Amino-terminal truncation of procalcitonin, a marker for systemic bacterial infections, by dipeptidyl peptidase IV (DP IV)

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Abstract Increased concentrations of procalcitonin (PCT) are found in the plasma of patients with thermal injury and in patients with sepsis and severe infection, making this molecule important as a diagnostic and prognostic marker in these diseases. Interestingly, only the truncated form of PCT, PCT(3-116), is present in the plasma of these patients. The enzyme responsible for this truncation is unknown as yet. Here, using capillary zone electrophoresis, mass spectrometry and Edman sequence analysis, we demonstrate that dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) is capable of catalyzing the hydrolysis of PCT(1-116), releasing the N-terminal dipeptide Ala-Pro. We hypothesize that PCT(3-116) is the result of the hydrolysis of PCT(1-116) by soluble DP IV of the blood plasma or by DP IV expressed on the surface of cells.

Key words: Procalcitonin; Dipeptidyl peptidase IV

1. Introduction

Procalcitonin (PCT), a species-specific propeptide of calcitonin, is a protein of 116 amino acids with a molecular weight of 13 kDa. Under physiological circumstances, PCT is produced and then cleaved by a specific protease to calcitonin and katacalcin in C-cells of the thyroid gland. However, strongly increased plasma concentrations of PCT were detected in patients with thermal injury, in children with bacterial meningitis, and in patients with sepsis and severe infection [1–4].

In comparison to that of sepsis patients (1000–100 000 pg/ml), the concentrations of PCT in the plasma of healthy blood donors were found to be very low (around 40 pg/ml) [5,6]. In in vivo studies, increased PCT levels could be measured after endotoxin injections in healthy volunteers [7]. Due to these characteristics and to its very long half-life time in the blood (25–30 h), PCT is routinely used as a parameter for the diagnosis of severe bacterial and fungal infections and for mediator-directed therapy of sepsis [4,6].

Recently, it was found that not PCT(1-116) but only the truncated form, lacking the dipeptide Ala-Pro, PCT(3-116), is present in the serum of patients with bacterial sepsis (Dr. Andreas Bergmann, BRAHMS Diagnostica GmbH, personal communication). The function of serum PCT(3-116) in septical and healthy status as well as the importance of the N-terminal truncation of PCT and the identity of the enzyme responsible for that truncation are completely unknown as yet. However, it is highly probably that the dipeptidyl peptidase IV (DP IV, EC 3.4.14.5, CD26) cleaves PCT(1-116). DP IV is a 110 kDa glycoprotein, which catalyzes the release of N-terminal dipeptides from oligo- and polypeptides preferentially with proline, hydroxyproline and, with less efficiency, alanine in the penultimate position [8–10]. In the blood circulation, this enzyme is expressed on the surface of endothelial and different hematopoietic cells and is also present in a soluble form in the plasma [11]. Since DP IV has this unique enzymatic specificity compared with other exopeptidases and it has access to serum PCT, we investigated whether PCT(1-116) is a substrate of DP IV.

2. Materials and methods

2.1. Cloning and expression of PCT116 cDNA

The cDNA fragment encoding human PCT(1-116) was amplified by PCR from human thyroid gland cDNA (Clontech, Palo Alto, CA, USA) using the following oligonucleotide primers: 5′-CCG GGA ATT CAG CTG CAC CAT TCA GGT CTG CCC TGG-3′ and 5′-CCG GGA ATT CGG AGG AGT TTA GTT GGC ATT CTG GGG C-3′ (restriction enzyme recognition sites for *Eco*RI and *Pvu*II are underlined). Amplification was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the purified PCR product was cloned into pET-26b (Novagen, Madison, WI, USA) containing the T7lac promoter and a pelB leader peptide. The resulting plasmid was verified by sequencing and used to transform BL21(DE3) (Novagen). Expression of PCT(1-116) was induced at a cell density of 0.6 A_{588} by addition of IPTG (1 mM final concentration). After 3 h, cells were harvested and the periplasmic fraction containing PCT(1-116) was isolated by osmotic shock [12].

