

Anthocyanin supplementation improves HDL-associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia

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Context and Objective: Paraoxonase 1 (PON1), an enzyme associated with high-density lipoprotein (HDL-PON1), is reported to have antioxidant and cardioprotective properties. The aim of the present study was to investigate the effects of anthocyanins on the HDL-PON1 activity and cholesterol efflux capacity in hypercholesterolemic subjects.

Design and Participants: A total of 122 hypercholesterolemic subjects were given 160 mg of anthocyanins twice daily or placebo ($n = 61$ of each group) for 24 wk in a double-blind, randomized, placebo-controlled trial. Participants and investigators were masked to treatment allocation.

Results: Anthocyanin consumption significantly increased HDL cholesterol and decreased LDL cholesterol concentrations compared with placebo ($P < 0.018$ and $P < 0.001$, respectively). Anthocyanin supplementation also increased the activity of HDL-PON1 compared with placebo ($P < 0.001$). Furthermore, cholesterol efflux capacity was increased more in the anthocyanin group (20.0% increase) than in the placebo group (0.2% increase) ($P < 0.001$). The negative correlations established between HDL-PON1 activity and the levels of lipid hydroperoxides associated with HDL confirm the relationship between PON1 activity and lipid peroxidation of lipoproteins. Furthermore, a strong positive correlation was noted between increased HDL-PON1 activity and improved cholesterol efflux capacity both before and after adjustment for HDL cholesterol and apolipoprotein AI in anthocyanin-treated subjects (both $P < 0.001$). Inhibition of HDL-PON1 activity strongly prevented the antioxidant ability of HDL and attenuated the cholesterol efflux capacity of subjects from anthocyanin group.

Conclusions: Our observations suggest that the alterations of PON1 activity by anthocyanin observed in hypercholesterolemic HDL reflect a shift to an improvement of cholesterol efflux capacity of HDL and may provide a link between anthocyanin and cardioprotective effects.

The inverse association between low levels of high-density lipoprotein (HDL) cholesterol and an increased risk for cardiovascular disease has been well established through epidemiological and clinical studies (1–3). These findings have promoted intensive research seeking to target HDL metabolism for therapeutic intervention. However, emerging evidence suggests that raising HDL cholesterol plasma concentrations alone is insufficient and

indicates that the functionality of HDL rather than the mere quantity determines its potential beneficial effects against atherosclerosis (4–6). In addition to promoting macrophage-specific reverse cholesterol transport (RCT), HDL is also reported to promote systemic anti-inflammatory (7) and antioxidant effects (8), and protect endothelial cells function (9), which are thought to be one of the

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Abbreviations:

most important HDL-mediated cardioprotective mechanisms.

The HDL associated esterase/lactonase paraoxonase 1 (PON1) is a calcium-dependent esterase able to hydrolyze oxidized phospholipids and thus protect lipoproteins (LDL and HDL) and membranes from oxidative modifications (9–11). It has been suggested that subjects with lower PON1 activity may have a greater risk of developing diseases in which oxidative damage and lipid peroxidation are involved compared with subjects with higher PON1 activity (12). Moreover, previous studies have shown that the antioxidant properties of HDL and its susceptibility to atherogenic modifications, such as oxidation, glycation, and homocysteinylolation, are related to HDL-PON1 activity (13, 14). Intervention to increase serum PON1 activity enhanced HDL-mediated macrophage cholesterol efflux from arterial macrophage foam cells and hence contributed to the atherosclerosis regression (15).

Many studies have shown that anthocyanins, a category of phenolic flavonoids, are the largest group of water-soluble pigments responsible for the blue, purple, and red color of many plant foods, such as blueberries, cranberry, black raspberry, red raspberry, purple grapes, muscadine grape, black rice and red cabbage. Today, interest in anthocyanin pigments has intensified because of their possible health-promoting benefits as dietary antioxidants. Our previous study showed that anthocyanins exerted beneficial properties in vitro by the promotion of cholesterol efflux from lipid-loaded macrophage foam cells (16). This effect may attribute to the improvement in serum HDL cholesterol levels in animal models (17) and human individuals (18). However, the effect of anthocyanin on HDL function and cholesterol efflux capacity in vivo remain unknown. The aim of this study was to evaluate the improved antioxidant activity of HDL by anthocyanins and its relationship with the cholesterol efflux capacity in subjects with hypercholesterolemia.

