Plasma apolipoprotein O level increased in the patients with acute coronary syndrome

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Abstract Apolipoprotein (apo) O is a novel apolipoprotein that is present predominantly in high density lipoprotein (HDL). However, overexpression of apoO does not impact on plasma HDL levels or functionality in human apoA-I transgenic mice. Thus, the physiological function of apoO is not yet known. In the present study, we investigated relationships between plasma apoO levels and high-sensitive C-reactive protein (hs-CRP) levels, as well as other lipid parameters in healthy subjects (n = 111) and patients with established acute coronary syndrome (ACS) (n = 50). ApoO was measured by the sandwich dot-blot technique with recombinant apoO as a protein standard. Mean apoO level in healthy subjects was 2.21 ± 0.83 µg/ml whereas it was 4.94 ± 1.59 µg/ml in ACS patients. There were significant differences in plasma level of apoO between two groups (P < 0.001). In univariate analysis, apoO correlated significantly with lg(hsCRP) (r = 0.48, P < 0.001) in ACS patients. Notably, no significant correlation between apoO and other lipid parameters was observed. Logistic regression analysis showed that plasma apoO level was an independent predictor of ACS (OR = 5.61, 95% CI 2.16–14.60, P < 0.001). In conclusion, apoO increased in ACS patients, and may be regarded as an independent inflammatory predictor of ACS patients.—Y. Yu, B-L., C-L. Wu, and S-P. Zhao. Plasma apolipoprotein O level increased in the patients with acute coronary syndrome. J. Lipid Res. 2012. 53: 1952–1957.

Supplementary key words apolipoproteins • atherosclerosis • high density lipoprotein • inflammation • lipoproteins

HDL is endowed with antiatherogenic effects. In addition to its role in reverse cholesterol transport, HDL exerts several other beneficial effects, including the ability to induce endothelial nitric oxide synthase, to protect LDL against oxidation and to inhibit immune and inflammatory responses (1–4). Protein cargo of HDL accounts for its major function. Proteomics analysis revealed the presence of 56 HDL-associated proteins including all known apolipoproteins and lipid transport proteins (5). However, the precise function of each HDL-associated protein and the factors responsible for the protective effects of HDL are still incompletely understood.

Apolipoprotein (apo) O is a new member of the apolipoprotein family, which was first discovered in 2006 (6). It is mainly present in HDL and in a much lower amount in LDL and VLDL. Its physiologic role remains incompletely known. Comparable to other α-helix-containing proteins, purified recombinant apoO has been shown to elicit cholesterol efflux from J774 cells in vitro. However, an in vivo study has shown that high level overexpression of apoO resulted in a substantial increase in plasma apoO level as well as HDL apoO content but left plasma HDL cholesterol level and HDL functionality essentially unchanged (7), suggesting this newly identified apolipoprotein might not be a major modulator of HDL under normal physiological conditions. Moreover, apoO was not detected in the comprehensive proteomics study aiming at the identification of HDL-associated proteins (5), conceivably because the abundance of this apolipoprotein is low. However, until now, little was known about its plasma level either in normal subjects or in pathophysiological conditions such as acute coronary syndrome (ACS).

Therefore, the aim of the present study was to measure the plasma levels of apoO and explore the association between apoO and other lipid parameters. We have synthesized apoO protein and raised a monoclonal antibody (Mab) against human apoO and used them to develop a new dot-blot sandwich analysis. We then used the assay to study plasma total apoO concentrations in healthy subjects and ACS patients.

Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease; CV, coefficient of variation; FPLC, fast protein liquid chromatography; hs-CRP, high-sensitive C-reactive protein; MAb, monoclonal antibody.

