

Females with angina pectoris have altered lipoprotein metabolism with elevated cholesteryl ester transfer protein activity and impaired high-density lipoproteins-associated antioxidant enzymes

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Abstract. In order to investigate non-invasive biomarkers for angina pectoris (AP), we analyzed the lipid and protein composition in individual lipoproteins from females with angina pectoris (n=22) and age- and gender-matched controls (n=20). In the low-density lipoprotein (LDL) fraction, the triglycerides (TG) and protein content increased in the AP group compared to the control group. The AP group had lower total cholesterol (TC) and elevated TG in the high-density lipoprotein (HDL) fraction. In the AP group, cholesteryl ester transfer protein (CETP) activity was enhanced in HDL and LDL, while lecithin:cholesterol acyltransferase (LCAT) activity in HDL₃ was almost depleted. Antioxidant activity was significantly decreased in the HDL₃ fraction, with a decrease in the HDL₂ particle size. In the HDL₃ fraction, paraoxonase and platelet activating factor-acetylhydrolase (PAF-AH) activity were much lower and the levels of CETP and apoC-III were elevated in the AP group. The LDL from the AP group was more sensitive to cupric ion-mediated oxidation with faster mobility. In conclusion, the lipoprotein fractions in the AP group had impaired antioxidant activity and increased TG and apoC-III with structural and functional changes.

Introduction

Coronary artery syndrome is the most common cause of death among women in developed countries (1). The coronary artery syndrome is initiated by atherosclerosis, which is complexed with dyslipidemia and an inflammatory process.

Angina pectoris (AP) is paroxysmal thoracic pain that is sometimes accompanied by a feeling of suffocation (2).

AP is most often due to ischemia of the myocardium and is precipitated by effort or excitement. Several biomarkers have been developed to diagnose coronary artery disease, including lipid and inflammatory markers (3), although the markers are not prognostic. It is well-known that the apoB/apoA-I ratio is important to predict the risk of coronary artery disease (CAD) (4). There have been many non-invasive biochemical measures used to predict cardiovascular risk, such as lipid and lipoprotein metabolism, inflammation, and oxidative stress (5-7). Recently, the apolipoprotein composition in lipoprotein and high-density lipoprotein (HDL) sub-fractions has been shown to change in the sera from patients with acute coronary syndrome (ACS). Huang *et al* (8) reported that plasma ApoAV is associated with ACS. Tashiro *et al* (9) reported that pre β 1-HDL is elevated in patients with unstable angina pectoris. Furthermore, we recently reported an increase in apoC-III in HDL₂ from a male with a myocardial infarction (10). Similarly, Lee *et al* (11) reported that low-density lipoprotein (LDL)-containing apoC-III is an independent risk factor for coronary events in diabetic patients. These findings collectively raised the possibility of a relationship between increased lipid and apoC-III, oxidative modification, and inflammatory processes. In ACS, oxidative stress constitutes an integral part of plaque rupture and platelet activation (12). The oxidative modification of LDL, which is considered a strong risk factor for atherosclerosis and ACS, occurs through the release of pro-inflammatory and oxidative signals. The composition and functional correlations of HDL is also associated with the incidence of metabolic syndrome as described in our previous report (13). Elevated triglycerides (TG) and low cholesterol (C) content in HDL is a major characteristic of the metabolic syndrome (14) and of myocardial infarction (MI) (10). A low HDL-C level is the most common lipid abnormality observed in families with premature coronary heart disease (CHD) (15).

There have been many studies attempting to establish non-invasive biomarkers for the early detection of risk for CHD, including AP and MI. In the current study, to detect unique parameters in lipoprotein levels, lipid and apolipoprotein compositions, and enzyme activities were analyzed between females with AP and controls.

