Plasma Prekallikrein is Associated with Carotid Intima-Media Thickness in Type 1 Diabetes

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Short Title: Plasma prekallikrein and diabetic vascular disease

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Abstract

The hypothesis that plasma prekallikrein (PK) is a risk factor for the development of vascular complications was assessed in a study using the Diabetes Control and Complications Trial/Epidemiology and Diabetes Intervention and Complications cohort of type 1 diabetic subjects (DCCT/EDIC). The circulating levels of plasma PK activity were measured in the plasma of 636 type 1 diabetic subjects (EDIC years 3-5). Common and internal carotid intima-media thickness (IMT) was measured by B-mode ultrasonography in EDIC years 1 and 6. Plasma PK levels were positively and significantly associated with body mass index, hemoglobin A1c, systolic blood pressure, total cholesterol, LDL-cholesterol and triglycerides, but not with age, gender, duration of diabetes and HDL-cholesterol. Univariate and multivariable statistical models after controlling for other risk factors, consistently demonstrated a positive association between plasma PK and progression of internal carotid IMT. Multivariate analysis using general linear model showed plasma PK to be significantly associated with progression of both internal and combined IMT (Wilks’ Lambda P-value of 0.005). In addition, the mean internal carotid IMT levels were higher in subjects with plasma PK levels in the highest 10th percentile compared to subjects with plasma PK levels in the lower 10th percentile, P=0.048. These novel findings implicate plasma PK as a risk factor for vascular disease in type 1 diabetes.
Introduction

Atherosclerosis is a leading cause of morbidity and mortality in diabetes, but the accelerated vascular pathology associated with diabetes is not fully explained by the coexistence of traditional cardiovascular risk factors such as hypertension, hyperlipidemia, smoking and a positive family history for cardiovascular disease. Early atherosclerotic lesions are characterized by endothelial dysfunction, accumulation of inflammatory cells, vascular smooth muscle cell (VSMC) proliferation and migration, and extracellular matrix deposition in the vessel wall (1,2).

The localization of all the components of the kallikrein-kinin system (KKS) within the vessel wall suggests a role for this system in the regulation of ultrastructure and vascular tone (3-7). Moreover, plasma PK has been implicated as a modulator of diabetic microvascular complications (nephropathy and retinopathy), with higher plasma PK activity associated with higher blood pressure and greater albumin excretion rates in type 1 diabetic patients (8,9). Additionally, plasma PK has a role in vascular remodeling by promoting growth of vascular smooth muscle cells through transactivation of epidermal growth factor (EGF) receptors (10). Finally Klkb1\(-/-\) null mice (prekallikrein deficient) have delayed arterial thrombosis by increasing protective vascular transcription factors Sirt1 and KLF4 to reduce vessel wall tissue factor and inflammation a forerunner of vessel atherothrombosis (11).

The contribution of plasma PK to vascular disease in diabetic subjects has been incompletely explored. Increased circulating levels of KKS components in subjects at risk for vascular disease would provide evidence for heightened system activity and support their potential role in vascular disease. In the present study, we evaluated whether elevated plasma PK levels are associated with vascular disease in the Diabetes Control and Complications Trial/Epidemiology and Diabetes Intervention and Complications (DCCT/EDIC)-cohort of type 1 diabetic subjects.
Methods

Study population. The study was conducted on a subset of 636 subjects from the DCCT/EDIC cohort. The DCCT (Diabetes Control and Complications Trial) cohort included 1441 subjects and consisted of men and women between the ages of 13-40 with 1-15 years of diabetes at study entry (12). The patients enrolled in the DCCT between 1983 and 1989 and the randomized clinical trial ran for approximately 6.5 years. Half of the subject population was randomly assigned to conventional diabetes treatment and the other half was assigned to intensive diabetes treatment. In 1993, the DCCT was stopped one year ahead of its scheduled end, when intensive treatment was clearly shown to reduce the risks of retinopathy, nephropathy and neuropathy (13). The subjects were invited to enroll in EDIC (Epidemiology of Diabetes Interventions and Complications), a multicenter longitudinal observational study of the development of macrovascular complications and further progression of microvascular complications (14). The DCCT and EDIC were approved by all Institutional Review Board of all participating DCCT/EDIC centers and all participants provided written informed consent.

Assessment of Carotid Intima-Media Thickness (IMT)

Carotid IMT (common and internal) were measured by B-mode ultrasound, 1 to 2 years after start of EDIC (Year 1) and repeated 5 years later (EDIC year 6) as previously described in detail (15).