2.2. Purification of PCT(1-116)

The supernatant containing PCT(1-116) was filtered (0.2 μm) and applied onto a 10×100 mm anion exchange chromatography column (Poros HQ, Perseptive Biosystems, Framingham, MA, USA). The protein was eluted with a 0–250 mM NaCl gradient in 20 mM Tris/bis-Tris-Propan pH 7.0. Fractions were analyzed using the LUMItest® PCT kit (BRAHMS Diagnostica GmbH, Hennigsdorf, Germany); those containing PCT(1-116) were pooled and finally purified by reversed phase chromatography using a 4.6×100 mm Poros R2 column (Perseptive Biosystems) and a gradient of 5% acetonitrile, 0.1% trifluoroacetic acid to 90% acetonitrile, 0.1% trifluoroacetic acid. After analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), fractions containing PCT(1-116) without any contaminations were pooled and lyophilized. The identity of the purified PCT(1-116) was confirmed by subjecting it to N-terminal sequence analysis and mass spectrometry.

2.3. Human kidney DP IV

DP IV was purified from human kidney cortex. Membrane proteins were solubilized by the addition of 1% Triton X-100 to the homogen-

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ized tissue for 1 h. Subsequently, a fractionated ammonium sulfate precipitation was performed. The fraction between 50% and 65% saturation, containing most of the DP IV, was used for further purification by means of three different steps of liquid chromatography. After gel chromatography on Sepharose 6B, the pooled DP IV$^+$ fractions were applicated on a Sephadex A50 ion exchange column and eluted with an increasing gradient of NaCl. The final polishing step was performed on a FPLC Poros HQ ion exchange column. Resulting DP IV has a specific activity of 32 U/mg and shows no contamination in a silver-stained PAGE.

2.4. Synthetic DP IV inhibitors, PCT(2-116)

The reversible DP IV inhibitor Lys[Z(NO_2)]-thiazolidide and the irreversible inhibitor Pro-Pro$^{(P)}$[OPh-4Cl]$_2$ were kindly provided by Dr. K. Neubert (Martin-Luther-University Halle/Wittenberg, Germany). PCT(2-116) was a generous gift from BRAHMS Diagnostica GmbH (Hennigsdorf, Germany).

2.5. Cleavage of PCT(1-116) by human kidney DP IV

Stock solutions of 1 mM PCT(1-116) and PCT(2-116) contained 1% human serum albumin (HSA, protease-free, Sigma, Deisenhofen, Germany) to reduce adsorption processes.

Samples of a total volume of 16 μl, containing 15.5 pkat of human kidney DP IV and 4 nmol PCT(1-116) peptide, or PCT(2-116) as control, in 10 mM sodium phosphate assay buffer pH 7.4, were incubated at 37°C for up to 24 h. For the starting value, DP IV activity in 2 μl aliquots was stopped immediately by addition of 3 μl 30 mM phosphoric acid, pH 2.0 (stopping buffer). After 0.5, 2 and 9.5 h in 3 μl of the reaction mixture, the DP IV activity was also stopped with 3 μl stopping buffer. To achieve complete degradation of the substrate, further DP IV (5.5 pkat) was added to the residual reaction mixture and incubation was continued for further 14.5 h. All samples were stored at −20°C.

The formation of the dipeptide product Ala-Pro as a consequence of PCT(1-116) degradation was determined by the method of capillary zone electrophoresis (CE) using Biofocus 3000 (Bio-Rad, München, Germany). The samples were injected by pressure onto a LPA coated silica capillary (30 cm length, 50 μm ID, Bio-Rad) filled with 0.1 M phosphate buffer pH 2.5 with linear polymer (Bio-Rad). The separation was performed with a voltage of 17 kV (positive to negative) and monitored with UV absorption at 200 nm. For examination of the influence of DP IV-specific, synthetic inhibitors, samples were preincubated with the inhibitor specified for 30 min at 37°C before PCT(1-116) was added and kinetics were performed as described.

