Coffee Consumption Enhances High-Density Lipoprotein–Mediated Cholesterol Efflux in Macrophages

Harumi Uto-Kondo, Makoto Ayaori, Masatsune Ogura, Kazuhiro Nakaya, Mai Ito, Atsushi Suzuki, Shun-ichi Takiguchi, Emi Yakushiji, Yoshio Terao, Hideki Ozasa, Tetsuya Hisada, Makoto Sasaki, Fumitaka Ohsuzu, Katsunori Ikewaki

Rationale: Association of habitual coffee consumption with coronary heart disease morbidity and mortality has not been established. We hypothesized that coffee may enhance reverse cholesterol transport (RCT) as the antiatherogenic properties of high-density lipoprotein (HDL).

Objective: This study was to investigate whether the phenolic acids of coffee and coffee regulates RCT from macrophages in vitro, ex vivo and in vivo.

Methods and Results: Caffeic acid and ferulic acid, the major phenolic acids of coffee, enhanced cholesterol efflux from THP-1 macrophages mediated by HDL, but not apoA-I. Furthermore, these phenolic acids increased both the mRNA and protein levels of ATP-binding cassette transporter (ABC)G1 and scavenger receptor class B type I (SR-BI), but not ABCA1. Eight healthy volunteers were recruited for the ex vivo study, and blood samples were taken before and 30 minutes after consumption of coffee or water in a crossover study. The mRNA as well as protein levels of ABCG1, SR-BI, and cholesterol efflux by HDL were increased in the macrophages differentiated under autologous sera obtained after coffee consumption compared to baseline sera. Finally, effects of coffee and phenolic acid on in vivo RCT were assessed by intraperitoneally injecting [3H]cholesterol-labeled acetyl low-density lipoprotein–loaded RAW264.7 cells into mice, then monitoring appearance of [3H]tracer in plasma, liver, and feces. Supporting in vitro and ex vivo data, ferulic acid was found to significantly increase the levels of [3H]tracer in feces.

Conclusions: Coffee intake might have an antiatherogenic property by increasing ABCG1 and SR-BI expression and enhancing HDL-mediated cholesterol efflux from the macrophages via its plasma phenolic acids. (Circ Res. 2010;106:779-787.)

Key Words: HDL cholesterol ■ efflux ■ coffee ■ phenolic acid

High-density lipoproteins (HDLs) have been shown to be inversely associated with the risk of atherosclerotic cardiovascular disease (CVD) and thus considered as antiatherogenic lipoproteins. Among antiatherogenic mechanisms including beneficial effects on inflammation, impaired endothelial function or hypercoagulation, HDL exerts the antiatherogenic property primarily by facilitating the efflux of cholesterol from peripheral tissues and transport it back to the liver in a process called reverse cholesterol transport (RCT). A major breakthrough in the understanding of the mechanism of RCT came with the discovery of the ATP binding cassette transporter (ABC)A1. Lipid-poor apolipoprotein (apo)A-I contributes to ABCA1-mediated cholesterol efflux from cells, but not HDL. Another ABC transporter, ABCG1, is also involved in the cholesterol efflux from macrophages mediated by HDL, but not apoA-I. Scavenger receptor class B type I (SR-BI) is also known to promote HDL-mediated cellular cholesterol efflux. Furthermore, the pivotal roles of these molecules on intracellular cholesterol homeostasis, associated with reverse cholesterol transport, have also been confirmed by animal studies where deletions of ABCA1, ABCG1, and SR-BI in macrophages accelerated the development of atherosclerosis.

Coffee is among the most widely consumed beverages worldwide. The relationship between coffee consumption and the incidence of CVD has been studied extensively. In addition, a recent study reported that regular coffee consumption might be associated with a decreased cardiovascular mortality rate. Coffee beans are known to be abundant in antioxidant phenolic acids, chlorogenic acid. Indeed, Xia M et al also reported that anthocyanins enhance cholesterol efflux from macrophages by increasing ABCA1 expression.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.109.206615

779
via peroxisome proliferator-activated receptor γ and LXRα pathways.14 Resveratrol regulates the expression of LXR-alpha in human macrophages.15

Chlorogenic acid is rapidly metabolized to caffeic acid or ferulic acids after coffee consumption in rats and humans.16,17 Although caffeic acids inhibit the development of atherosclerosis in mice by attenuating inflammation,18 it remains unclear whether the phenolic acids of coffee affect cholesterol efflux from macrophages.

Therefore, the present study was undertaken to investigate the effects of coffee intake or the phenolic acids of coffee on HDL-mediated cholesterol efflux from the macrophages and ABCA1, ABCG1, and SR-BI expression. The results show for the first time that the elevated plasma phenolic acids after coffee consumption may be attributable to the enhanced ABCG1 and SR-BI expression and HDL-mediated cholesterol efflux from the macrophages in vitro and ex vivo and macrophage RCT in vivo.

Methods

Materials

Caffeic acid, ferulic acid, actinomycin D, and human apoA-I were purchased from Sigma (St Louis, Mo). Phorbol 12-myristate 13-acetate (PMA) was from Wako Pure Chemical (Tokyo, Japan). Human apoA-I was purchased from Sigma. HDL was isolated by sequential ultracentrifugation, and acetylated LDL was prepared according to the methods as previously reported.19

Cell Culture

THP-1 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 (Sigma) containing 10% FBS. The differentiation of THP-1 monocytes into macrophages was induced in the presence of 320 mmol/L PMA (Wako, Tokyo, Japan) for 72 hours.

Determination of Cholesterol Efflux

Cholesterol efflux experiments were performed as previously described.9,20 The cells were labeled with [3H]cholesterol (1.0 μCi/mL) in media containing 0.1% BSA for 24 hours. The cells were washed with PBS and incubated in RPMI 1640 containing 0.1% BSA in the presence and absence of apoA-I (10 μg/mL) or HDL (50 μg/mL) for 24 hours. The percentage cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the cells.