Assessment of Components of the KKS: Plasma PK, Factor XII:coagulant and high molecular weight kininogen (HK):coagulant were measured as previously reported (8). KKS components were measured in 636 subjects (EDIC years 3-5) out of a total of 905 EDIC subjects who participated in our ancillary longitudinal study. They were selected sequentially as they appeared at study sites for scheduled visits. The clinical characteristics of the subjects in which KKS components were measured were compared to those of the remaining EDIC subjects. No differences in age, gender, hemoglobin A1c (HbA1c), systolic blood pressure (SBP), DCCT treatment group, duration of diabetes, or body mass index (BMI) were observed between the two groups (8).
Assessment of coagulation/fibrinolysis factors. Plasma concentrations of fibrinogen and Plasminogen activator inhibitor (PAI-1) activity levels were determined as previously described (16,17).

Statistical Analysis

Plasma PK was hypothesized to be associated with carotid IMT in type 1 diabetic patients. To examine this hypothesis, univariate and multivariable regression analysis as well as a General Linear Model multivariate analysis were utilized. Linear regression analyses with plasma PK as the dependent variable were initially performed to assess whether changes in the levels of plasma PK (upregulated or downregulated) are influenced by changes in the levels of cardiovascular risk factors such as HbA1c, BMI, lipids and blood pressure, in addition to associations with other components of the KKS (FXII, HK, and plasma tissue kallikrein). The association between plasma PK and progression of internal carotid IMT (year 6 - year 1) was assessed using multivariable regression adjusted for all covariates listed in Table 2. To assess the multivariate effect of plasma PK on carotid IMTs (internal and combined), the General Linear Model multivariate analysis was performed and the significance of the Wilks Lambda P-value pertaining to plasma PK was determined. The effect of each component of the KKS (FXII and tissue kallikrein) was evaluated in the model to determine whether it had a direct significant association with carotid IMTs or whether these covariates modified the relationship between plasma PK and carotid IMTs. Risk factors that had a P-value ≤ 0.2 in the simple linear regression were included in the multivariable linear analysis in addition to other covariates that we considered of clinical relevance such as the components of the KKS, treatment, gender, age, Lipoproteins and Blood Pressure (Table 2).
Results

Plasma PK levels in DCCT/EDIC-cohort of type 1 diabetic subjects.

The circulating levels of plasma PK were measured in 636 type 1 diabetic subjects from plasma collected in years 1997-1999 (EDIC years 3-5). The PK levels were symmetrically distributed and ranged from 0.2-3.0 units/ml, with a mean value of 1.29 U/ml. The univariate analysis of PK with vascular disease risk factors in the cohort are shown in Table 1. The cross-sectional data showed a positive and significant association between PK levels and body mass index (BMI), and with HbA1c, a marker of metabolic control and with SBP. A positive and significant association was also observed between PK levels and total cholesterol, LDL-cholesterol and triglycerides. With respect to components of the KKS and coagulation factors, a positive and significant association was also detected with FXII, HK, fibrinogen and plasminogen activator inhibitor (PAI-1) activity (Table 1). No association was observed between PK and age, gender, duration of diabetes, angiotensin converting enzyme inhibitor (ACEI) use, DCCT treatment group, high density lipoprotein (HDL)-cholesterol and tissue kallikrein (Table 1).

Plasma PK and Carotid IMT. We evaluated whether PK levels were associated with subclinical macrovascular disease by examining the relationship between plasma PK activity and common, internal and/or combined carotid IMT. The combined IMT was defined as the sum of the intima-media measurements of the common and internal carotid arteries (18). Plasma PK levels were positively and significantly associated with the internal (p<0.001) and combined (p=0.011) carotid IMT but not with the common (p=0.384) carotid IMT (Table 1).

We next determined if plasma PK levels were associated with progression of carotid IMT by evaluating the association of plasma PK with changes in carotid IMT from EDIC year 1 to EDIC year 6. Variations in internal carotid IMT was assessed by fitting the model with the difference (Δ change) between internal carotid IMT measurements at year 6 and year 1 as the outcome of interest. The effect of plasma PK on the Δ change in IMT was determined using multivariable analysis adjusted for Factor XII, tissue kallikrein, duration of diabetes, age, gender, DCCT treatment group, systolic blood pressure, log AER, current smoker, BMI, HbA1c, ACE inhibitors, total cholesterol, LDL-cholesterol, triglycerides, fibrinogen, P1 activity and ultrasonography.
equipment used at EDIC year 6 (Table 2). Our results demonstrated a significant positive association between plasma PK on the Δ difference in internal IMT (year 6- year 1), p= 0.017. Furthermore, the mean internal carotid IMT levels were highest in subjects with plasma PK levels in the upper 10th percentile (0.87±0.07 mm) compared to subjects with plasma PK levels in the lower 10th percentile (0.72±0.04 mm), p=0.048, Figure 1.

