Plasma Prolylcarboxypeptidase (Angiotensinase C) is Increased in Obesity and Diabetes Mellitus and Related to Cardiovascular Dysfunction

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BACKGROUND: Prolylcarboxypeptidase (PRCP) (angiotensinase C) has 3 major targets, angiotensin II, prekallikrein, and α-melanocyte stimulating hormone1–13. The truncation of the latter leads to loss in appetite regulation and obesity in experimental animals. The objectives of this study were to purify PRCP from a native source, establish a sensitive immunoassay for PRCP, and relate plasma PRCP concentrations to signs and symptoms of obesity, diabetes mellitus, and cardiovascular dysfunction.

METHODS: Purification of PRCP from human neutrophils and establishment of a sensitive ELISA was carried out with the use of samples from study participants. Three cohorts were studied: healthy individuals (n = 40); a chest pain cohort (Fast Assessment of Thoracic Pain by Neural Networks) (n = 165); and a community-based cohort [Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS)] (n = 1004).

RESULTS: PRCP was purified to homogeneity. Mean (SD) plasma concentrations in healthy individuals were 12.9 (3.2) μg/L and were increased in patients with chest pain and in patients with obesity and/or diabetes mellitus (P < 0.0001). In the PIVUS cohort the concentrations were related to several measures of arterial plaque formation, thickness of arterial intima media and posterior wall of the heart (P = 0.04–0.000005); the Framingham score (r = 0.14, P < 0.0001); and concentrations of C-reactive protein (r = 0.16, P < 0.0001) and N-terminal pro B-type natriuretic peptide (r = −0.13, P < 0.0001).

CONCLUSIONS: Plasma concentrations of PRCP may be used to reflect metabolic conditions in individuals with obesity and diabetes mellitus. The associations of PRCP concentrations with signs of cardiovascular dysfunction and cardiovascular abnormalities suggest a pivotal role of the enzyme in disease.

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Prolylcarboxypeptidase (PRCP), also called angiotensinase C, is an enzyme with potentially important activities (1, 2). The gene of PRCP is located on chromosome 11 and consists of 9 exons (3) and may be produced by several different cells. It was shown to inactivate angiotensin II and III, activate prekallikrein to kallikrein with the consequent production of bradykinin, and truncate α-melanocyte stimulating hormone (α-MSH) (4–8). The biological consequences of these activities may have an impact on blood flow and blood pressure, inflammatory activities, and regulation of appetite and obesity. PRCP has been suggested as a new target for obesity treatment, because the truncation of α-MSH by PRCP leads to increased food intake (2). This effect is believed to take place at the site of hypothalamic neurons. However, recent studies showed that treatment of experimental animals with inhibitors of PRCP, which did not penetrate the brain, led to weight loss (9). These experimental results suggest that PRCP may also have an effect on the energy expenditure of peripheral tissues. Our hypothesis, therefore, was that increased plasma concentrations of PRCP might play a role in this proposed cause of obesity. To test this hypothesis we purified human PRCP from a native source and established a sensitive immunoassay that allows the measurement of PRCP in human body fluids. We observed for the first time that the concentrations of PRCP in plasma are strongly associated not only with obesity, but also with diabetes mellitus, plaque formation in blood vessels, and thickness of the myocardium.
Materials and Methods

PATIENTS

The Fast Assessment of Thoracic Pain by Neural Networks cohort. Chest pain patients were participants from the FASTER I (Fast Assessment of Thoracic Pain by Neural Networks) study that was conducted at 3 investigational centers in Sweden between October 2002 and August 2003 and enrolled 380 study participants (10). The sole criterion for inclusion of patients in the study was their admission to the coronary care unit because of chest pain lasting for ≥15 min within the previous 8 h. The study exclusion criteria were pathological ST-segment elevation on the admission 12-lead electrocardiogram or strong suspicion of acute myocarditis.

