Circulating Levels of Secretory Type II Phospholipase A₂ Predict Coronary Events in Patients with Coronary Artery Disease

Kiyotaka Kugiyama, MD; Yasutaka Ota, MD; Keiji Takazoe, MD; Yasushi Moriyama, MD; Hiroaki Kawano, MD; Yuji Miyao, MD; Tomohiro Sakamoto, MD; Hirofumi Soejima, MD; Hisao Ogawa, MD; Hideki Doi, MD; Seigo Sugiyama, MD; Hirofumi Yasue, MD

Background—The circulating levels of secretory nonpancreatic type II phospholipase A₂ (sPLA₂) are increased in various chronic inflammatory diseases and the increase in the levels correlates with the disease severity. sPLA₂ may possibly play a role in atherogenesis and is highly expressed in atherosclerotic arterial walls that are known to have inflammatory features. Thus, this study prospectively examined whether circulating levels of sPLA₂ may have a significant risk and prognostic values in patients with coronary artery disease (CAD).

Methods and Results—Plasma levels of sPLA₂ were measured in 142 patients with CAD and in 93 control subjects by a radioimmunoassay. The sPLA₂ levels had a significant and positive relations with serum levels of C-reactive protein, a marker of systemic inflammation, and with the number of the traditional coronary risk factors associated with individuals. Multivariate logistic regression analysis showed that higher levels of sPLA₂ (>246 ng/dL; 75th percentile of sPLA₂ distribution in controls) were a significant and independent risk factor for the presence of CAD. In multivariate Cox hazard analysis, the higher levels of sPLA₂ were a significant predictor of developing coronary events (ie, coronary revascularization, myocardial infarction, coronary death) during a 2-year follow-up period in patients with CAD independent of other risk factors, including CRP levels, an established inflammatory predictor.

Conclusions—The increase in circulating levels of sPLA₂ is a significant risk factor for the presence of CAD and predicts clinical coronary events independent of other risk factors in patients with CAD; these results may reflect possible relation of sPLA₂ levels with inflammatory activity in atherosclerotic arteries. (Circulation. 1999;100:1280-1284.)

Key Words: atherosclerosis • coronary disease • lipids • prognosis • risk factors

Phospholipases A₂ (PLA₂) are ubiquitous enzymes that hydrolyze the sn-2-acyl bond of phospholipids of cell membrane and lipoproteins and yield free fatty acids and lysophospholipids, precursors of various proinflammatory lipid mediators including leukotrienes, eicosanoids, prostaglandins, and platelet-activating factors.¹⁻³ Several studies showed that secretory nonpancreatic type II phospholipase A₂ (sPLA₂) might importantly contribute to the pathogenesis of various inflammatory diseases.²,³ Recently, sPLA₂ was found to be highly expressed in human atherosclerotic arterial walls,⁴⁻⁶ where the inflammatory process is known to have a pathogenetic role.⁷⁻⁹ The lipid products generated through sPLA₂ and their related products, including modified LDLs, participate in the development of atherosclerosis and play a significant role in the pathogenesis of coronary artery disease (CAD).⁸⁻¹² There is growing evidence²,³,¹³,¹⁴ that the circulating levels of sPLA₂ are increased in various inflammatory diseases and that the levels reflect severity of these diseases, including rheumatoid arthritis, inflammatory bowel diseases, and sepsis. Thus, it is expected that the circulating levels of sPLA₂ may also be increased in patients with CAD. In this study, we prospectively examined whether the circulating levels of sPLA₂ may have a significant risk and prognostic values in patients with CAD.

Methods

Study Patients

This study enrolled 196 consecutive patients with CAD who underwent elective and diagnostic cardiac catheterization for chest pain or ischemic changes in ECG in Kumamoto University Hospital. The patients with CAD who were admitted for the planned revascularization therapy were not eligible for this study. All patients had angiographic documentation of organic stenosis of >70% of at least one major coronary artery. Forty-nine patients were excluded because they had one of the following exclusion criteria: myocardial infarction, major surgery and trauma, and serious infectious diseases within previous 4 weeks; malignancies; and chronic inflammatory diseases, including rheumatoid arthritis, osteoarthritis, and inflammatory bowel disease. Ultimately, 147 (75%) of the 196 patients were included in this study. Patients’ characteristics are shown in Table 1.