A multivariate regression model was implemented using general linear model (GLM) with IMT internal and combined year 6 both as multivariate outcomes and plasma PK as the key risk factor, adjusting for combined IMT year 1 along with other covariates listed in Table 3. Specifically the global association of plasma PK and progression of carotid IMT attained by accounting for year 1 IMT in the model, was evaluated simultaneously using year 6 internal and combined IMT as multivariate outcomes. Here plasma PK was shown to be significantly associated with both internal and combined carotid IMT concurrently with a global test effect of Wilks’ Lambda P-value of 0.005. Statistical tests between individual subjects effects revealed a significant and positive association between plasma PK and internal and combined carotid IMT with P-values of 0.001 and 0.042, respectively.

**Discussion**

Data in the current study demonstrate that circulating levels of plasma PK are associated with carotid intima-media thickness and its progression in type 1 diabetic subjects. Progression of internal and combined carotid IMT are increased in subjects with higher levels of plasma PK, and this is consistently demonstrated in the univariate and multivariable analysis as well as in the multivariate model after controlling for other risk factors. The association of plasma PK with the combined carotid IMT is driven by the strength of the association between plasma PK and internal carotid IMT. These findings support plasma PK as an independent risk factor for cardiovascular complications in type I diabetic subjects.

Plasma PK is predominately synthesized in the liver and secreted into the circulation where it circulates as a bimolecular complex bound to its substrate HK (19). Under physiological conditions, the assembly of the plasma PK-HK complex on the surfaces of endothelial cells is facilitated by the binding of domains 3, 4 and 5 of HK to a multiprotein receptor complex that
consists of cytokeratin 1 (CK1), the receptor for globular head of the C1q (gC1qR) and the urokinase plasminogen activator receptor (u-PAR) (20-22). Endothelial cell prolylcarboxypeptidase, which is bound to the complex, activates plasma PK to activate kallikrein, which in turn cleaves HK to release bradykinin (BK). The generated, BK acts on its B2-receptors in an autocrine and or paracrine manner to initiate a multitude of cellular signals that influence vascular structure and tone (23).

However, when endothelial injury and dysfunction occur under pathologic conditions such as atherosclerosis or diabetes, circulating plasma PK is in constant contact with VSMC leading to its direct activation by VSMC through a novel yet an unidentified putative plasma PK activator (24). Once activated on the surface of exposed VSMC, Plasma PK not only generates BK, but also activates protease activated receptors 1 and 2, leading to transactivation of EGF receptors, release of proinflammatory cytokines and proliferation of VSMC that comprise the main cellular component in atherosclerotic lesions, contributing to the thickness of the intima (10). Moreover our results demonstrate that plasma PK positively associates with fibrinogen and PAIact, factors that promote thrombogenesis and increase vascular disease risk. In this regard, it is important to point out that Klkb1−/− null mice are protected against thrombosis by both 1) reduced contact activation and 2) reduced vessel wall tissue factor (11).

Our findings point to a role for Plasma PK as an independent risk factor in vascular disease, but they do not demonstrate whether the increase in the levels of plasma PK we observed are the result of, or the root of vascular disease. To investigate a causal role for plasma PK in vascular disease, longitudinal studies should focus at determining whether prior increases in the levels of plasma PK are predictive of future increases in measures of subclinical vascular disease as well as developments of future cardiovascular event rates in type 1 diabetes. Furthermore, the human population studies should be translated into animal models for type 1 diabetes that will evaluate the role of plasma PK as a pathogenic risk factor for vascular disease progression by utilizing plasma PK inhibitors as a therapeutic approach to ameliorate vascular disease.
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**Table 1.** Univariate Linear Regression with Plasma PK as dependent Variable and Risk Markers and Clinical Parameters as Covariates