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) cohort. All individuals who were 70 years of age and living in the community of Uppsala, Sweden, were eligible for this study. The participants were chosen from the register of community living, and were invited in a randomized order. The participants received an invitation by letter within 2 months of their 70th birthday. Of the 2025 individuals invited, 1016 (50.1%) participated. The baseline investigation was started in April 2001 and has been described in detail elsewhere (11). The cohort was extensively investigated with regard to the cardiovasculature, as described in several previous publications (12–14).

Both studies were approved by the ethics committee of Uppsala University, and the participants gave informed consent.

CLINICAL MEASUREMENTS

C-reactive protein was measured by an immunoturbidimetric assay on the Architect instrument (Abbott Diagnostics), and N-terminal pro B-type natriuretic peptide (NT-proBNP) was measured on the Modular Diagnostics), and N-terminal pro B-type natriuretic peptide (NT-proBNP) was measured on the Modular (Invitrogen Corporation) at 4 °C overnight. Additional binding sites were blocked with carbonate–bicarbonate buffer containing 1% BSA (200 μL/well) (Sigma-Aldrich) at 37 °C for 40 min. We then added 100 μL standards (0.156 to 10 μg/L) or samples diluted in assay solution (PBS containing 0.2% BSA, 0.1% Tween-20, 0.05% cetrimonium bromide and 0.02% NaN3) in duplicates and incubated the mixtures at room temperature for 1.5 h. Subsequently, 100 μL of diluted biotinylated rabbit polyclonal antibody against human PRCP was added to each well and incubated at room temperature for 60 min, followed by the addition of 100 μL/well of diluted streptavidin-conjugated horseradish peroxidase (GE Healthcare) incubated for 30 min at room temperature. The plates were washed 4 times in a washing buffer (PBS containing 0.05% Tween-20) with a microplate washer (Anthos Fluido) between all steps. The enzyme reaction was visualized by use of a 3,3′,5,5′-tetramethylbenzidine solution (100 μL/well) (Sigma-Aldrich) as substrate at room temperature for 15 min, and the reaction was stopped by adding 1 mol/L H2SO4 (100 μL/well). Absorbance was measured at 450 nm with a reference reading at 540 nm in blank wells by a microplate reader (SPECTRAMax 250, GMI).

STATISTICS

The Mann–Whitney test was used for comparison between groups, and Spearman rank correlation coefficients were used to test associations between variables. Multiple regression analysis and logistic regression analysis were used to analyze for dependent associations of PRCP to continuous and dichotomous variables, respectively. The statistical programs Statistica for Windows v 10 and MedCalc were used throughout. P values <0.05 were considered significant. Numerical variables are given as medians and 95% CI of medians unless otherwise stated.

Results

PURIFICATION AND CHARACTERIZATION OF PRCP

PRCP was purified by a 4-step procedure (for details see the online Supplemental Data file). The SDS-electrophoresis patterns of the proteins in the 4 steps are shown in Fig. 1A. The final purified product showed only 1 band at a molecular weight of 55 kDa under nonreducing (lane 6) and reducing conditions.
Plasma Concentrations of PRCP (Angiotensinase C)

ENZYME-LINKED IMMUNOSORBENT ASSAY
We developed an ELISA for the measurement of PRCP using polyclonal antibodies to PRCP. We used purified native PRCP as a standard. The range of measurement was 0.156–10 μg/L with a detection limit of 0.07 μg/L (defined as 3 SD of the blank). A parallelism was observed between the standard curve and serially diluted serum (serial dilution: mean 99.47%, range 92.47%–105.80%, n = 5). Cross-reactivities with granule proteins such as lysozyme, lactoferrin, human neutrophil lipocalin, myeloperoxidase, elastase, eosinophil cationic protein, and eosinophil protein X were tested in the assay by measuring these proteins up to 100 μg/L. None of these proteins were detected, indicating high assay specificity. A recovery of 99% (range 93%–105%) was obtained when purified PRCP at 3 different concentrations (1.25, 2.5, and 5 μg/L) was added to serum. The precision of the assay was assessed by repeated measurements of 3 different concentrations of sera. The mean within- and between-assay CVs were <6% (n = 10) and 10% (n = 10), respectively. We assessed the stability of PRCP in serum by measuring PRCP in serum samples with or without freezing and thawing 5 times. No differences in serum concentrations of PRCP were seen after 5 freeze–thaw cycles.

