

Anthocyanins Induce Cholesterol Efflux from Mouse Peritoneal Macrophages

THE ROLE OF THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ -LIVER X RECEPTOR α -ABCA1 PATHWAY*

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It is widely accepted that stimulation of reverse cholesterol transport, the efflux of excess cholesterol from peripheral tissues and transferring it to the liver for biliary excretion, is becoming an important component in reducing excess cholesterol deposition in atherosclerotic plaques. The ATP-binding cassette transporter has been identified as a key regulator of macrophage cholesterol efflux and apoAI-mediated reverse cholesterol transport. *In vivo* studies have documented anthocyanins, a large group of naturally phenolic compounds rich in plants, possess substantial capacities in improving plasma cholesterol levels. In this study, we investigated the potential role of anthocyanins in modulating cholesterol efflux from mouse peritoneal macrophages and macrophage-derived foam cells and the possible molecular mechanism linking ABCA1 to cholesterol efflux. Incubation of the mouse peritoneal macrophages and macrophage-derived foam cells with cyanidin-3-*O*- β -glucoside and peonidin-3-*O*- β -glucoside led to dose-dependent (1–100 μ M) induction in cholesterol efflux and ABCA1 mRNA expression, and this effect could be blocked by the ABCA1 inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt, and a general inhibitor of gene transcription actinomycin D. Treatment of the macrophages with anthocyanins also activated peroxisome proliferator-activated receptor γ , liver X receptor α mRNA expression, and their mediated gene expression. Addition of geranylgeranyl pyrophosphate ammonium salt or GW9662 markedly inhibited the anthocyanin-induced increase of ABCA1 gene expression and apoAI-mediated cholesterol efflux. These data demonstrated that anthocyanin induces cholesterol efflux from mouse peritoneal macrophages and macrophage-derived foam cells and that stimulation of cholesterol efflux by anthocyanin is mediated, at least in part, by peroxisome proliferator-activated receptor γ -liver X receptor α -ABCA1 signaling pathway activation.

Atherosclerosis (AS)² is a multifactorial cardiovascular disease, and its pathogenesis is not fully demonstrated (1, 2). Many studies (3, 4)

suggested that macrophages played critical pathogenic roles in the formation of atherosclerotic lesions. Fatty streaks of atherosclerosis contain large numbers of macrophage foam cells derived from circulating monocytes that adhere to activated endothelium and migrate into the artery wall (5). These cells subsequently differentiate into macrophages that express the scavenger receptor A gene, as well as other scavenger receptors that mediate the uptake of large amounts of cholesterol (6). As these receptors are not subject to negative regulation by high levels of intracellular cholesterol, massive accumulation of cholesterol esters can occur in macrophages, resulting in foam cell formation. This pathological phenomenon is the typical character of the early stage in the development of AS. Now, it is widely accepted that the removal of excess free cholesterol from arterial cells is very important for maintaining cellular cholesterol homeostasis, decreasing the size of atherosclerotic plaque, and protecting against AS.

Although there are multiple mechanisms involved in the efflux of cellular cholesterol, recent work (7) suggested that the ATP-binding cassette A1 (ABCA1) mediates this process. ABCA1 is a member of the ATP-binding cassette transporter family that is involved in the control of high density lipoprotein and apolipoprotein AI (apoAI)-mediated cholesterol efflux from macrophages (7–9). Mutation in the ABCA1 gene causes Tangier disease, which is marked by severe accumulation of cholesterol in macrophages and other tissues (10), suggesting that the ABCA1 gene plays an integral role in modulating cellular cholesterol transport (11).

Expression of the ABCA1 gene is transcriptionally regulated. PPAR γ was shown recently to induce the expression of the cholesterol transporter, ABCA1, in macrophages through a transcriptional cascade mediated by the nuclear receptor, liver X receptor (LXR) (12–14). Moreover, transplantation of PPAR γ -null bone marrow into mice lacking low density lipoprotein receptor resulted in a significant increase in atherosclerotic lesion size. The implication of these findings is that PPAR γ exerts anti-atherogenic effects by facilitating the removal of cholesterol from macrophages via cholesterol transporter proteins such as ABCA1. Parallel studies (12) demonstrated that the ligand of PPAR γ leads to induction of LXR α and enhanced ABCA1 gene expression, and this induction was significantly less in macrophages from PPAR γ knock-out mice. These combined findings illustrate a complex pathway of PPAR γ -LXR α -ABCA1 in the cellular regulation of cholesterol transport.

diphenyltetrazolium bromide; PBS, phosphate-buffered saline; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt; GGPP, geranylgeranyl pyrophosphate ammonium salt; Ros, rosiglitazone; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LXRE, liver X receptor-response element; AcLDL, acylated LDL.

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² The abbreviations used are: AS, atherosclerosis; Cy-3-g, cyanidin-3-*O*- β -glucoside; Pn-3-g, peonidin-3-*O*- β -glucoside; ABCA1, ATP-binding cassette transporter A1; PPAR γ , peroxisome proliferator-activated receptor γ ; LXR, liver X receptor; apoAI, apolipoprotein AI; BSA, bovine serum albumin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-

TABLE ONE

Sequences of primers and probes for real time quantitative PCR

Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')
PPAR γ	TCTTAAC TGCCGGATCCACAA	GCCCAAACCTGATGGCATT	TCCGGTTTCAGAAGTGC
LXR α	AGCGTCCATT CAGAGCAAGTG	CTCGTGGACATCCCAGATCTC	CAGGACAAAAGCTTC
ABCA1	TGGAAACTCACCAGCAACA	GGCAGGACAATCTGAGCAAAG	TTGCCAGACGGAGCC

Anthocyanins are naturally occurring pigments in the plant kingdom, and they are widely distributed in nature. *In vivo* and *in vitro* studies suggested that anthocyanins have an array of health-promoting benefits besides anti-oxidative and anti-inflammatory actions; however, anthocyanins have received less attention. We have demonstrated previously that black rice pigment fraction attenuated atherosclerotic plaque formation in apolipoprotein E-deficient mice (15). We also have shown that black rice pigment fraction significantly ameliorated hypercholesterolemia and suppressed cholesterol accumulation in liver and aorta, implying the pigment has a great potential function in removal of cholesterol away from tissues. The anthocyanins rich in the black rice pigment may contribute to the cardiovascular health-promoting effects (15).

This study was designed to investigate the effect of anthocyanin on ABCA1-mediated cholesterol efflux and to explore its possible mechanisms related to ABCA1 transporter and its regulation.

EXPERIMENTAL PROCEDURES

Materials—Anthocyanin standards cyanidin-3-*O*- β -glucoside (Cy-3-g) and peonidin-3-*O*- β -glucoside (Pn-3-g) were purchased from Polyphenol AS (Sandnes, Norway). Defined fetal bovine serum was provided by HyClone (Logan, UT). RPMI 1640 culture medium was obtained from Invitrogen. Bovine serum albumin (BSA), penicillin/streptomycin, cholesterol standard (high pressure liquid chromatography grade), cholesterol oxidase, cholesterol esterase, peroxidase, *p*-hydroxyphenylacetic acid, apolipoprotein AI, 1 α ,2 α -[³H]cholesterol (250 μ Ci), and actinomycin D were purchased from Sigma. The ABCA1 inhibitor-4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt (DIDS), the PPAR γ antagonist GW9662, and the LXR α antagonist geranylgeranyl pyrophosphate ammonium salt (GGPP) were purchased from Molecular Probes (Eugene, Oregon), Sigma, and Calbiochem, respectively.

Isolation of Mouse Peritoneal Macrophages—To harvest mouse peritoneal macrophages, the pathogen-free NIH mice were sacrificed, and ice-cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity of each mouse. This fluid was carefully collected and centrifuged at 3000 rpm. The supernatant was then withdrawn, and the cell pellet was resuspended in RPMI 1640 medium, allowed to adhere for 3 h, and then washed three times with pre-warmed PBS to remove nonadherent cells. The medium was then replaced with fresh RPMI 1640 medium supplemented with 10% fetal bovine serum (16). The cells were incubated for the specific times as indicated in the text and legends.