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Materials and methods

Patients and controls. Female patients with stable AP (n=22) were selected using the following criteria: the presence of chest or arm discomfort that is rarely described as pain, but is reproducibly associated with physical exertion or stress and relieved within 5-10 min of rest and/or administration of sublingual nitroglycerin. The diagnosis was confirmed with a treadmill exercise test and coronary angiography in all patients. Patients did not take any medications, except for statins, prior to hospitalization. Age- and gender-matched reference subjects (n=20) were recruited from healthy volunteers who underwent regular health evaluations at the Health Center of Yeungnam University Hospital (Daegu, Korea). They had unremarkable medical records without a history of endocrinological disorders. Heavy alcohol consumers (>30 g EtOH/day) and those who had taken prescribed drugs to treat hyperlipidemia, diabetes mellitus, or hypertension were excluded. Informed consent was obtained from all patients and the control group prior to enrollment in the study. The Institutional Review Board at the Medical Center of Yeungnam University approved the protocol.

Isolation of lipoproteins. After overnight fasting, blood was collected using a vacutainer (BD Bio Sciences, Franklin Lakes, NJ, USA) containing EDTA (final concentration, 1 mM). Plasma was isolated by low-speed centrifugation and stored at -80°C until analysis.

Very low-density lipoproteins (VLDL, $d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$), HDL₂ ($1.063 < d < 1.125$) and HDL₃ ($1.125 < d < 1.225$) were isolated from individual patient and control sera via sequential ultracentrifugation (16), with the density adjusted by the addition of NaCl and NaBr in accordance with standard protocols. Samples were centrifuged for 24 h at 10°C at 100,000 x g using a Himac CP 90α (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center of Yeungnam University.

For each of the lipoproteins which were individually purified, total cholesterol (TC) and TG measurements were obtained using commercially available kits (cholesterol, T-CHO, and TG, Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). The protein concentrations of lipoproteins were determined via the Lowry protein assay, as modified by Markwell *et al* (17) using the Bradford assay reagent (Bio-Rad, Seoul, South Korea) with bovine serum albumin (BSA) as a standard. To assess the degree of oxidation of individual LDL, the concentration of oxidized species in LDL was determined by the thiobarbituric acid reactive substances (TBARS) method using malondialdehyde (MDA) as a standard (18).

Ferric reducing ability of plasma assay. The ferric reducing ability of plasma (FRAP) was determined using the method described by Benzie and Strain (19) with a slight modification, as described previously (20). The antioxidant activities of the individual HDL fractions (20 μg each) were then estimated by measuring the increase in absorbance induced by the generated ferrous ions.

Cholesteryl ester conversion assay. Cholesteryl ester conversion was performed via lecithin:cholesterol acyltransferase (LCAT) assays, as previously described (21). An equal amount

of individual lipoproteins (in 50 μl) from each patient was utilized as the enzyme source. ApoA-I-rHDL containing radiolabeled cholesterol (1 μCi of [¹⁴C]-4-cholesterol/69 μg of cholesterol/1 mg of apoA-I) was used as a substrate, and the apoA-I was then expressed using an *E. coli* expression system, as described previously (21). Discoidal rHDL was prepared via the sodium cholate dialysis method using initial molar ratios of palmitoyl-oleoyl phosphatidylcholine (POPC)-cholesterol-apoA-I-sodium cholate at a ratio of 95:5:1:150 (wt/wt/wt/wt). The reaction was initiated by the addition of individual serum, and the mixture was then incubated for 1 h at 37°C. Next, the esterified cholesterol and free cholesterol were separated via thin layer chromatography, and the activity was expressed as the percentage conversion rate of cholesteryl ester from free cholesterol.

Cholesteryl ester transfer assay. An rHDL-containing apoA-I and cholesteryl oleate was synthesized in accordance with the method described by Cho (20) using trace amounts of [³H]-cholesteryl oleate (TRK886, 3.5 μCi/mg of apoA-I; GE Healthcare) with a slight modification (22). The CE-transfer reaction was allowed in 300 μl reaction mixtures that contained equal amounts of the individual lipoproteins (20 μl, 10-20 μg of protein) as a cholesteryl ester transfer protein (CETP) source and rHDL-agarose (50 μl, 0.25 mg/ml) and human LDL (50 μl, 0.25 mg/ml) as a CE-donor and CE-acceptor, respectively. After incubation at 37°C, the reaction was halted via brief centrifugation (10,000 x g) for 3 min at 4°C. The supernatant (150 μl) was then subjected to scintillation counting, and the percentage transfer of [³H]-CE from rHDL to LDL was calculated.