PLASMA MEASUREMENTS OF PRCP
Mean (SD) concentrations in plasma of PRCP measured in 40 healthy reference individuals with a mean (SD) age of 27 (6.7) years were 12.9 (3.2) μg/L. In a cohort of 165 patients referred to the hospital because of chest pain [mean (SD) age 65 (12) years] the mean (SD) concentrations were 15.3 (6.7) μg/L (P = 0.02 compared to the healthy individuals). However, no differences were observed between patients with various underlying causes of chest pain such as acute myocardial infarction and unstable angina pectoris. In a cross-sectional community-based study of 1004 men and women of mean age 70 years (the PIVUS study cohort) the mean (SD) concentrations were 14.2 (4.7) μg/L, with minimum and maximum concentrations of 4.3 and 64.1 μg/L, respectively. Men had higher mean concentrations than women, 14.0 μg/L, 95% CI 13.5–14.5 μg/L vs 13.4 μg/L, 95% CI 13.1–13.9 μg/L, P = 0.028. On average no difference vis-à-vis the younger reference population was seen, whereas the concentrations in the chest pain patients were significantly higher than those seen in the PIVUS cohort (P < 0.01) and in the healthy reference population (P < 0.03).

In the PIVUS cohort the PRCP concentrations were significantly correlated (P < 0.00001) to several symptoms and signs of obesity such as weight, body mass index, abdominal circumference, and waist/hip ratio, and also to blood glucose and markers of lipid metabolism (Table 1). The concentrations of PRCP were significantly higher in patients with diabetes mellitus and those with obesity and in patients with the metabolic syndrome (P < 0.0001). In nonobese, nondiabetic patients with the metabolic syndrome, however, the concentrations of PRCP were not different.

Fig. 1. Purification of PRCP.
(A), SDS-PAGE of materials from each step of the chromatographic purification procedure. From each purification step, approximately 0.5–20 μg of protein was applied to SDS-PAGE under nonreducing conditions, and analyzed by silver staining as described in the Materials and Methods section. Marker proteins and their corresponding molecular weights are indicated in lane 1. Lane 2: material from the acid extracts of granules. Lane 3: material from the Sephadex G-75 purification step. Lane 4: material from the first mono-S purification step at pH 4.0. Lane 5: material from the second mono-S purification step at pH 7.4. Lane 6: material from the hydroxyapatite purification step. (B), Western blotting of postnuclear supernatant of neutrophil granulocytes with polyclonal anti-PRCP antibodies. A postnuclear supernatant (25 μg) was separated on SDS-PAGE and immunoblotted with polyclonal antibodies against PRCP. Lane 1, marker proteins; lane 2, postnuclear supernatant.
from those in healthy individuals. In patients with both obesity and diabetes mellitus the concentrations were higher than among those who had obesity (Fig. 2) \((P < 0.001)\). The plasma concentrations of PRCP were correlated with the Framingham score \((r = 0.14, P < 0.0001)\).

In the PIVUS cohort positive associations were found between the PRCP concentrations in blood and several signs of cardiovascular abnormalities. Those individuals who had any plaque formation in the carotid arteries had significantly higher concentrations of plasma PRCP (median 13.1 µg/L, 95% CI 12.5–13.5, \(n = 323\) vs 14.1 µg/L, 95% CI 13.7–14.5, \(n = 609\)).

