Elevated secretory non-pancreatic type II phospholipase A2 serum activity is associated with impaired endothelial vasodilator function in patients with coronary artery disease

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ABSTRACT

Low-grade inflammatory activity is associated with an increased risk for ischaemic coronary events. sPLA2 (secretory non-pancreatic type II phospholipase A2) serum activity is increased in chronic inflammatory diseases and may also contribute to atherogenesis. Since the endothelium is a major target for inflammatory cytokines, we hypothesized that elevated serum activity of sPLA2 is associated with an impaired vasodilator function in patients with documented CAD (coronary artery disease). Endothelium-dependent (acetylcholine, 10–50 µg/min) and endothelium-independent (sodium nitroprusside, 2–8 µg/min) FBF (forearm blood flow) responses were measured by venous occlusion plethysmography in 50 male patients with angiographically documented CAD. sPLA2 serum activity was inversely correlated with acetylcholine-induced FBF responses ($r = -0.36; P < 0.05$). In addition, there was a significant correlation between sPLA2 and CRP (C-reactive protein; $r = 0.33, P < 0.02$). In contrast, FBF responses to sodium nitroprusside did not correlate with sPLA2 serum activity. In order to identify independent predictors of an impaired endothelium-dependent vasodilator function in patients with CAD, a multivariate analysis was performed including the inflammatory serum markers as well as classical risk factors of CAD. This analysis demonstrated that both sPLA2 ($P < 0.05$) and CRP serum levels ($P < 0.05$) were the only significant independent predictors of an impaired acetylcholine-induced FBF response. In conclusion, elevated sPLA2 serum activity is associated with a significant impairment in systemic endothelial vasodilator function in patients with CAD. The identification of sPLA2 as a novel independent predictor for endothelial dysfunction provides another important clue to link a systemic marker of inflammation with coronary atherosclerotic disease.

INTRODUCTION

Atherosclerosis is thought to be a chronic inflammatory disease of the vessel wall [1]. Different proinflammatory cytokines have been suggested to be promoter, actor and markers in patients with established CAD (coronary artery disease) as well as in patients with risk factors for CAD [2,3]. Recently, circulating levels of sPLA2

Key words: coronary artery disease, C-reactive protein (CRP), endothelial function, forearm blood flow, inflammation, secretory non-pancreatic type II phospholipase A2 (sPLA2).

Abbreviations: ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome; AUC, area under the curve; CAD, coronary artery disease; COX, cyclo-oxygenase; CRP, C-reactive protein; FBF, forearm blood flow; HDL, high-density lipoprotein; IL-1β, interleukin-1β; IL-6, interleukin-6; LDL, low-density lipoprotein; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; sPLA2, secretory non-pancreatic type II phospholipase A2; TNF-α, tumour necrosis factor-α.

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(secretory non-pancreatic type II phospholipase A2) have been described as an independent risk factor for the presence of CAD and future clinical coronary events [4].

PLA2 are ubiquitous enzymes that hydrolyse the sn-2-acyl bond of cell membrane phospholipids and lipoproteins and yield free fatty acids and lysophospholipids, precursors of various proinflammatory lipid mediators, including leukotrienes, prostaglandins and platelet-activating factor [5].

Proinflammatory cytokines lead to an increase in expression and secretion of sPLA2 from different organs and tissues, including atherosclerotic plaques [6], where sPLA2 was found to be highly expressed [6,7]. Extracellular sPLA2 is mainly localized at sites where it hydrolyses phospholipids from lipoproteins and lipid aggregates retained in the extracellular matrix of the arterial wall. This may be a potential mechanism for in situ release of proinflammatory lipids, free fatty acids and lysophosphatidylcholine in regions of apolipoprotein B accumulation, which are abundant in atherosclerotic lesions [8]. Importantly, we demonstrated recently [9] that sPLA2 induces the expression of chemokines and adhesion molecules in microvascular endothelium.

Thus, since the endothelium is a major target for inflammatory cytokines and different cytokines have been shown to be associated with an impaired endothelial function [10–14], we hypothesized that elevated serum activity of sPLA2, as an acute-phase reactant with properties to catalyse the production of lipid mediators, is associated with an impaired vasodilator function in patients with documented CAD.

METHODS

Patients

Fifty male patients with angiographically documented CAD were studied. Patients with inflammatory disease or malignancy, troponin T levels > 0.1 ng/ml and left ventricular ejection fraction < 45 % were excluded. Vasodilator medications, including calcium-channel blockers, ACE (angiotensin-converting enzyme)-inhibitors and long-acting nitrates, were withheld at least 24 h prior to the study. All patients were on chronic aspirin (100 mg/day) and chronic β-blocker therapy. The clinical characteristics of these patients are summarized in Table 1.