Paraoxonase assay. Paraoxonase-1 (PON-1) activity toward paraoxon was determined by evaluating the hydrolysis of paraoxon into *p*-nitrophenol and diethylphosphate, which was catalyzed by the enzyme (23). PON-1 activity was then determined by measuring the initial velocity of *p*-nitrophenol production at 37°C, as determined by measuring the absorbance at 405 nm (microplate reader, Bio-Rad model 680; Bio-Rad, Hercules, CA, USA), as described previously (13).

Platelet activating factor-acetylhydrolase (PAF-AH) assay. The individual lipoprotein fractions (10 μl, 20 μg) were used as an enzyme source for the PAF-AH reaction with an Lp-PLA₂ assay conducted according to the method described by Boyd *et al* (24). Briefly, [³H]-platelet activating factor (hexadecyl-2-acetyl sn-glycerol-3-phosphorylcholine, NET910, 0.1 mCi/ml; Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA) and 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocholine were used as substrates for the reaction. A substrate solution containing 10 μl of [³H]-PAF (1 μCi, 50 μM) and 12 μM of cold PAF was incubated using each HDL solution as a source for 30 min. The reaction was then stopped by vortexing the solutions with 600 μl of CHCl₃:MeOH (2:1, v/v), after which the aqueous layer (150 μl) was removed. The aqueous layer was then vortexed again with CHCl₃, after which it was centrifuged and the upper phase was used for scintillation counting.

Electromobility of lipoproteins. In order to compare the electromobility of the patient and control samples, the migration of each lipoprotein (LDL, HDL₂ and HDL₃) was evaluated by

Table I. Lipid and protein composition of lipoproteins from patients.

	Angina pectoris (n=22)			Control (n=20)		
	TC (mg/dl)	TG (mg/dl)	TP (mg/ml)	TC (mg/dl)	TG (mg/dl)	TP (mg/dl)
VLDL	120±64	203±140	2.8±0.1	130±31	243±46	2.8±0.1
LDL	1095±231	256±65 ^a	6.6±0.2	909±177	165±15	5.5±1.3
HDL ₂	46±14 ^a	82±24 ^a	2.0±0.1	75±14	52±28	1.8±0.1
HDL ₃	64±16	16±7	3.6±0.5	58±22	24±22	3.8±0.1

^aP<0.05 vs. control. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; TG, triacylglycerol; TP, total protein.

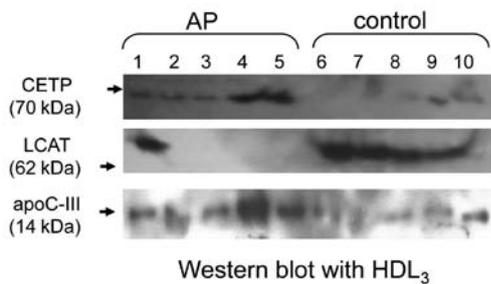


Figure 1. Immunodetection of apolipoproteins and enzymes in HDL₃ between the angina pectoris (AP) and control groups. Equal amounts of HDL₃ (6 µg of protein) from an individual subject were loaded per lane. Polyclonal CETP antibody (Abcam, ab19012), apoC-III antibody (Chemicon, AB821), and LCAT antibody (Abcam, ab786) were used as primary antibodies.

agarose electrophoresis. The gels were then dried and stained with 0.125% Coomassie Brilliant Blue, after which the relative band intensities were compared via band scanning using Gel Doc[®] XR (Bio-Rad) with Quantity One software (version 4.5.2).

Western blot analysis. The apolipoprotein/lipoprotein compositions were compared via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with identical protein loading quantities (6 µg of total protein per lane) from individual HDL₃, and the levels of expression of apolipoprotein were analyzed via immunodetection. Anti-human apoC-III antibody (AB821) was purchased from Chemicon (Temecula, CA, USA). Anti-human CETP antibody (ab19012) and LCAT antibody (ab786) were purchased from Abcam (Cambridge, UK). The relative band intensity (BI) was compared via band scanning with Gel Doc[®] XR (Bio-Rad) using the Quantity One software (version 4.5.2).