LDL Preparation and Acetylated Modification—LDL was isolated from the fresh plasma of healthy subjects. Briefly, after density adjustment to 1.200 g/ml with sodium bromide (NaBr), the plasma was separated by preparative ultracentrifugation at 60,000 rpm for 5.5 h on a Beckman L-80 ultracentrifuge, using a type Ti 90 rotor (17). The LDL was collected, sterilized, and stored in the dark at 4 °C. Acetylation of LDL was performed by the addition of 4 aliquots each of 1 μ l of acetic anhydride at 10-min intervals to 2 mg of LDL in 600 μ l of ice-cold 50% saturated sodium acetate. LDL was aggregated by vortexing a 1 mg/ml solution at low speed for 15 s. The acylated LDL (AcLDL) was dialyzed

against PBS overnight, and the protein concentration was determined after by a Lowry assay as described previously (18).

Cholesterol Loading and Efflux Assay—Adherent peritoneal macrophages were incubated in RPMI 1640 with 50 μ g of protein/ml of AcLDL (containing or not [³H]cholesterol) (19) at 37 °C for 24 h to induce macrophage foam cells. The cells were then washed in ice-cold PBS and incubated with Cy-3-g or Pn-3-g in medium containing 1.0 mg/ml BSA for the indicated time. After this incubation period, cells were washed three times in PBS, and apoAI-mediated cholesterol efflux studies were immediately performed by adding fresh medium with or without 10 μ g/ml apoAI for 24 h. Since in macrophages the equilibrium between esterified and free cholesterol is not obtained even after an additional 24-h incubation period (20), the experiments were performed in the absence of equilibrium. At the end of this incubation, lipids of cells and media were separately extracted in chloroform and methanol, and then the samples were dried under nitrogen, and free cholesterol and total cholesterol were measured by enzymatic assays. Esterified cholesterol was measured as the difference between total and free cholesterol. Cellular proteins were collected by digestion in NaOH and were measured by using the Bradford method. The percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free culture medium was determined as the percent counts in medium over counts in medium + cells. Each assay was performed in triplicate (21).

In the experiments with [³H]cholesterol, we measured radioactivity by scintillation counting in centrifuged medium and in cellular lipids extracted with hexane/isopropyl alcohol. ApoAI-induced [³H]cholesterol efflux was measured as the fraction of total radiolabeled cholesterol appearing in the medium in the presence of apoAI after subtraction of values for apoAI-free medium.

Cytotoxicity Tests—Cells were grown in microtiter plates and subjected to the experimental culture conditions and treatments as described for efflux experiments. 0.5 mg/ml MTT was added to each well and incubated for 4 h in the cell culture incubator. Solubilization buffer (10% SDS in 0.01 M HCl) was added to each well and incubated in a cell culture incubator overnight. Absorbance was measured at 550 nm on a microtiter plate reader. Percent MTT cleavage was determined as follows: (treatment value – media with vehicle value)/(0.1% Triton X-100 value – media with vehicle value) \times 100 (22). A lactate dehydrogenase release assay was performed according to the manufacturer's instructions (BioVision).

Real Time PCR-based Quantitative Gene Expression Analysis—Oligonucleotide primers and TaqMan probes were designed by using Primer Express software 2.0 (PE Biosystems) and were synthesized by Takara Biotechnology Inc. Sequences of probes and primers were listed in TABLE ONE. Total RNA was extracted from the cultured cells using TRIzol reagent according to the protocol provided by the manufacturer (Invitrogen). Real time quantitative TaqMan PCR analysis was used to measure the relative levels of PPAR γ , LXR α , and ABCA1 mRNA expression. The PCRs were performed according to the manufacturer's instructions (TaqMan Gold RT-PCR protocol, PE Biosystems). Sequence-specific amplification was detected with an increased fluorescent signal of 6-carboxyfluorescein (reporter dye) during the amplifica-

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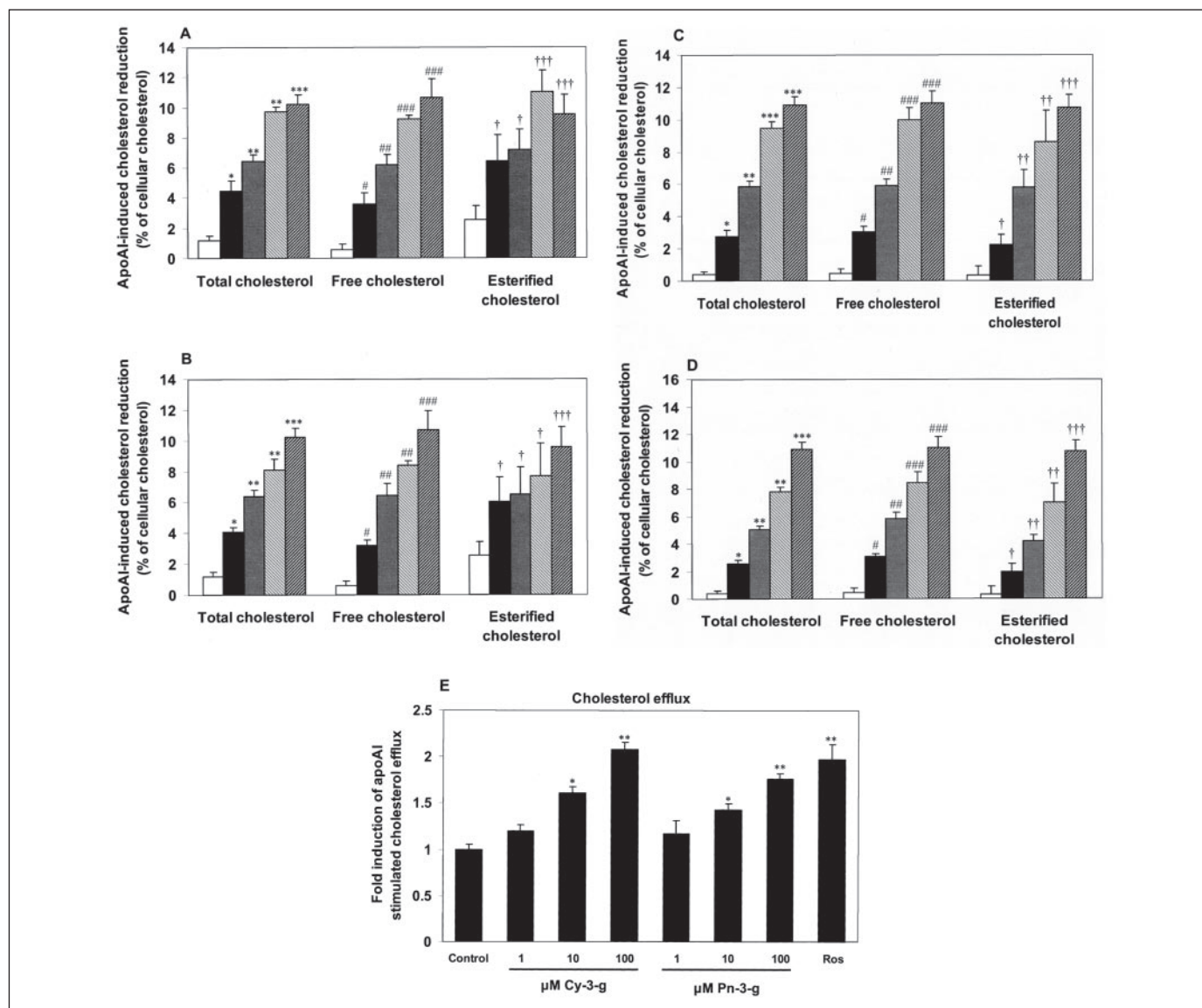