All patients gave written informed consent. The study protocol was approved by the Ethical Committee of the Johann Wolfgang Goethe University of Frankfurt/Main.

Study protocol

FBF (forearm blood flow) measurements were performed in the morning in a quiet and temperature-controlled (22 °C) laboratory as described previously [12]. Patients were asked to refrain from smoking and alcohol and caffeine intake for 12 h before the study. After local anaesthesia (< 1.5 ml of 2 % Mepivacain; Astra Pharma, Wedel, Germany), a 22-gauge catheter (Braun, Melsungen, Germany) was inserted under sterile conditions into the arteria brachialis of the non-dominant arm for infusion of vasoactive substances and saline solution. After arterial puncture, patients were allowed to rest for at least 20 min. FBF (measured as ml·min⁻¹·100 ml⁻¹ of forearm tissue) was measured with venous occlusion plethysmography (model EC-4; D. E. Hokanson, Bellevue, WA, U.S.A.), with calibrated mercury-in-Silastic strain gauges applied to the widest part of the forearm. Upper arm cuffs were intermittently inflated to 40 mmHg for 10 s every 15 s to temporarily prevent venous outflow (Rapid cuff inflator E-10; D. E. Hokanson). To exclude hand circulation from the blood flow, a wrist cuff was inflated to suprasystolic pressure 1 min before the measurements. FBF measurements were recorded, and six readings were obtained for each measurement. Basal FBF was measured under infusion of physiological saline solution with a constant-rate infusion pump (1 ml/min; Braun, Melsungen, Germany). For assessment of endothelium-dependent vasodilation, acetylcholine (Ciba Vision GmbH, Wessling, Germany) was infused intra-arterially in increasing doses of 10, 20, 30, 40 and 50 µg/min. To assess endothelium-independent vasodilation, sodium nitroprusside (Schwarz Pharma, Mohnheim, Germany) was infused intra-arterially in increasing doses of 2, 4, 6 and 8 µg/min.

Laboratory analysis

At the time of the FBF study, blood samples were collected, and serum and plasma were aliquoted and stored at −80 °C until analysed for the measurement of sPLA2 activity in serum and plasma high-sensitivity CRP (C-reactive protein) levels.

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
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<tr>
<td>Values are means ± S.E.M., and number of cases (%)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>ACS (n)</td>
</tr>
<tr>
<td>Current smoking (n)</td>
</tr>
<tr>
<td>Hypertension (n)</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
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<tr>
<td>Mean arterial blood pressure (mmHg)</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
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<tr>
<td>LDL-cholesterol (mmol/l)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
</tr>
<tr>
<td>Concurrent medication</td>
</tr>
<tr>
<td>β-Blockers</td>
</tr>
<tr>
<td>Aspirin (100 mg/d)</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
</tr>
<tr>
<td>Statin therapy</td>
</tr>
<tr>
<td>Calcium-channel blockers</td>
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<tr>
<td>Nitrates</td>
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sPLA2 activity

sPLA2 activity in serum samples was determined with [1-14C] oleate-labelled *Escherichia coli* as a substrate as described previously [15]. Briefly, assay mixtures (150 µl) contained 100 mM Tris/HCl (pH 7.0), 1 mM CaCl2, [1-14C]oleate-labelled *E. coli* (≈10 000 c.p.m.), and 5 µl of the serum diluted (1:1000) in assay buffer [100 mM Tris/HCl (pH 7.0), 10 mM CaCl2 and 0.1 % fatty-acid-free BSA], which is sufficient to produce less than 5 % substrate hydrolysis and to be in the linear range. Reaction mixtures were incubated for 1 h at 37°C in a thermomixer. The extraction of the lipids was performed by the Dole method exactly as described. Free [1-14C]oleate was measured in a β-counter. Inter assay coefficient of variation is < 5 % with intra-assay coefficients of variation of 2.0–3.5 %.

CRP levels

CRP levels were determined with an ultrasensitive CRP test (N Latex CRP mono; Behring, Marburg, Germany). The measurement range is 0.2–10.0 mg/l (for 1:20 dilution; higher concentrations were determined after appropriate dilution), with intra-assay coefficients of variation of 1.7–2.5 % and inter assay coefficients of variation of 1.7 %–3.6 %.