Data analysis. All data are expressed as the mean ± SD from at least three independent experiments with duplicate samples. Data comparisons were assessed by the Student's t-test using the SPSS program (version 14.0; SPSS, Inc., Chicago, IL, USA).

Results

Lipid and protein composition in lipoprotein. The serum TC concentrations were similar between the groups (204±57 and 200±32 mg/dl, respectively), which remained in the normal

range, as suggested by the guidelines of the National Cholesterol Education Program (NCEP)-adult treatment panel (ATP)-III. The LDL-C level was similar between the groups (105±38 and 108±33 mg/dl for the AP patients and controls, respectively). However, the HDL-C level was slightly lower in the AP patients than the controls. The ratio of HDL-C-to-TC was significantly lower in the AP patients (34±2%) compared to the control group (40±4%). The serum TG level was not significantly different between the groups (136-175 mg/dl).

Properties of lipoprotein are good biomarkers which reflect the progress of cardiovascular and renal disease. As shown in Table I, the AP group had a similar composition of lipid and protein in VLDL with the control group. Although the TC and protein content in LDL was similar between the groups, the TG content in LDL was significantly higher in the AP group (38 mg of TG/mg of TP) compared to the control group (30 mg of TG/mg of TP). In the HDL₂ fraction, the AP group had a much lower TC content and a higher TG content (44% higher TC and 32% lower TG) than the control group. Immunodetection revealed that the level of expression of apoC-III was elevated in the HDL₃ fraction of the AP group (Fig. 1).

CETP and LCAT activity. As shown in Table II, although the CE-transfer activity of the VLDL fraction was similar between the groups (~2-3% CE-transfer), the LDL fraction of the AP group had 2-fold increased CE-transfer activity. The CETP activity of the HDL fraction also increased in the AP group (a 70 and 34% increase for HDL₂ and HDL₃, respectively), compared to the control. Immunodetection revealed that CETP was highly expressed in the HDL₃ fraction of the AP group (Fig. 1).

The LCAT activity was significantly lower in the HDL₃ fraction of the AP group, while no difference existed in the HDL₂ fraction between the groups. The LCAT activity for CE-conversion from FC was lowered in the HDL₂ fraction in the AP and control groups, as shown in Table II. The level of expression of LCAT was nearly undetectable in the AP group (lane 1-5) except in one patient (Fig. 1).

Antioxidant activity of HDL₂ and HDL₃ was decreased in the AP group. The HDL₃ from the AP group had weaker antioxidant activity (172% increase from the initial level) than the control

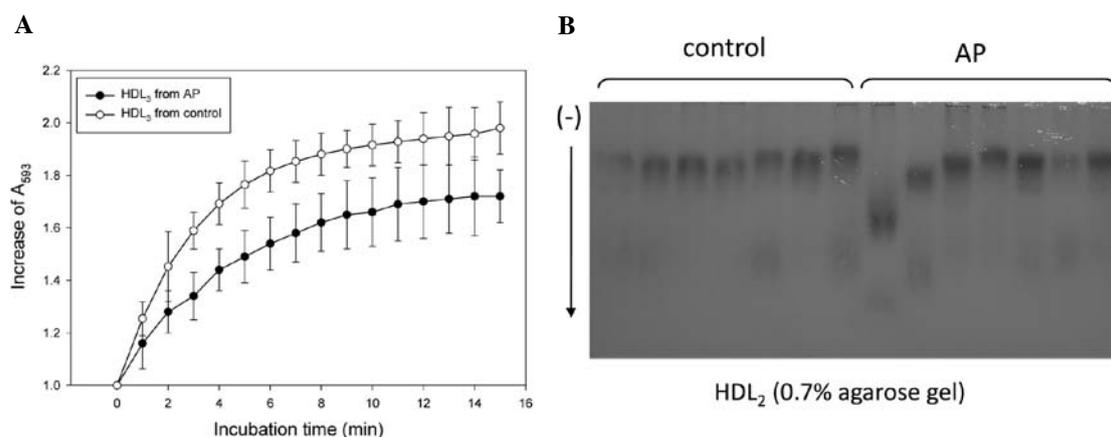


Figure 2. Comparison of the antioxidant activity of HDL. Reduction potential of HDL₃ based on the ferric-reducing ability of plasma (FRAP). The same amount of HDL₃ (0.05 ml, 2 mg/ml) was added to the substrate solution. Comparison of electrophoretic mobility of HDL₂ on 0.7% agarose gels without cupric ion treatment.

