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Hesperetin Upregulates ABCA1 Expression and Promotes Cholesterol Efflux from THP-1 Macrophages

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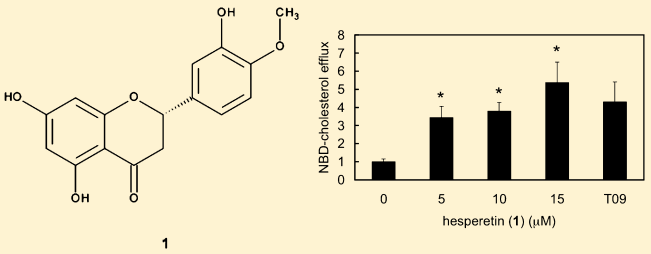
Supporting Information

ABSTRACT: ABCA1, a member of the ATP-binding cassette transporter family, regulates high-density lipoprotein (HDL) metabolism and cholesterol transport. Its expression is upregulated mainly by the activation of the liver X receptor (LXR). Since ABCA1 plays a pivotal role in cholesterol and HDL metabolism, identification of a compound capable of increasing its expression may be beneficial for the prevention and therapy of atherosclerosis. Firefly luciferase reporter assays were developed for human ABCA1 promoters and LXR enhancers, and an in-house phytochemical library was screened. It was found that a citrus flavonoid, hesperetin (**1**), increased ABCA1 promoter and LXR enhancer activities in THP-1 macrophages. It was also found that this flavonoid promoted PPAR-enhancing activity. In accordance with these findings, **1** increased mRNA and protein expression of ABCA1 and consequently upregulated ApoA-I-mediated cholesterol efflux. These results provide evidence that **1** promotes ApoA-I-mediated cholesterol efflux from macrophages by increasing ABCA1 expression through the activation of LXRα and PPARγ.



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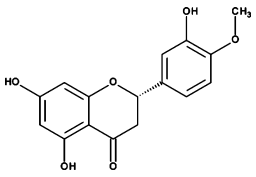
Hypercholesterolemia is the presence of high levels of cholesterol in the blood and a major risk factor in the development and progression of coronary atherosclerosis.[1] High-density lipoprotein (HDL) cholesterol plays a critical role in the removal of excess cholesterol from peripheral tissues including the arterial wall to the liver.[2] The ATP-binding cassette transporter A1 (ABCA1) is a membrane transporter that directly contributes to HDL biogenesis by mediating the cellular efflux of cholesterol to apolipoprotein A-I (ApoA-I).[3] Expression of the ABCA1 gene is regulated transcriptionally by a definite liver X receptor (LXR) both in vitro and in vivo.[4,6] Identification of compounds capable of increasing ABCA1 expression and accelerating the reversal of cholesterol transport may lead to a therapeutic benefit by reducing cardiovascular diseases.

Hesperetin (**1**) is one of the major citrus flavonoids that also include naringenin, nobiletin, and tangeretin.[7] Oral administration of **1** decreases plasma cholesterol and triglyceride levels in hypercholesterolemic rats[8] and hamsters.[9] In humans, a cohort study found that the intake of **1** reduces the risk of chronic disease such as cerebrovascular disease.[10] However, the potential antiatherogenic effects of **1** remain largely uninvestigated.

Our group has developed firefly luciferase reporter assays for human ABCA1 promoters and LXR enhancers and has used these for screening an in-house phytochemical library. In the present study, it was found that **1** can increase the ABCA1-promoting and LXR-enhancing effects in THP-1 macrophages. The effect of **1** on ABCA1 expression and cholesterol efflux was also investigated, and the underlying molecular mechanisms have been characterized, as described in this report.



hesperetin (**1**)

RESULTS AND DISCUSSION

To determine the effects of hesperetin (**1**) on the cell viability of THP-1 macrophages, the WST-8 assay was performed, which measures the mitochondrial activity of viable cells. When cells were treated with various concentrations of **1** (0−100 μM), cell viability was not affected (Figure S1, Supporting Information). Thus, **1** was not cytotoxic at a concentration of <100 μM, which was consistent with previous reports that showed no cytotoxic effect in other primary cells and cell lines at this same concentration level of **1**.[11−13]

LXRs play an important role in cholesterol metabolism and homeostasis by regulating a number of genes, including cholesterol transporters, cholesterol metabolizing enzymes, and apolipoproteins

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in cells.[14] A luciferase reporter gene assay was performed to test whether **1** can increase LXR enhancer activity. As shown in Figure 1A, **1** dose-dependently (5−15 μM) increased