Materials and Methods

Materials

Anthocyanin (also called Medox and placebo capsules were provided by Biolink Group (Sandnes, NORWAY). Each capsule of Medox contains 80 mg anthocyanin which was comprised of 17 different natural anthocyanins purified from the bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) (18). Anthocyanin capsules also contained pullulan, maltodextrin, and citric acid (which occupied for 4% per capsule and was helpful to maintain stability of anthocyanins), whereas the placebo capsules only contained pullulan and maltodextrin. The anthocyanin and placebo capsules were identically packaged. The dose of anthocyanins was referred to our previous human studies (18, 19).

Subjects and design

A total of 122 hypercholesterolemic subjects aged 40–65 years were recruited into this clinical trial between November 2008 and December 2010 from physical examination centers in 3 hospitals in Guangzhou, Guangdong, China. Women and men represented 58% and 42% of this cohort, respectively. Participants selected for inclusion in the study had a fasting total cholesterol concentration between 200 and 310 mg/dl (5.2 and 8.0 mM). Exclusion criteria included a history of cardiovascular disease, diabetes mellitus, hypertension, thyroid disorders, smoking, or the use of any drugs that could influence the measurement of lipid parameters or inflammatory markers. This study was approved by the ethics committee of Sun Yat-Sen University, and all participants gave their informed consent.

For the intervention study, eligible participants were randomized in a double-blind, placebo-controlled, parallel, 24-week trial and assigned to either the anthocyanin group ($n = 61$; 26 males and 35 females, 55.3 ± 5.0 years) or the placebo group ($n = 61$; 24 males and 37 females; 55.1 ± 5.4 years). During the trial period, the participants were instructed to consume two anthocyanin capsules or placebo capsules twice daily (30 minutes after breakfast and supper). The anthocyanin capsules provided a total daily intake of 320 mg of anthocyanins. They were also asked to maintain their habitual diet and lifestyle. Each of the participants attended a follow-up session every 4 wks. During these visits, their adherence to the protocol was assessed by collecting the empty packages and obtaining related information. Meanwhile, the capsules were dispensed, and the body weight, blood pressure (BP), and circumferences of the waist and hip of each participant were measured. Moreover, energy and macronutrient intakes of the participants during the intervention periods were assessed with 3 days weighed dietary records and with a 24 hours dietary recall. All subjects randomly assigned to the two intervention groups completed the study. According to the count of the recalled capsules at every visit, compliance was very well.

Laboratory Measurements

Fasting blood samples were obtained at baseline and at the end of the intervention period. Except for immediate lipoprotein determinations, serum and plasma samples were stored at -80°C and analyzed at the end of the study. Serum levels of total cholesterol, HDL cholesterol, and triglycerides were measured enzymatically using an automatic analyzer (Hitachi Co Ltd). LDL cholesterol was calculated according to the Friedewald formula: $\text{LDL cholesterol} = \text{total cholesterol} - (\text{triglycerides}/5 + \text{HDL cholesterol})$. Serum levels of apolipoprotein AI and apolipoprotein B were measured by enzyme-linked immunosorbent assays (Abcam). The intra-assay and interassay coefficient of variation for all of the measured biochemical parameters was $< 5\%$.

Isolation of plasma HDL

Blood samples were obtained after overnight fasting and plasma was prepared by centrifugation at 3000 rpm for 20 minutes and was thereafter used for the preparation of lipoproteins. HDL (density, 1.063–1.210 g/ml) was isolated by single vertical spin density gradient ultracentrifugation for 1.5 hours at 65,000 rpm (20) and dialyzed at 4°C for 24 hours against PBS (pH 7.4). The lipoproteins were used immediately within 24 hours or stored at -70°C after isolation. The protein concentration of the

HDL was determined by the method of Lowry et al (21), using serum albumin as the standard.

Measurement of the antioxidative capacity of HDL

The antioxidative activity of HDL was determined as previously described with minor modifications (22). Briefly, dihydrorhodamine (DHR) was suspended in dimethyl sulfoxide (DMSO) as a stock solution at the concentration of 50 mM, which was diluted in HEPES (20 mM HEPES, 150 mM NaCl, pH 7.4) containing 1 mM 2,2'-azobis-2-methylpropanimidamidedihydrochloride (AAPH) to a 50 μ M working reagent. In a 96-well plate, 15 μ g of HDL protein and 30 μ l of DHR working reagent were added, and the volume was diluted to 200 μ l with HEPES buffer. The increase in fluorescence due to the oxidation of DHR was measured every 2 minutes for 1 hour at 538 nm. The increase in fluorescence per minute was determined for samples containing only DHR and for samples containing DHR and individual HDL samples from placebo- or anthocyanin-treated subjects.