Table II. LCAT and CETP activities in lipoprotein fractions.

	Angina pectoris (n=22)	Control (n=20)
CETP activity ^a		
VLDL	2.6±0.6	3.1±0.1
LDL	5.5±0.3 ^c	2.0±1.0
HDL ₂	17±2.1 ^c	10±3.6
HDL ₃	35.4±6.6 ^c	26.8±2.1
LCAT activity ^b		
HDL ₂	1.2±0.7	2.0±1.5
HDL ₃	3.5±1.3 ^c	12.3±2.1

^aCETP activity is expressed as % CE transfer/4 h. ^bLCAT activity is expressed as % CE conversion/h/100 μg of protein in HDL. ^cP<0.05 vs. control. LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

group (198% increase) when the same amount of protein in HDL (1.5 mg/ml) was used as an antioxidant source (Fig. 2A). The extent of oxidation in the native state was compared by relative electrophoretic mobility on 0.7% agarose gel electrophoresis. The HDL₂ from the AP group migrated faster than the control group without cupric ion treatment, indicating that HDL₂ of the AP group was more oxidized in the native state (Fig. 2B). More highly oxidized HDL has a faster mobility due to a smaller particle size and an increase in charge. In particular, HDL₂ from the AP group was 2-fold more susceptible to cupric ion-mediated oxidation, as shown in Fig. 3, indicating that the antioxidant potential was significantly decreased in the AP group. Specifically, electron microscopy revealed that HDL₂ from the AP group had a smaller particle size than the control; HDL₂ from the AP group was 18–20 nm in width and length, while HDL₂ from the control group was 22–25 nm in width and length. These results suggest that more highly oxidized HDL has faster electromobility and reduced particle size.

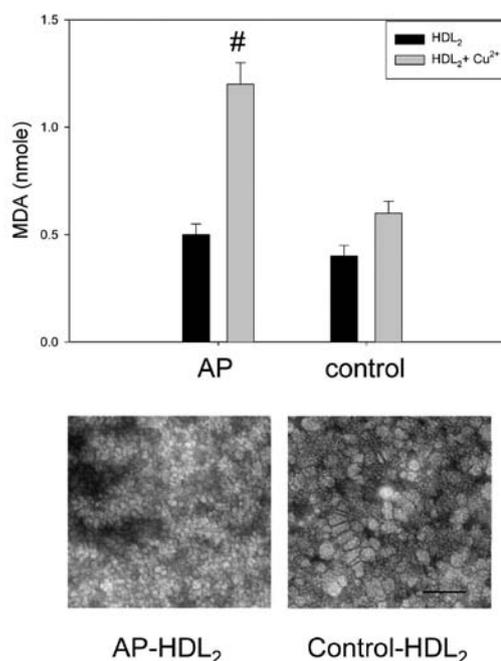


Figure 3. Properties of HDL₂ from the AP and control groups. Susceptibility to oxidation in HDL₂ by cupric ion treatment (graph). Representative picture of negatively-stained HDL₂ from the angina pectoris (AP) and control groups (electron microscopy, bottom photo). All micrographs are shown at a magnification of ×40,000. The scale bar corresponds to 100 nm. MDA, malondialdehyde. [#]P<0.01 vs. the control in the presence of cupric ion.

HDL-associated paraoxonase and PAF-AH. The HDL₂-associated PON activity was lower in the AP group than in the control group (112±10 vs. 164±25 μU/mg of protein) (Fig. 4A). Moreover, the AP group had a 3-fold lower HDL₃-associated PON activity than the control group (109±16 vs. 561±36 μU/mg of protein, respectively).