FIGURE 1. Anthocyanin Cy-3-g and Pn-3-g stimulate apoAI-mediated cholesterol efflux from mouse peritoneal macrophages and macrophage-derived foam cells. Primary mouse peritoneal macrophages (A and B) and cholesterol-loaded macrophage foam cells (C and D) were incubated with anthocyanin Cy-3-g (A and C) or Pn-3-g (B and D) with 1 μM (■), 10 μM (▤), 100 μM (▥), or 100 nM Ros (▧), respectively, and subsequently incubated with RPMI 1640 medium with or without apoAI (10 μg/ml). Intracellular lipids and lipids in culture medium were determined. Results are the mean ± S.E. of triplicate determinations of four experiments and are expressed as the percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium. Untreated cells were used as control (□). Statistically significant differences are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control (total cholesterol); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with control (free cholesterol); †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with control (esterified cholesterol). E. [^3H]cholesterol-loaded macrophages were treated with Cy-3-g or Pn-3-g and subsequently incubated with RPMI 1640 medium with or without apoAI (10 μg/ml) where indicated. ApoAI-induced [^3H]cholesterol efflux was measured as described. Values are expressed relative to the untreated control, set as 1. Results are the mean ± S.E. of triplicate determinations, representative of three independent experiments. Statistically significant differences are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$, or **, $p < 0.01$ compared with control.

tion cycle. Amplification of the murine GAPDH gene was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Levels of the different mRNAs were subsequently normalized to GAPDH mRNA levels. The amplification was performed on ABI Prism 7000 TaqMan real time fluorescent thermal cycler (PerkinElmer Life Sciences). The thermal cycling conditions included 2 min at 93 °C, 1 min at 93 °C, and 1 min at 55 °C. Thermal cycling proceeded with 40 cycles.

Presence of ABCA1 on the Cell Surface—Cholesterol-loaded macrophages grown on a 35-mm Petri dish (MatTek Corp.) were rinsed, fixed for 15 min with paraformaldehyde (4%), and incubated successively in PBS supplemented with 10% normal serum for 20 min. Then the cells were incubated for 60 min at 37 °C with primary goat anti-human monoclonal

ABCA1. After rinsing, the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody at 37 °C for 45 min. Finally, the cells were examined using a Zeiss Axiophot microscope (Zeiss LSM510, Carl Zeiss). Cells in all steps were rinsed with PBS. Replacement of the primary antibody with PBS, 10% normal serum was used to control the specificity of the immunolabeling of the cells (23). Scanned images were acquired using a laser-scanning spectral confocal microscope system.

Cell Surface Binding of ^{125}I -Labeled ApoAI—ApoAI binding to cells was performed as described previously (24). Pretreated cholesterol-loaded macrophages were chilled on ice and rinsed with cold PBS. ^{125}I -ApoAI was exposed to the cells at 2 μg/ml in HEPES-buffered RPMI 1640/BSA media with or without a 50-fold excess of unlabeled apoAI (100 μg/ml). Cells were incubated on ice for 1 h and rinsed with cold

PBS containing 0.1% BSA, then twice with PBS alone. Cells were solubilized in 0.1 N NaOH, and aliquots were taken for scintillation counting. Results are expressed as nanograms of ^{125}I -apoAI per mg of cell protein after subtraction of values in the presence of unlabeled apoAI.

Nuclear Protein Extraction and PPAR γ Transcription Factor Activity Assay—Nuclear extracts of cells were prepared as described previously (25). Briefly, monolayers (2×10^6 cells) were harvested by scraping, washed in cold PBS, and incubated in two packed cell volumes of buffer A (10 mM HEPES (pH 8.0), 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, 200 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin and aprotinin, and 0.5% Nonidet P-40) for 5 min at 4 °C. The nuclei were collected by microcentrifugation, rinsed once in buffer A, and resuspended in two-thirds packed cell volume of buffer C (20 mM HEPES (pH 7.9), 1.5 mM MgCl_2 , 420 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, 1.0 $\mu\text{g}/\text{ml}$ leupeptin and aprotinin). Nuclei were incubated at 4 °C for 20 min and clarified by microcentrifugation for 5 min. The resulting supernatants were used as the nuclear fraction, and protein concentration was determined by the Bradford method (26). PPAR γ transcription factor activity was assayed by using enzyme-linked immunosorbent assay-based PPAR γ transcription factor activity assay kit to detect and qualify transcription PPAR γ factor activation (Active Motif Inc.). The measurement was done according to the protocol of the kit. This assay is specific for PPAR γ activation, and it will not cross-react with PPAR α or PPAR β .

Transient Transfections and Reporter Gene Assays—LXR-response element (LXRE)-driven luciferase reporter vector (LXRE-tk-Luc) was kindly provided by David J. Mangelsdorf (University of Texas Southwestern Medical Center). For LXR activation studies, 0.75 μg of LXRE-driven luciferase reporter vector (LXRE-tk-Luc) and 0.75 μg of β -galactosidase control vector (Promega) were used. Six hours after transfection, cells were treated with Cy-3-g or Pn-3-g for 12 h. Luciferase and β -galactosidase activities were determined in cell lysate. The amount of luciferase activity was normalized for β -galactosidase and reported as relative light units.

RESULTS

Anthocyanin Induces Cholesterol Efflux to ApoAI in Macrophages—As stated above, the previous study (15) has suggested the possible role of black rice pigment rich in anthocyanins in reverse cholesterol efflux. This relationship led us to hypothesize that anthocyanin might play a role in the cellular regulation of cholesterol efflux. Because of its relevance close to the atherosclerotic process, mouse peritoneal macrophages were selected for observation on cholesterol efflux *in vitro*. To examine the potential effect of anthocyanin on cholesterol efflux, the mouse peritoneal macrophages were loaded with 50 $\mu\text{g}/\text{ml}$ AcLDL for 24 h to promote cholesteryl ester accumulation inducing the formation of macrophage-derived foam cells. After that the primary mouse macrophages (Fig. 1, A and B) and macrophage-derived foam cells (Fig. 1, C and D) were treated with 1, 10, and 100 μM Cy-3-g or Pn-3-g or with 100 nM Ros, the PPAR γ ligand, for 24 h, respectively, and subsequently exposed to apoAI in order to promote cholesterol efflux. After 24 h, cholesterol efflux was measured by the change in cellular cholesterol levels from cells. Both Cy-3-g (Fig. 1, A and C) and Pn-3-g (Fig. 1, B and D) treatment reduced intracellular cholesterol concentrations in macrophages and macrophage-derived foam cells in a dose-dependent manner, and these two anthocyanins reduced the esterified cholesterol pool and free cholesterol levels substantially under these conditions.

To demonstrate that the variation of intracellular lipids was not because of the action of anthocyanin on *de novo* cholesterol synthesis, we loaded mouse peritoneal macrophages with 250 μCi of [^3H]choles-

terol plus AcLDL (50 $\mu\text{g}/\text{ml}$) for 24 h and determined the apoAI-mediated efflux of cholesterol by measuring the appearance of cholesterol in the medium. Anthocyanin Cy-3-g, Pn-3-g, and Ros treatment in the cells loaded with cholesterol increased [^3H]cholesterol release approximately from 1- to 2-fold, respectively, compared with untreated cells (Fig. 1E). These results indicate that Cy-3-g and Pn-3-g enhanced apoAI-mediated cholesterol efflux from mouse macrophages and macrophage-derived foam cells. Anthocyanins Cy-3-g and Pn-3-g exhibited similar effects on cholesterol efflux as PPAR γ agonist rosiglitazone.