Measurement of PON1 activity on HDL

PON1 activity was assayed in HDL isolated from plasma of subjects from placebo and anthocyanin group by using UV spectrophotometry in a 96-well plate format using phenyl acetate or paraoxon as substrates (10, 23). Briefly, for the paraoxonase activity assays, the rate of generation of para-nitrophenol was determined at 405 nm in HDL (50 μ g) in reaction mixtures composed of 1.5 mM paraoxon, 10 mM Tris-HCl (pH 8.0), 1 M NaCl, and 2 mM CaCl_2 at 24°C. An extinction coefficient (at 405 nm) of 17 000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ was used for calculating units of paraoxonase activity, which are expressed as the amount of para-nitrophenol produced in nanomoles per minute per milliliter of HDL. Paraoxonase assays for each sample were performed in duplicate, and the average measurement of enzyme activity for each sample was calculated. Each 96-well plate included blank samples to monitor spontaneous hydrolysis of substrates. The intra-assay and interassay coefficients of variance for the performance of the paraoxonase activity assays were 2.0% and 5.6%, respectively.

Measurement of Lp-PLA2 activity on HDL

Peripheral blood samples were collected from patients after an overnight fast. Lp-PLA2 activity in apoB-depleted plasma after the sedimentation of all apoB-containing lipoproteins with dextran sulfate-magnesium chloride (HDL-Lp-PLA2 activity) was determined using the trichloroacetic acid precipitation procedure using 2-thio PAF (100 μ M final concentration) as a substrate (24). Total plasma (50 μ l) diluted 1:50 v/v with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) or the apoB-depleted plasma (diluted 1:3 v/v with 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) was used as a source of the enzyme. The reaction was performed for 10 minutes at 37°C, and Lp-PLA2 activity was expressed as nanomoles of PAF degraded per minute per milliliter of plasma. The intra-assay and interassay coefficients of variation of the Lp-PLA2 activity measurements in the apoB-depleted plasma were 5.7% and 6.6%, respectively.

Assessment of Cholesterol Efflux Capacity

The cholesterol efflux capacity of two groups at baseline and after intervention was quantified by a modified method (6). J774

mouse macrophages were plated and radiolabeled with 2 μ Ci of ^3H -cholesterol per milliliter. ATP binding cassette transporter A1 (ABCA1) was up-regulated by means of a 6 hours incubation with 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP. Subsequently, efflux mediums containing 2.8% apoB-depleted serum were added for 4 hours. All steps were performed in the presence of the acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor CP113818 (2 μ g/ml). Liquid scintillation counting was used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated by means of isopropanol extraction of the control wells not exposed to subjects serum. The percent efflux was calculated by the following formula: $[(\text{microcuries of } ^3\text{H}\text{-cholesterol in medium containing 2.8\% apoB-depleted serum} - \text{microcuries of } ^3\text{H}\text{-cholesterol in serum-free medium}) \div \text{microcuries of } ^3\text{H}\text{-cholesterol in cells extracted before the efflux step}] \times 100$. All assays were performed in duplicate. To correct for interassay variation across plates, a pooled serum control from five healthy volunteers was included on each plate, and values for serum samples from patients were normalized to this pooled value in subsequent analyses.

Statistical analysis

Continuous variables are presented as mean \pm SD, while non-normally distributed variables are presented as medians and interquartile ranges. We used Student *t* tests for independent samples to compare means for normally distributed variables. The Shapiro-Wilk test for normality was used to evaluate the *t* test assumption. Bivariate correlations were performed using Pearson correlation coefficients and linear regression analysis using beta regression coefficients with SE using SPSS 17.0. A *P* value < 0.05 was considered significant.