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**MATERIALS AND METHODS**

**Study groups**

Subjects for the present study consisted of 50 ACS patients (32 males and 18 females with a mean age of 64.6 ± 9.3 years) and 111 unrelated individuals who were enrolled as control group (71 males and 40 females with a mean age of 63.2 ± 5.8 years). Diagnostic criteria for unstable angina and acute myocardial infarction were taken from the related guidelines from American College of Cardiology/American Heart Association published in 2007 (for details, please see: [1]). Briefly, the patients were diagnosed as ACS who had acute chest pain symptoms, changes in serial ECG tracings with (acute myocardial infarction) or without (unstable angina) abnormalities of serum cardiac biomarkers such as creatine kinase, creatine kinase-MB, and/or troponins. Exclusion criteria were: (1) severe hepatic or/and renal disease; (2) severe heart failure; (4) other diseases, including severe hypertrophy or diabetes; (3) severe angina; (4) recent (<2 months) infection, immunologic disorders, thyroid diseases, recent major trauma, and cancer; and (5) treatment with immunosuppressive or anti-inflammatory agents. All individuals in both ACS and control groups were Han Chinese. This study was approved by the ethical committee of the Second XiangYa Hospital. Written informed consent was obtained from all subjects.

**Strains and plasmids**

ApoO cDNA generated from reverse transcription of mRNA from human normal liver L-02 cells was PCR-amplified using Taq polymerase (Takara) and the following primers: forward, 5′-GTC TCC TCG AGC TTA GTT CCA GGT GAA TTC-3′, and reverse, 5′-GTC TCC TCG AGC AAC TTA GTT CCA GGT GAA TTC-3′. The sequence was inserted into pET-32a (+) expression vector (Qiagen) using the restriction sites BamHI and XhoI. Competent DH10B and BL21 (DE3) cells (Novagen) were prepared using Transformation and Storage Solution, essentially as described previously. The DH10B transformants obtained were subjected to sequence analysis to verify the presence of the expected sequence. The constructed pET-apoO plasmid was isolated and transformed into BL21 (DE3) cells for protein expression.

**Protein expression and purification**

ApoO protein was expressed in BL21 (DE3) cells, which were grown on Luria-Bertani medium with 50 µg/ml ampicillin. The culture was incubated at 37°C with constant shaking at 200 rpm until the absorbance at 600 nm (A600 nm) reached 0.5-1.0. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM to induce protein expression, and the culture was incubated at 37°C with shaking for 4 h. Cells were pelleted at 5,000 g for 15 min at 4°C and resuspended in 40 ml of ice-cold imidazole buffer (20 mM imidazole, 500 mM NaCl, and 20 mM phosphate buffer, pH 6.6) supplemented with 1 mM phenylmethanesulfonyl fluoride (Amresco), 1 mM MgCl2, and 0.2 mg/ml lysozyme. The cells were disrupted by sonication and insoluble proteins were pelleted by centrifugation at 5,000 g for 30 min at 4°C. Supernatant contained soluble apoO conjugated with thioredoxin (Trx). Purified Trx-apoO was purified using an IMAC (Immobilized Metal Affinity Chromatography) column HiTrap FF (Amersham-Phar-macia Bio-Science) following the manufacturer’s recommendations. The elution fractions containing Trx-apoO were dialyzed in PBS (pH 7.4) and passed through a Protein A column (Clontech) and a Protein G column (Amersham) to remove Trx. The elution fractions containing apoO were pooled and subjected to size-exclusion chromatography (Superose 6 column, GE Healthcare) equilibrated in PBS. The purity of apoO was assessed by SDS-PAGE and Western blot analysis.

**Measurement of plasma apoO concentration**

Plasma apoO concentration was determined with a dot-blot sandwich technique. Each plasma sample was diluted in PBS (dilution ratio 1:20). The protein standard, recombinant human apoO with a Trx-tag at a concentration of 0.1 µg/µl, was diluted in PBS, in ratios of 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200. 5 µl of the plasma specimens was placed on the polystyrene membrane (PVDF) membrane (Bio-rad) in three repetitions and 5 µl of the recombinant apoO dilutions was placed in two repetitions. Distilled water and a 3% solution of BSA were used as a negative control. The membrane was blocked with a 5% solution of nonfat dry milk. The membranes were then incubated at room temperature in a 1:1000 dilution of HRP conjugated mouse anti-human apoO monoclonal antibody in Tween-TBS overnight. Membranes were washed three times in Tween/TBS, and then developed using Western/Pico chemiluminescent substrate (Pierce), and signal detection was made by Kodak Image Station 4000MM. The mean signal densities within the repetitions of each specimen and protein standards were measured with Carestream Molecular Imaging software (Version 5.0.2.30 Kodak) and presented as net intensity. ApoO concentration was calculated from the standard curves computed on the basis of recombinant apoO dilutions.