When sex, diabetes, and obesity were included in the logistic regression analysis model, the PRCP concentrations were independently associated with any plaque formation \((\text{OR} = 1.034, 95\% \text{ CI} \,1.001–1.067 \, (P = 0.04))\) and to greatest plaque size as measured in millimeters on either side \((\text{OR} = 1.060, 95\% \text{ CI} \,1.015–1.107 \, (P = 0.008))\) (see online Supplemental Table 4, a and b). In Spearman correlation analysis PRCP showed significant correlations to intimal media thickness of the brachial artery \((r = 0.10, P = 0.01)\) and to the brachial artery intimal media grey-scale median \((r = 0.18, P < 0.000005)\). In the multivariate regression analysis these associations were independent of sex, diabetes, and obesity (see online Supplemental Table 5, a and b). The results indicated an increased vessel wall stiffness owing to increased fibrosis and collagen deposition. In the univariate regression analysis plasma concentrations of PRCP showed significant correlations to posterior wall thickness and relative wall thickness of the heart \((P = 0.0002\) and \(P = 0.005\), respectively). By multiple logistic regression analysis including diabetes, obesity, sex, blood pressure, and heart rate in the model the associations were shown to be independent and significant \((P = 0.002\) and \(P = 0.005\), respectively) (see online Supplemental Table 6, a and b). In Spearman correlation analysis ankle–brachial index, a sign of peripheral vessel disease, was correlated with PRCP \((r = 0.12, P = 0.002)\). This association was confirmed in the multivariate regression analysis including sex, diabetes mellitus, and obesity in the model with an OR of 1.065, 95% CI 1.015–1.118 (see online Supplemental Table 7).

### Table 1. Correlations between plasma concentrations of PCRP and measures of obesity, lipids, blood sugar, and biomarkers of myocardial function and inflammation in the PIVUS cohort.

<table>
<thead>
<tr>
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<th>Spearman (r)</th>
<th>(P)</th>
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<tr>
<td>Body mass index</td>
<td>0.23</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.22</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>0.12</td>
<td>0.00009</td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum HDL</td>
<td>−0.09</td>
<td>0.005</td>
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<tr>
<td>Serum LDL</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum triglycerides</td>
<td>0.20</td>
<td>&lt;0.00001</td>
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<tr>
<td>NT-proBNP</td>
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<td>0.00004</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>0.16</td>
<td>0.0000001</td>
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**Fig. 2.** Plasma concentrations of PRCP in the PIVUS cohort with and without diabetes mellitus or obesity.

The concentrations in nonobese, nondiabetic individuals were significantly lower than in the cohorts with diabetes mellitus or obesity \((+++\, P < 0.001)\). The concentrations in the cohort with both diabetes mellitus and obesity were significantly higher than in the cohort with obesity only \((**\, P < 0.01)\). The data are shown as medians with 95% CIs.
Discussion

In this investigation we purified human PRCP to homogeneity from a native source, i.e., the neutrophil granule. This purification allowed us to produce specific antibodies against PRCP and for the first time establish a sensitive immunoassay for the measurement of PRCP in various body fluids. Earlier assays of PRCP have been based on enzymatic activity (15, 16). In this study we measured PRCP in plasma obtained from 3 different cohorts, i.e., from a group of young healthy individuals, from a cohort of patients admitted to the emergency room with chest pain, and from the PIVUS community-based cohort of more than one thousand men and women of mean age 70 years (17). We obtained several intriguing results that suggest a pivotal role of PRCP in metabolic processes related to obesity, diabetes mellitus, and cardiovascular disorders. Our results support the notion of PRCP as an important player in appetite regulation, possibly via its truncation of α-MSH (2). The strong associations with diabetes mellitus and signs of arteriosclerosis may be secondary to this activity, although PRCP has been shown to have several other relevant activities such as conversion of both angiotensin II and III and the formation of kallikrein from prekallikrein. Theoretically the effects of PRCP on the angiotensins should result in vasodilatation and lowered blood pressure, whereas the effects on prekallikrein suggest a proinflammatory activity of PRCP that also might be reflected by its significant association with C-reactive protein concentrations.