Results

Clinical, laboratory, and demographic characteristics of the dyslipidemic patients

Supplemental Table 1 summarizes the biochemical data obtained from subjects of placebo and anthocyanin group. The distribution of anthropometric features was uniform between the two groups. There were also no significant differences in daily mean energy and energy-producing nutrients intakes, or dietary habits (data not shown) between the placebo and anthocyanin group. No subjects reported any adverse events resulting from the consumption of either the placebo or anthocyanin capsules throughout the intervention study.

Effects of anthocyanin on serum lipid profile, glucose and hormone levels

Serum lipid and glucose concentrations at baseline and after the 24-week intervention are summarized in Supplemental Table 2. The mean serum concentrations of the lipids and glucose did not differ significantly between the two groups at the start of intervention. The serum HDL cholesterol concentration increased significantly more in

the anthocyanin group than in the placebo group [11.39% (95% CI: 9.64%, 13.13%) and -1.46% (95% CI: -3.19%, 0.27%), respectively; $P < .018$] after the 24-week intervention. Serum LDL cholesterol concentration was significantly decreased by 9.72% (95% CI: -13.80%, -5.63%) in the anthocyanin group and reduced by 2.18% (95% CI: -5.85%, 1.50%) in the placebo group by the end of the intervention. The change in HDL cholesterol and LDL cholesterol was significantly different between the two groups (both $P < .05$). However, no significant differences in total cholesterol, triglycerides, apoAI, apoB, glucose, insulin or adiponectin concentrations were observed between the two groups at baseline and after intervention.

Effects of anthocyanin on PON1 and Lp-PLA2 activity in HDL

Previous data suggested that HDL-PON1 and/or Lp-PLA2 contribute to the antioxidant activity of HDL (25, 26). We found that the HDL-PON1 activity values increased by 17.4% (95% CI: 6.7%, 14.1%) in the anthocyanin group and increased by 0.5% (95% CI: 23.6%, 10.5%) in the placebo group after intervention. The change in HDL-PON1 activity was significantly different between the two groups ($P < .001$). However, the Lp-PLA2 activity was not altered significantly at baseline or after intervention (Table 1). In addition, we observed that at baseline, the HDL from two groups inhibited DHR oxidation to a similar extent. After 24 weeks of intervention, the ability of HDL to inhibit DHR oxidation in anthocyanin-treated subjects was significantly enhanced by 20.8%; however, it was decreased by 2.3% in placebo-treated subjects (Table 1). We further measured the levels of lipid hydroperoxides associated with HDL isolated from placebo- and anthocyanin-treated patients. The lipid hydroperoxides in HDL did not differ between the two groups at baseline. After intervention, the lipid hydroperoxides levels decreased by 23.7% in the subjects from the

anthocyanin-treated group but increased by 2.5% in the subjects from the placebo-treated group (Table 1).

Effects of anthocyanin on cholesterol efflux capacity

At baseline, there were no differences in normalized cholesterol efflux capacity between the anthocyanin and placebo groups (0.68 ± 0.11 and 0.68 ± 0.10 , respectively). After intervention, cholesterol efflux capacity was significantly increased by 17.7% in the anthocyanin group ($P < .001$ compared to baseline) but slightly and insignificantly increased by 1.5% in the placebo group ($P = .148$ compared to baseline). The change in the cholesterol efflux capacity was significantly different between the two groups ($P < .001$, Table 2). These findings indicate clear differences between the control subjects and the anthocyanin subjects in the regulation of macrophage cholesterol efflux capacity.

Association between changes in PON1 activity and antioxidant activity of HDL

A negative correlation between the changes in HDL-PON1 activity and the alterations of lipid hydroperoxides levels associated with HDL (Figure 1A) and a positive association between HDL-PON1 changes and the variation of DHR oxidation ability of HDL (Figure 1C) were observed in subjects from the anthocyanin group. However, this association was not found in the placebo-treated subjects (Figure 1B and 1D). These results demonstrate that anthocyanin treatment improved HDL-PON1 activity and attenuated the oxidative damage of HDL in hypercholesterolemic subjects.