Western Blot Analyses

The cells were harvested and protein extracts prepared as previously described.19,20 They were then subjected to Western blot analyses (10% SDS-PAGE; 30 μg protein per lane) using rabbit anti-ABCG1–specific (Novus Biologicals, Littleton, Colo), SR-BI–specific (Novus Biologicals), and β-actin–specific (Santa Cruz Biotechnology) antibodies. The proteins were visualized and quantified using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare UK Ltd) and the NIH image analysis software program.

Real-Time Quantitative RT-PCR

At the indicated hours after treatment with the compounds, total RNA was extracted from the cells, and first-strand cDNA was synthesized from the total RNA (250 ng) by placing in a Reverse Transcription Reagent (Applied Biosystems, Foster City, Calif). Quantitative PCR was performed using an ABI 7900 PCR machine, TaqMan PCR master mix, and FAM-labeled TaqMan probes (Assays-on-Demand, Applied Biosystems) for human ABCA1, ABCG1, SR-BI, and 18S ribosomal RNA. The expression data were normalized for 18S levels.

Construction of Human ABCG1 and SR-BI 3’ Untranslated Region Constructs

Segments of the human ABCG1 and SR-BI 3’ untranslated region (UTR) were PCR-amplified using oligonucleotide primers containing flanking SpeI (for ABCG1) and XbaI (for SR-BI) recognition sequences and human genomic DNA as a template. The PCR products were gel-purified and ligated downstream of the firefly luciferase coding region of the pGL3-Control vector (Promega, Madison, Wis). The pGL3-Control vector was chosen because it contains the SV40 promoter without enhancers; therefore, changes in luciferase activity can be attributed to the effect of 3’-UTR insertions. The following primers were used to amplify the 3’-UTR of human ABCG1: forward, 5’-GACTAGTGCTAATAATAATCGGCCAGAAGT-3’; reverse, 5’-GACTAGTCTATAAAATTAGAAAGACGTGGA-3’. SR-BI: forward, 5’-GCTCTAGGGTCTGAGGAGAACAA3’; reverse, 5’-GCTCTAGATCCCATTTTTTTAAACG-3’. All constructs were confirmed by direct sequencing.

DNA Transfection and Luciferase Assays

RAW264.7 cells cultured in 24-well plates were transfected with 450 ng of luciferase reporter plasmids and 50 ng of phRL-TK (Promega) per well using Lipofectamine LTX and plus reagent (Invitrogen, Carlsbad, Calif), according to the instructions of the manufacturer. HEK293 cells were also transfected with 190 ng of luciferase reporter plasmids and 10 ng of phRL-TK per well using FuGene 6 transfection reagent (Roche, Indianapolis, IN). At 10 hours after transfection, the media was replaced with DMEM containing the indicated amount of coffee polyphenols or vehicle and incubated for an additional 24 hours. Luciferase assay was performed as previously described.19,20

Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Analysis of the Phenolic Acids of Coffee in Plasma

The apparatus and analytic conditions were adopted from a previous report.21 Preparation procedures were performed as follows. An aliquot of 100 μL of plasma was placed in 1.5-mL sample tubes, and 70 μL 0.1 mol/L sodium acetate (pH 5.0) and 70 μL 0.05 mol/L perchloric acid were added. The mixtures were vigorously mixed for 30 seconds with a vortex mixer and combined with 25 μL of 6 mol/L acetic acid. The mixtures were vigorously mixed for 30 seconds with a vortex mixer. Each mixture was kept at 5°C for 30 minutes.
Next, 125 µL of acetonitrile was added and the mixtures were vigorously mixed for 2 minutes with a vortex mixer. Each mixture was kept at 5°C for 30 minutes, and 85 µL water was added. The mixtures were centrifuged at 20,000g for 5 minutes at 5°C in a centrifuge (Eppendorf 5417R, Hamburg, Germany). Each supernatant was combined with 15 µL 3 mol/L potassium carbonate solution and mixed moderately for 30 seconds and then kept at 5°C for 30 minutes. Insoluble potassium perchlorate was precipitated by centrifugation at 20,000g for 5 minutes at 5°C. The resulting supernatants were then collected and subjected to liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS).

Human Study
Eight healthy volunteers (6 males), 31 to 48 years of age, who were moderate coffee drinkers (1 to 2 cups/day) were recruited to participate in a crossover study where coffee was compared with water. Subjects were instructed to maintain their usual habits except that coffee drinking was prohibited for 3 days before the experiment. After an overnight (12 hours) fast, the study subjects consumed 350 mL of freshly prepared 10 g of instant coffee or water within 5 minutes of brewing. Venous blood samples were taken at time 0 and 30 minutes after the consumption. Timing of the postconsumption blood sampling was determined by the pharmacokinetic data that concentration of caffeic and ferulic acid in circulation peak at 0.5 to 1 hour after oral administration of chlorogenic acid in rats or humans.16,22 All subjects provided their written informed consent. The study was approved by the Ethical Committee of the National Defense Medical College.

Human Monocyte Isolation and Macrophage Differentiation
Human monocytes were isolated as previously described.19,20 The remaining blood was drawn into heparinized blood collection tubes. Human peripheral blood monocytes were isolated using the method of Fogelman et al21, with Ficoll/Hypaque gradient centrifugation. The mononuclear cells were resuspended in RPMI 1640 (Sigma) supplemented with 20% autologous serum, plated on to serum-coated 10-cm dishes and incubated for 2 hours. Nonadherent cells were removed by washing 3 times with PBS, and adherent cells were then detached by incubation in PBS containing 10% autologous serum and 0.02% EDTA at 4°C for 30 minutes. The adherent cells were then washed extensively and resuspended in RPMI 1640 supplemented with 10% autologous serum. They were then plated on 12-well plates and incubated for 10 days so that they would differentiate into macrophages.