Anthocyanin Treatment Does Not Cause Significant Toxicity in Macrophages—Because few studies had reported that anthocyanin caused cytotoxicity and the cells released cholesterol during death, we characterized whether the anthocyanin induction of cholesterol efflux is relevant to its toxicity on macrophages. To address this possibility, we used two different approaches: the MTT test to measure overall metabolic activity of the cell and a lactate dehydrogenase release assay to assess cellular membrane integrity. The results show that none of these tests revealed significant cytotoxicity (MTT and lactate dehydrogenase release) when cells were treated with 100 μM Cy-3-g or 100 μM Pn-3-g (results not shown). These data show that both Cy-3-g and Pn-3-g do not cause significant cytotoxicity, and anthocyanin-induced cholesterol efflux is not relevant to the cell cytotoxicity.

Anthocyanin Induces ABCA1 Gene Expression—By having established that anthocyanin is a specific inducer of apoAI-mediated cholesterol efflux, we next determined the effects of anthocyanin on ABCA1 transporter gene expression, which controls the first steps of apoAI-mediated cholesterol efflux and reverses the cholesterol transport pathway. Treatment with 1–100 μM Cy-3-g or Pn-3-g enhanced ABCA1 gene expression in a dose-dependent manner in primary mouse peritoneal macrophages and macrophage-derived foam cells (Fig. 2A). Moreover, in macrophage-derived foam cells, we found that ABCA1 gene expression induced by 100 μM Cy-3-g or 100 μM Pn-3-g reached a high level at 24 h and then decreased (Fig. 2B). To address whether the induction of gene expression for ABCA1 by the anthocyanin was secondary to enhanced gene transcription, the macrophages were incubated with 100 μM Cy-3-g or 100 μM Pn-3-g or 100 nM Ros, respectively, in the presence of a general inhibitor of gene transcription actinomycin D. The addition of actinomycin D completely abolished the increase of ABCA1 mRNA expression in response to anthocyanins and the PPAR γ agonist rosiglitazone (Fig. 2C).

Anthocyanin Increases Cellular ABCA1 and Binding of ApoAI—Because previous studies (28, 29) have proposed that ABCA1 may be most active at the plasma membrane, presumably to allow interactions with extracellular apolipoproteins, which controls the rate of apoAI-mediated lipid efflux, the effects of anthocyanins on the cell membrane content of ABCA1 were determined. The confocal images showed a significantly increased degree of ABCA1 content (green) at the surfaces of the cells in the presence of anthocyanins *versus* untreated cells (Fig. 3, A–H).

Next we tested the effect of anthocyanins on apoAI cell surface binding to ABCA1, which could account for the higher cholesterol efflux. Cholesterol-loaded macrophages were treated with 1, 10, and 100 μM Cy-3-g or Pn-3-g and 100 nM Ros for 24 h, and ^{125}I -apoAI binding was measured. As shown in Fig. 4, binding was increased significantly after treatment with anthocyanins. Taken together, all these studies showed that anthocyanins increase the total membrane content of ABCA1 in macrophages, leading to more plasma membrane ABCA1 available for interactions with apoAI.

Inhibition of ABCA1 Prevents Cholesterol Efflux Induced by Anthocyanins—To determine whether enhanced cholesterol efflux following ABCA1 activation requires an increase in transcription, cells

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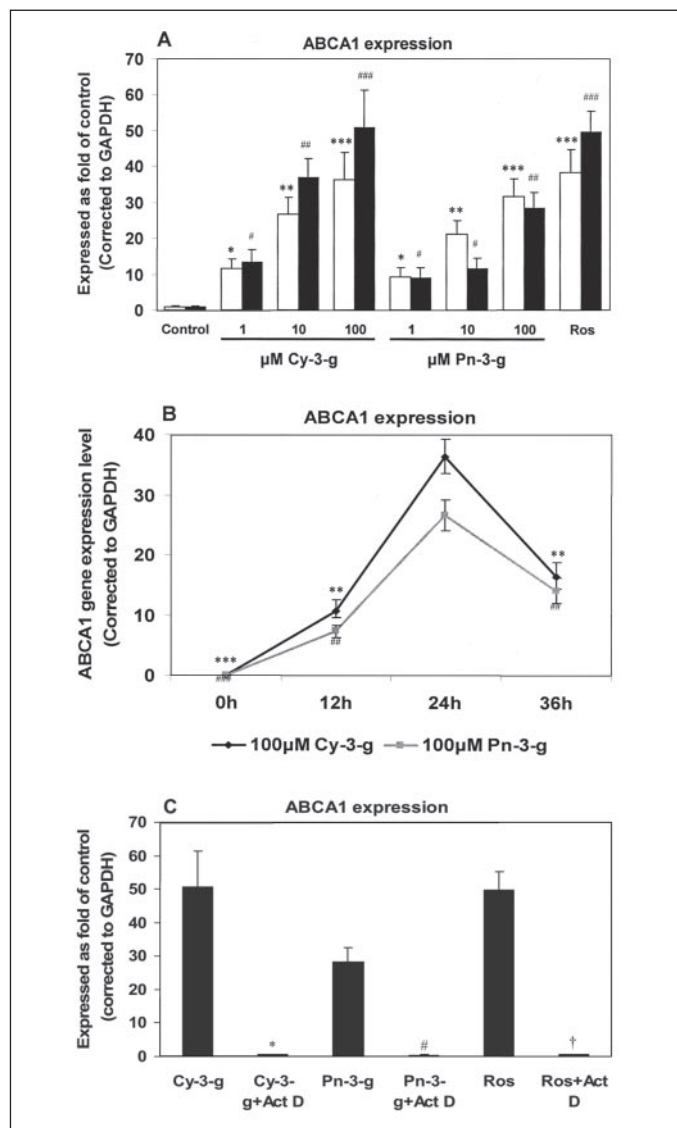


FIGURE 2. Anthocyanin Cy-3-g and Pn-3-g stimulate ABCA1 gene expression. A, primary mouse peritoneal macrophages (□) or cholesterol-loaded macrophage foam cells (■) were treated with 1, 10, and 100 μM Cy-3-g or Pn-3-g or 100 nM Ros for 24 h. ABCA1 mRNA expression was measured by real time quantitative PCR. Values are expressed relative to the untreated control, set as 1. Results are the mean \pm S.E. of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control (untreated primary macrophages); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with control (untreated macrophage-derived foam cells). B, cholesterol-loaded macrophages were treated with 100 μM Cy-3-g or 100 μM Pn-3-g for 0–36 h, and ABCA1 mRNA expression was determined, and results are expressed as mean \pm S.E. of the ratio of ABCA1 relative to GAPDH of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: **, $p < 0.01$; ***, $p < 0.001$ compared with 24 h (Cy-3-g-treated cells); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with 24 h (Pn-3-g-treated cells). C, cholesterol-loaded mouse peritoneal macrophages were treated with 100 μM Cy-3-g or Pn-3-g or 100 nM Ros in the presence of 5 $\mu\text{g}/\text{ml}$ actinomycin D (Act D) for 24 h. ABCA1 mRNA expression was determined, and results are expressed relative to the untreated control, set as 1. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.001$ compared with Cy-3-g; #, $p < 0.001$ compared with Pn-3-g; †, $p < 0.001$ compared with Ros.

were incubated with anthocyanin together with actinomycin D. The addition of actinomycin D completely prevented the increase of cholesterol efflux into medium (Fig. 5).

DIDS is an inhibitor of the transport activities of ABCA1 and blocker of apoAI-mediated cholesterol efflux. When DIDS was added simultaneously with anthocyanins, it markedly reduced the apoAI-mediated

efflux of tritiated cholesterol and completely abolished the positive effects of Cy-3-g and Pn-3-g on apoAI-mediated cholesterol efflux (Fig. 5). This finding strongly suggests that ABCA1 transporter is responsible for facilitating cholesterol efflux.