Association between changes in HDL levels or PON1 activity and cholesterol efflux capacity

To investigate the relationship between HDL levels and the cholesterol efflux capacity, we studied the correlation between the HDL levels and the cholesterol efflux capacity

Table 1. Anti-oxidative capacity of HDL at baseline and after the 24-week intervention

	Placebo group (n = 61)			Anthocyanin group (n = 61)			P
	Baseline	24 week	Change, % (95% CI)	Baseline	24 week	Change, % (95% CI)	
HDL-Lp-PLA2 activity, nm/min/ml	5.31 ± 0.53	5.29 ± 0.63	0.43 (-3.13, 3.98)	5.26 ± 0.60	5.40 ± 0.65	2.92 (0.67, 5.17)	0.239
HDL-PON1 paraoxonase activity, nm/min/ml	620.67 ± 96.02	601.22 ± 98.64	-1.75 (-6.32, 2.81)	627.43 ± 115.47	745.28 ± 134.49	22.25 (18.03, 26.47)	<0.001
DHR oxidation of HDL, fluorescence/min	3097.7 ± 474.9	3027.5 ± 474.6	-0.90 (-5.32, 3.53)	3136.6 ± 573.6	3788.3 ± 664.0	21.48 (18.60, 24.35)	<0.001
Lipid hydroperoxides in HDL, nm/100 µg	4.05 ± 0.44	3.94 ± 0.55	-2.71 (-3.45, -1.96)	4.01 ± 0.53	3.06 ± 0.55	-23.97 (-26.30, -21.65)	<0.001

Table 2. Effect of anthocyanin interventions on cholesterol efflux capacity

Group	Percent Change in Cholesterol Efflux Capacity (95% CI)	P	
		vs. Baseline	vs. Placebo
Placebo	0.68 (-0.16, 1.52)	0.148	—
Anthocyanin	16.60 (14.87, 18.33)	<0.001	<0.001

in placebo- and anthocyanin-treated subjects. Although both treatments were associated with small increases in the level of HDL cholesterol, a positive association was noted between a change in the HDL cholesterol concentration and a change in the cholesterol efflux capacity in the anthocyanin group ($r = 0.315$ and $P = .013$) but not in the placebo group ($r = 0.043$ and $P = .74$).

We further assessed that HDL-PON1 activity alterations are involved in the improvement of cholesterol efflux capacity. Statistical analysis showed that the change in HDL-PON1 activity strongly correlated with the improved cholesterol efflux capacity in the anthocyanin group ($r = 0.489$ and $P < .001$) but not in the placebo group ($r = 0.161$ and $P = .264$).

In the logistic-regression analysis adjusted for age, sex,

and traditional cardiovascular risk factors, we found that increased HDL-PON1 activity by anthocyanin was strongly associated with an enhanced cholesterol efflux capacity. This relationship remained robust even after the addition of HDL cholesterol as a covariate (Table 3).

Role of PON1 in HDL-mediated activation of cholesterol efflux

To further determine the functional relevance of PON1 activity for HDL-mediated cholesterol efflux, we examined the effects of PON1 inhibition by either hydroxyquinoline or EDTA (15, 27) on cholesterol efflux. Paraoxonase activity measurements revealed effective inhibition of PON1 activity after incubation of HDL with hydroxyquinoline ($81\% \pm 9\%$, $P < .01$) or EDTA (74%