2.6. Mass spectrometry analysis of DP IV-catalyzed PCT(1-116) cleavage

Aliquots (2 μl) of the 2 and 9.5 h sample were evaporated and mass spectrometry analysis was performed by the Max-Delbrück-Centrum (MDC, Berlin-Buch, Germany) using a Q-Tof mass spectrometer (Micromass, UK).

2.7. Edman sequence analysis of DP IV-catalyzed PCT(1-116) cleavage

N-terminal Edman sequence analysis was performed by WITA GmbH (Teltow, Germany). The 24 h sample was evaporated and prior to the application onto a with polybrene (ABI Bio Brene Plus, Applied Biosystems, Weiterstadt, Germany) pretreated Micro TFA glass fiber filter (Applied Biosystems) dissolved in 50% acetonitrile/H_2O. Six cycles of N-terminal Edman degradation were performed with ABI Procice sequencer (Applied Biosystems).

3. Results

3.1. Hydrolysis of PCT(1-116) by purified human kidney DP IV

Due to its N-terminal sequence with proline in the penultimate position, PCT(1-116) is a putative substrate for DP IV. Based on the method of capillary electrophoresis, we established an assay for the examination of PCT(1-116) hydrolysis by purified human kidney DP IV. Neither HPLC nor capillary electrophoresis could separate PCT(1-116) from PCT(3-116). Therefore, we monitored the formation of the N-terminal dipeptide Ala-Pro. This product could be properly separated from the large peptides PCT(1-116) and PCT(3-116) as well as from the carrier protein HSA by means of CE (Fig. 1A). However, since the UV absorption of the dipeptide Ala-Pro is low, we needed to use high non-physiological concentrations of PCT in the assay. For quantification of the enzymatic reaction, the amount of Ala-Pro in the sample was set in relation to the amount of Ala-Pro formed by complete degradation after 24 h.

In Fig. 1A, the time course of the DP IV-catalyzed degradation of PCT(1-116) is shown. After 9.5 h, 70% of the PCT(1-116) was hydrolyzed. With the molar ratio of 1:250 for DP IV to substrate, used in our assay, the maximum turnover rate was calculated to 135 pmol/h. The incubation of PCT(2-116) with DP IV revealed no cleavage products in the electropherograms, indicating the specificity of the assay used (Fig. 1B).

Furthermore, analysis of the 24 h sample by N-terminal Edman sequence analysis clearly demonstrated that DP IV cleaves full-length PCT(1-116) and that Ala-Pro is not released from shorter intermediary products. As expected, due to the N-terminal sequence of PCT (Ala-Pro-Phe-Arg-Ser-Ala-Leu-Glu), Ala (from Ala-Pro) and Phe (from PCT(3-116)) were detected in the first cycle as the main products. Pro and Arg were clearly detected in the second cycle and Ser, Ala, Leu and Glu, corresponding to the sequence of PCT(3-116), in the following cycles. In the third step, no Phe was detected, indicating that PCT(1-116) was completely hydrolyzed after 24 h of incubation (data not shown).

Moreover, mass spectrometry analysis confirmed these results. Two proteins with masses of 12 791.5 Da and 12 623.5 Da were found in the 9.5 h sample (theoretical masses of PCT(1-116) and PCT(3-116) are 12 796.2 Da and 12 628 Da, respectively), which shows a partial DP IV-catalyzed hydrolysis of PCT(1-116) (data not shown).