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TABLE 1. Baseline Characteristics of Study Patients and Controls

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=93)</th>
<th>Patients With Coronary Artery Disease (n=142)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60 (50, 69)</td>
<td>62 (52, 66)</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>63 (68)</td>
<td>99 (70)</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>40 (42)</td>
<td>65 (46)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>9 (10)</td>
<td>53 (37)†</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>26 (27)</td>
<td>66 (46)†</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>198±39</td>
<td>201±38</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>50±14</td>
<td>41±11†</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>121±38</td>
<td>131±35*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>118 (77, 173)</td>
<td>118 (88, 181)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23±3</td>
<td>24±3</td>
</tr>
<tr>
<td>No. of risk factors</td>
<td>1.3±1.0</td>
<td>2.2±1.1†</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.24 (0.08, 0.25)</td>
<td>0.25 (0.09, 0.56)‡†</td>
</tr>
<tr>
<td>Unsuitable angina (%)</td>
<td>...</td>
<td>54 (38)</td>
</tr>
<tr>
<td>Unstable angina (%)</td>
<td>...</td>
<td>34 (24)</td>
</tr>
<tr>
<td>Peripheral artery (%)</td>
<td>...</td>
<td>10 (7)</td>
</tr>
</tbody>
</table>

This study initially included 147 patients with coronary artery disease. Five patients with coronary artery disease were lost to follow-up and were excluded. Values in parentheses represent median (25th and 75th percentiles) except where noted.

BMI indicates body mass index; CRP, C-reactive protein; and MI, myocardial infarction. *P<0.05, †P<0.01 vs control subjects (χ² test or unpaired t test). ‡P=0.02 vs control subjects (Mann-Whitney U test).

Biomedical Measurements

Venous blood was taken from all of the study patients and control subjects after an overnight fast. Blood samples, anticoagulated with EDTA or citrate, were immediately centrifuged at 3000 rpm at 4°C for 10 minutes. The plasma was aliquoted and stored at −80°C until analyzed. Levels of immunoreactive sPLA₂ in EDTA-plasma were measured by a radioimmunoassay using a monoclonal antibody developed against membrane-associated PLA₂, which was purified from human spleen and was identical with type IIA PLA₂ purified from rheumatoid arthritic synovial fluid (Shionogi Pharmaceutical Ltd., Osaka, Japan).15,16 This monoclonal antibody had no detectable cross-reactivity with human pancreatic PLA₂ (type IB).14,15 The radioimmunoassay gave a linear response in a range from 78 to 5000 ng/dL of sPLA₂.14,15 The interassay and intra-assay coefficients of variation were <8%.14,15 The plasma levels of the immunoreactive sPLA₂ had a significant correlation with the calcium-dependent PLA₂ activity in the citrated plasma (r=0.923, P<0.0001, n=88 by Spearman’s rank correlation test), a result which is compatible with that in previous reports.13,14 Serum levels of C-reactive protein (CRP) were measured in all of the control subjects and the patients with CAD using an N Latex CRP immunodetection kit (Dade Behring).14,15 Serum levels of total cholesterol, triglycerides, and HDL-cholesterol were measured by the enzymatic methods.17 and LDL-cholesterol levels were calculated as previously described.17

Follow-Up Study

After laboratory samples and catheterization data were obtained, the 147 patients with CAD (1-vessel disease, 70 patients; 2-vessel disease, 34 patients; three-vessel disease, 27 patients; left main coronary stenosis, 16 patients) were followed up every month in hospital or with a clinic visit for a maximum of 24 months or until occurrence of one of the following clinical coronary events: recurrent or refractory angina pectoris requiring coronary revascularization by PTCA or CABG, nonfatal myocardial infarction, and cardiac death. Time from the day when blood sampling was performed to first coronary event was prospectively evaluated. Diagnosis of myocardial infarction was made by chest pain, appearance of new Q wave on the ECG, and elevation of creatine kinase enzyme to more than twice the upper limit of normal. Cause of death was determined from hospital records. For the study, revascularization therapy based only on angiographic data were not counted as a coronary event. All of the patients received standardized medical therapy. The patients with high extent of CAD (3-vessel disease or left main coronary stenosis) who were included in this study were also followed up without revascularization therapy immediately after the inclusion because of diffuse peripheral CAD. Cutoff a high (≥46) between previous CAD or repeated PTCA, or unwillingness for the revascularization therapy. The attending physician and interventional cardiology independent of this prospective study decided the need for and timing of revascularization.