Although there was no significant difference in the HDL₂ fraction used as the PAF-AH source, the activity was significantly lower in the AP group when the HDL₃ fraction was used (Fig. 4). HDL₃ from the AP group showed 40% less activity than the control group (15±2 and 26±3 pmole PAF/h/mg of protein for the AP and control groups, respectively).

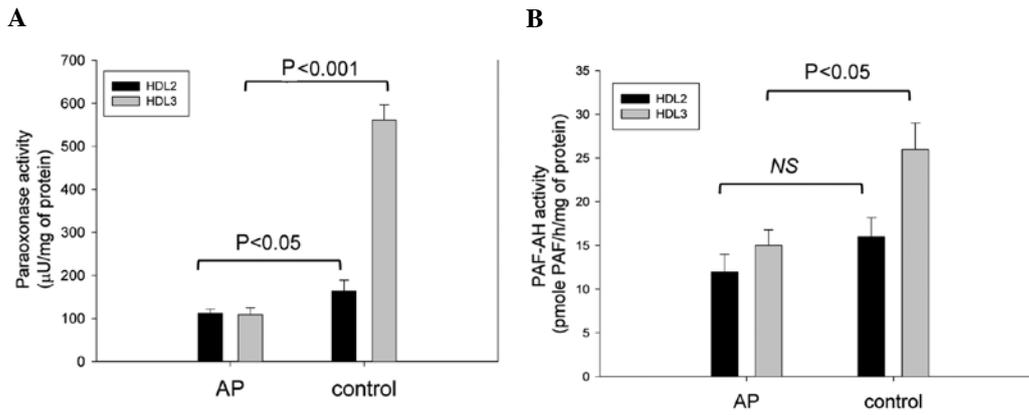


Figure 4. Activities of HDL-associated antioxidant enzymes, (A) paraoxonase and (B) platelet activating factor-acetylhydrolase (PAF-AH), between the angina pectoris (AP) and control groups. An equal amount of individual HDL₂ and HDL₃ were used as each enzyme source.

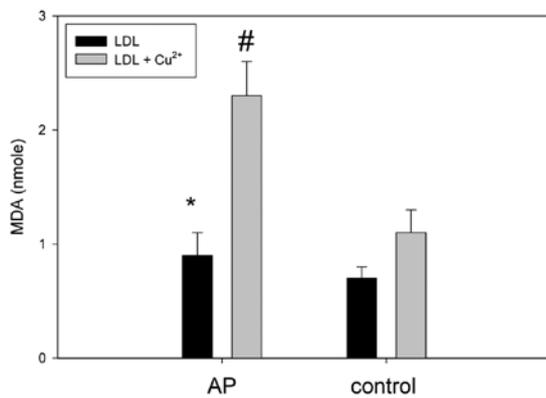


Figure 5. Oxidized extent of LDL with or without cupric ion treatment between the angina pectoris (AP) and control groups. *P<0.05 and #P<0.01 vs. the control.

LDL from AP patient was more oxidized. The LDL from the AP patient had ~1.8-fold higher levels of MDA than the control without cupric ion treatment, indicating a greater extent of oxidation of LDL in the AP group in the native state (Fig. 5). Under treatment with cupric ion (final 10 µM), the LDL from the AP and control group showed 2.3 and 1.1 nmole of MDA, respectively, suggesting that the AP group LDL was more sensitive to cupric-ion mediated oxidation.

Discussion

In addition to a change in serum lipid parameters, lipid and protein compositions in lipoproteins have emerged as a parameter which is associated with the progress of metabolic diseases, such as metabolic syndrome (13,14) and CHD (15). In fact, structural and functional changes in HDL are more dramatic in the acute phase, such as viral infections (25) and after cardiac surgery (26).