Anthocyanin Induces LXR α Expression—Many studies (17, 19) implied that induction of ABCA1 occurred through the nuclear orphan receptors LXR. LXR ligands (30) can also modulate cholesterol levels through enhancing ABCA1 gene expression. To investigate the potential mechanism by which anthocyanins induce ABCA1 mRNA expression, we examined the effect of anthocyanins on LXR α expression. Real time PCR analysis demonstrated that Cy-3-g and Pn-3-g induce LXR α mRNA in cholesterol-loaded macrophages in a dose-dependent manner (Fig. 6A). The effect of 100 μM Cy-3-g on the induction of LXR α expression was nearly identical with 100 nM rosiglitazone. Furthermore, we found that LXR α expression induced by Cy-3-g or Pn-3-g arrived at a high level at 12 h (Fig. 6B).

Anthocyanin Induces PPAR γ Expression—Recent reports (13, 14) showed that PPAR γ was relevant to mediating the expression of the cholesterol transporter ABCA1 and cholesterol efflux. To investigate whether PPAR γ was involved in anthocyanin-induced cholesterol efflux, we studied the effect of these compounds on PPAR γ expression. The results of real time PCR showed that Cy-3-g or Pn-3-g induces PPAR γ mRNA expression in cholesterol-loaded macrophages in a dose-dependent manner (Fig. 7A). The effect of 100 μM Cy-3-g on the induction of PPAR γ expression was in the same manner with 100 nM rosiglitazone. PPAR γ mRNA expression was obtained at a high level after the cells were treated with Cy-3-g or Pn-3-g for 12 h.

Treatment of Anthocyanin Alone Does Not Influence Foam Cell Formation—PPARs have been documented to regulate macrophage scavenger receptor expression such as CD36 (4, 6, 7), and this led to the supposition that PPAR γ activation may promote foam cell formation, a known atherogenic process characterized by cholesterol ester droplet accumulation in macrophages (27). In this experiment, we further investigated the influence of both Cy-3-g and Pn-3-g on AcLDL-induced transformation of mouse peritoneal macrophages into foam cells. We loaded mouse peritoneal macrophages with AcLDL for 48 h and then treated them with 100 μM Cy-3-g or Pn-3-g. The results showed that Cy-3-g (Fig. 8A) and Pn-3-g (Fig. 8B) at a concentration of 100 μM did not influence AcLDL-induced cholesterol accumulation in macrophages. The observation that neither Cy-3-g nor Pn-3-g stimulates esterified or free cholesterol accumulation indicates that PPAR γ activation is not implicated in foam cell formation.

Increased ABCA1 Gene Expression and Cholesterol Efflux Induced by Anthocyanins Are Dependent on PPAR γ -LXR α Activation—Previous studies have documented that PPAR γ and LXR α were upstream of ABCA1, and PPAR γ induces ABCA1 gene expression through a molecular transcription pathway involving LXR α activation. Therefore, we determined whether the anthocyanin-mediated increases in ABCA1 mRNA expression and cholesterol efflux were dependent on the activation of PPAR γ and/or LXR α . Initially, the effect of the antagonists of PPAR γ and LXR α on ABCA1 mRNA expression and cholesterol efflux was determined. Co-incubation of the LXR α antagonist GGPP and the PPAR γ antagonist GW9662 blocked the anthocyanin-enhanced ABCA1 gene expression (Fig. 9A) and the cholesterol efflux to apoAI (Fig. 9B). PPAR γ contained in nuclear extracts is a member of the nuclear transcription factors and can specifically bind to peroxisome proliferator-response elements. We next detected the PPAR γ transcriptional activity with its specific oligonucleotide by using enzyme-linked immunosorbent assay-based PPAR γ transcription factor assay kit. Fig. 10 shows that anthocyanin enhanced PPAR γ activity in a dose-depend-

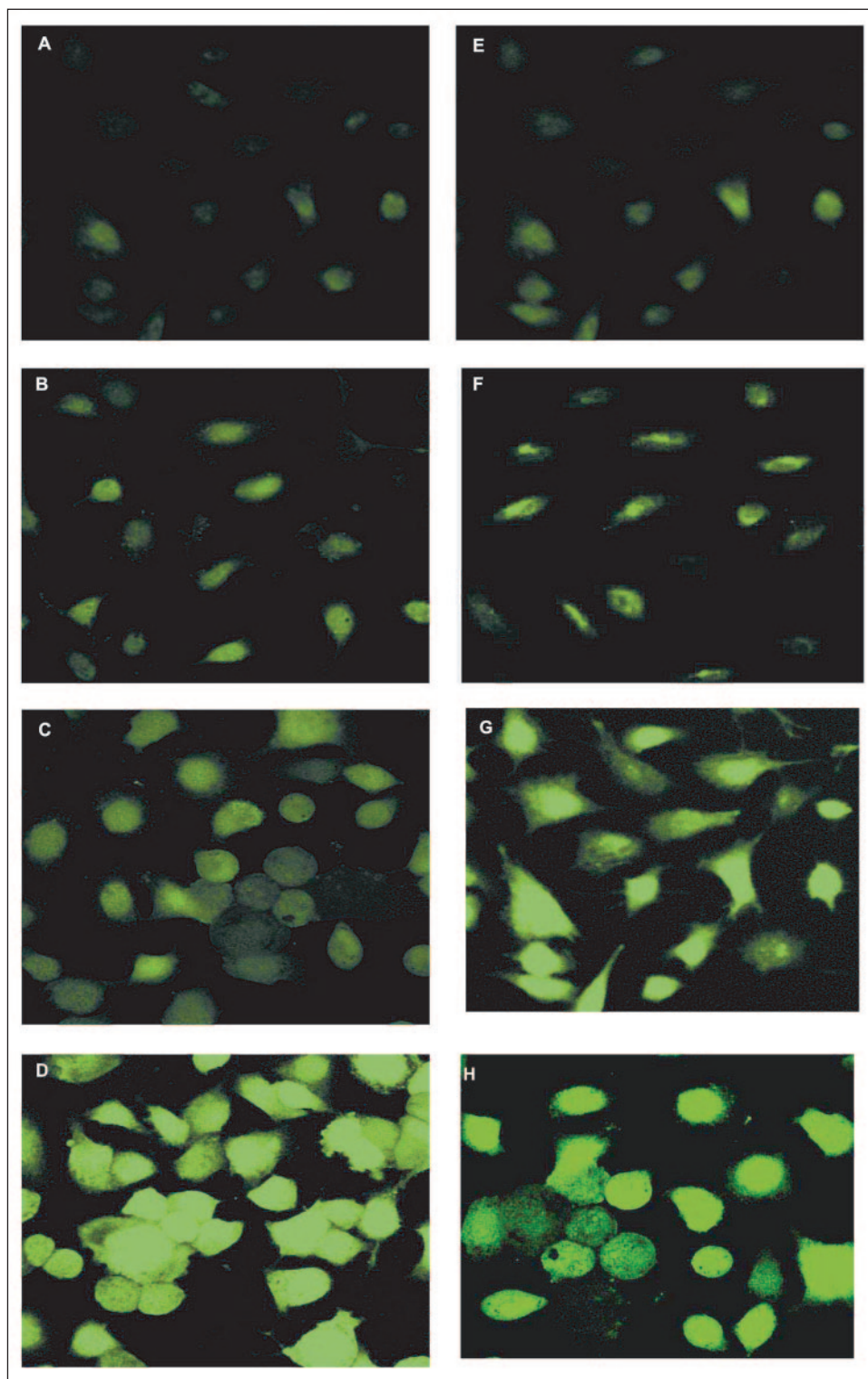


FIGURE 3. Fluorescent confocal microscopy analysis of ABCA1 presence in mouse macrophages. Cholesterol-loaded macrophages were plated in RPMI 1640 medium with 0.1% BSA and incubated with vehicle or Cy-3-g or Pn-3-g of 1, 10, 100 μM for 24 h. Cells were incubated successively in PBS supplemented with 10% normal serum. After 20 min, cells were rinsed and incubated with the primary goat monoclonal antibody to ABCA1. Cells were then extensively washed and incubated with a fluorescent secondary antibody. After that, the cells were coverslipped for imaging on a confocal microscope. Images are representative fields from the same experiment. *A*, untreated control; *B*, 1 μM Cy-3-g; *C*, 10 μM Cy-3-g; *D*, 100 μM Cy-3-g; *E*, 1 μM Pn-3-g; *F*, 10 μM Pn-3-g; *G*, 100 μM Pn-3-g; *H*, 100 nM Ros.

ent manner, and the action of 100 μM Cy-3-g was approximate to 100 nM rosiglitazone.

