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Dipeptidyl peptidase IV activity in commercial solutions of human serum albumin ☆

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ABSTRACT

Due to the heterogeneous nature of commercial human serum albumin (cHSA), other components, such as the protease dipeptidyl peptidase IV (DPP-IV), possibly contribute to the therapeutic effect of cHSA. Here, we provide evidence for the first time that DPP-IV activity contributes to the formation of aspartate–alanine diketopiperazine (DA-DKP), a known immunomodulatory molecule from the N terminus of human albumin. cHSA was assayed for DPP-IV activity using a specific DPP-IV substrate and inhibitor. DPP-IV activity was assayed at 37 and 60 °C because cHSA solutions are pasteurized at 60 °C. DPP-IV activity in cHSA was compared with other sources of albumin such as a recombinant albumin (rHSA). In addition, the production of DA-DKP was measured by negative electrospray ionization/liquid chromatography mass spectrometry (ESI^-/LCMS). Significant levels of DPP-IV activity were present in cHSA. This activity was abolished using a specific DPP-IV inhibitor. Fully 70 to 80% DPP-IV activity remained at 60 °C compared with the 37 °C incubate. No DPP-IV activity was present in rHSA, suggesting that DPP-IV activity is present only in HSA produced using the Cohn fractionation process. The formation of DA-DKP at 60 °C was observed with the DPP-IV inhibitor significantly decreasing this formation. DPP-IV activity in cHSA results in the production of DA-DKP, which could account for some of the clinical effects of cHSA.

The use of commercial human serum albumin (cHSA)[1] solutions in the critically ill is sometimes indicated for blood volume restoration in certain conditions such as burn, acute lung injury, and shock [1–3]. For these patients, cHSA administration is controversial, with recent evidence demonstrating at best no reduction in mortality rates in comparison with cheaper alternatives such as saline [3]. In addition, the heterogeneity of cHSA solutions has been demonstrated and includes oxidation and truncation of the HSA molecule [4]. During processing and storage of cHSA solutions, the protein truncation occurs at the N terminus of the protein and results in the cleavage of the first two amino acids of HSA, Asp-Ala [5,6]. Due to the unique nature of the N terminus of HSA, this dipeptide is further converted to a cyclic dipeptide termed aspartate–alanine diketopiperazine (DA-DKP) [5]. In addition, the N terminus of HSA, in conjunction with the nearby reduced cysteine-34 residue, is involved in redox chemistry and the binding of redox-active metals such as copper [7,8]. DA-DKP has been found in significant quantities in cHSA solutions, and DA-DKP itself has immunosuppressive effects on activated peripheral blood mononuclear cells (PBMCs) and T-lymphocytes in vitro [9].

The mechanism of formation of DA-DKP from cHSA is currently unknown, but auto-degradation of the N terminus and/or an enzymatic reaction involving a peptidase is possible. Dipeptidyl peptidase IV (DPP-IV), or CD26, is a peptidase that preferentially cleaves Xaa-Pro and Xaa-Ala dipeptides from the N terminus of proteins [10]. DPP-IV activity has been reported on the cell surface of immune and endothelial cells [11] as well as in blood serum as a soluble form [12]. The main function of DPP-IV is thought to be the modification of biologically active peptides, cytokines, and other cell surface proteins for the purpose of regulating the immune response and cell differentiation [10]. In addition, a novel mechanism

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[1] *Abbreviations used:* cHSA, commercial human serum albumin; DA-DKP, aspartate-alanine diketopiperazine; PBMC, peripheral blood mononuclear cell; DPP-IV, dipeptidyl peptidase IV; *p*NA, *p*-nitroaniline; rHSA, recombinant HSA; MWCO, molecular weight cutoff; < 5-kDa, less than 5-kDa; LCMS, liquid chromatography mass spectrometry; HPLC, high-performance liquid chromatography; ESI^-, negative electrospray ionization; NAT, *N*-acetyl-tryptophan; ROS, reactive oxygen species.

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has been elucidated involving the DPP-IV-mediated degradation of the extracellular matrix (ECM), leading to the invasion of endothelial cells into collagenous matrices [13].

Recently, an exhaustive proteomic analysis was performed on cHSA solutions in order to understand the therapeutic effects, adverse reactions, and mechanisms involved in treatments using cHSA solutions [14]. In this study, a total of 1219 peptides corresponding to 141 proteins different from HSA were identified. More important, the peptidase DPP-IV was positively identified in the cHSA solution. Therefore, due to its ability to cleave peptides after an alanine residue, it is conceivable that DPP-IV is involved in the formation of DA-DKP in cHSA solutions. To test this hypothesis, commercially available solutions of HSA were assayed for DPP-IV activity using a chromogenic substrate and a known DPP-IV inhibitor [10]. The presence of DPP-IV activity was also tested in a recombinant HSA source not produced via the Cohn fractionation process. Finally, the effect of temperature on DPP-IV activity as well as DA-DKP production in commercial solutions of HSA was assessed.