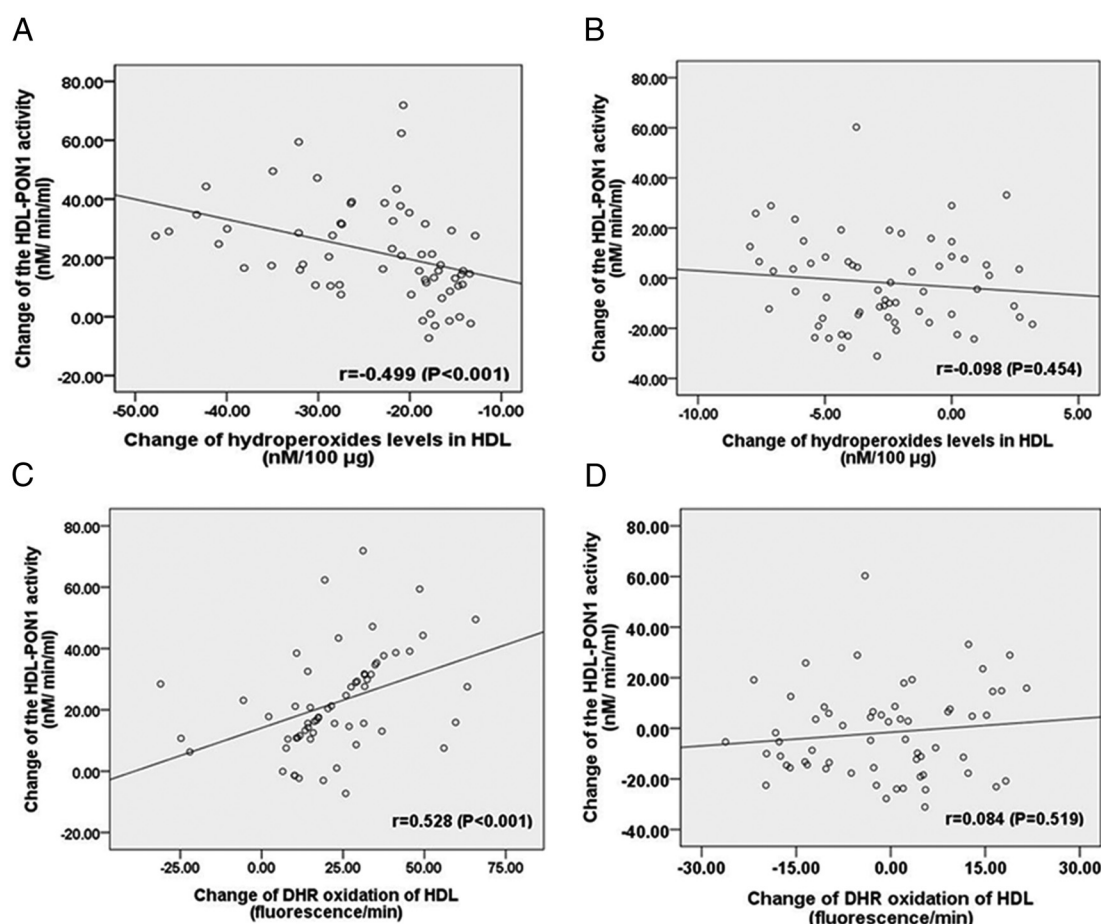


Figure 1. Correlation between changes in PON1 activity and antioxidant activity of HDL. Correlation between the changes of HDL-PON1 activity and hydroperoxides levels associated with HDL (A, B) and the variation of DHR oxidation ability of HDL (C, D). Pearson's correlation coefficients are noted for each plot. A, C: anthocyanin group; B, D: Placebo group.

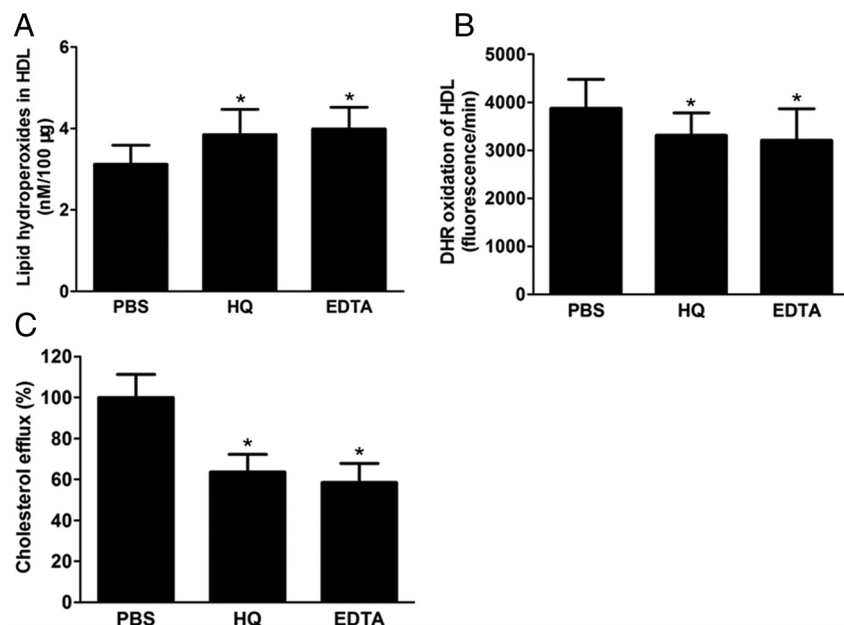


Figure 2. Effect of inhibition of HDL-associated PON1 on the improved HDL function in subjects from anthocyanin group. Human isolated HDL (100 μ g protein/ml) was incubated for 2 hours at 37°C with no addition (Control-HDL), with 200 μ M of the PON1 specific inhibitor 2-hydroxyquinoline (HQ), or with 5 mM Na₂EDTA (EDTA). The hydroperoxides levels on HDL (A), DHR oxidation ability of HDL (B) and HDL-mediated cholesterol efflux (C) were determined as indicated methods, respectively. * $P < .05$ vs. PBS. $n = 12$ of each group.

