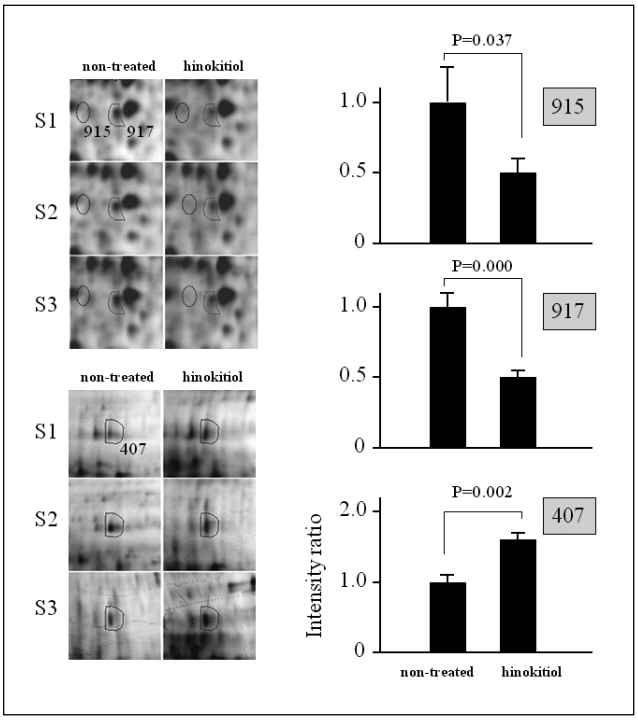

Fig.3 Representative protein spots on the 2DE gels whose intensity were altered by the stimulation with hinokitiol

The results of spots 915, 917, and 407 are shown. In the right three figures, the average of the spot intensity among the three donors (S1-3) was defined as 1.0 in each figure.

detected by Cy3 in the internal control sample, after which the normalized intensity of the protein spots was compared between the hinokitiol-stimulated and non-treated samples. As a result, we detected 2157 protein spots in total. Interestingly, the intensity of 150 protein spots was increased or decreased by the hinokitiol stimulation with the significant difference ($p<0.05$). Out of them, the intensity of 63 protein spots was increased to more than 1.5 folds or was decreased to less than 1/1.5 folds by the hinokitiol stimulation, as representative spots were shown in Fig.3. Further, out of the 63 protein spots, the intensity of 18 protein spots was increased to more than 2.0 folds or decreased to less than 1/2.0 folds. Out of the 18 proteins, the intensity of 6 protein spots was increased to more than 3.0 folds or decreased to less than 1/3.0 folds. The result was summarized in Table 1. These data indicate that hinokitiol greatly influenced the protein profile of PBMCs.

2) Identification of proteins influenced by hinokitiol

We then tired to identify the proteins whose intensity was increased or decreased by the hinokitiol stimulation. Focusing on the 63 protein spots whose intensity was increased to more than 1.5 folds or was decreased to less than 1/1.5 folds by the hinokitiol stimulation, we recovered peptides from each of the protein spots after trypsin digestion and then the recovered peptides were subjected to mass spectrometry and protein data base searching. As a result, 22 out of the 63 protein spots were successfully identified (Table 1 and Fig.4). The 22 protein spots consisted of 4 cytoskeleton-related proteins, 2 RNA binding proteins, 3 enzymes, 1 enzyme inhibitor, 2 translation-related proteins, 2 transcription-related proteins, 1 extracellular matrix, 1 extracellular matrix-binding protein, 1 stress protein, 1 chemokine, 1 tumor-related protein, and 1 pro-

Table 1 Numbers of the hinokitiol-affected protein spots

Difference ratios of spot intensity (hinokitiol-stimulated/non-treated, folds)	Number of spots
>3.0	5
>2.0	8
>1.5	17
<1/1.5	46
<1/2.0	10
<1/3.0	1

By the 2D-DIGE analysis, 2157 protein spots were detected in total, out of which 150 protein spots changed their intensity significantly ($p<0.05$) by the hinokitiol. Out of the 150 protein spots, 63 spots changed their intensity up to more than 1.5 folds or down to less than 1/1.5 fold as summarized.