Materials and methods

Materials

Three commercially available, 250-ml, 5% (w/v) HSA products (CSL Behring, Kankakee, IL, USA; Grifols Biologicals, Los Angeles, CA, USA; and Octapharma USA, Hoboken, NJ, USA) were used throughout the study. The N-terminal HSA peptide (DAHK) was manufactured by Diosynth (The Netherlands). Recombinant HSA (ecoHSA) was obtained from Genlantis (San Diego, CA, USA) and was produced in the seeds of Asian rice (*Oryza sativa*). Synthetic DA-DKP was produced by Syngene International (India). All other reagents, including the DPP-IV substrate and inhibitor, were obtained from Sigma–Aldrich (St. Louis, MO, USA).

DPP-IV assay

DPP-IV activity was assayed by using a chromogenic substrate, Gly-Pro-*p*NA (*p*-nitroaniline), as described previously [10]. All reactions were carried out in DPP-IV assay buffer (pH 7.6) consisting of 0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 8 mM glucose, and 10 mg/ml bovine serum albumin (BSA). cHSA (5%), recombinant HSA (rHSA), and 0.9% NaCl (no-HSA control) were combined with 1 mM Gly-Pro-*p*NA (DPP-IV substrate) in assay buffer. Incubations were performed at 37 or 60 °C for 2 to 24 h. For DPP-IV inhibition studies, 1 mM diprotin A in assay buffer was preincubated with the HSA solutions for 15 min at 37 °C prior to DPP-IV substrate addition. All incubations were read at 405 nm (SpectraMax M2 spectrophotometer, Molecular Devices, Sunnyvale, CA, USA). Each reading at 405 nm was corrected by subtracting the A_{405} for the DPP-IV substrate-containing incubation from the corresponding A_{405} for the negative control incubation for each HSA solution tested.

Isolation of < 5-kDa HSA fraction

For the analysis of DA-DKP formation, an aliquot was added to a microcentrifugal filter (Vivaspin 2, molecular weight cutoff [MWCO] = 5000, Sartorius Stedim Biotech, Germany). Filters were centrifuged at 3500 rpm for 30 min at room temperature. The less than 5-kDa (< 5-kDa) fraction was collected and transferred to a separate storage tube for liquid chromatography mass spectrometry (LCMS) analysis.

Table 1
HPLC gradient used in separation of DA–DKP in < 5-kDa HSA solutions.

Time (min)	A (%)	B (%)	C (%)
0	25	40	35
10	10	40	50
15	10	40	50
15.01	25	40	35
20	25	40	35

LCMS assay

Each < 5-kDa fraction and DA-DKP synthetic standard (20–2000 ng/ml) was spiked with 0.01 mM L-tryptophan-d_5 (indole-d_5), which was used as an internal standard. An internal standard was used to correct for any small changes in the consistency and sensitivity of the LCMS between runs. Here, 50 μl was injected into a strong anion exchange column (Spherisorb S5 SAX, 250 × 4.0 mM, Waters, Milford, MA, USA) connected to high-performance liquid chromatography (HPLC) instrument (Waters 2795 Separations Module) coupled to a mass spectrometer (LCT-TOF, Micromass, UK). A ternary mobile phase consisting of distilled water (dH_2O, solvent A), methanol (solvent B), and 200 mM ammonium formate (pH 5.4, solvent C) was used at a flow rate of 0.5 ml/min using the gradient shown in Table 1.

The output of the HPLC instrument was split 1:20 (v/v) and injected into the mass spectrometer using negative electrospray ionization (ESI^-) with a scan range of m/z 80 to 1000, cone voltage of 30 eV, source temperature of 100 °C, and gas temperature of 300 °C. DA-DKP was measured by monitoring m/z 185, which corresponds to DA-DKP minus a single proton ($-H^+$). The straight chain of DA-DKP, Asp-Ala, can also be analyzed with this method by monitoring m/z 203.

Statistical methods

The amount of *p*NA produced (in μM) was calculated based on the *p*NA molar extinction coefficient in Hepes buffer [15]. Statistical analysis was performed using the software packages Excel (Microsoft) and Matlab R13 (MathWorks). Groups were compared using a two-tailed Student's t test with a significance level at $P < 0.05$. All data are reported as means ± standard deviations.

Results

DPP-IV activity was assessed in commercial preparations of HSA. The activity assay chosen is well documented in the literature and involves the cleavage of a known DPP-IV substrate, Gly-Pro-*p*NA. The resulting liberation of a chromogen, *p*NA, was measured spectrophotometrically at 405 nm. Three commercially available solutions of 5% HSA were chosen with no particular manufacturer preference. The only requirements were that the solutions were unexpired and were produced by different manufacturers using the Cohn fractionation process. For the incubation temperatures of the enzyme assay, 37 and 60 °C were chosen because the former represents physiological conditions and the latter represents the pasteurization temperature of cHSA solutions.

DPP-IV activity at 37 °C was measured in all three 5% cHSA solutions. All three cHSA solutions contained significant DPP-IV activity, with the CSL Behring HSA having slightly less activity than the Octapharma and Grifols HSAs (Fig. 1). The amount of DPP-IV activity did not correlate with the expiration dates of the cHSA sources. DPP-IV was completely suppressed in the presence of a known DPP-IV inhibitor, diprotin A. This resulted in no additional chromogen production during the entire incubation. In one of the