| Variable                                      | Estimate | SE     | P      |
|-----------------------------------------------|----------|--------|--------|
| Factor XII (U/ml)                             | 0.040    | 0.019  | 0.033  |
| High Molecular Weight Kininogen (U/ml)        | 0.118    | 0.022  | 0.001  |
| Tissue Kallikrein (ng/ml)                     | 0.006    | 0.015  | 0.676  |
| Duration of Diabetes (yrs)                    | 0.002    | 0.004  | 0.612  |
| Hemoglobin A1c (%)                            | 0.049    | 0.013  | 0.001  |
| Age (yrs)                                     | 0.001    | 0.003  | 0.875  |
| Body Mass Index (kg/m\(^2\))                 | 0.112    | 0.004  | 0.006  |
| Systolic Blood Pressure (mmHg)                | 0.005    | 0.001  | <0.001 |
| Total Cholesterol (mg/dl)                     | 0.002    | 0.001  | <0.001 |
| LDL-cholesterol (mg/dl)                       | 0.002    | 0.001  | <0.001 |
| HDL-Cholesterol (mg/dl)                       | 0.000    | 0.001  | 0.466  |
| Triglycerides (mg/dl)                         | 0.001    | 0.000  | <0.001 |
| ACE Inhibitor                                 | 0.005    | 0.050  | 0.090  |
| Gender (male)                                 | 0.005    | 0.034  | 0.879  |
| DCCT treatment Group                          | -0.001   | 0.033  | 0.965  |
| Log AER                                       | 0.046    | 0.012  | <0.001 |
| Fibrinogen (mg/dl)                            | 0.001    | 0.000  | <0.001 |
| P1 Activity (U/ml)                            | 0.008    | 0.003  | 0.004  |
| Intima-Media Thickness Internal (mm)           | 0.111    | 0.035  | 0.001  |
| Intima-Media Thickness Common (mm)             | 0.011    | 0.012  | 0.384  |
| Intima-Media Thickness Combined (mm)           | 0.450    | 0.176  | 0.011  |

ACE (angiotensin converting enzyme inhibitors), AER (albumin excretion rate), P1 activity (plasminogen activator inhibitor activity).
Table 2. Multiple Linear Regression with Outcome Δ Change in Internal Carotid IMT from EDIC Year 1 to EDIC Year 6 and Risk Markers and Clinical Parameters as Covariates.

| Variable                                | Estimate | STD Error | P    |
|-----------------------------------------|----------|-----------|------|
| Plasma PK (U/ml)                        | 0.146    | 0.060     | 0.017|
| Factor XII (U/ml)                       | 0.019    | 0.018     | 0.312|
| Tissue Kallikrein (ng/ml)               | 0.004    | 0.015     | 0.796|
| Duration of Diabetes (yrs)              | 0.004    | 0.005     | 0.415|
| Age (yrs)                               | 0.006    | 0.004     | 0.094|
| Gender (male)                           | -0.022   | 0.049     | 0.655|
| DCCT treatment Group                    | 0.023    | 0.045     | 0.615|
| Systolic Blood Pressure (mmHg)          | 0.003    | 0.002     | 0.132|
| Log AER (mg/24h)                        | 0.029    | 0.023     | 0.220|
| Current smoker                          | 0.013    | 0.065     | 0.846|
| Body Mass Index (kg/m2)                 | -0.003   | 0.006     | 0.578|
| Hemoglobin A1c (%)                      | -0.009   | 0.018     | 0.622|
| ACE inhibitors                          | 0.038    | 0.061     | 0.529|
| Total Cholesterol (mg/dl)               | 0.000    | 0.002     | 0.871|
| LDL-Cholesterol (mg/dl)                 | 0.000    | 0.002     | 0.898|
| Triglycerides (mg/dl)                   | -0.001   | 0.001     | 0.240|
| Fibrinogen (mg/dl)                      | 0.000    | 0.000     | 0.218|
| P1 Activity (U/ml)                      | 0.007    | 0.005     | 0.213|
| Ultra sonography Equipment used         | 0.001    | 0.001     | 0.428|

ACE (angiotensin converting enzyme inhibitors), AER (albumin excretion rate), P1 activity (plasminogen activator inhibitor activity).
Table 3. Multivariate Regression using General Linear Model with Internal and Combined Carotid Intima-Media Thickness for EDIC Year 6 as Multivariate Outcomes and Risk markers and Clinical Parameters as Covariates.

| Variable                        | Wilks’ Lambda P-value |
|---------------------------------|-----------------------|
| Plasma PK (U/ml)                | 0.005                 |
| Factor XII (U/ml)               | 0.727                 |
| Tissue Kallikrein (ng/ml)       | 0.909                 |
| Duration of Diabetes (yrs)      | 0.175                 |
| Age (yrs)                       | 0.000                 |
| Gender (male)                   | 0.403                 |
| DCCT treatment Group            | 0.425                 |
| Current smoker                  | 0.964                 |
| Hypertension#                   | 0.603                 |
| Total Cholesterol (mg/dl)       | 0.940                 |
| Triglycerides (mg/dl)           | 0.390                 |
| Combined IMT (year 1)           | 0.000                 |
| Ultrasonography Equipment used  | 0.525                 |

#blood pressure ≥ 140/90 or antihypertensive medications used.
Figure Legends

Figure 1. Internal carotid intima media thickness (mean±SE) stratified by plasma PK levels (lower 10\textsuperscript{th} percentile and upper 10\textsuperscript{th} percentile), * P=0.048.
Internal Carotid IMT (mm)

Lower 10 Percentile

Upper 10 Percentile

Plasma PK (U/ml)

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