Statistical analysis

Data are expressed as means ± S.D. Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test and compared by one-way ANOVA. Categorical variables were compared by the χ2 test and Fishers-exact test. In the case of non-normal distribution, non-parametric tests were used (Mann–Whitney U test or Kruskal–Wallis ANOVA on ranks). Differences between the group FBF measurements are presented as means ± S.E.M. FBF responses to acetylcholine and sodium nitroprusside were calculated as the AUC (area under the curve) and expressed in arbitrary units [16]. Linear regression analysis and non-parametric bivariate correlation [Spearman rank correlation coefficient (r)] were used to compare FBF responses with the serum markers of inflammation. Multivariate analysis was performed with the logistic regression model. Probability values < 0.05 were considered statistically significant. All statistical analysis was performed with SPSS for Windows 9.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

The patient characteristics are shown in Table 1. Twenty-eight patients were studied within 5 days of a troponin T-negative ACS (acute coronary syndrome), whereas 22 patients had stable CAD. CRP serum levels were significantly elevated in patients with ACS (14.9 ± 9.5 mg/l compared with 4.9 ± 4.7 mg/l; P < 0.01). There were no systemic effects observed related to the infused substances.

sPLA2 and vasodilator function

sPLA2 serum activity ranged from 405.5–1427.5 c.p.m./µl (mean ± S.D., 829.7 ± 266.1 c.p.m./µl; median, 822.0 c.p.m./µl). Figure 1(A) shows that the FBF dose–response curves to acetylcholine, expressed as quartiles of the AUC, were associated with sPLA2 serum

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**Figure 1** Relationship between FBF dose–response curves to acetylcholine (A) and sodium nitroprusside (B) expressed as the AUC and sPLA2 activity categorized into quartiles

Values are means ± S.E.M. Quartile 1 (Q1), < 600 c.p.m./µl; quartile 2 (Q2), 601–800 c.p.m./µl; quartile 3 (Q3), 801–1040 c.p.m./µl; quartile 4 (Q4), > 1041 c.p.m./µl. ACH, acetylcholine; SNP, sodium nitroprusside.
Table 2  sPLA₂ serum activity, concurrent medication and risk factors for CAD
Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>sPLA₂ serum activity (c.p.m./µl)</th>
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<tbody>
<tr>
<td>ACS</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>845.0 ± 54.8</td>
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<tr>
<td>No</td>
<td>811.6 ± 45.4</td>
</tr>
<tr>
<td>Current smoking</td>
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<tr>
<td>Yes</td>
<td>684.9 ± 72.5</td>
</tr>
<tr>
<td>No</td>
<td>822.3 ± 49.3</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>837.3 ± 71.5</td>
</tr>
<tr>
<td>No</td>
<td>758.9 ± 50.8</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td></td>
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<tr>
<td>Yes</td>
<td>837.3 ± 71.5</td>
</tr>
<tr>
<td>No</td>
<td>788.5 ± 43.9</td>
</tr>
<tr>
<td>Statin therapy</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>846.8 ± 48.3</td>
</tr>
<tr>
<td>No</td>
<td>683.3 ± 77.4</td>
</tr>
<tr>
<td>ACE-inhibitor therapy</td>
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<tr>
<td>Yes</td>
<td>792.9 ± 52.1</td>
</tr>
<tr>
<td>No</td>
<td>795.3 ± 73.6</td>
</tr>
</tbody>
</table>

Correlation of sPLA₂ and risk factors for CAD
As shown in Table 2, sPLA₂ serum activity did not differ with respect to the presence or absence of the classical risk factors, such as diabetes, hypertension or smoking, or with respect to clinical symptomatology.

Moreover, there was no correlation between serum activity of sPLA₂ and total cholesterol, LDL (low-density lipoprotein)- or HDL (high-density lipoprotein)-cholesterol serum levels. There were no significant differences in sPLA₂ activity with respect to the measured lipid profiles or CRP serum level (Table 3). However, there was a significant correlation between sPLA₂ and CRP (r = 0.33, P < 0.05).

Multivariate analysis including risk factors of CAD
In order to identify independent predictors of an impaired endothelium-dependent vasodilator function in patients with CAD, a multivariate analysis was performed including the inflammatory serum markers as well as classical risk factors for CAD. As shown in Table 4, multivariate analysis revealed that only sPLA₂ and CRP as markers of inflammation were identified as major independent predictors for an impaired endothelium-dependent vasodilator response (P < 0.05). In order to that the results of the present study are not driven by those patients with major elevation of CRP serum levels, we additionally performed a multivariate analysis restricted to patients (n = 34) with CRP serum levels < 10.0 mg/l. This analysis gave identical results with both markers being independent predictors of an impaired vasodilator function (P < 0.05).

The incremental value of sPLA₂ serum activity to predict impaired endothelium-dependent vasodilator function is shown further in Figure 2. Elevation of both inflammatory serum markers, sPLA₂ and CRP, was associated with the worst endothelium-dependent FBF response, whereas endothelium-dependent blood flow responses did not differ between patients with elevation of either parameter alone. However, in these patients, endothelium-dependent vasodilator capacity was significantly decreased compared with patients with low levels of both markers (P < 0.05).