Statistical Analysis

Because sPLA₂ levels were not distributed normally, results of sPLA₂ levels were expressed as median and range (25th and 75th percentiles) and nonparametric analyses were used. Mann-Whitney U test was used to evaluate difference in sPLA₂ levels between the 2 groups. Spearman’s rank correlation test was used for relations of sPLA₂ levels with CRP levels and number of the coronary risk factors associated with individuals. To evaluate sPLA₂ levels as an independent risk factor differing between the patients with CAD and the control subjects, forward, stepwise, multiple logistic regression analysis was performed using the following factors as categorical covariates: smoking history (defined as smoking at least 10 cigarettes per day for ≥10 years), hypertension (≥140/90 mm Hg or requiring antihypertensive medication), diabetes mellitus (according to World Health Organization criteria,18 hypercholesterolemia (≥220 mg/dL or the use of lipid-lowering medications), high LDL-cholesterol (≥130 mg/dL), low HDL-cholesterol (<35 mg/dL), and high CRP levels (>0.48 mg/dL). 90th percentile of the distribution of CRP levels in the control subjects). Kaplan-Meier method (log-rank test) was applied in survival analysis according to the levels of sPLA₂. The predictive value for coronary events during the follow-up period was assessed by Cox proportional hazard analysis. The multivariate Cox analysis always included the following factors as categorical covariates: sPLA₂ levels, stenosis of the left main coronary artery, number of coronary arteries with stenosis, low left ventricular ejection fraction (LVEF) on baseline left ventriculography (<50%), age (<70 years), sex (male), smoking history, hypertension, diabetes mellitus, hypercholesterolemia, high LDL-cholesterol, and low HDL-cholesterol. Cox hazard model and Kaplan-Meier analyses, sPLA₂ levels were divided into tertiles that were based on 90th and 75th percentiles of the distribution of the sPLA₂ levels in the control subjects. In Cox hazard model and Kaplan-Meier analyses, sPLA₂ levels were divided into tertiles that were based on the number of coronary arteries with stenosis, stenosis of the left main coronary artery was counted as 2-vessel disease. Mean values of continuous variables with normal distribution and frequencies among subgroups were compared by unpaired t test and χ² analysis, respectively. Statistical significance was defined as P<0.05. The analyses were performed partly using SPSS Professional Statistics 6.1 for the Macintosh (SPSS Japan Inc.).
**Results**

**sPLA₂ as a Coronary Risk Factor**

The distribution of the levels of sPLA₂ immunoreactivity in patients with CAD was skewed and shifted to lower levels with a median level of 286 (218 and 386) ng/dL, which was significantly higher than that in control subjects (191 [154 and 246] ng/dL) \(P<0.0001\). The levels of sPLA₂ had a significant and positive correlation with CRP levels (Figure 1). As shown in Figure 2, the levels of sPLA₂ were significantly related with the number of the traditional coronary risk factors associated with individuals. There was no significant association of higher sPLA₂ levels \( (>246 \text{ ng/dL}) \) with stable angina (median [25th and 75th percentiles], 309 ng/dL [243, 461] versus 268 ng/dL, 10th and 90th percentiles, respectively; \(P=0.03\)). The sPLA₂ levels were higher in patients with peripheral artery disease (diagnosed by angiography) than those without it (median [25th, 75th], 377 ng/dL [311, 583] versus 279 ng/dL, [217, 365], n=10 and 132, respectively; \(P=0.02\)).

**sPLA₂ as a Predictor of Coronary Events in Patients with CAD**

All of the patients received the standard medical therapy consisting of a combination of calcium channel blockers (used in 82% of patients), \(\beta\)-blockers (41%), nitrates (70%), angiotensin-converting enzyme inhibitor (38%), aspirin (93%), and lipid-lowering drugs (34%) during the follow-up study. Seven patients with unstable angina and high extent of CAD had intravenous infusion of heparin and nitrates for several days (2 to 7 days) after the inclusion. Only 5 patients with CAD were lost to follow-up. The remaining 142 patients with CAD were followed for a mean duration of 17.2 months (range, 0.5 to 24 months). The patients with higher levels of sPLA₂ had more frequent coronary events during the follow-up period (data not shown). Kaplan-Meier analysis demonstrated a significantly higher probability of developing the clinical coronary events in the patients with the higher levels of sPLA₂ than those with the lowest levels, as shown in Figure 3. In univariate Cox proportional hazard model analysis, higher levels of sPLA₂ (Table 3), higher levels of CRP \( (>0.48 \text{ mg/dL}) \), and low HDL-cholesterol \( (<35 \text{ mg/dL}) \) were the variables differing significantly and independently between the patients with CAD and the control subjects, as shown in Table 2. The sPLA₂ levels were significantly higher in patients with unstable angina (the class B of Braunwald’s classification) than stable angina (median [25th and 75th percentiles], 309 ng/dL [243, 461] versus 268 ng/dL [217, 338], n=34 and 108, respectively; \(P=0.03\)). The sPLA₂ levels were higher in patients with peripheral artery disease (diagnosed by angiography) than those without it (median [25th, 75th], 377 ng/dL [311, 583] versus 279 ng/dL, [217, 365], n=10 and 132, respectively; \(P=0.02\)).