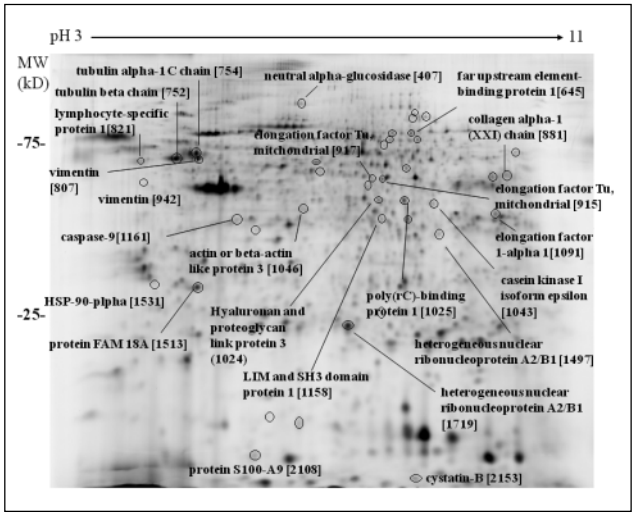

Fig.4 The location of identified proteins on the 2DE gel

The numbers in [ ] indicate the spot number described in Table 2. The circled spots without protein names indicate those which were not able to be identified.

Table 2 Identification of proteins whose expression was significantly altered by the stimulation with hinokitiol

Spot no.	Difference rate (p-value)	MW (observed)	pI (observed)	protein name	Mascot scores	Accession no.	MW (calculated)	pI (calculated)
942	3.65 (p=0.020)	47	4.5	vimentin (VIME_HUMAN)	68	gi 62414289	53.6	5.06
1531	3.06 (p=0.006)	27	4.7	heat shock protein HSP 90-alpha (HS90A_HUMAN)	101	gi 92090606	84.6	4.94
2108	1.97 (p=0.023)	19	6.0	protein S100-A9(S10A9_HUMAN)	170	gi 115444	13.2	5.71
				beta-actin-like protein 3 (ACTBM_HUMAN)	121	gi 74739412	42.0	5.91
1046	1.80 (p=0.037)	40	6.6	actin, cytoplasmic 1 (ACTB_HUMAN)	121	gi 46397333	41.7	5.29
				actin, cytoplasmic 2 (ACTG_HUMAN)	121	gi 113278	41.8	5.31
407	1.51 (p=0.002)	120	6.6	neutral alpha-glucosidase (GANAB_HUMAN)	58	gi 54037162	106.8	5.74
1024	1/1.51 (p=0.000)	42	7.7	hyaluronan and proteoglycan link protein 3 (HPLN3_HUMAN)	64	gi 47605734	40.9	6.07
1043	1/1.52 (p=0.008)	41	8.4	casein kinase I isoform epsilon (KC1E_HUMAN)	58	gi 1346169	47.3	9.68
807	1/1.53 (p=0.010)	57	5.2	vimentin (VIME_HUMAN)	263	gi 62414289	53.6	5.06
1091	1/1.54 (p=0.008)	38	9.3	elongation factor 1-alpha 1 (EF1A1_HUMAN)	88	gi 55584035	50.1	9.1
				tubulin beta chain (TBB5_HUMAN)	106	gi 56757569	49.6	4.78
				tubulin beta-2A chain (TBB2A_HUMAN)	90	gi 74762137	49.6	4.78
				tubulin beta-2B chain (TBB2B_HUMAN)	89	gi 74761283	49.9	4.78
752	1/1.56 (p=0.006)	57	5.2	tubulin beta-3 chain (TBB3_HUMAN)	77	gi 20455526	50.4	4.83
				tubulin beta-2C chain (TBB2C_HUMAN)	74	gi 55977480	49.8	4.79
				tubulin beta-4 chain (TBB4_HUMAN)	72	gi 93141323	49.6	4.78
				tubulin beta-8 chain B (TBB8B_HUMAN)	65	gi 187761884	49.5	4.75
645	1/1.58 (p=0.000)	68	8.2	far upstream element-binding protein 1 (FUBP1_HUMAN)	109	gi 116241370	67.5	7.18
1161	1/1.58 (p=0.031)	37	5.7	caspase-9 (CASP9_HUMAN)	60	gi 28558771	46.2	5.74
917	1/1.61 (p=0.000)	49	7.7	elongation factor Tu, mitochondrial (EFTU_HUMAN)	92	gi 1706611	49.5	7.26
1513	1/1.75 (p=0.002)	27	5.2	protein FAM18A (FA18A_HUMAN)	61	gi 182649415	24.1	6.51
881	1/1.78 (p=0.003)	50	9.4	collagen alpha-1(XXI) chain (COLA1_HUMAN)	60	gi 74752071	29.7	6.61
1158	1/1.83 (p=0.008)	37	7.7	LIM and SH3 domain protein 1 (LASP1_HUMAN)	73	gi 3122342	29.7	6.61
821	1/1.87 (p=0.010)	55	4.5	lymphocyte-specific protein 1 (LSP1_HUMAN)	88	gi 462553	37.2	4.69
754	1/1.94 (p=0.002)	59	5.2	tubulin alpha-1C chain (TBA1C_HUMAN)	85	gi 20455322	49.9	4.96
1025	1/1.95 (p=0.001)	42	8.0	poly(rC)-binding protein 1(PCB1_HUMAN)	75	gi 42560548	37.5	6.66
915	1/2.00 (p=0.037)	49	7.6	elongation factor Tu, mitochondrial (EFTU_HUMAN)	105	gi 1706611	49.5	7.26
1497	1/2.02 (p=0.004)	28	9.2	heterogeneous muclear ribonucleoproteins A2/B1 (ROA2_HUMAN)	98	gi 133257	37.4	8.97
2153	1/2.11 (p=0.000)	19	8.2	cystatin-B (CYTB_HUMAN)	101	gi 1706278	11.1	9.96
1719	1/2.16 (p=0.004)	25	7.3	heterogeneous muclear ribonucleoproteins A2/B1 (ROA2_HUMAN)	76	gi 133257	37.4	8.97