The purified protein had a molecular weight of 55 kDa on SDS-PAGE. There are differences in the apparent molecular weight of PRCP as purified from other sources, such as kidney and human umbilical vein endothelial cells, in which PRCP has been reported to have molecular weights of 58 kDa (18) and 73 kDa (7), respectively. The enzyme was first purified from kidney in 1978 (6). It was reported that the enzyme contained 2 subunits with molecular weights of 45 kDa and 66 kDa, respectively, on SDS-PAGE. It was concluded that the 45-kDa subunit was due to partial proteolysis of the enzyme during purification and that the 66-kDa subunit was equal to the 58-kDa protein (18). Thus, different cells in the body appear to express different sizes of PRCP. These differences in molecular weights are likely due to differences in posttranslational modifications. Whether such modifications affect the enzyme activity remains to be shown. Having purified the protein, we raised antibodies and developed a sensitive immunoassay with low mean within- and between-assay CVs. The cross-reactivity studies confirmed the specificity of the assay, because various other proteins from neutrophils and eosinophils were not detected in the assay. The accuracy of the assay was demonstrated by a 100% recovery after the addition of known amounts of the purified protein. Thus, the assay was found suitable for the measurements of the protein in various body fluids under physiological and pathological conditions.

The finding that our emergency room patients with chest pain diagnosed as acute myocardial infarction or unstable angina pectoris had increased concentrations of PRCP may suggest a link to arteriosclerosis, because the increased concentrations could not be explained by differences in age or sex. A possible link to arteriosclerosis was further supported by the examination of the vasculature and the heart in the PIVUS cohort, with associations to plaque formation, peripheral vessel function, and arterial wall thickness independent of sex, obesity, or diabetes. The mechanisms behind these associations are a matter of speculation at present, but correlations with markers of lipid and glucose metabolism suggest that these processes are involved. The raised concentrations might also be signs of endothelial dysfunction with increased production and release of PRCP owing to arteriosclerotic activities. Indeed overexpression of angiotensin II type 2 receptor in mouse coronary artery endothelial cells has been shown to lead to increased expression of PRCP and increased bradykinin release (19).

Our findings of strong associations of PRCP to all considered measures of obesity support our hypothesis and the notion of others that PRCP is involved in appetite regulation through the metabolism of α-MSH1–13 (2). Thus, the loss of the C-terminal amino acid of α-MSH1–13 results in the loss of appetite regulation (8), most likely at the hypothalamic neuron level. PRCP-null mice were also shown to have a leaner body mass and be resistant to high-fat diet–induced obesity, findings that support the importance of PRCP in this regard. PRCP has consequently been proposed as a new target for obesity treatment. One question raised by our results is whether the increased plasma concentrations of PRCP reflect the processes active in the hypothalamus or whether α-MSH1–13 may also be active in the energy expenditure of peripheral tissues, or might PRCP even have other targets outside the central nervous system that are involved in mechanisms of obesity? In support of these latter speculations are findings that inhibitory peptides of PRCP reduced weight in experimental animals even if the peptides were not brain penetrating (9).

Invariably the plasma concentrations of PRCP were independently associated with diabetes mellitus and blood glucose concentrations. These associations are not readily explained by the known activities of PRCP. However, a genome-wide scanning study showed associations of PRCP gene polymorphisms with the metabolic syndrome (20), and in our study the...
PRCP concentrations were increased in patients with the metabolic syndrome. In those patients who had diabetes mellitus and the metabolic syndrome the concentrations were similar to those in patients having only the metabolic syndrome.

NT-proBNP is generally found to be increased in patients with myocardial disease and in particular in patients with heart failure (21). We rather unexpectedly found a significant negative association between PRCP and NT-proBNP. In a recent review PRCP was advocated as a cardioprotective enzyme by its metabolism of angiotensin II and by the production of bradykinin (1). Our findings could lend some support to this notion. It was also shown that PRCP may act as a regulator of vascular reactive oxygen species homeostasis and that depletion of PRCP leads to endothelial cell dysfunction and loss of anticoagulant properties leading to thrombosis (22).

In conclusion, we have shown that PRCP concentrations in blood are strong indicators of metabolic conditions related to obesity and diabetes mellitus. Blood concentrations of PRCP may also prove useful for monitoring the arteriosclerotic process and the condition of the heart. Future studies may demonstrate the possible clinical value of PRCP measurements in such conditions.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.

Consultant or Advisory Role: B. Lindahl, Beckman Coulter, Siemens, Radiometer, Phillips, bioMerieux, and Roche Diagnostics.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: Uppsala University and Uppsala University Hospital through ALF fundings; B. Lindahl, Roche Diagnostics.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References