**TABLE 2. Multiple Logistic Regression Analysis: Final Significant Variables Differing Between Patients with CAD and Control Subjects**

<table>
<thead>
<tr>
<th>Variables</th>
<th>(\beta)-Coefficient</th>
<th>SE</th>
<th>(P)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>sPLA₂ (&gt;246 \text{ ng/dL})</td>
<td>1.67</td>
<td>0.36</td>
<td>&lt;0.00001</td>
<td>5.3</td>
<td>1.6–17.3</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.76</td>
<td>0.48</td>
<td>0.0002</td>
<td>5.8</td>
<td>1.1–30.1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.08</td>
<td>0.37</td>
<td>0.004</td>
<td>3.0</td>
<td>1.3–6.6</td>
</tr>
<tr>
<td>Constant</td>
<td>-1.48</td>
<td>0.40</td>
<td>0.0002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Forward, stepwise, multiple logistic regression analysis was performed using the following factors as categorical covariates: sPLA₂, smoking history, hypertension, diabetes mellitus, hypercholesterolemia, high levels of LDL-cholesterol, low levels of HDL-cholesterol, and high CRP levels \( (>0.48 \text{ mg/dL}) \).

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Figure 1. Relation between levels of sPLA₂ and C-reactive protein in patients with CAD.

Figure 2. Box and whisker plots showing relation between sPLA₂ levels and the number of the traditional coronary risk factors associated with individuals, including age (\(\geq 70\) years), histories of smoking, diabetes mellitus, hypertension, hypercholesterolemia (\(>220 \text{ mg/dL}\)), and low HDL-cholesterol (\(<35 \text{ mg/dL}\)) in patients with CAD and control subjects. Lines within boxes represent median values; upper and lower lines of boxes, 75th and 25th percentiles, respectively; upper and lower bars outside of boxes, 90th and 10th percentiles, respectively. \(n=256\), \(P<0.0005\).
sPLA2 was an independent risk factor for the presence of CAD and that the increase in the levels predicted the development of clinical coronary events in patients with CAD. There is increasing evidence that atherosclerosis is an inflammatory disease that develops in response to the coronary risk factors.\(^{7,9,12}\) In this context, the present study further showed that the circulating levels of sPLA2 were significantly correlated with the levels of CRP, a marker of systemic inflammation, and with the number of the traditional coronary risk factors associated with individuals. These imply that the increase in sPLA2 levels in patients with CAD may reflect the inflammatory activity in the development of atherosclerosis. sPLA2 may be one of the inflammatory markers, but sPLA2 is a better inflammatory predictor of coronary events than CRP, a well-established inflammatory prognostic marker because sPLA2 but not CRP remained a significant and independent predictor in the multivariate Cox hazard analysis when addition of both factors into the covariates.

sPLA2 is shown to be induced in vascular cells by cytokines such as interleukin-1 and tumor necrosis factor-\(\alpha\),\(^ {2,3,19–21} \) which abundantly exist in atherosclerotic arterial walls and have crucial roles in the inflammatory and immunological features in atherosclerotic development.\(^ {7,9,11} \) These cytokines could stimulate synthesis of sPLA2 in the atherosclerotic arterial walls and release into circulation, resulting in the elevation of the sPLA2 levels in patients with CAD.