Human PBMCs were cultured in the presence or absence of hinokitiol. Protein profiles of the cells were compared by 2D-DIGE. 63 protein spots whose intensity was significantly altered to more than ± 1.5 folds (p<0.05) by the stimulation with hinokitiol were subjected to mass spectrometric analysis. 22 out of the 63 protein spots were identified as above. MW, molecular weight; pI, isoelectric point.

tein with unknown function. Almost all of the identified proteins were found to be influenced by hinokitiol for the first time through this study.

Discussion

This study analyzed comprehensively the effects of hinokitiol on the human PBMCs by a proteomic approach. We detected 150 proteins significantly influenced by the stimulation with hinokitiol. Further, we identified 22 proteins out of them. As shown in Table 2, a variety of proteins were found to be influenced by the stimulation with hinokitiol. Functionally, the following points would be of interest.

First, hinokitiol was found to decrease significantly the intensity of protein spot 1161, assigned to caspase-9, an essential enzyme for apoptosis of cells. Doulias et al reported that hinokitiol and its structurally related compound of tropolone protected Jurkat cells from hydrogen peroxide-induced DNA damage and that tropolone inhibited apoptosis induced by hydrogen peroxide[8]. Thus, the mechanisms how hinokitiol protects cells from apoptosis would include the down-regulation of caspase-9.

Second, hinokitiol up-regulated significantly the intensity of protein spot 1531, assigned to heat shock protein 90 (HSP90). HSP90 is a molecular chaperon critical for the maintenance of protein conformation and function[12]. HSP90 was reported to be essential for the induction of inducible nitric oxide synthase (iNOS) in mouse macrophages[13], which is known to produce NO to kill bacteria and viruses. Thus, the increase of HSP90 by the hinokitiol stimulation may increase cellular resistance to infection. However, on the other hand, HSP90 plays an important role in maintenance of transformed phenotypes of cells[14], and thus, HSP90 inhibitors have been investigated as an anti-cancer agent recently[15]. In this context, use of hinokitiol in some people such as patients with malignancy may bring about adverse consequence. This point should be elucidated in the future.

Third, hinokitiol down-regulated significantly the inensity of protein spots 915, 917, and 1091, two of which were assigned to mitochondorial elongation factor Tu (EF-Tu-mt), and the remaining one of which was assigned to elongation factor 1-alpha. EF-Tu-mt promotes the binding of the aminoacyl-tRNA to the A-site of the ribosome[16]. A gene mutation of EF-Tu-mt was discovered in an infant with lactic acidosis and fetal encephalopathy, who later died at an age of 14 months[17]. The gene mutation of EF-Tu-mt was later found to inactivate mitochondrial polypeptide elongation[18]. Thus, the decrease of EF-Tu-mt may bring about disadvantage to the cell homeostasis.

Lastly, in the case of vimentin, vimentin with an observed molecular weight (MW) of 57 kD was decreased and vimentin with an observed MW of 47 kD was increased. This indicates degradation of vimetin. Also, hinokitiol decreased several other cytoskeleton proteins of tubulin beta chain, tubulin alpha chain, and cytoplasmic actin. Thus hinokitiol would greatly alter the cytoskeleton profile, which may reflect functional changes of the cells. This point should be investigated in the future.

In addition, it is interesting that cystatin B was decreased by the hinokitiol stimulation, since it was reported that down-regulation of cystatin B reduced replication of human immunodeficiency virus-1 (HIV-1) in macrophages[19]. This indicates the potential of hinokitiol to suppress HIV-1 infection.

In conclusion, we here identified more than 20 proteins affected by hinokitiol in human PBMCs by a proteomic approach. Our data would provide basic but useful knowledge to utilize hinokitiol more safely and effectively in our life.

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