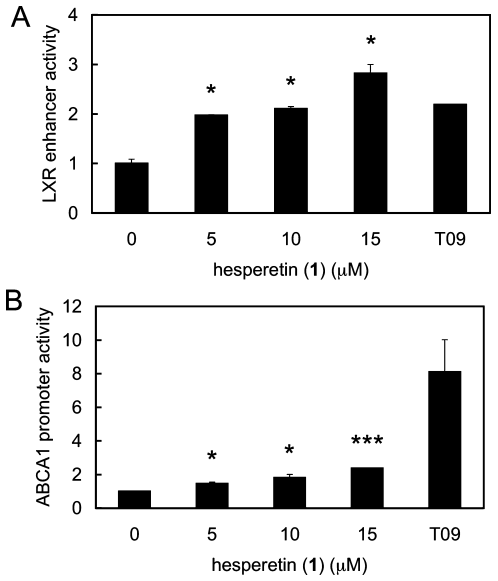

Figure 1. Effects of hesperetin (**1**) on LXR enhancer and ABCA1 promoter activities. THP-1 macrophages were transfected with 3xLXRE-luc (450 ng) and TK-RL (50 ng) vectors (A) or the pGL3-ABCA1 promoter reporter (450 ng) and TK-RL (50 ng) vectors (B). Twenty-four hours later, cells were treated with **1** (0−15 μM) or T09 (1 μM; LXRs agonist for positive control) for an additional 4 h (A) or 24 h (B). LXR enhancer and ABCA1 promoter activities were measured by dual-luciferase assay. Firefly luciferase activity was normalized to Renilla luciferase activity (*p < 0.05, ***p < 0.001).

expression of the LXR luciferase reporter gene 4 h after treatment. In addition, **1** enhanced the ABCA1 promoter activity 24 h after treatment (Figure 1B). Since LXR has been shown to bind to the LXRE in the ABCA1 promoter and activate its transcription,[4] these results suggested that **1** may upregulate ABCA1 transcription through increasing the LXR enhancer activity. Although a direct binding assay was not performed between **1** and the LXRs, it is possible that **1** acts as an LXR agonist, as demonstrated with the grapefruit flavonoids naringenin and a flavone derivative, CM108.[15,16]

To investigate whether **1** can increase mRNA and protein levels of ABCA1, a quantitative real-time RT-PCR and Western blot analysis were performed. Consistent with the effect on the ABCA1 promoter activity, **1** increased both mRNA (Figure 2A) and protein (Figure 2B) expression of ABCA1.

A previous report has shown that **1** promotes adiponectin expression through PPARγ activation in adipocytes.[17] Many studies have indicated that PPARγ regulates the expression of ABCA1 through induction of LXRs.[18−20] Moreover, PPARγ functions by forming obligate heterodimers with LXRs,[21] and LXRs interact with coactivators (PGC-1, SRC-1, and CBP/p300) that bind PPARs.[22] These previous findings suggest that PPARγ and LXRα might upregulate ABCA1 transcription in a cooperative manner. To examine the effects of **1** on PPARγ enhancer activity in THP-1 macrophages, a luciferase reporter gene assay was performed using PPREx3-tk and PPARγ expression vectors. As shown in Figure 3, **1** dose-dependently increased PPARγ enhancer activity in macrophages, as observed

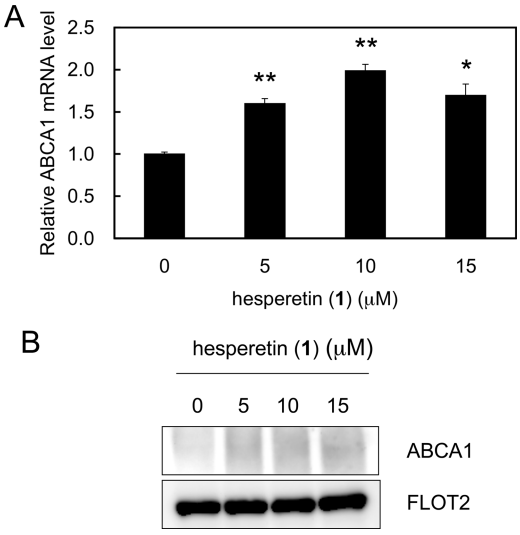

Figure 2. Effects of hesperetin (**1**) on ABCA1 mRNA and protein expression in THP-1 macrophages. (A) THP-1 macrophages were cultured in the presence of **1** (0−15 μM) for 24 h, and then quantitative real-time RT-PCR was performed for ABCA1 (*p < 0.05, **p < 0.01). (B) THP-1 macrophages were cultured in the presence of **1** (0−15 μM) for 48 h. Then, the membrane fraction was harvested and subjected to Western blotting analysis to detect ABCA1 and integral membrane protein, flotillin-2.