To determine whether anthocyanin treatment results in LXR α -mediated gene expression, we examined the effect of anthocyanin on LXR response element-dependent transcriptional activity. In cells transfected with an LXRE-driven luciferase-reporter vector (LXRE-tk-Luc), LXR activity was dose-dependently increased in cells treated with anthocyanin (Fig. 11). Thus, the data clearly demonstrated that anthocyanin up-regulated the LXR-dependent transcription.

Collectively, these results demonstrate that anthocyanin enhances LXR activation, subsequently resulting in increased *ABCA1* gene expression and macrophage cholesterol efflux. These activations by anthocyanin may be mediated through increased gene expression and activity of PPAR γ .

Anthocyanin Cy-3-g and Pn-3-g Do Not Synergistically Induce ABCA1 Gene Expression—To demonstrate cross-talk between Cy-3-g and Pn-3-g activation on *ABCA1* gene regulation, we performed real time PCR analysis to determine quantitatively *ABCA1* gene expression

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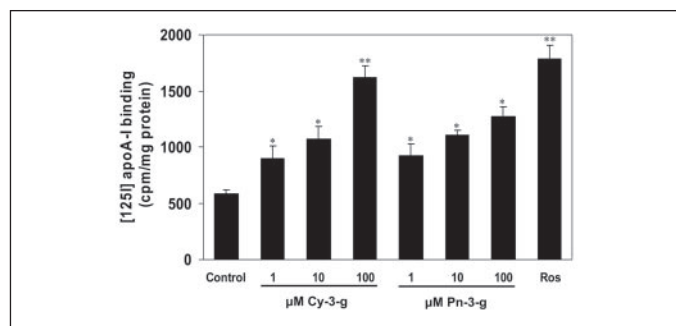


FIGURE 4. Anthocyanin treatment increases apoA-I-binding of macrophages. Cholesterol-loaded macrophages were incubated in RPMI 1640 medium. After exposure to 1, 10, and 100 μM Cy-3-g or Pn-3-g or 100 nM Ros for 24 h, cells were chilled on ice and then exposed to 2 $\mu\text{g}/\text{ml}$ ^{125}I -apoA-I, with or without a 50-fold excess of unlabeled apoA-I. After 1 h on ice, cells were washed, lysed, and taken for γ -counting and protein mass assay. The ^{125}I -apoA-I binding was calculated as described under "Experiment Procedures." Results are presented as mean \pm S.E. from three determinations from three experiments. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$, or **, $p < 0.01$ compared with control.

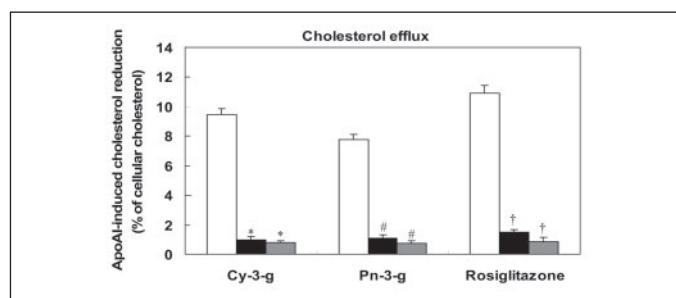


FIGURE 5. ABCA1 antagonist DIDS and actinomycin D prevent cholesterol efflux induced by anthocyanins. Cholesterol-loaded macrophages were incubated with 100 μM Cy-3-g or Pn-3-g or 100 nM Ros alone (□) or in the presence of 400 μM DIDS (■) or with 5 $\mu\text{g}/\text{ml}$ actinomycin D (Act D, ▨). After 24 h, the percent of cholesterol efflux was estimated as described. The data represent the mean \pm S.E. from four separate experiments. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.001$ compared with cells treated with Cy-3-g alone; #, $p < 0.001$ compared with cells treated with Pn-3-g alone; †, $p < 0.001$ compared with cells treated with Ros alone.

in macrophage foam cells. The results confirm that *ABCA1* gene expression is induced by either Cy-3-g or Pn-3-g alone and that combination treatment with Cy-3-g and Pn-3-g does not result in further induction, indicating no functional positive cross-talk between two anthocyanins (data not shown).

DISCUSSION

Cholesterol-loaded macrophages are present at all stages of atherogenesis, and recent *in vivo* studies indicate that macrophage-derived foam cells play important roles in early lesion development and complicated lesion and plaque vulnerability (31). Nowadays, it is widely accepted that high density lipoprotein protects against atherosclerosis by removing excess cholesterol from these cells by an active process mediated by a cell-membrane transporter called ABCA1. Thus, the apolipoprotein/ABCA1 pathway efficiently clears cells of excess cholesterol that would otherwise accumulate as intracellular lipid droplets (32). Some drugs have been shown atheroprotective functions in human disease and animal models through enhancing macrophage ABCA1 activity (33, 34). However, there has been little evidence that food factors directly modulate the cholesterol metabolism, including cholesterol efflux or some specific regulatory gene expression. In the present study, we show for the first time that anthocyanin Cy-3-g or Pn-3-g produced the effect of promoting apoA-I-mediated cholesterol efflux from mouse peritoneal macrophages and macrophage-derived foam cells through a

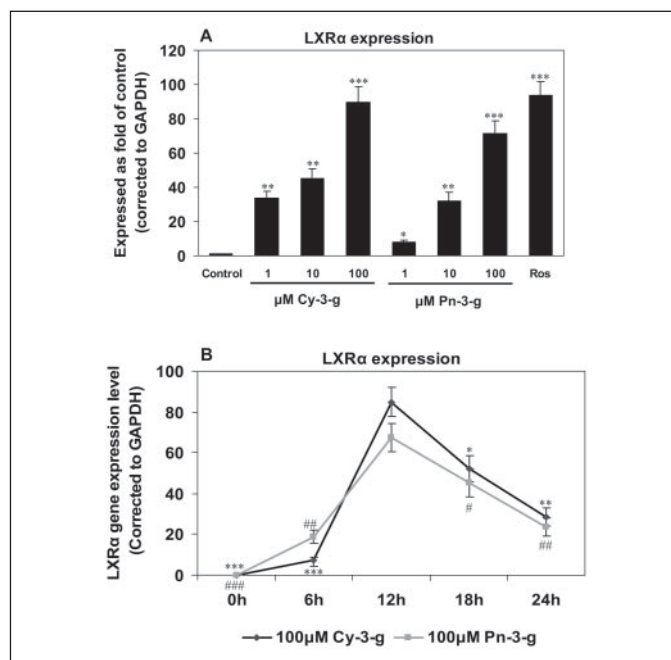


FIGURE 6. Anthocyanin Cy-3-g and Pn-3-g stimulate LXRα gene expression. A, cholesterol-loaded macrophages were treated with 1, 10, and 100 μM Cy-3-g or Pn-3-g or 100 nM Ros for 12 h. LXRα mRNA expression was measured by real time PCR. Values are expressed relative to the untreated control, set as 1. Results are the mean \pm S.E. of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ compared with control. B, cholesterol-loaded macrophages were treated with Cy-3-g or Pn-3-g of 100 μM for 0–24 h. LXRα mRNA expression was assayed, and results are the mean \pm S.E. of the ratio of LXRα relative to GAPDH of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with 12 h (Cy-3-g-treated cells); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with 12 h (Pn-3-g-treated cells).

transcriptional regulation pathway, PPAR γ and LXR α activation, and following increased expression ABCA1.