The inter- and intra-assay imprecision of the measurements were evaluated with 10 and 20 repeated measurements respectively for three samples. The precision was presented as coefficient of variation (CV).

To test for the specificity of the detection, a Western blot assay was performed with 10 µl of 1:20 dilutions of plasma samples, 1:1000 recombinant apoO dilution, 0.3% BSA, and distilled water. The proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membranes using the wet tank transfer technique. The membranes were then processed as described above.

**Lipid measurements**

Blood samples were obtained after an overnight fast and stored at −80°C until analysis. Plasma was isolated from blood coagulation with centrifugation for 20 min at 4°C. Lipids were measured enzymatically by a Hitachi 7060 analyzer using available commercial kits for cholesterol and triglycerides. C-reactive protein levels were analyzed with a high-sensitivity assay (hs-CRP; N Latex CRP mono).

**Lipoprotein separation by FPLC**

Size-fractionation of lipoproteins was performed by fast protein liquid chromatography (FPLC). In brief, a Pharmacia BioSciences FPLC equipped with a Superose 6 column (GE Healthcare Europe GmbH, Munich, Germany) was used with Dulbecco’s...
RESULTS

Characterization of anti-apoO MAb

The bacterial Trx-apoO purified from the lysate of Escherichia coli showed a major band of 32 kDa (Fig. 1A). This represented 99% of the total protein after scanning the gel. The MAb specific for apoO was established. When human plasma and culture medium of HepG2 cells were subjected to SDS-PAGE, the MAb reacted with a single protein (Fig. 1B), the molecular mass of which (55 kDa, Fig. 1B, lanes 1 and 2) was similar to that previously reported for human plasma apoO (6). Because of the cellular glycosylation process, the molecular weight of bacterial recombinant apoO (rhapoO) conjugated with Trx appeared to be less than those in plasma and culture medium. There was no evidence of recognition of other plasma proteins. Neither distilled water nor albumin produced a detectable signal in Western blot and dot-blot (data not shown).

Distribution of apoO on lipoprotein subclasses

To characterize the apoO distribution in plasma lipoprotein subclasses, lipoproteins from healthy subjects or ACS patients were separated by FPLC for subsequent dot-blot analysis of apoO expression. ApoO distribution in lipoprotein subclasses are shown in Fig. 2. In healthy subjects, apoO mainly exists in HDL particles; we could not detect apoO in LDL and VLDL particles. However, in ACS patients, apoO was detected in both LDL and HDL particles.

Standardization of dot-blot sandwich analysis for plasma apoO concentration

To avoid potential nonlinearity caused by very low or high intensity, the apoO concentrations in plasma samples were measured using several dilutions (1:5 to 1:5,120). The analysis system we developed showed a dose-dependent response to human plasma in the dilution range of 1:5 to 1:160 (Fig. 3). Thus, a 20-fold dilution of plasma was chosen for routine use.

For calibration of the analysis, purified bacterial rhapoO was used as the calibrator. Serial dilutions (1:100 to 1:3200) of 0.1 µg/µl rhapoO were made to obtain a calibration curve (Fig. 4). The dot-blot was linear up to 1,000 ng/ml and suitable for quantifying apoO concentrations as low as 31.25 ng/ml.

Mean inter-assay imprecision of dot-blot, evaluated as CV, was 7.9% and intra-assay imprecision (CV) was 7.4%.

Clinical characteristics of CAD patients and control group

The clinical characteristics of coronary artery disease (CAD) patients and control group are shown in Table 1. There were no significant differences in gender, age, body mass index, and blood glucose between these groups. ACS patients had higher hypertension rates. As expected, triglycerides (1.85 ± 0.97 mmol/L vs. 1.27 ± 0.72 mmol/L, P < 0.027) were significantly higher in the ACS group whereas HDL cholesterol level (1.00 ± 0.22 mmol/L vs. 1.48 ± 0.34 mmol/L, P < 0.001) was markedly lower. Surprisingly, the plasma TC level in ACS patients (4.27 ± 1.33 mmol/L) was significantly lower than that in control subjects (4.85 ± 0.63 mmol/L, P < 0.001), conceivably because the modifications in lipids occur after ACS (8, 9). Hs-CRP concentration was significantly higher in patients with ACS (11.84 ± 21.30 mmol/L) than in controls (0.75 ± 0.36 mmol/L) (P < 0.001). In addition, five patients in ACS group took statins before admission.