3.2. Influence of synthetic DP IV inhibitors on PCT(1-116) cleavage

To clarify whether the cleavage of PCT(1-116) is done exclusively by DP IV and not by a putative impurity in the DP IV preparation used, further experiments were performed in the presence and absence of two synthetic, highly specific DP IV inhibitors. Lys[Z(NO_2)]-thiazolidide, a competitive inhibitor with $IC_{50} = 2.7 \pm 0.3$ μM [13], and the irreversible inhibitor Pro-Pro$^{(P)}$[OPh-4Cl]$_2$ ($K_i = 18.6$ μM) were used in concentrations of 20 μM and 4 μM, respectively. As shown in Fig. 2, both inhibitors were capable of preventing the DP IV-catalyzed hydrolysis of PCT(1-116) almost completely.

4. Discussion

Different groups have shown the ubiquitous distribution of the DP IV on the membrane of a variety of cells including epithelial and endothelial cells and the exceptional high expression in intestine, kidney and liver [11]. In the circulation, DP IV, which is identical with the T cell activation marker CD26, is expressed on the surface of resting and activated T cells, activated B cells and activated NK cells [10]. Furthermore, soluble forms of DP IV are described in the human plasma [14,15].

DP IV is a type II membrane enzyme, which has an extracellularly orientated catalytic domain with unique enzymatic