Anthocyanins are the most important plant pigments visible to the human eye. They belong to the widespread class of phenolic compounds named flavonoids (35). They are widely distributed in the human diet through crops, beans, fruits, and vegetables (36), suggesting that we ingest considerable amounts of anthocyanin pigments from plant-based daily diets. Cy-3-g and Pn-3-g are among the only six anthocyanins that have higher contents in plants. In recent years, many reports (37, 38) have been published on the important role of anthocyanins as dietary antioxidants for the prevention of oxidative damage caused by active oxygen radicals in living systems. However, due to limited knowledge, other biochemical functions of anthocyanins have been less thoroughly reported. In this study, we made the novel observation that treatment of macrophages and cholesterol-loaded macrophages with anthocyanins significantly enhance cholesterol efflux to its acceptor-apoA-I. This effect of anthocyanins was not because of its cytotoxicity as proved by two different assays. The induction of cholesterol removal was consistent with the anthocyanin-induced up-regulation of ABCA1 mRNA expression. Moreover, we found that increased exogenous concentrations of anthocyanins resulted in enhanced cellular presence of ABCA1 content, increased binding of isotope-labeled apoA-I, and elevated cholesterol efflux. Anthocyanin-induced cholesterol efflux was almost completely abolished by the ABCA1-specific inhibitor DIDS. Actinomycin D is a widely used intercalating transcription inhibitor that acts by binding to DNA (39). In the present study, we also found that addition of actinomycin D completely prevented anthocyanin-induced ABCA1 mRNA expression and cholesterol efflux. Overall, these find-

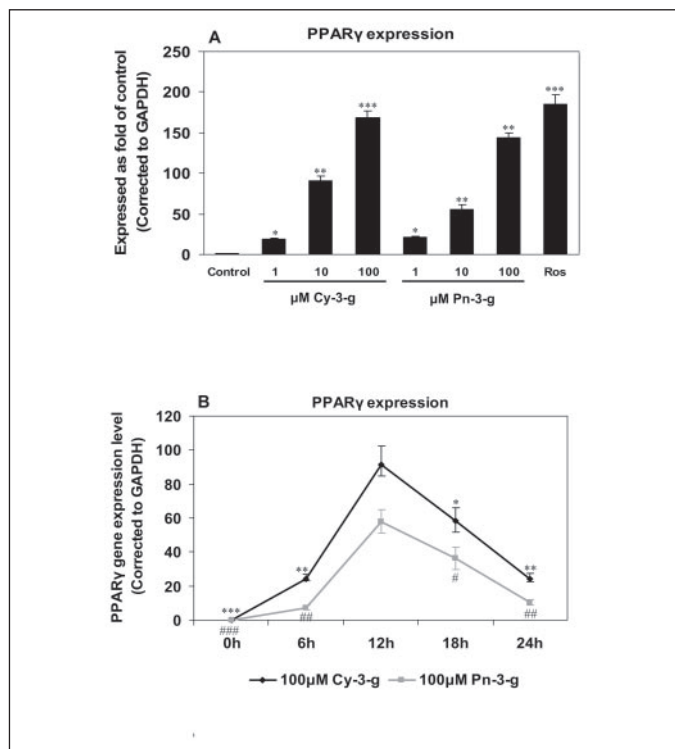


FIGURE 7. Anthocyanin Cy-3-g and Pn-3-g induce PPAR γ gene expression. A, cholesterol-loaded macrophages were treated with 1, 10, and 100 μ M Cy-3-g or Pn-3-g or 100 nM, Ros for 12 h. PPAR γ mRNA expression was measured by real time PCR. Values are expressed relative to the untreated control, set as 1. Results are the mean \pm S.E. of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ compared with control. B, cholesterol-loaded macrophages were treated with 100 μ M Cy-3-g or Pn-3-g for 0–24 h. PPAR γ mRNA expression was assayed, and results are the mean \pm S.E. of the ratio of PPAR γ relative to GAPDH of triplicate determinations. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with 12 h (Cy-3-g-treated cells); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with 12 h (Pn-3-g-treated cells).

ings strongly suggest that anthocyanins induced cholesterol efflux by regulating ABCA1 transcription.

Transcription of ABCA1 is markedly induced by activation of the nuclear receptors liver X receptor (LXR α and/or LXR β) and retinoid X receptor (20, 40). Here we reveal that anthocyanins induce expression of the gene encoding LXR α in mouse macrophages and macrophage-derived foam cells. LXR α is implicated in anthocyanin-induced ABCA1 gene expression and cholesterol efflux because the LXR α antagonist GGPP significantly abolishes the increase of both ABCA1 mRNA expression and apoAI-mediated cholesterol efflux by anthocyanins. Furthermore, the treatment of the cells with anthocyanin increased the activity of an LXRE-reporter construct. These data imply that anthocyanin-induced up-regulation of ABCA1 gene expression and cholesterol efflux is dependent on the activation of LXR α . LXR α may regulate ABCA1 gene expression through binding to an LXR element of the ABCA1 promoter.

One of the important transcriptional factors for upstream regulation of LXR γ and ABCA1 is PPAR γ . PPAR γ is a member of the nuclear receptor superfamily that regulate genes involved in lipid homeostasis. As PPAR γ has also been demonstrated to stimulate cholesterol efflux in cultured macrophages by inducing the expression of LXR, which in turn activates expression of ABCA1 and other genes involved in cholesterol efflux (12), we supposed that PPAR γ participated in mediating cholesterol efflux and ABCA1 gene expression by anthocyanins. The hypothesis is also supported by our observations that the PPAR γ antagonist

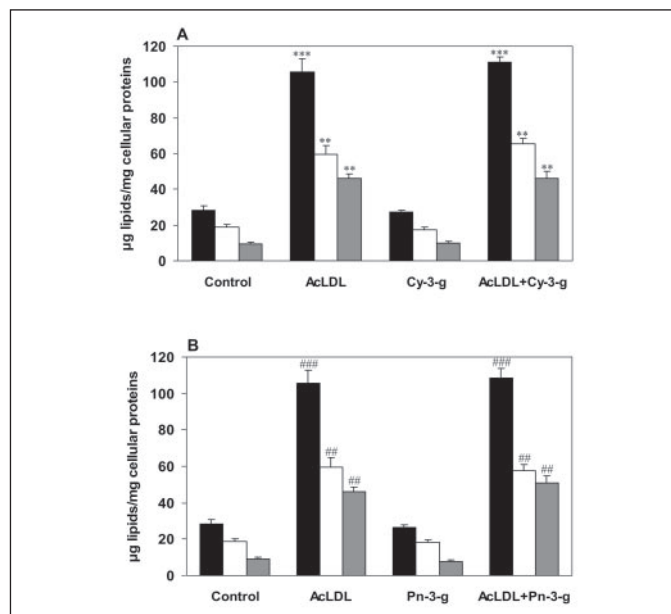


FIGURE 8. Cy-3-g and Pn-3-g do not influence foam cell transformation of mouse peritoneal macrophages. Cholesterol-loaded mouse peritoneal macrophages were treated with 100 μ M Cy-3-g (A) or 100 μ M Pn-3-g (B). Intracellular total cholesterol (■), free cholesterol (□), and esterified cholesterol (▨) were enzymatically determined. Results are the means \pm S.E. of triplicate determinations, representative of three independent experiments. Statistically significant differences between treatments are indicated. ANOVA was followed by Mann-Whitney's test: **, $p < 0.01$; ***, $p < 0.001$ compared with control and Cy-3-g. ##, $p < 0.01$; ###, $p < 0.001$ compared with control and Pn-3-g.