In Vivo Macrophage RCT Studies
We divided 7-week-old male C57BL/6 mice (Clea Japan, Tokyo, Japan) into 3 groups (n=6 each): vehicle, ferulic acid (as a representative of phenolic acids), and coffee groups. Ferulic acid was chosen based on a greater HDL-mediated cholesterol efflux capacity23 at lower concentration compared to caffeic acid (Figure 1A and 1B). Ferulic acid (5 mg/kg per day) or the vehicle were added to the cultures after [3H]cholesterol labeling, which were then incubated in the presence of 50 µg/mL of HDL (A and B) or 10 µg/mL of human apoA-I (C and D) for 24 hours. Determination of cholesterol efflux from the cells was performed as described in Methods. The results are expressed as the means±SEM from 6 replicate wells. *P<0.05 vs vehicle. Results are representative of 3 or more experiments. Data were analyzed by 1-way ANOVA, Bonferroni–Dunn post hoc test.

Liver, Bile, and Fecal Analyses
Liver lipids were extracted as described previously.26 Briefly, a 50-mg piece of tissue was homogenized in water, and lipids were extracted with a 2:1 (vol/vol) mixture of chloroform/methanol. The lipid layer was collected, evaporated, resuspended in a 3:2 (vol/vol) mixture of hexane/isopropanol, and counted in an LSC. The bile was directly counted in an LSC. The total feces collected from 0 to 48 hours were weighed and soaked in Millipore water (1 mL of water per 100 mg of feces) overnight at 4°C. An equal volume of ethanol was added on the next day, and the samples were homogenized; 200 µL of the homogenized samples was counted in an LSC. Results were expressed as percentage of cpm injected.

Statistical Analysis
Statistical analyses were performed using the Stat View Version 5.0 software package (SAS Institute Inc). Paired t test was used for paired sample (human study) and one-way ANOVA, followed by post hoc analysis using Bonferroni–Dunn test for cell culture and animal studies, with a value of P<0.05 considered to be significant. All results are expressed as the means±SEM.

Results
Caffeic and Ferulic Acid Enhanced HDL-Mediated Cholesterol Efflux in Macrophages
The effect of the phenolic acids of coffee on cholesterol efflux from THP-1 macrophages mediated by HDL or apoA-I was first investigated. As shown in Figure 1A and 1B, both caffeic acid and ferulic acid dose-dependently enhanced HDL-mediated cholesterol efflux from THP-1 macrophages up to 1.4±0.2-fold (P<0.01) and 1.4±0.3-fold (P<0.01), respectively. In contrast, apoA-I-mediated cholesterol efflux was unchanged (Figure 1C and 1D). We further confirmed that both caffeic acid and ferulic acid significantly enhanced HDL-mediated efflux by 1.3±0.1-fold (P<0.01) and 1.3±0.3-fold (P<0.01), respectively, but not apoA-I.
mediated efflux in human monocyte-derived macrophages (MDMs) (Online Figure I, A and B [Online Data Supplement, http://circres.ahajournals.org]). Given the heterogeneous nature of HDL, the HDL subtraction that contributed to the enhanced HDL-mediated efflux was determined. Both HDL2 and HDL3 were found to promote cholesterol efflux in the presence of caffeic acid (HDL2: 1.3±0.2-fold, \(P<0.01\); HDL3: 1.1±0.2-fold, \(P<0.05\)) or ferulic acid (HDL2: 1.7±0.1-fold, \(P<0.01\); HDL3: 1.2±0.2-fold, \(P<0.01\)) (Online Figure II, A through D).

**Caffeic and Ferulic Acid Increased ABCG1 and SR-BI Expression in Macrophages**

The expression of ABCA1, ABCG1, and SR-BI mRNA was determined to elucidate molecular mechanism underlying the enhanced cholesterol efflux by phenolic acids. As shown in Figure 2A and 2B, both caffeic acid and ferulic acid (\(=1\) mM) increased ABCG1 mRNA levels up to 1.7±0.5-fold (\(P<0.05\)) and 2.4±0.4-fold (\(P<0.05\)), respectively, in THP-1 macrophages. Similar dose-dependent increases were also evident in SR-BI mRNA levels (1 mM of caffeic acid: 3.2±1.2-fold; \(P<0.01\), ferulic acid: 2.8±0.9-fold; \(P<0.01\)) (Figure 2C and 2D). In contrast, the phenolic acids did not affect ABCA1 expression in THP-1 macrophages (Figure 2E and 2F), thus supporting the observation of unchanged apoA-I-mediated cholesterol efflux. Mirroring the increased mRNA levels, both caffeic and ferulic acid increased the protein levels of ABCG1 (1 mM/mL of caffeic acid: 2.9±0.8-fold, \(P<0.01\); ferulic acid: 2.9±0.9-fold, \(P<0.01\)) and SR-BI (1 mM/mL of caffeic acid: 3.3±0.6-fold, \(P<0.01\); ferulic acid: 3.5±0.9-fold, \(P<0.01\)) in a dose-dependent manner (Figure 2G and 2H) except a modest increase in the ABCG1 protein level at 1 mM/mL of caffeic acid. Similar stimulatory effects of these phenolic acids were observed on ABCG1 and SR-BI mRNA levels in human MDMs (Online Figure I, C and D). To further explore the mechanisms by which the phenolic acids increased ABCG1 and SR-BI mRNA levels in the macrophages, we investigated whether they transcriptionally induced these genes using actinomycin D. As shown in Online Figure III (A and B), treatment with actinomycin D did not abolish the inducible effects of the phenolic acids on ABCG1 and SR-BI expression in THP-1 macrophages. Further, ABCG1 and SR-BI mRNA decay induced by actinomycin D were inhibited in the presence of the phenolic acids (Online Figure III, C and D), thus indicating they enhanced mRNA stability of ABCG1 (1.5±0.1-fold) and SR-BI (1.5±0.1-fold). To further explore the stabilization mechanisms, we investigated whether phenolic acids modulates 3′-UTRs, region known to destabilize mRNA. As shown in Online Figure IV (A and B), the presence of both ABCG1 and SR-BI 3′-UTR attenuated the baseline luciferase activities compared with empty vectors, implying that these regions indeed destabilize mRNA of these genes. Coffee phenolic acids did not restore the attenuated luciferase activity induced by the presence of 3′-UTR, indicating that these regions of human ABCG1 and SR-BI were not involved in mRNA stabilization induced by phenolic acids. We also obtained similar results by using HEK293 cells in case of RAW cells (data not shown).