Although the AP group had similar levels of TC and LDL-C, the AP group had a lower ratio of HDL-C/TC. While the TC content was lower in HDL₂ from the AP group, the TG content was significantly elevated. An increase in TG in the serum is a potent inflammatory factor and is associated

with the incidence of CAD (27). Accumulation of serum TG in HDL has been correlated with the incidence of cardiovascular disease (4,10). TG-enriched lipoprotein is more inflammatory in vascular events (28). An elevated TG/HDL-C ratio is associated with increased insulin resistance and cardiovascular events (29). The current report suggests that the serum TG was more highly accumulated in the LDL and HDL₂ fractions, rather than in the VLDL fraction, which is similar to the results of a previous report involving a male MI patient (10) that showed a strong and consistent association of hypertriglyceridemia with enriched LDL fraction. Recently, TG/HDL-C was shown to be a strong independent predictor of mortality in women with an ischemia syndrome (4). Several reports have suggested that an increased TG level is associated with elevation of apoC-III in lipoproteins; apoC-III in VLDL and LDL is linked with CHD and senescence (11,30). In the current study, TG in HDL₂ and LDL, and CETP activity were elevated in AP patients, suggesting that apoC-III in HDL is also a risk factor for coronary events in female AP patients (Table I and Fig. 1).

It is known that serum CETP is an atherogenic factor. CETP promotes the transfer of CE from HDL to VLDL and LDL in exchange for TG, which moves in the opposite direction. The exchange of CE and TG between lipoproteins is linked to elevated levels of TG-enriched lipoprotein, which is pro-inflammatory and pro-atherogenic (31). CETP is an independent risk factor for CHD and metabolic syndrome (32). In addition, we recently reported that the metabolic syndrome in male patients is characterized by a 38% higher serum cholesteryl ester transfer protein (CETP) activity than the control group (10). The increase in TG is also associated with elevated level of apoC-III in the serum and lipoproteins in male MI patients (10). Furthermore, CETP activity is not decreased when apoC-III-enriched HDL is used as a CETP source (20). The current report showed that the AP group had an elevated level of apoC-III in HDL₃.

With the alteration in the lipid content in HDL, many reports have suggested that HDL particle size is associated with cardiovascular events (9). Zeller *et al* (33) proposed that the smaller particle size of HDL is associated with young age in patients with acute MI. In addition, Arsenault *et al* (34) reported that a decreased HDL particle size is associated with

an adverse cardiometabolic risk profile. They also proposed that a small HDL particle size was associated with an increased CHD risk. Interestingly, the HDL particle size was inversely related to CETP activity, serum TG concentration, body mass index, and C-reactive protein.

One of the beneficial virtues of HDL is exerting anti-oxidant activity. The increase in oxidation susceptibility in the AP group might be linked to alteration of lipid and protein composition in HDL. In the AP group, HDL₂-TC was ~40% lower than the control, while HDL₂-TG was elevated by 60%. Moreover, LCAT activity in HDL₂ and HDL₃ was 40 and 72% lower in the AP group, respectively, compared to the control. Using immunodetection techniques, LCAT expression was undetectable in the HDL₃ fraction of the AP group with the exception of one patient, while the LCAT band was detected in the control (Fig. 1). The decrease in LCAT activity and expression may contribute to the loss of antioxidant activity and oxidation sensitivity.

In addition, human serum PON (EC 3.1.1.2) is an HDL-associated calcium-dependent enzyme, and has strong antioxidant activity. It catalyzes the hydrolysis of oxidized fatty acids from phospholipids and prevents the accumulation of oxidized lipids in lipoproteins, particularly LDL (23). PON activity and -SH levels have been shown to be lower in CAD patients (35), which suggests that reduced PON activity may contribute to the severity of CAD. PAF-AH (EC 3.1.1.47) is also involved in the antioxidant and anti-inflammatory functions associated with the surfaces of HDL (36), and is a Ca²⁺-independent enzyme belonging to group 7 of the PLA₂ family (37). PAF-AH degrades oxidized phospholipids and platelet activating factor, which is a pro-inflammatory factor. Thus, PAF-AH may function as a profoundly anti-atherogenic enzyme. These three enzymes were coincidentally lowered in the HDL fraction of the AP group, which is in good agreement with decreased antioxidant activity.

In conclusion, the current results strongly support the inter-relationship between CETP activity, the serum TG level and its distribution, apoC-III expression, and that the change in HDL particle size and antioxidant ability are intimately correlated, especially in the onset of the female with AP.

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