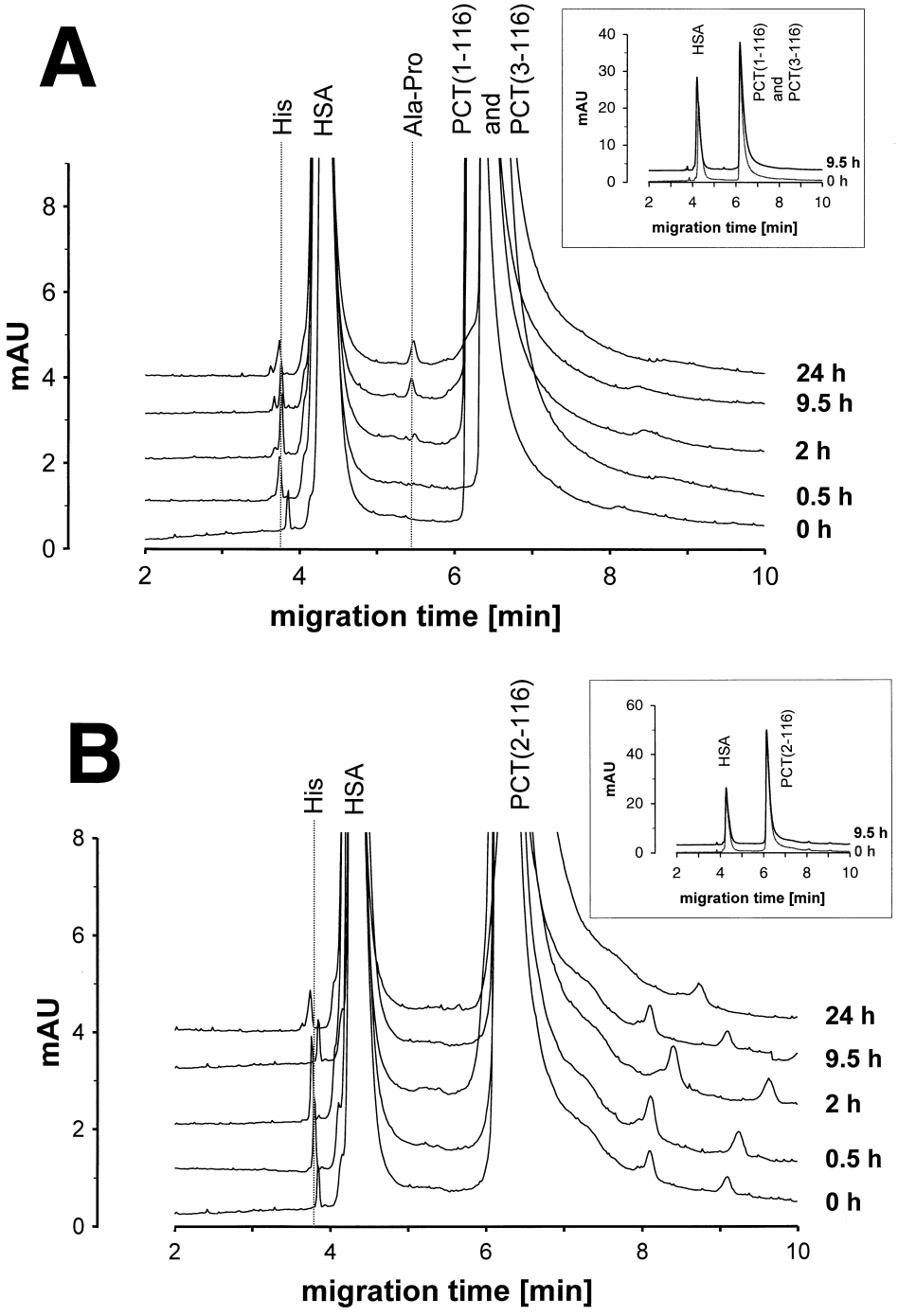

Fig. 1. Incubation of PCT(1-116) (A) and PCT(2-116) (B) with DP IV. Aliquots of the reaction mixture were taken and applied to CE after the incubation times indicated. Total cleavage was achieved by addition of further DP IV to the residual reaction mixture after 9.5 h and incubation for a further 14.5 h (indicated as 24 h). The inset shows the complete scale of the *y*-axis (HSA, carrier protein; His, histidine, standard for CE separation).

specificity [8,9,16]. The penultimate N-terminal proline is present in a number of peptides originating from the neuroendocrine system (e.g. substance P, β-casomorphine, neuropeptide Y, peptide YY, growth hormone-releasing factor) and of cytokines and growth factors, such as IL-3, IL-5, IL-10, IL-11, IL-13, GM-CSF, G-CSF, RANTES, LIF and thrombopoietin [10,16,17]. This proline residue protects these molecules from degradation by most aminopeptidases [8]. Short peptides as substance P and gastrin-releasing peptide have been known as effective substrates for DP IV [9]. In contrast, none of the intact, full-length cytokines with proline in the penultimate position has been identified as a DP IV substrate although smaller peptides up to 24 amino acids, containing the N-terminal sequence of cytokines (e.g. IL-1β, IL-2, murine IL-6), were cleaved. The rate of DP IV-catalyzed hydrolysis was negatively correlated with their chain length [17].