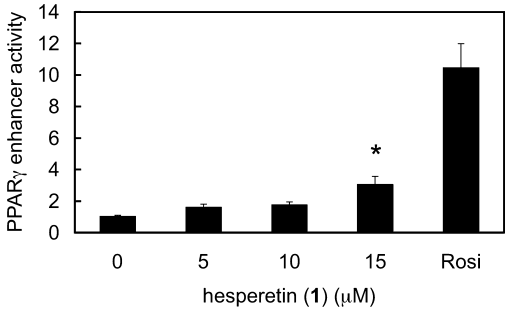

Figure 3. Effects of hesperetin (**1**) on PPARγ enhancer activity. THP-1 macrophages were transfected with PPREx3-tk (450 ng) and TK-RL (50 ng) vectors along with the PPARγ expression vector (pCMX-hPPARγ1). Twenty-four hours later, cells were treated with **1** (0−15 μM) or Rosi (2 μM; PPARγ agonist for positive control) for an additional 24 h. The PPARγ enhancer activity was measured by a dual-luciferase assay. Firefly luciferase activity was normalized to Renilla luciferase activity (*p < 0.05).

in adipocytes.[17] It is therefore suggested that **1** increases the PPARγ enhancer activity and thereby upregulates ABCA1 transcription possibly in a cooperative manner with LXRs.

Since ABCA1 expression was upregulated by **1**, its effect on cholesterol efflux from THP-1 macrophages was determined by measuring the fluorescent-labeled cholesterol content (Figure 4). The cholesterol efflux to ApoA-I was found to be increased significantly in a dose-dependent manner in response to treatment with **1**.

In the present study, it has been demonstrated that hesperetin (**1**) enhanced ApoA-I-mediated cholesterol efflux in THP-1 macrophages significantly, which was accompanied by an induction of the *ABCA1* gene, which is critical for cholesterol metabolism. The effect of hesperetin (**1**) on ABCA1-dependent cholesterol efflux may be explained in part by its potency of activation of LXRα and PPARγ enhancers. These results

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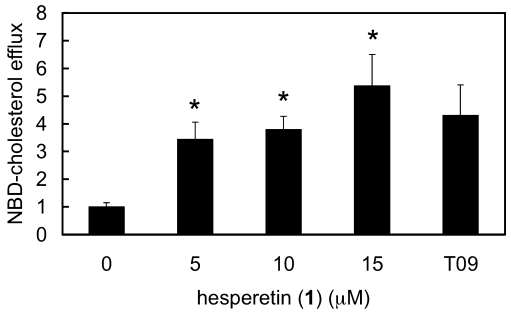

Figure 4. Effects of hesperetin (**1**) on cholesterol efflux in THP-1 macrophages. THP-1 macrophages were labeled for 12 h with NBD-cholesterol (1 μg/mL) in the presence of **1** (0−15 μM) or T09 followed by mesurement of cholesterol efflux as described in the Experimental Section (*$p < 0.05$).

suggest that hesperetin (**1**) has the potential as a dietary supplement for the prevention and treatment of cardiovascular disease such as atherosclerosis.

EXPERIMENTAL SECTION

Reagents. RPMI1640 medium, the cell counting kit-8, Isogen II, and Pikkagene Dual-SeaPancy luminescence kits were purchased from Wako (Tokyo, Japan). Charcoal-stripped fetal bovine serum (FBS) was obtained from Biological Industries (Kibbutz Beit-Ha'Emek, Israel). PrimeScript RT reagent kit, SYBR Premix Ex *Taq*II, and RNase-free DNase I were from Takara (Otsu, Japan). Apolipoprotein A-I and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma (St Louis, MO, USA). The firefly luciferase reporter vector pGL4.27 was obtained from Promega (Madison, WI, USA). Anti-ABCA1 antibody was from Novus Biologicals (Littleton, CO, USA). Horseradish peroxidase-conjugated donkey anti-rabbit/mouse IgG and ECL Plus Western blotting detection reagents were from GE (Fairfield, CT, USA). Oligonucleotide primers were from Rikaken (Nagoya, Japan). 22-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bis-nor-5-cholen-3β-ol (NBD-cholesterol) and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). T0901317 (T09) and rosiglitazone maleate (Rosi) were purchased from Alexis (Lausen, Switzerland). FuGENE6 and fatty acid-free BSA were obtained from Roche (Penzberg, Germany). ProteoJET membrane protein extraction kit was purchased from Fermentas (Vilnius, Lithuania). Anti-flotillin-2 antibody was from BD Biosciences (Franklin Lakes, NJ, USA). The PPREx3-tk reporter gene and PPARγ expression vector (pCMX-hPPARγ1)[23] were kind gifts from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan). Hesperetin (**1**) (purity >98%) prepared from hesperidin isolated from *Citrus* species[24] was donated by Alps Pharmaceutical Industry Co. Ltd. (Furukawa, Japan).