$\pm 11\%$, $P < .01$). Notably, inhibition of PON1 activity in HDL from anthocyanin-treated subjects by hydroxyquinoline and EDTA resulted in a loss of the antioxidant capacity of HDL (Figure 2A and 2B) and impaired the ability of HDL to stimulate cholesterol efflux from cholesterol-preloaded macrophages (Figure 2C), supporting the interpretation that HDL-associated PON1 determines the HDL capacity needed to stimulate cholesterol efflux effects by anthocyanin.

Discussion

Accumulating evidence has revealed that consuming anthocyanin-rich foods and beverages has vasoprotective effects in humans (28, 29). Oxidative stress is thought to play a pivotal role in the pathogenesis of a number of chronic inflammatory disease processes including atherosclerosis. In this study, we demonstrated that anthocyanin supplementation enhanced the HDL-associated protein PON1 activity, promoted pronounced antioxidant effects on HDL, and promoted the cholesterol efflux capacity in subjects with hypercholesterolemia. Furthermore, we confirmed that increased HDL-PON1 activity was strongly correlated with the improved antioxidant ability of HDL and the cholesterol efflux capacity in subjects with hypercholesterolemia. These results suggest that the modulation of PON1 activity is a potential novel mechanism of an-

thocyanin to regulate cholesterol efflux capacity (Figure 3).

HDL is well-known to protect against the development of atherosclerosis, and the primary function of HDL is the promotion of reverse cholesterol transport (RCT) (30, 31). Cholesterol efflux is the first and most critical step of RCT. Because HDL and apoA1 are major receptors of cholesterol in the cholesterol efflux pathway, elevated HDL cholesterol concentrations may facilitate this process. In recent years, increasing HDL cholesterol concentrations has been considered an important strategy for preventing and treating cardiovascular diseases (32). We found that anthocyanin treatment resulted in a dual beneficial effect in raising HDL-cholesterol and lowering LDL-cholesterol concentrations.

Currently, the static measurement of HDL cholesterol levels has inherent limitations as a metric of the functional effects of HDL in vivo (33). A substantial body of evidence suggests that cholesterol efflux capacity, an integrated measure of HDL quantity and quality, is reflective of the role of HDL in atheroprotection (34, 35). We previously reported that anthocyanin treatment induced cholesterol efflux from macrophage-derived foam cells in vitro (16) and in vivo (36). Furthermore, anthocyanin supplementation in humans increased the HDL cholesterol concentrations and enhanced cellular cholesterol efflux to serum ex vivo (29). However, the effect of anthocyanin on cholesterol efflux capacity was not evaluated. Because plasma concentrations of HDL do not predict RCT in mouse models (3), we further investigated whether increased HDL by anthocyanins contributed to the promotion of RCT using an established assay that integrates the pathways known to mediate cholesterol efflux from macrophages (ie, ABCA1, ABCG1, scavenger receptor B1, and aqueous diffusion). Notably, we observed that long-term intervention by anthocyanin elicited an improvement in the cholesterol efflux capacity.

Although the antiatherogenic activity of HDL is principally attributable to the reverse transport of cholesterol, the beneficial effects of anthocyanin on different disease models are also mostly dependent on its antioxidative, anti-inflammatory and antiapoptotic properties (37). It has been speculated that PON1 contributes to the atheroprotective properties of HDL via the promotion of anti-

Table 3. β coefficients for the association between HDL-PON1 activity and cholesterol efflux capacity

Linear-Regression Covariates*	β Coefficient per 1-SD Increase in Efflux Capacity (95% CI)	P Value
Age and sex	0.022 (0.014 to 0.029)	0.030
Age, sex, and cardiovascular risk factors	0.025 (0.017 to 0.033)	0.030
Age, sex, cardiovascular risk factors, and ApoA1	0.038 (0.021 to 0.041)	0.006
Age, sex, cardiovascular risk factors, and HDL-C	0.031 (0.019 to 0.037)	0.012

* Cardiovascular risk factors were systolic blood pressure, glucose, total cholesterol, triglycerides, LDL cholesterol.