**Coffee Consumption Resulted in Increased Levels of Phenolic Acids in Human Serum**

Next, the question of whether these in vitro observations above can translate into human physiology was addressed. Ex vivo experiments were performed using healthy volunteers to assess the effects of phenolic acids on cholesterol efflux. As shown in Figure 3A, serum concentrations of both phenolic acids and their metabolites are either not detectable or very low at baseline. The consumption of coffee resulted in marked increases in blood phenolic acids/metabolites levels in all subjects (9.4±1.2-fold, \(P<0.01\)) (Figure 3A). A detailed analysis revealed that ferulic acid accounted for 63±8% of the total increase in phenolic acids/metabolites, followed by caffeic acid (22±3%)>the ferulic acid conjugated form (14±4%)>the caffeic acid conjugated form (1±1%). Interestingly, the average ferulic acid level after coffee consumption (0.22 ±μmol/L) is comparable to the dose that promoted HDL-mediated cholesterol efflux from THP-1 macrophages (Figure 1B). As expected, water consumption did not cause any changes.
HDL-Mediated Cholesterol Efflux Is Enhanced in Macrophages in the Presence of Serum Obtained After Coffee Consumption by Increasing ABCG1 and SR-BI Expression

Next, an HDL-mediated cholesterol efflux assay was conducted using human MDMs cultured in media containing sera obtained before and after coffee consumption. Monocytes and sera were isolated before and 0.5 hour after consumption of coffee or water from 8 subjects. The monocytes were cultured and differentiated into macrophages (MDMs) under pooled sera obtained before (pre) and after (post) coffee or water as described in Methods. The results are expressed as the means±SEM. *P<0.05 vs precoffee plasma. B, Monocytes and sera were isolated before and 0.5 hour after consumption of coffee or water from 4 subjects. The monocytes were cultured and differentiated into macrophages (MDMs) under pooled sera obtained before (pre) and after (post) coffee or water consumption as described in Methods. After 3Hcholesterol labeling, MDMs were incubated in the presence of 50 μg/mL of HDL for 24 hours. Determination of cholesterol efflux from the cells was performed as described in Methods. The results are expressed as the means±SEM from 4 to 6 replicate wells. *P<0.05 vs precoffee cells and sera. Results are representative of 3 or more experiments. Data were analyzed using paired t test.

Individual changes in ABCG1 and SR-BI mRNA levels in the MDMs differentiated using autologous sera obtained before and after coffee or water consumption are shown in Figure 4. When consumed coffee, the ABCG1 and SR-BI mRNA levels were increased in most study subjects, resulting in significant 1.7±0.4-fold (P<0.05) and 3.9±0.7-fold (P<0.01) increases by postcoffee MDMs treated with postcoffee sera in comparison to precoffee cells and sera combinations. As expected, water did not affect the expression of the MDM genes. Parallel increase in ABCG1 and SR-BI protein levels were confirmed as shown in Figure 5. ABCG1 and SR-BI protein levels in postcoffee MDMs cultured under postcoffee sera were significantly increased up to 2.3±0.4-fold (P<0.05) and 2.5±0.6-fold (P<0.05) in comparison to precoffee MDMs and sera in 3 representative subjects (Figure 5A). Again, there was no difference between pre- and postwater cells and sera (Figure 5B).

Sera, Rather Than Cells, Contribute to Enhanced HDL-Mediated Cholesterol Efflux by Coffee Consumption

The next question was which factor, namely the sera or the cells, was essential for the enhanced HDL-mediated cholesterol efflux following coffee consumption. Based on a cholesterol efflux assay using THP-1 macrophages incubated with either pre- or postcoffee sera, treatment with postcoffee sera pooled from 4 subjects indeed promoted HDL-mediated cholesterol efflux by 1.2±0.3-fold (P<0.05) in THP-1 macrophages in comparison to precoffee sera (Figure 6A). Likewise, both ABCG1 and SR-BI mRNA levels in THP-1 macrophages were significantly increased by the treatment with individual postcoffee sera in comparison to the precoffee sera (ABCG1: 1.5±0.4-fold, P<0.05; and SR-BI: 1.5±0.2-fold, P<0.05) (Figure 6B and 6C). In contrast, no differences were observed in the ABCG1 or SR-BI mRNA levels between in pre- and post-MDMs cultured under precoffee...
sera (Figure 6D and 6E). To further assess the possibility that HDL in the sera may account for the enhanced cholesterol efflux, cholesterol efflux assays were conducted using HDL isolated from before- and after-coffee consumption sera in THP-1 macrophages. Both HDL isolated from before- and after-coffee consumption sera, however, yielded similar ABCG1 and SR-BI mRNA levels (Online Figure V). Overall, these results strongly indicate that the coffee derived compounds, most likely phenolic acids, rather than cells or HDL, induced ABCG1 and SR-BI expression, thus promoting the HDL-mediated cholesterol efflux.