Recently, DP IV/CD26 has been shown also to process a

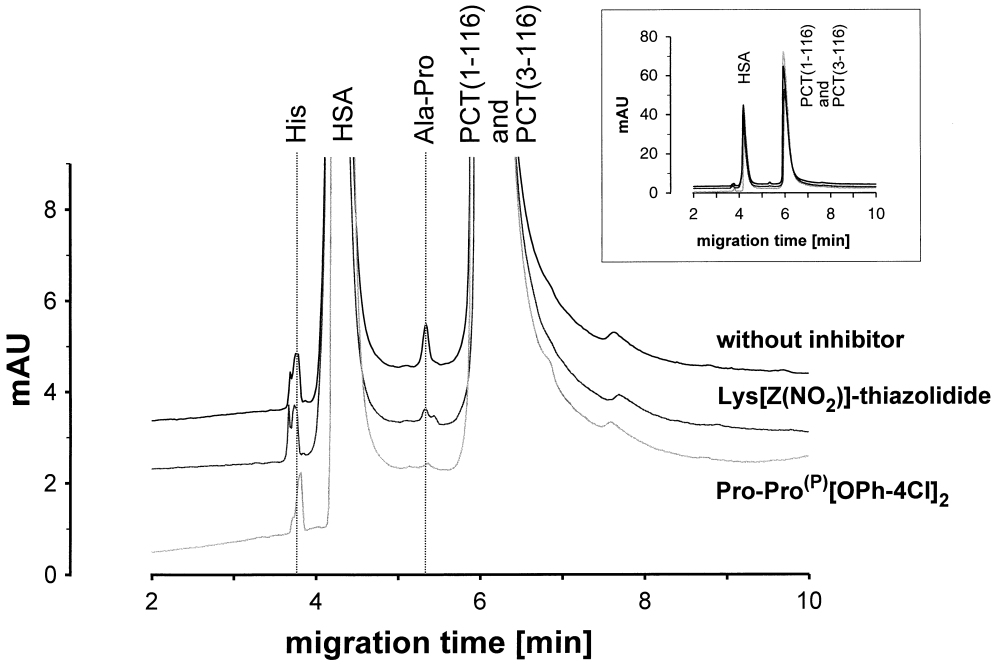

Fig. 2. DP IV-catalyzed hydrolysis of PCT(1-116) in presence and absence of the reversible DP IV inhibitor Lys[Z(NO_2)]-thiazolidide (20 μM) and the irreversible inhibitor Pro-Pro$^{(P)}$[OPh-4Cl]$_2$ (4 μM): the electropherograms of aliquots taken after 8 h of incubation are shown. The inset shows the complete scale of the *y*-axis (HSA, carrier protein; His, histidine, standard for CE separation).

number of chemokines including RANTES (regulated on activation normal T cell expressed and secreted), SDF-1 (stromal cell-derived factor-1), GCP-2 (granulocyte chemotactic protein-2), MDC (macrophage-derived chemokine) and others, generating naturally occurring truncated molecules with a significantly altered biological activity [16]. These DP IV substrates are proteins with a chain length between 69 and 75 amino acids.

PCT(1-116), a parameter for the diagnosis of severe bacterial and fungal infections with the N-terminal sequence Ala-Pro-Phe-Arg-Ser-Ala-, is also a potential substrate of DP IV. Interestingly, only the N-terminal truncated PCT(3-116), lacking the N-terminal dipeptide Ala-Pro, is found in high concentrations in patients with severe sepsis. Here, using the method of CE, we demonstrated for the first time that human DP IV is capable of catalyzing the hydrolysis of PCT(1-116) in vitro. This was confirmed by mass spectrometry and N-terminal sequence analysis. To our knowledge, this is the first report about DP IV-catalyzed hydrolysis of a protein greater than 75 amino acids.

Due to the length of 116 amino acids, the turnover rate of this cleavage is relatively low (135 pmol/h for the molar ratio 1:250 for DP IV to PCT in the assay). In vivo, however, a molar excess of DP IV compared to PCT is present. We measured in the plasma of eight healthy volunteers DP IV concentrations of 16.5 ± 2.9 nmol/l (data not shown). In patients with sepsis, together with membrane-bound DP IV, highly expressed on different activated inflammatory and endothelial cells, the total DP IV activity in the circulation should be high enough to be responsible for the truncation of even a long substrate like PCT(1-116).

However, the biological relevance and the origin of the N-terminal-truncated form of PCT, which is present in the serum of patients with bacterial infections and sepsis, are unknown as yet. Recently, PCT mRNA and protein expression was proved in peripheral blood mononuclear cells [18]. Lipopolysaccharide, phytohemagglutinin and various proinflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, IL-2), which have pronounced stimulatory effects on the PCT mRNA expression of these cells [18], are also potent stimulators of DP IV expression and activity on T cells, B cells and NK cells [10]. These facts suggest the involvement of DP IV, expressed on these activated immune cells as well as on endothelial cells, and soluble DP IV of the serum, in the truncation of PCT(1-116) in patients with bacterial infection and sepsis.

Current work is designed to locate the origin of PCT and to correlate the occurrence of truncated PCT with that of DP IV expression.

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