oxidant effect (38); however, there has been no definitive in vivo evidence that anthocyanin promotes antioxidant effects in humans. We observed that HDL-PON1 activity was significantly enhanced in the anthocyanin group compared with the baseline and that this effect was not found in the placebo group. A significant negative correlation between the alteration of HDL-PON1 activity and the levels of lipid hydroperoxides of HDL and the positive association with DHR oxidation of HDL isolated from placebo and anthocyanin subjects demonstrate that anthocyanin intervention improves the antioxidant ability and limits the oxidative damage of HDL from hypercholesterolemic subjects. These results confirm the relationship between PON1 activity and its protective role against

lipoprotein peroxidation. While the mechanism(s) for anthocyanin on PON1-mediated antioxidant effects remains to be determined, the present findings strongly support a role for anthocyanin in modulating HDL-associated protein oxidative stress in humans. Moreover, the present study observed that PON1 activity was required for the stimulatory effect on HDL-mediated macrophage cholesterol efflux by anthocyanin, as addition of the PON1 specific inhibitor 2-hydroxyquinoline, or EDTA blocked PON1 paraoxonase activity, attenuated the antioxidant capacity of HDL and abolished the ability of HDL to stimulate cholesterol efflux from cholesterol accumulated macrophages, indicating a potential link between PON1 and cholesterol efflux capacity. A few studies revealed the mechanism of linkage between PON1 and macrophage cholesterol efflux. HDL-associated PON1 may contribute to the macrophage cholesterol efflux by its ability to act on macrophage phospholipids, to form lysophosphatidylcholine, in turn, stimulates HDL binding to the cells (39). The increased HDL binding to macrophages induced by PON1 was not associated with upregulation of scavenger receptor BI (SR-BI) or ABCA1 expression (15).

One limitation of this study is that although our assessment of cholesterol efflux capacity reflects the ability to mobilize free cholesterol from macrophages in vivo, it does not capture variation in the RCT pathway in terms of cellular components (ie, the hydrolysis of cholesteryl esters and the status of endogenous macrophage cholesterol transporters) or terminal components (ie, uptake into the liver and biliary excretion) (40).

In conclusion, our results suggest that anthocyanin supplementation in dyslipidemic patients has a beneficial effect on the lipoprotein profile, which includes a decrease in LDL-cholesterol and an increase in HDL-cholesterol concentrations. These beneficial effects may be partially explained by the improvement of cholesterol efflux capacity, a key metric of HDL function, via enhancing HDL-PON1 activity. These findings reinforce the concept that assessment of HDL function rather than HDL level may prove informative in refining our understanding of anthocyanin-mediated atheroprotection.

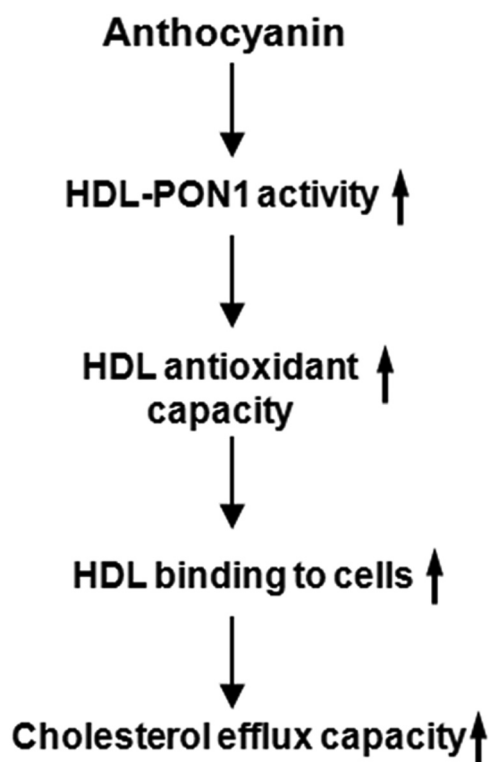


Figure 3. Anthocyanin regulation of HDL-PON1. The diagram shows the regulatory circuitry of the responses of HDL-PON1 to atheroprotective anthocyanin. Anthocyanin with a forward direction regulates the activation of PON1 activity through unknown mechanism. Serving as a transcription factor, PON1 prevents HDL from oxidative damage which in turn maintains HDL-mediated cholesterol efflux.

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