**Ferulic Acid Promotes Macrophage RCT In Vivo**

Finally, we performed an in vivo RCT assay to assess whether in vitro observations above can extend to in vivo circumstances in mice. As shown in Figure 7A through 7C, there were no differences in $^3$H tracer levels in plasma, liver, or bile among 3 groups. In contrast, coffee and ferulic acid administration resulted in enhanced fecal excretion of the $^3$H tracer as compared to the control group by $14 \pm 1.2\%$ and $37 \pm 1.4\%$ ($P<0.05$), respectively (Figure 7D). These results indicate that the increased initial step of RCT (cholesterol efflux) indeed translated into the increased overall RCT in mice in vivo.

**Discussion**

Plasma concentrations of HDL-cholesterol have strong inverse correlations with risk of atherosclerotic cardiovascular disease. Although the mechanism by which HDL may exert a direct protective effect against development of atherosclerosis is not yet well understood, HDL has been postulated to facilitate the efflux of cholesterol from peripheral tissues and transport it back to the liver in a process called RCT. Recently, several key molecules have been identified to play pivotal roles on RCT. $^3$ ABCA1 facilitates cholesterol efflux...
Coffee is one of the most popular habitual beverages in the world and is the major source of dietary antioxidants because coffee contains phenolic acids with antioxidant properties. The Zutphen Elderly Study demonstrated that a flavonoid-rich (another phenolic acid derivatives) diet reduced the rate of atherosclerosis in vivo. This led to the hypothesis that coffee exerts its antiatherosclerotic effects via antioxidative and/or antiinflammatory functions. Indeed, several studies reported that coffee intake is inversely associated with inflammatory biomarkers. Although numerous studies reported the association between coffee consumption and CVD, the information remains inconsistent. Some authors have reported a positive association between coffee intake and coronary heart disease, whereas others have reported J-shaped and no relationship. Moreover, the increase in coronary heart disease risk may be the result of elevated blood pressure and cholesterol levels attributable to unhealthy dietary habits or increased smoking habits associated with coffee consumption. It seems that an independent association between coffee consumption and the risk of developing coronary heart disease has not yet been established. Thus, investigations of “the role of coffee consumption on CVD incidence” have been very scarce. The present study therefore sheds a new light into the potential antiatherogenic properties of coffee in additional to antioxidative/antiinflammatory functions. Interestingly, the phenolic acids of coffee selectively induced ABCG1 and SR-BI, but not ABCA1, expressions. Although we are not certain of the cause for this discriminative function of phenolic acids, it appears to be unlikely that phenolic acids activate liver X receptor-alpha (LXRα) expression, because LXRs, in turn, transactivates both ABCA1 and ABCG1. Indeed, we showed that caffeic and ferulic acids enhanced ABCG1 and SR-BI mRNA stability (Online Figure III, C and D), thus resulting in increased steady-state expression of these genes in the macrophages. Based on recent evidences, 3’-UTRs play an important role in mRNA degradation by interacting with microRNA. We therefore performed a reporter gene assay using the luciferase constructs containing human ABCG1 and SR-BI 3’-UTRs to assess a possibility whether phenolic acids affect these regions. As shown in Online Figure IV (A and B), coffee phenolic acids did not restore the attenuated luciferase activity induced by the presence of 3’-UTR, indicating that these regions of human ABCG1 and SR-BI were not involved in mRNA stabilization of these genes. Further investigations are therefore needed to explore the precise mechanisms for these effects of phenolic acids.

Coffee also contains caffeine which may exert acute neurohumoral effects by increasing catecholamine, and cardiac output. Caffeine, a potent inhibitor of insulin activity, and acute increase in blood pressure and homocysteine levels. Thus, these mechanisms also support our finding of an inverse association between coffee and all-cause mor-

Figure 7. Ferulic acid promotes macrophage RCT in mice. Ferulic acid (5 mg/kg per day) or the vehicle was given by oral gavage; these 2 groups were fed with water. The vehicle was also given to coffee group, which was fed with cooled coffee liquid (10 mg/mL, instant coffee prepared with hot water) ad libitum. Seven days after administration. One week after the initial administration, [3H]cholesterol-labeled RAW264.7 cells were injected intraperitoneally (5 × 10⁶ cells containing 7.5 × 10⁶ cpm in 0.5 mL medium), A, Blood samples obtained from mice 24 and 48 hours after injection were subjected to 3H tracer analysis. B through D, Forty-eight hours after injection, mice were euthanized, and liver (B) and bile (C) were isolated, prepared as described in Methods, and then subjected to 3H tracer analysis. Feces (D) collected continuously from 0 to 48 hours were prepared as described in Methods and were then subjected to 3H counting. Data are expressed as percent counts relative to total injected tracer (means±SEM; n=6 for each group). *P<0.05 vs vehicle. Data were analyzed by 1-way ANOVA, Bonferroni–Dunn post hoc test.

from cells to lipid-poor apoA-1, but not HDL₃, whereas another ABC transporter, ABCG1, and SR-BI are involved in the cholesterol efflux from macrophages mediated by HDL₃, but not apoA-I. One human study revealed that single oral administration of coffee raised plasma levels of phenolic acids and their metabolites and, again, induced ABCG1 and SR-BI expression, thus leading to the enhanced cholesterol efflux. Additionally, in line to the in vitro finding, ferulic acid was found to promote overall RCT in vivo in mice. These comparable results obtained from in vitro, ex vivo, and in vivo studies, therefore, indicate that the potential antiatherogenic properties of coffee proposed from previous studies could be explained, at least in part, by the upregulated cholesterol efflux from macrophages mediated through ABCG1 and SR-BI pathways.