Plasma apoO concentration in healthy subjects and CAD patients

As shown in Fig. 5, the average plasma apoO concentration was 2.21 ± 0.83 µg/ml, ranging from 1.05 to 5.47 µg/ml in healthy subjects, whereas it was 4.94 ± 1.59 µg/ml in
CAD patients. There were significant differences in plasma level of apoO between two groups ($P<0.001$). Sex did not influence apoO level in healthy subjects ($2.29$ vs. $2.15$ µg/ml, $p=0.893$ for males and females). In CAD patients, it was higher in males ($5.09 \pm 1.51$ µg/ml) than in females ($4.39 \pm 1.77$ µg/ml) although without statistic significance ($P=0.356$).

**Plasma apoO concentrations in relation to lipid profiles and hs-CRP**

To investigate the relationship between plasma apoO level and other lipid parameters, a correlation analysis was performed in both controls and ACS patients. To our surprise, no significant associations were observed between plasma apoO level and all other lipid parameters in either controls or ACS patients. However, significant positive correlation was detected between apoO and lg(hsCRP) in ACS patients ($r=0.480$, $P<0.001$) (Fig.6).

**Relationship between plasma apoO level and the risk of ACS**

To determine whether plasma apoO level is an independent predictor of ACS, a multiple logistic regression analysis was performed. After adjustment for confounding factors including age, gender, smoking, blood pressure, blood glucose, triglyceride, HDL cholesterol, LDL cholesterol, statins treatment, and hs-CRP, we demonstrated that apoO was an independent predictor of ACS (OR = 5.61, 95% CI 2.16–14.60, $P<0.001$).

**Lipopolysaccharide stimulates apoO expression in adipocytes and HepG2 cells**

To explore the effect of inflammatory stimulus on the expression of apoO, we detected the apoO mRNA expression using real-time PCR in adipocytes and HepG2 cells pretreated with lipopolysaccharide (LPS, 1000ng/ml). It has been shown that incubation of adipocytes and HepG2 cells with lipopolysaccharide for 24 h can cause a 7-fold and 2.5-fold increase in apoO mRNA expression, respectively, as compared with the control ($P<0.001$ for both) (Fig.7).

**DISCUSSION**

We developed a dot-blot sandwich analysis for plasma apoO concentration using a monoclonal antibody. The specificity of the antibody was confirmed by immunoblotting. It could react with purified rhapoO from *E. coli*. Furthermore, it could similarly react with a single protein of 55 kDa in human plasma and culture medium from HepG2 cells, which is the same as that reported previously for secreted apoO. The applied sandwich analysis can be used to measure up to 1,000 ng/ml plasma apoO with linearity. The lowest concentration of apoO detectable was about 31.25 ng/ml.

Fig. 3. Titration curve of human plasma. The dot-blot sandwich analysis was performed as described in Methods. The titration curve was made using serial dilutions (1:5 to 1:100, 5120) of human plasma. Each point represents the mean of triplicate determinations.

Fig. 4. Standard curve for purified rhapoO concentration. The standard curve was made using serial dilutions (1:100 to 1:3200) of 0.1 µg/µl purified rhapoO. Each point is the mean of triplicate determinations.
nese individuals and failed to find a significant correlation between plasma apoO concentrations in 111 healthy Chinese subjects showed that apoO mainly exists in nonlipoprotein fraction and HDL particles (6). Hence, its level in HDL particles is lower than that in plasma. This explains why apoO was not detected in the comprehensive protemics study aiming at the identification of HDL-associated proteins.