DISCUSSION

The results of the present study demonstrate that elevated sPLA₂ activity in serum of patients with established CAD...
is associated with an impairment in systemic endothelial vasodilator function. Confirming our previously described observations [12], elevated CRP levels were also associated with a blunted endothelium-mediated vasodilator response. Although sPLA2 activity and CRP serum levels were significantly associated with each other, after multivariate analysis including the classical risk factors for CAD, both sPLA2 and CRP remained the only independent statistically significant predictors of the vasodilator response to acetylcholine. Thus our data reveal a further link between endothelial vasodilator function and inflammatory markers in addition to the previously established relationship between CRP serum levels and endothelial function, independent of classical risk factors of CAD or clinical symptomatology [12,17,18]. However, studying patients in a clinical setting without the capacity to acutely modulate sPLA2 serum activity precludes the reporting of a cause-and-effect relationship between sPLA2 serum activity and endothelial function.

Since impaired endothelial vasodilator function [19] as well as increased serum levels of markers of inflammation [20–22] have been shown to predict further cardiovascular events in patients at risk, it is tempting to speculate that endothelial dysfunction represents a functional readout integrating the various injurious insults acting on the endothelial monolayer. As such, the identification of sPLA2 serum activity as an independent risk factor for endothelial dysfunction provides a further clue to link a systemic marker of inflammation to atherosclerotic disease.

The association between elevated sPLA2 serum activity and impaired endothelium-dependent vasodilator function can be rationalized well by sPLA2 being an acute-phase reactant in response to a variety of inflammatory cytokines, such as IL-1β (interleukin-1β), IL-6 (interleukin-6) and TNF-α (tumour necrosis factor-α) [5,23,24]. Elevated TNF-α serum levels are known to impair endothelium-mediated vasodilator functions in humans [14]. Likewise, IL-1β and IL-6 have been shown experimentally to activate endothelial cells [25], leading to an impaired NO (nitric oxide) bioavailability, which is the major determinant of endothelial vasodilator function in the human forearm circulation in response to acetylcholine infusion [26,27]. Furthermore, sPLA2 has not only been shown to be highly expressed in normal and atherosclerotic arteries in human arterial walls [7], but, more importantly, directly induces the expression of chemokines and adhesion molecules in microvascular endothelium [9]. Finally, Webb et al. [28] demonstrated elegantly that group IIA sPLA2 overexpression contributes to atherosclerotic lesion development in mice through a mechanism that is independent of systemic lipoprotein metabolism. These data indeed suggest a causal link between endothelial activation and PLA2 activity.

sPLA2 and CRP serum levels might be regarded as surrogate parameters of vascular inflammation, thereby reflecting inflammatory activation of the endothelium with ensuing endothelial dysfunction. Indeed, the correlation between sPLA2 and CRP levels observed in the present study suggests that these acute-phase reactants might serve as systemic markers for endothelial dysfunction secondary to inflammatory responses of the vascular wall. However, in analogy with CRP, which was very recently shown to interfere directly with endothelial NO production by degrading NOS (NO synthase) [29,30], sPLA2 might also directly impair endothelial vasodilator function, since sPLA2 is capable of hydrolysing phospholipids to generate lysophosphatidylcholine [31]. In addition, sPLA2 was shown to accelerate HDL catabolism [32] and to modify LDL particles [33]. Lysophosphatidylcholine and oxidized LDL are known to directly inhibit the activity of eNOS (endothelial NOS) [34]. HDL was recently shown to activate eNOS via Akt-dependent phosphorylation [35]. Thus sPLA2 might also interfere with eNOS activity by critically influencing local levels of lipid messengers. Since sPLA2 is highly expressed in human atherosclerotic tissue and co-localizes with monocyte-derived macrophages [7,36], it may causally contribute to the impaired endothelial vasodilator function observed in the present study. Indeed, our finding that both sPLA2 and CRP serum levels independently predicted an impaired blood flow response to acetylcholine suggests that both parameters do not merely reflect systemic inflammatory responses, but may also play a role as mediators of endothelial dysfunction.

Another potential mechanism involved in the sPLA2-mediated propagation of inflammatory reactions in the vascular wall could be the up-regulation of the COX (cyclo-oxygenase)-2 pathway, leading to increased prostaglandin biosynthesis [37,38]. However, since all
patients in the present study were chronically treated with aspirin, COX-dependent effects on endothelial vasodilator function might have been blunted [39]. Indeed, a very recent study [40] suggested that COX-2 inhibition in patients chronically treated with aspirin improves, rather than impairs, endothelial function.

Taken together, the identification of sPLA₂ as a novel independent predictor for systemic endothelial dysfunction provides another important clue to link a systemic marker of inflammation to coronary atherosclerotic disease. Future therapeutic interventions, such as specific inhibitors of human serum sPLA₂ activity, should be evaluated in order to elucidate the potential of anti-inflammatory substances for patients at risk.

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REFERENCES


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