Coffee is one of the most popular habitual beverages in the world and is the major source of dietary antioxidants because coffee contains phenolic acids with antioxidant properties. The Zutphen Elderly Study demonstrated that a flavonoid-rich (another phenolic acid derivatives) diet reduced the rate of atherosclerosis in vivo. This led to the hypothesis that coffee exerts its antiatherosclerotic effects via antioxidative and/or antiinflammatory functions. Indeed, several studies reported that coffee intake is inversely associated with inflammatory biomarkers. Although numerous studies reported the association between coffee consumption and CVD, the information remains inconsistent. Some authors have reported a positive association between coffee intake and coronary heart disease, whereas others have reported J-shaped and no relationship. Moreover, the increase in coronary heart disease risk may be the result of elevated blood pressure and cholesterol levels attributable to unhealthy dietary habits or increased smoking habits associated with coffee consumption. It seems that an independent association between coffee consumption and the risk of developing coronary heart disease has not yet been established. Thus, investigations of “the role of coffee consumption on CVD incidence” have been very scarce. The present study therefore sheds a new light into the potential antiatherogenic properties of coffee in additional to antioxidative/antiinflammatory functions. Interestingly, the phenolic acids of coffee selectively induced ABCG1 and SR-BI, but not ABCA1, expressions. Although we are not certain of the cause for this discriminative function of phenolic acids, it appears to be unlikely that phenolic acids activate liver X receptor-alpha (LXRα) expression, because LXRs, in turn, transactivates both ABCA1 and ABCG1. Indeed, we showed that caffeic and ferulic acids enhanced ABCG1 and SR-BI mRNA stability (Online Figure III, C and D), thus resulting in increased steady-state expression of these genes in the macrophages. Based on recent evidences, 3’-UTRs play an important role in mRNA degradation by interacting with microRNA. We therefore performed a reporter gene assay using the luciferase constructs containing human ABCG1 and SR-BI 3’-UTRs to assess a possibility whether phenolic acids affect these regions. As shown in Online Figure IV (A and B), coffee phenolic acids did not restore the attenuated luciferase activity induced by the presence of 3’-UTR, indicating that these regions of human ABCG1 and SR-BI were not involved in mRNA stabilization of these genes. Further investigations are therefore needed to explore the precise mechanisms for these effects of phenolic acids.

Coffee also contains caffeine which may exert acute neurohumoral effects by increasing catecholamine, and cardiac output. Caffeine, a potent inhibitor of insulin activity, and acute increase in blood pressure and homocysteine levels. Thus, these mechanisms also support our finding of an inverse association between coffee and all-cause mor-

Figure 7. Ferulic acid promotes macrophage RCT in mice. Ferulic acid (5 mg/kg per day) or the vehicle was given by oral gavage; these 2 groups were fed with water. The vehicle was also given to coffee group, which was fed with cooled coffee liquid (10 mg/mL, instant coffee prepared with hot water) ad libitum. Seven days after administration. One week after the initial administration, [3H]cholesterol-labeled RAW264.7 cells were injected intraperitoneally (5 × 10⁶ cells containing 7.5 × 10⁶ cpm in 0.5 mL medium), A, Blood samples obtained from mice 24 and 48 hours after injection were subjected to 3H tracer analysis. B through D, Forty-eight hours after injection, mice were euthanized, and liver (B) and bile (C) were isolated, prepared as described in Methods, and then subjected to 3H tracer analysis. Feces (D) collected continuously from 0 to 48 hours were prepared as described in Methods and were then subjected to 3H counting. Data are expressed as percent counts relative to total injected tracer (means±SEM; n=6 for each group). *P<0.05 vs vehicle. Data were analyzed by 1-way ANOVA, Bonferroni–Dunn post hoc test.

from cells to lipid-poor apoA-1, but not HDL₃, whereas another ABC transporter, ABCG1, and SR-BI are involved in the cholesterol efflux from macrophages mediated by HDL₃, but not apoA-I. One human study revealed that single oral administration of coffee raised plasma levels of phenolic acids and their metabolites and, again, induced ABCG1 and SR-BI expression, thus leading to the enhanced cholesterol efflux. Additionally, in line to the in vitro finding, ferulic acid was found to promote overall RCT in vivo in mice. These comparable results obtained from in vitro, ex vivo, and in vivo studies, therefore, indicate that the potential antiatherogenic properties of coffee proposed from previous studies could be explained, at least in part, by the upregulated cholesterol efflux from macrophages mediated through ABCG1 and SR-BI pathways.

Coffee is one of the most popular habitual beverages in the world and is the major source of dietary antioxidants because coffee contains phenolic acids with antioxidant properties. The Zutphen Elderly Study demonstrated that a flavonoid-rich (another phenolic acid derivatives) diet reduced the rate of atherosclerosis in vivo. This led to the hypothesis that coffee exerts its antiatherosclerotic effects via antioxidative and/or antiinflammatory functions. Indeed, several studies reported that coffee intake is inversely associated with inflammatory biomarkers. Although numerous studies reported the association between coffee consumption and CVD, the information remains inconsistent. Some authors have reported a positive association between coffee intake and coronary heart disease, whereas others have reported J-shaped and no relationship. Moreover, the increase in coronary heart disease risk may be the result of elevated blood pressure and cholesterol levels attributable to unhealthy dietary habits or increased smoking habits associated with coffee consumption. It seems that an independent association between coffee consumption and the risk of developing coronary heart disease has not yet been established. Thus, investigations of “the role of coffee consumption on CVD incidence” have been very scarce. The present study therefore sheds a new light into the potential antiatherogenic properties of coffee in additional to antioxidative/antiinflammatory functions. Interestingly, the phenolic acids of coffee selectively induced ABCG1 and SR-BI, but not ABCA1, expressions. Although we are not certain of the cause for this discriminative function of phenolic acids, it appears to be unlikely that phenolic acids activate liver X receptor-alpha (LXRα) expression, because LXRs, in turn, transactivates both ABCA1 and ABCG1. Indeed, we showed that caffeic and ferulic acids enhanced ABCG1 and SR-BI mRNA stability (Online Figure III, C and D), thus resulting in increased steady-state expression of these genes in the macrophages. Based on recent evidences, 3’-UTRs play an important role in mRNA degradation by interacting with microRNA. We therefore performed a reporter gene assay using the luciferase constructs containing human ABCG1 and SR-BI 3’-UTRs to assess a possibility whether phenolic acids affect these regions. As shown in Online Figure IV (A and B), coffee phenolic acids did not restore the attenuated luciferase activity induced by the presence of 3’-UTR, indicating that these regions of human ABCG1 and SR-BI were not involved in mRNA stabilization of these genes. Further investigations are therefore needed to explore the precise mechanisms for these effects of phenolic acids.