The lipid products generated through PLA2 and their related products, such as lysophosphatidylcholine and modified LDL, are proatherogenic and proinflammatory\(^ {2,3,10,11} \) and they activate vascular cells to produce plasminogen activators (PA), PA inhibitor-1, adhesion molecules, various proatherogenic cytokines and growth factors, and oxygen free radicals,\(^ {10,22–24} \) leading to atherothrombotic development and plaque instability in the atherosclerotic arterial walls.\(^ {9,12,25} \) This atherothrombogenic role of sPLA2 may result in the association of the increased levels of sPLA2 with the high frequency of future coronary events in patients with CAD, as observed in our study. The causative role of sPLA2 levels in human plaque unstabilization is now under investigation in our laboratory.

**Discussion**

The study demonstrated that the increase in plasma levels of sPLA2 was an independent risk factor for the presence of CAD and that the increase in the levels predicted the development of clinical coronary events in patients with CAD. There is increasing evidence that atherosclerosis is an inflammatory disease that develops in response to the coronary risk factors.\(^ {7,9,12} \) In this context, the present study further showed that the circulating levels of sPLA2 were significantly correlated with the levels of CRP, a marker of systemic inflammation, and with the number of the traditional coronary risk factors associated with individuals. These imply that the increase in sPLA2 levels in patients with CAD may reflect the inflammatory activity in the development of atherosclerosis. sPLA2 may be one of the inflammatory markers, but sPLA2 is a better inflammatory predictor of coronary events than CRP, a well-established inflammatory prognostic marker because sPLA2 but not CRP remained a significant and independent predictor in the multivariate Cox hazard analysis when addition of both factors into the covariates.

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**TABLE 3. Cox Proportional Hazard Model Analysis of Risks of Developing Coronary Events According to the Levels of sPLA2 in Patients with CAD**

<table>
<thead>
<tr>
<th>sPLA2 levels (ng/dL)</th>
<th>No. of Patients with Coronary Events (%</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Odds Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>&gt;366 ng/dL (n=37)</td>
<td>18 (49)</td>
<td>3.88</td>
<td>1.7–9.0</td>
</tr>
<tr>
<td>247–366 ng/dL (n=58)</td>
<td>23 (39)</td>
<td>3.04</td>
<td>1.2–7.4</td>
</tr>
<tr>
<td>≤246 ng/dL (n=47)</td>
<td>7 (15)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

sPLA2 levels were divided into tertiles based on 90th and 75th percentiles (366 and 246 ng/dL, respectively) of the distribution of sPLA2 levels in control subjects. sPLA2 levels, stenosis of left main coronary artery, number of diseased coronary arteries, age (≥70 y), male sex, histories of smoking, diabetes mellitus, and hypertension, hypercholesterolemia, high levels of LDL-cholesterol, low levels of HDL-cholesterol, and low LV ejection fraction were included as categorical covariates on multivariate analysis.
Recent reports have demonstrated that the increase in levels of CRP was associated with CAD. CRP is heptically derived and has an uncertain physiological role in atherosclerotic development and in CAD. sPLA2 could be also one of acute phase reactants. However, unlike CRP, the lipid mediators produced through sPLA2 and their related lipids in the arterial walls can stimulate T cells and macrophages to synthesize and release the proatherogenic and proinflammatory cytokines. This positive feedback mechanisms could amplify this sequence of events in the atherosclerotic arterial walls, thereby sPLA2 present in the arterial walls may play an important role in the pathogenesis of CAD, not simply as a marker of inflammation.

This study showed that diabetes mellitus, hypertension, and high sPLA2 levels were significant and independent not simply as a marker of inflammation. These positive feedback mechanisms did not necessarily be provoked at the sites of traditional risk factors during the follow-up by medications and improvement of lifestyle. Our study also showed that extensive coronary diseases (left main coronary disease and 3-vessel disease) had weak but significant probability for clinical coronary events in the univariate analysis. However, presence of the extensive coronary diseases alone may not be related directly to the significant probability of coronary events, which will not necessarily be provoked at the sites of severe coronary stenosis on baseline angiograms. Other associated risk factors may additionally contribute to the significant probability because the extensive coronary diseases did not remain significant in the multivariate analysis.

This study is limited by the small number of the studied patients. Also, a case-control study has cross-sectional nature and it may have inherent selection bias of cases and controls. A trial with specific inhibitors of sPLA2 activity in a large number of study patients with homogeneous risk is required to assess the precise role of sPLA2 in the pathogenesis of CAD.

In conclusion, high levels of sPLA2 in the circulation have an independent risk factor for the presence of CAD and predict future coronary events in patients with CAD.

Acknowledgments

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References

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