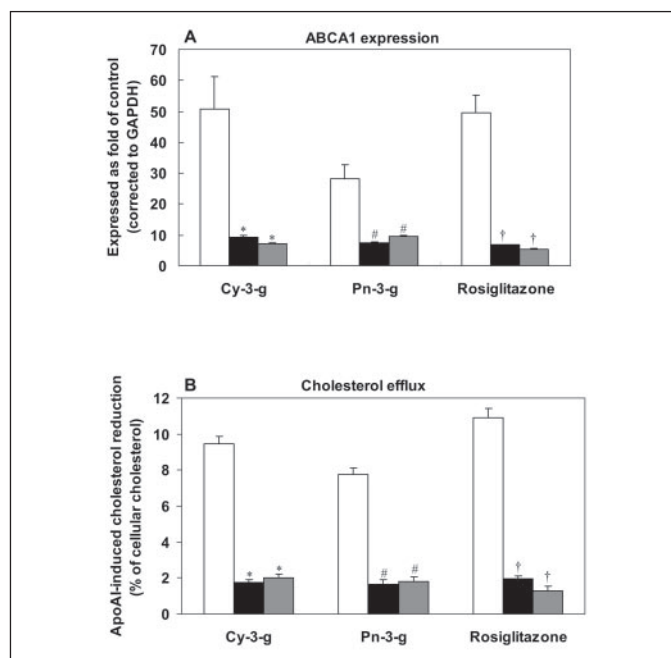


FIGURE 9. LXR α antagonist GGPP and PPAR γ antagonist GW9662 block the anthocyanin-mediated increase in ABC expression and cholesterol efflux. A, ABCA1 mRNA expression was measured after cholesterol-loaded cells were incubated with 100 μ M Cy-3-g or Pn-3-g or 100 nM Ros alone (□), in the presence of a LXR α antagonist GGPP (10 μ M, ■), or with a PPAR γ antagonist GW9662 (10 μ M, ▨) for 24 h. Values represent the mean \pm S.E. of three separate experiments with triplicate measurements. ANOVA was followed by Mann-Whitney's test: *, $p < 0.01$ compared with cells treated with Cy-3-g alone; #, $p < 0.01$ compared with cells treated with Pn-3-g alone; †, $p < 0.01$ compared with cells treated with Ros alone. B, mouse macrophages were cholesterol-loaded, and the percent of cholesterol efflux to apoAI (10 μ g/ml) was determined following a 24-h preincubation with 100 μ M Cy-3-g or Pn-3-g or 100 nM Ros alone (□), in the presence of 10 μ M GGPP (■), or with 10 μ M GW9662 (▨) for 24 h. Values represent the mean \pm S.E. ANOVA was followed by Mann-Whitney's test: *, $p < 0.01$ compared with cells treated with Cy-3-g alone; #, $p < 0.01$ compared with cells treated with Pn-3-g alone; †, $p < 0.01$ compared with cells treated with Ros alone.

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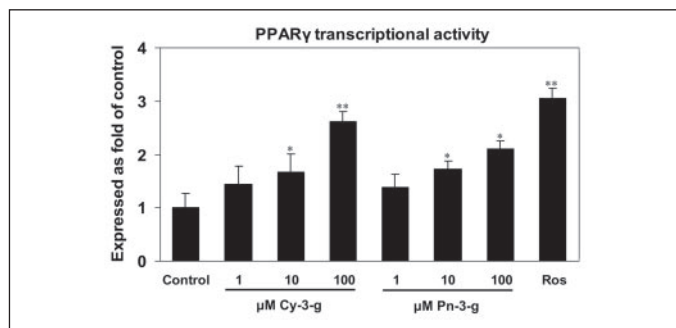


FIGURE 10. Anthocyanin Cy-3-g and Pn-3-g enhance PPAR γ transcriptional activity. Cholesterol-loaded cells were treated by 1, 10, and 100 μ M Cy-3-g or Pn-3-g or 100 nM Ros for 12 h. The cells were then rinsed, and nuclear protein was extracted. The protein concentration was measured by the Bradford method, and 10 μ g of nuclear extracts were added to a 96-well plate that has been immobilized by an oligonucleotide that contained peroxisome proliferator-response elements (5'-AACTAGTGCAAGGTCA-3'). After 1 h, the wells were incubated with diluted primary PPAR γ antibody to recognize the accessible epitope on PPAR γ protein upon DNA binding. The horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 h. At the end, the reaction was stopped, and absorbance was read at 450 nm on a spectrophotometer. The values represent the means \pm S.E. relative to untreated cells (taken as 1) from three independent experiments. *, $p < 0.05$; or **, $p < 0.01$ compared with control.

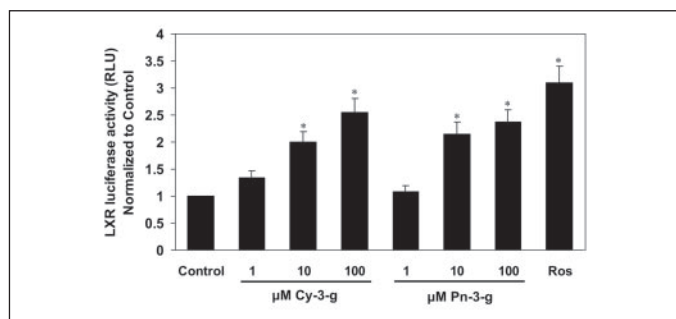


FIGURE 11. Anthocyanin Cy-3-g and Pn-3-g increase LXRE-dependent transcriptional activity. Macrophages were transfected with a luciferase reporter plasmid of LXREs upstream of the thymidine kinase promoter (LXRE-tk-Luc) in the presence of β -galactosidase as a reference plasmid. Cells were treated as described under "Experimental Procedures," and luciferase activity in cholesterol-loaded macrophages were measured and normalized to the β -galactosidase activity. The values represent the means \pm S.E. relative to untreated cells (taken as 1) from two independent transfections performed in triplicate. *, $p < 0.05$ compared with control. RLU, relative light units.

GW9662 significantly attenuated the anthocyanin-mediated increase in cholesterol efflux and ABCA1 mRNA expression. Our findings also show that the effect of anthocyanins on regulating ABCA1 gene expression was similar with the widely used PPAR γ agonist rosiglitazone. These data provide a plausible mechanism for explaining the action of anthocyanins on ABCA1 gene expression.

The increased cholesterol efflux occurred within 18–24 h of the cell being treated with anthocyanins. As it was well known that ABCA1 facilitates the initial step of cholesterol transfer from macrophage foam cells, we presumed that the possibility for this phenomenon of anthocyanins promoting cholesterol efflux may be attributed to transcriptional regulation of ABCA1 gene expression. The results showed that the highest expression of ABCA1 was obtained when the cells were treated with anthocyanins for 24 h, whereas higher induction of PPAR γ and LXR α expression and activity occurred 12 h after the cells were incubated with anthocyanins. The difference in gene expression among PPAR γ , LXR α , and ABCA1 suggested the anthocyanins may first activate the nuclear receptors as PPAR γ and LXR α and then in turn enhance the ABCA1 gene expression through the transcriptional pathway. This evidence supports that anthocyanin leads a gene transcriptional regulation in induction of ABCA1 expression and cholesterol efflux.

In summary, we provide evidence that anthocyanins increased ABCA1-mediated cholesterol efflux in macrophages, suggesting that anthocyanins rich in plant food may have therapeutic implications in the treatment of cardiovascular diseases by enhancing cellular cholesterol removal via apolipoproteins. The present study indicated that the mechanism by which anthocyanin promotes cholesterol efflux from macrophages is relevant to the regulation of PPAR γ -LXR α -ABCA1 activation. However, more work is needed to demonstrate whether anthocyanin directly or indirectly induced activation of the PPAR γ -LXR α -ABCA1 pathway.

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