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Characterization of PLAC® tests in the quantization of lipoprotein associated phospholipase A_2 for assessment of cardiovascular diseases

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A B S T R A C T

Background: PLAC® mass test (diaDexus, Inc.) does not detect all Lp-PLA_2 proteins in the circulation. The total circulating Lp-PLA_2 mass can be quantized by using the CHAPS modified PLAC® mass test. To compare the difference of the PLAC® mass, CHAPS modified PLAC® mass and PLAC® activity tests in risk assessment of CVD, the 3 Lp-PLA_2 quantization methods were characterized using a collection of serum and plasma from CVD patents and matched non-symptomatic controls. Improvement on risk assessment for ischemic stroke by Lp-PLA_2 and lipids were also investigated.

Methods: Ninety one human sera and plasma from elderly patients with first CVD incidents and 78 matched controls were collected at clinics. Lp-PLA_2 was assessed by PLAC® mass, CHAPS modified PLAC® mass and PLAC® activity tests and data were subjected to statistical analyses. Correlation with lipid cholesterols or Apo proteins was compared for all formats of PLAC® tests. Ratios of Lp-PLA_2 by different PLAC® tests to different lipids were assessed for synergistic enhancement in the indication of ischemic stroke.

Results: The PLAC® mass test was superior to other formats of PLAC® tests in the assessment of CVD and is independent of lipids. The Lp-PLA_2 by the CHAPS modified PLAC® mass test has no separation between the CVD and control groups.

Conclusions: Both PLAC® mass and PLAC® activity tests are effective but the CHAPS modified PLAC® mass test has no or less utility in the risk assessment of CVD. The ratio of Lp-PLA_2 by either PLAC® mass or PLAC® activity over ApoA1 or (Apo A1 + Apo B) synergistically enhance the risk assessment power for ischemic stroke.

1. Introduction

Atherosclerosis and cardiovascular diseases (CVD) have remained the leading causes of morbidity and mortality globally [1]. According to World Health Organization (WHO), ischemic heart disease and stroke are the world's biggest killers, accounting for a combined 15 million deaths in 2015 [1]. Risk prediction for the population and stratification of CVD patients for targeted therapy are keys to reduce the morbidity and mortality rates. The traditional Framingham Risk Score includes age, sex, blood pressure, serum total cholesterol or low-density lipoprotein (LDL) cholesterol concentrations, high-density lipoprotein (HDL) cholesterol concentrations, smoking, and diabetes which together account for most of the excess risk for incident coronary heart disease (CHD) [2]. However, the Framingham Risk Score does not explain the entire excess risk, and approximately 40% of CHD deaths occur in individuals with cholesterol concentrations lower than the population average [3]. In the search for other biomarkers, it has been demonstrated that the elevated circulating activity or mass (concentration) of lipoprotein associated phospholipase A_2 (Lp-PLA_2) is independently associated with the risk of adverse outcomes in patients with CHD and ischemic stroke [4–10]. The enzyme was found to hydrolyze glycerophospholipids such as platelet-activating factor (PAF) containing short and/or oxidized functionalities at the sn-2 position and generate lysoPAF/lyso phosphatidylcholine (lysoPC) and short and/or oxidized fatty acids [11], many of which also have been reported to have pro-inflammatory and pro-oxidative activities. Based on this mechanism, Lp-PLA_2 was thought to actively contribute to vascular inflammation and atherogenesis [12]. However, a potent Lp-PLA_2

Abbreviations: ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; IHD, ischemic heart disease; Lp-PLA_2, lipoprotein associated phospholipase A_2; PAF, platelet-activating factor; PC, phosphatidylcholine; RMSE, root mean square error; TGLIP, triglyceride lipase

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inhibitor, darapladib, failed two pivotal trials to meet both primary and secondary endpoints [13,14]. In patients with stable coronary heart disease, darapladib did not significantly reduce the risk of the primary composite end point of cardiovascular death, myocardial infarction, or stroke. Lp-PLA_2 is thus unlikely to represent a causal factor in atherogenesis but remains a biomarker for CVD. Recent publication suggested that increased enzyme activities actually reflected a response to the pro-inflammatory/pro-oxidative stress that is typical of atherosclerosis [15].