Although apoO was reported to have an apparent association with plasma HDL, the impact of apoO expression on plasma lipid and lipoprotein levels as well as on HDL functional properties still need to be further investigated. The availability of dot-blot sandwich analysis, which is used to detect apoO concentration in human plasma, will contribute to gaining insight into either the function of apoO or the mechanism involved. However, in our study, we detected plasma apoO concentrations in 111 healthy Chinese individuals and failed to find a significant correlation between apoO plasma level and HDL cholesterol or other lipid parameters in either males or females. Thus, we were unable to confirm the influence of apoO on plasma lipoprotein levels or HDL functionality. Earlier studies showed that purified apoO could elicit cholesterol efflux from macrophages in vitro (6), consistent with its role as an α-helix-containing apolipoprotein. However, Nijstad et al (7) recently demonstrated that high level overexpression of apoO by means of a recombinant adenovirus or AAV vector resulted in a substantial increase in plasma apoO levels as well as HDL apoO content in human apoA-I transgenic mice but left plasma HDL cholesterol levels and HDL functionality essentially unchanged. Hence, these data and the results of our study indicate that, in contrast to the reported efflux-eliciting properties in vitro, apoO as a constituent of HDL might not determine plasma HDL levels or function in vivo. The possibility remains that apoO has functions in the tissues it is expressed in. Therefore, further studies will be needed to investigate the precise functional importance of apoO in vivo.

More interestingly, our analysis showed that concentration of plasma apoO in ACS patients was higher than that in the control group. Despite part of apoO protein being distributed in LDL particles, we were unable to find a significant correlation between apoO plasma level and LDL cholesterol. Notably, correlation analysis between the concentration of plasma apoO and hs-CRP showed that plasma level of apoO is positively associated with hs-CRP. It is clear that inflammation play a vital role in the pathogenesis of ACS. In the vicinity of plaque formation, inflammation in ACS also occurs at a systemic level, not only from locally produced cytokines that are released into the circulation but also from liver proteins that are produced during the acute phase response. CRP is a pentraxin acute-phase protein and its level increases many hundred-fold in ACS (11, 12). Other apolipoproteins such as apoAIV and apoA-IV were demonstrated to be acute-phase proteins (13). In previous studies from our laboratory, we have shown that plasma apoAIV levels elevated in ACS patients (14). Furthermore, in vitro studies have shown that lipopolysaccharide, which is a kind of inflammatory stimulus, could significantly stimulate apoO expression in HepG2.

![Fig. 5. Plasma concentration of ApoO in CAD patients and the control group.](image)

**Table 1.** The clinical and biochemical data of the subjects

|                     | Control Group | ACS Group | P       |
|---------------------|---------------|-----------|---------|
| (n = 111)           | (n = 50)      |           |         |
| Gender (male/female)| 71/40         | 32/18     | NS      |
| Age (years)         | 63.2 ± 5.8    | 64.6 ± 9.3| NS      |
| BMI (kg/m²)         | 23.3 ± 2.8    | 23.5 ± 2.7| NS      |
| Hypertension (%)    | 10.1          | 33.9      | 0.000   |
| Blood glucose (mmol/L)| 4.82 ± 0.58| 4.78 ± 0.62| NS     |
| Triglycerides (mmol/L)| 1.27 ± 0.72| 1.85 ± 0.97| 0.027   |
| Total cholesterol (mmol/L)| 4.85 ± 0.63| 4.27 ± 1.33| 0.000   |
| HDL cholesterol (mmol/L)| 1.48 ± 0.34| 1.00 ± 0.22| 0.008   |
| LDL cholesterol (mmol/L)| 2.78 ± 0.58| 2.77 ± 1.25| NS      |
| hs-CRP (mg/L)       | 0.75 ± 0.36   | 11.84 ± 21.30| 0.000   |

ACS, acute coronary syndrome; BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein.
cells and adipocytes. Thus, even though we have no in vivo evidence, it is conceivable that apoO may also be a positive acute-phase protein that elevates during inflammation and the overproduction of apoO from liver and adipose tissue resulting from activated inflammation could be the reason for elevated apoO during ACS.

The systemic inflammatory mediators and acute phase reactants can act as biomarkers for ACS. In the present study, logistic regression analysis showed that plasma apoO level was an independent predictor of ACS. However, because of the limited samples, the predictive value of apoO as a biomarker for ACS and its potential functional role in plaque progression, instability, and rupture is to be further investigated.

In conclusion, using the dot-blot analysis system we developed, we detected plasma apoO concentration for the first time, and found that plasma apoO concentration was significantly increased and positively correlated with hs-CRP in ACS patients. However, no significant correlation between apoO and other lipid parameters was observed. These findings suggest that apoO might be regarded as an independent inflammatory predictor of ACS patients despite the precise functional importance of apoO remaining to be determined.

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