Coffee also contains caffeine which may exert acute neurohumoral effects by increasing catecholamine, and cardiac output. Caffeine, a potent inhibitor of insulin activity, and acute increase in blood pressure and homocysteine levels. Thus, these mechanisms also support our finding of an inverse association between coffee and all-cause mor-
tality independent of caffeine intake. In this experiment, the sera obtained at 30 minutes after coffee ingestion could contain caffeine and its effector substance. Therefore, there is a possibility that these compounds contribute to the increased ABCG1 and SR-BI expression and resultant enhancement of cholesterol efflux. However, it is very unlikely that this acute effect could modulate the monocyte characteristics to increase cholesterol efflux, because caffeine has not been reported to directly modulate cholesterol efflux in vitro. Nonetheless, experiments using decaffeinated coffee will be necessary to make any definitive conclusions. Likewise, coffee is a complex mixture of compounds that may have either beneficial or harmful effects on the cardiovascular system. For example, boiled coffee consumption is associated with increased cholesterol levels resulting from cafestol and kahweol, which are otherwise removed by filtering. In contrast and consistent to this study, filtered coffee decreases HDL cholesterol in human.

Whether the increased cholesterol efflux by the phenolic acids of coffee is attributable to its antioxidative property remains inconclusive. Firstly, probucol, a potent antioxidant, decreases cholesterol efflux with a degradation of the ABCA1 protein in macrophages. α-Tocopherol, a fat soluble antioxidant, disturbs macrophage LXR a regulation of ABCA1, ABCG1, and cholesterol handling. Overall, it is conceivable to state that not all antioxidants have the cholesterol efflux properties of HDL.

Ex vivo human study can be interpreted that, in addition to phenolic acids in postcoffee sera, postcoffee monocyte or HDL also contributes for the enhanced HDL-mediated cholesterol efflux. However, monocyte isolated from postcoffee consumption did not increase ABCG1 or SR-BI mRNA levels. Likewise, HDL isolated from pre- and postcoffee consumption sera yielded similar ABCG1 and SR-BI mRNA levels. Overall, these results strongly indicate that the coffee derived compounds, most likely phenolic acids, rather than cells or HDL, induced ABCG1 and SR-BI expression, thus promoting the HDL-mediated cholesterol efflux.

There are several limitations in the present study. First, as mentioned above, coffee contains compounds other than phenolic acids and potential effects of these compounds on cholesterol efflux were not assessed in this study. However, these compounds, in general, have been reported to deteriorate, rather than ameliorate, the lipoprotein profile, thus being very unlikely to enhance the cholesterol efflux. Second, the subjects in the human study were healthy volunteers. Therefore, it is difficult to generalize the findings to dyslipidemic patients who are at increased risk for atherosclerosis. Third, a single oral administration of coffee represents an acute effect which may differ from the chronic (habitual) effect of coffee. Finally, the present study did not test a possibility that coffee consumption affects HDL proinflammatory or antiinflammatory, a factor also potentially affecting cholesterol efflux. Likewise, we did not directly examine whether coffee consumption actually removed oxidized cholesterol, oxidized fatty acids, or oxidized phospholipids from peripheral tissues. These interesting posteriori hypotheses deserve future studies.

In conclusion, coffee intake has an antiatherogenic effect by increasing ABCG1 and SR-BI expression and enhancing HDL-mediated cholesterol efflux from the macrophages by its plasma phenolic acids. In addition to the antioxidative properties, the potential cardioprotective properties of coffee might therefore be associated with an enhanced antiatherogenic function of HDL.

Acknowledgments
We thank Yoshinori Masukawa, Naoki Kondo, and Tadashi Hase (Kao Corporation) for helpful discussions.

Sources of Funding
This research was supported, in part, by a research grant from the All Japan Coffee Association.

Disclosures
None.

References
Coffee Phenolic Acid and Cholesterol Efflux

Uto-Kondo et al


34. Wu CA, Tsujita M, Yokoyama S, Prokobolin inactivates ABCA1 in the plasma membrane with respect to its mediation of apolipoprotein binding and high density lipoprotein assembly and to its pro-atherogenic degradation. J Biol Chem. 2004;279:30168–30174.


Novelty and Significance

What Is Known?

- Antioxidants have been shown to be cardioprotective.
- However, epidemiological studies investigating the association of coffee, abundant in antioxidant phenolic acids, with cardiovascular diseases have yielded mixed results.

What New Information Does This Article Contribute?

- We show that coffee, or its major phenolic acids, increase cholesterol efflux from macrophage, an initial step of RCT.
- Additional in vivo experiments in mice and human supported this finding.