Two platforms of Lp-PLA_2 detection methods were used for clinical studies, either by immunological detection of mass or by enzymatic detection of catalytic activity. Majority of the clinical studies used the commercial PLAC® mass and PLAC® activity tests, manufactured by diaDexus Inc. The lack of a correlation between these 2 PLAC® tests were long recognized by the research community [16] and the biochemical difference and the cause of that have recently been illustrated [17]. Majority of Lp-PLA_2 concentration by PLAC® mass were significantly under estimated due to the matrix effect of lipoproteins. Dissociation of Lp-PLA_2 from lipoproteins by detergents substantially increased the values of Lp-PLA_2 concentration in serum or plasma and improved the correlation between the Lp-PLA_2 mass and activity assays. One emerging question is how the PLAC mass test reflects the elevation of Lp-PLA_2 despite it only partially detects the protein in serum or plasma. In order to further understand the molecular mechanism of Lp-PLA_2 in the association with CVD risk, the mass, the detergent (CHAPS) modified mass and the activity of PLAC® tests were characterized using a small collection of serum and plasma samples consisting of 91 CVD cases and 78 matched non-symptomatic controls all from elderly donors. The first goal of the study is aimed to investigate the effectiveness of total Lp-PLA_2 mass detected by the detergent (CHAPS) modified PLAC® mass test in the risk assessment of CVD and its relationship to the total Lp-PLA_2 activity. For cardiovascular disease, the individual components of the Framingham score, such as total and low-density lipoprotein cholesterol, systolic blood pressure, or even smoking, all have small hazard ratios, typically in the range 1.5 to 2.5 [18]. Recent studies discover that total cholesterol becomes less of a risk factor or not at all for all-cause and cardiovascular (CV) mortality with increasing age [19]. We also investigated the relationship between Lp-PLA_2 and lipoproteins and search for possible improvement in the assessment of ischemic risk, especially for elderly patients at age ≥ 70 and with normal or low total cholesterol concentrations.

2. Methods

2.1. Materials

PLAC® mass and PLAC® activity test kits were products of diaDexus, Inc. Modified PLAC® mass kits were prepared as described in by Zhuo, et al. [17].

2.2. Serum specimens

Human sera and plasmas were obtained as archival samples from a commercial vendor Proteogenex who collected blood samples from consenting donors of visiting patients and volunteers at undisclosed hospitals during the time period of 2010–2013. All donors were Caucasian. Details of specimen handling and storage are as described by Cerelli, et al. [20]. The 169 samples consisted of 33 (19.5%) ischemic stroke, 16 (9.5%) hemorrhagic stroke, 25 (14.8%) acute myocardial infarction (AMI), and 17 (10.0%) ischemic heart disease/hypertension (IHD) patients with 78 (46.2%) matched non-symptomatic controls. The 78 matched control donors had no obvious acute syndromes but some of the donors did have chronic conditions including ischemic, hypertension, cystitis, pancreatitis, gastritis, prostatitis, bronchitis, and cholecystitis. All stroke cases were first time occurrence and the diagnoses were confirmed by magnetic resonance imaging (MRI) with size of brain lesion ranging 0.04–0.24 cm^2. AMI diagnosis was confirmed by both electrocardiogram and troponin-l increase (2.5–10.5 μg/l of troponin-l). The 17 IHD patients were not clearly diagnosed due to hospital conditions but all had hypertension. No left ventricle ejection fraction (< 40%) was measured for IHD patients. No patients or non-symptomatic controls were treated with statin or Lp-PLA_2 inhibitors before blood donation. Bloods were collected between 12 and 24 h after CVD incidence. Efforts were made to match the control and CVD composite samples as much as possible.

2.3. Measurement of the Lp-PLA_2 activity and mass concentration, and lipid panel concentration in serum and plasma

Serum Lp-PLA_2 activity and mass concentration were measured by using PLAC® activity and mass test kits (diaDexus, Inc.) following the manufacturer's instruction. PLAC® mass measurements were performed by manual ELISA and PLAC® activity measurements were carried out by the Beckman Coulter AU400 analyzer as described by Cerelli et al. [20]. CHAPS-modified PLAC® ELISA test kits were made by adding solid CHAPS to the assay buffer to the final concentration of 10 mmol/l. The same procedures were followed as that of the unmodified PLAC® mass test kits. The lipid panel included total cholesterol, HDL cholesterol, calculated LDL cholesterol, VLDL cholesterol, triglyceride, Apo-AI, Apo-B, Lp(a) and triglyceride lipase (TGLIP) and was determined by Hunter Laboratories.

2.4. Statistics

Data were analyzed using JMP v12 and Graphpad Prism v6. Associations between Lp-PLA_2 levels and CVD/stroke cases or other demographics were assessed by linear or logistic regression models. Mean values of Lp-PLA_2 mass or activity and lipid panel levels were compared between CVD event cases and non-cases using *t*-tests or one-way ANOVA for continuous variables and χ^2 tests for categorical variables. A p-0.05 (Prob > F or Prob > ChiSq) for interaction was considered statistically significant.

3. Results

3.1. Baseline characteristics

Baseline characteristics of the study population are shown in Tables 1 and 2. The effects of demographics and other factors on the separation of Lp-PLA_2 mass or activity values for all 169 samples were assessed by One Way ANOVA and the mean Lp-PLA_2 value, SD or root mean square error (RMSE) and p-values are listed. All donors were generally elderly with average age of 73 y for CVD and 71 y for control groups and slightly overweight with average BMI of 26–27. Samples were matched with 58% female for non-symptomatic control and 64% female for CVD composite in this study. No significant correlation of all 3 formats of Lp-

Table 1
Matching of donor serum and plasma samples.

Sample donor	N	Female	Male	Ave. age	Ave. BMI	Hypertension	Smoking	Serum	Plasma
Non-symptomatic control	78	57.7%	42.3%	70.5 ± 7.6	26.1 ± 2.1	20.5%	15.4%	67.9%	32.1%
CVD composite	91	63.7%	36.3%	73.1 ± 6.9	26.7 ± 2.0	92.2%	1.1%	72.5%	27.5%