Cell Culture, Treatment, and Cytotoxic Assay. THP-1 cells were obtained from ATCC (Manassas, VA, USA) and grown in RPMI1640 supplemented with 10% (v/v) heat-inactivated FBS at 37 °C in an atmosphere containing 5% CO_2. To induce macrophage differentiation, THP-1 cells were stimulated for 4 days with TPA (80 nM). Cell viability was determined calorimetrically using the cell counting kit-8 according to the manufacturer's protocol (WAKO).

Measurement of LXR Enhancer and ABCA1 Promoter Activities. The LXR reporter gene, 3xLXRE-luc, was constructed by inserting three copies of the LXRE responsive element (LXRE; DR4) derived from the human ABCA1 promoter into the *Bgl*II site of the enhancerless minimal artificial promoter/luciferase expression vector, pGL4.27, using the following primer set: 5′-GATCGGCTTTGACC-GATAGTAACCTCTGCGCTCG-3′ and 5′- GATCCGAGCGCA-GAGGTTACTATCGGTCAAAGCC-3′. To measure the activities of LXR enhancer and ABCA1 promoter, the 3xLXRE-luc and pGL3-ABCA1 promoter reporter vectors (WT1)[25] were co-transfected with Renilla luciferase expression vector (TK-RL), respectively. To measure the PPARγ transcriptional activity of hesperetin (**1**), the PPREx3-tk reporter gene and PPARγ expression vector (pCMX-hPPARγ 1) were co-transfected with TK-RL as previously described.[26] Transfection into postdifferentiated THP-1 macrophages was carried out using FuGENE6. Compound **1** was added to the culture medium 24 h after transfection. Four or 24 h later, cells were lysed, and luciferase activities were measured using the Pikkagene Dual-SeaPancy luminescence kit according to the manufacturer's instructions (WAKO). Firefly luciferase activity was normalized to Renilla luciferase activity.

Real-Time RT-PCR. Total RNA extracted from cells using Isogen II was treated with RNase-free DNase I and then reverse transcribed using PrimeScript RT reagent kit. Quantitative real-time PCR was performed using SYBR Premix Ex *Taq*II on a Dice real-time thermal cycler (TP800, Takara). GAPDH was used as an internal control. Primer sets were as follows: GAPDH, 5′-CCACATCGCTCAGACAC-CAT-3′ and 5′- GCAACAATATCCACTTTACCAGAGTTAA-3′; ABCA1, 5′- GCCTGCTAGTGGTCATCCTG-3′ and 5′-CCA-CGCTGGGATCACTGTA-3′; LXRα, 5′-CAGGGCTCCAGAAAGA-GATG-3′ and 5′- ACAGCTCCACCGCAGAGT-3′; PPARγ, 5′- GA-CAGGAAAGACAACAGACAAATC-3′ and 5′- GGGGTGATGT-GTTTGAACTTG-3′. Crossing points for each transcript were determined using the second derivative maximum method, and quantification was performed using the comparative Ct ($\Delta\Delta$Ct) method according to the manufacturer's protocol.

Western Blot Analysis. The cell membrane fraction of THP-1 macrophages was prepared using ProteoJET membrane protein extraction kit according to the manufacturer's protocol. Ten micrograms of the cell membrane fraction was separated by SDS-PAGE on 5−12% polyacrylamide gel and then electroblotted onto a PVDF membrane. After blocking for 2 h by 5% skim milk, the membrane was incubated overnight at 4 °C with anti-ABCA1 or anti-flotillin-2 antibody and then reacted with horseradish peroxidase-conjugated anti-rabbit or -mouse secondary antibody. Immunoreactive protein bands were detected with the ECL kit and chemiluminescence detector LAS-4000 (Fujifilm, Tokyo, Japan).

Cholesterol Efflux Assay. Differentiated macrophages were equilibrated with NBD-cholesterol (1 μg/mL) for 12 h.[27] NBD-cholesterol-labeled cells were washed with PBS and incubated in RPMI1640 medium containing 0.2% (w/v) fatty acid-free BSA and 10 μg/mL ApoA-1 for 6 h. The fluorescence-labeled cholesterol released from cells into the medium was measured with a MT-600F fluorescence microplate reader (Corona Electric, Hitachinaka, Japan). Cholesterol efflux was expressed as a percentage of fluorescence in the medium relative to the total amounts of fluorescence detected in cells and the medium.

Statistical Analysis. Results were expressed as mean ± SD. Data were analyzed using Student's *t*-test. A value of $p < 0.05$ was considered significant.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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