Coffee consumption is inconsistently associated with decreased cardiovascular disease and the mechanisms by which coffee

exerts its antiatherogenic properties remain largely unknown. We hypothesized that coffee promoted cholesterol efflux from macrophages, leading to the enhanced reverse cholesterol transport (RCT) according to the antiatherogenic properties of high-density lipoprotein (HDL). Striking results of in vitro as well as ex vivo human studies consistently demonstrated that phenolic acids present in coffee increase HDL-mediated cholesterol efflux from macrophages by inducing proteins that regulate cholesterol transport. The increased in cholesterol efflux, an initial step of RCT, resulted in an overall increase in RCT in mice in vivo. To our knowledge, this is the first study to demonstrate the antiatherogenic property of Coffee both in vitro and in vivo. These results extend our knowledge of antiatherogenic properties of coffee, one of the most common beverages in the world, and have important clinical implications.
Coffee Consumption Enhances High-Density Lipoprotein-Mediated Cholesterol Efflux in Macrophages

Harumi Uto-Kondo, Makoto Ayaori, Masatsune Ogura, Kazuhiro Nakaya, Mai Ito, Atsushi Suzuki, Shun-ichi Takiguchi, Emi Yakushiji, Yoshio Terao, Hideki Ozasa, Tetsuya Hisada, Makoto Sasaki, Fumitaka Ohsuzu and Katsunori Ikewaki

Circ Res. 2010;106:779-787; originally published online January 14, 2010; doi: 10.1161/CIRCRESAHA.109.206615

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/4/779

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/01/14/CIRCRESAHA.109.206615.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Online Figure I. Caffeic and ferulic acid enhance HDL-mediated cholesterol efflux by increasing of ABCG1 and SR-BI expressions in human monocyte-derived macrophages (MDM). A,B, After \(^{3}H\)cholesterol labeling, caffeic, ferulic acid (1 µmol/L) or the vehicle (Cont) were added to the cultures which were then incubated in the presence of 50 µg/mL of HDL or 10 µg/mL of human apoA-I for 24 hours. Determination of cholesterol efflux from the cells was performed as described in Materials and Methods. The results for 4 samples are presented as mean ± SEM. * \(P<0.05\) vs vehicle. C,D, MDM treated with the vehicle (Cont) or caffeic acid or ferulic acid (1 µmol/L) for 6 hours. RNA extraction and real-time quantitative RT-PCR was performed as described in Methods. The mRNA levels of each gene were standardized for 18S ribosomal RNA levels. The results from 3 separately performed experiments are presented as mean ± SEM. * \(P<0.05\) vs vehicle. Data were analyzed using one-factor factorial ANOVA, Bonferroni/Dunn’s post hoc test.
Online Figure II. Coffee polyphenols increase cholesterol efflux to either HDL2 or HDL3 in THP-1 macrophages. After $[^3]$H]cholesterol labeling, indicated doses of caffeic, ferulic acid or the vehicle were added to the cultures which were then incubated in the presence of 50 µg/mL of HDL2 (A,B) or HDL3 (C,D) for 24 hours. Determination of cholesterol efflux from the cells was performed as described in Materials and Methods. The results for 4-6 samples are presented as mean ± SEM. * P<0.05 vs vehicle. Data were analyzed using one-factor factorial ANOVA, Bonferroni/Dunn’s post hoc test.
Online Figure III

A

Fold changes

- ActD  + ActD

ABCG1

! + ActD - ActD

Fold changes

B

Fold changes

- ActD  + ActD

SR-BI

! + ActD - ActD

Fold changes

C

Fold changes

0 1 3 6
time after the treatments (hour)

ABCg1

- ActD  + ActD

Fold changes

D

Fold changes

0 1 3 6
time after the treatments (hour)

SR-BI

- ActD  + ActD

Fold changes

* Cont  Caffeic acid  Ferulic acid

* Cont  Caffeic acid  Ferulic acid

* Cont  Caffeic acid  Ferulic acid

* Cont  Caffeic acid  Ferulic acid

* Cont  Caffeic acid  Ferulic acid
Online Figure III. ABCG1 and SR-BI mRNA stability enhanced by caffeic and ferulic acid. THP-1 macrophages were pretreated with 10 µg/mL of actinomycin D (Act D) 0.5 hour before the treatment with 1 µmol/L of caffeic acid, ferulic acid or the vehicle (Cont) for indicated periods. A, B, Total RNA was extracted 6 hours after the treatment with coffee phenolic acids. C, D, Total RNA was extracted 1, 3 and 6 hours after the treatment with coffee phenolic acids. RNA extraction and real-time quantitative RT-PCR was performed as described in Methods. The mRNA levels of each gene were standardized for 18S ribosomal RNA levels. The results from 3 separately performed experiments are presented as mean ± SEM. *P<0.05 vs vehicle. Data were analyzed using one-factor factorial ANOVA, Bonferroni/Dunn’s post hoc test.
Online Figure IV

A

![Bar chart showing fold changes for different treatments](chart_A.png)

B

![Bar chart showing fold changes for different treatments](chart_B.png)

**Online Figure IV.** Human ABCG1 and SR-BI 3'-untranslated regions (UTR) were not involved in caffeic and ferulic acid-mediated mRNA stabilization. Ten hours after transfection with pGL3control, pGL3control containing human ABCG1 (A) or SR-BI 3'-UTR (B), RAW264.7 cells were treated with 1 µmol/L of caffeic acid, 1 µmol/L of ferulic acid, or the vehicle (Control) as described in Methods. Twenty-four hours after the treatments, the cells were lysed and a luciferase assay performed. The results from 3 separately performed experiments are presented as mean ± SEM. Data were analyzed using one-factor factorial ANOVA, Bonferroni/Dunn’s post hoc test.
Online Figure V. Post-coffee HDL does not increase cholesterol efflux from THP-1 macrophages compared to pre-coffee HDL. After [3H]cholesterol labeling, the cultures were incubated in the presence of 50 µg/mL of HDL obtained from 4 subjects before (pre) and after (post) coffee consumption for 24 hours. Determination of cholesterol efflux from the cells was performed as described in Materials and Methods. The results for 4 samples are presented as mean ± SEM. Data were analyzed using paired t-test.