Analytical Biochemistry 441 (2013) 13–17

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Dipeptidyl peptidase IV activity in commercial solutions of human serum albumin

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ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 14 May 2013
Accepted 6 June 2013
Available online 14 June 2013

Keywords:
Human serum albumin
Dipeptidyl peptidase IV
Diketopiperazine
Cohn fractionation
Inflammation

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.
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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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http://dx.doi.org/10.1016/j.ab.2013.06.002

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

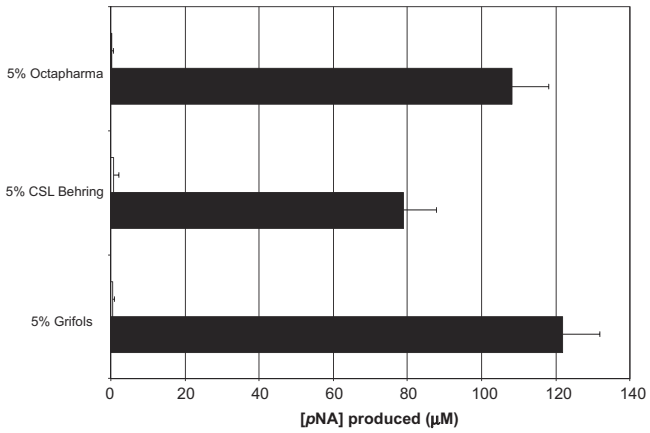

Fig.1. DPP-IV activity in 5% cHSA solutions. DPP-IV activity ($n = 3$) is represented as the total amount of *p*NA (in μM) produced during a 24-h incubation at 37 °C with (vertical lines) and without (solid bars) the DPP-IV inhibitor, diprotin A.

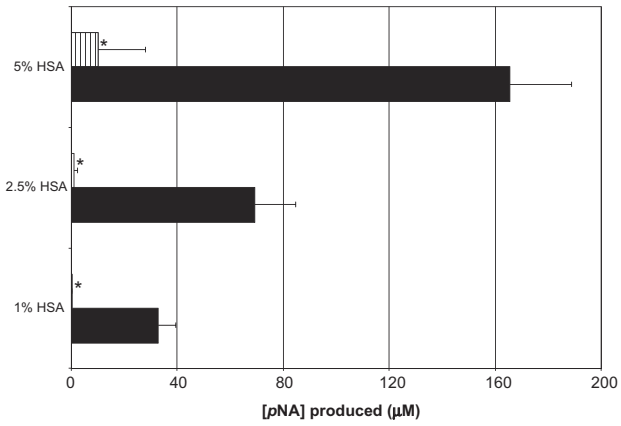

Fig.3. DPP-IV activity in HSA solutions produced by different manufacturing methods. DPP-IV activity ($n = 3$) is represented as the total amount of *p*NA (in μM) produced during a 24-h incubation at 37 °C. DPP-IV activity was measured in a cHSA solution produced using Cohn fractionation (solid bars) and in an rHSA solution (vertical lines). An asterisk (*) represents statistical significance ($P < 0.05$).

cHSA solutions (CSL Behring), DPP-IV activity at 60 °C was assayed. DPP-IV activity was present at significant levels (Fig. 2). However, DPP-IV activity at 60 °C was approximately 70 to 80% of the original DPP-IV activity at 37 °C. At both temperatures, a dose response in DPP-IV activity was observed with increasing concentrations of the cHSA solution.

To compare DPP-IV activity in HSA isolated using a non-Cohn fractionation process, an rHSA produced in rice was analyzed. One of the cHSA solutions produced by Cohn fractionation was also included in the DPP-IV activity assay. For both HSA types, concentrations ranged from neat solutions (5%, w/v) to diluted solutions (1 and 2.5%). At all three concentrations, the amount of DPP-IV activity in the cHSA solution was significantly higher than that in the rHSA solution (Fig. 3). In addition, DPP-IV activity in the rHSA solutions was not statistically significant from that in the assay buffer-only incubations. Therefore, no significant DPP-IV activity was present in the rHSA solution.

The formation of the diketopiperazine, DA-DKP, was measured in a cHSA solution heated at 60 °C in the presence or absence of a known DPP-IV inhibitor, diprotin A. The low-molecular-weight fraction of HSA containing DA-DKP was isolated using a 5-kDa MWCO spin column. The < 5-kDa fraction was assayed for DA-DKP content by LCMS using ESI^-. During the first 24 h, DA-DKP content in the incubations containing no inhibitor increased 30% from baseline DA-DKP levels (Fig. 4). In the presence of the DPP-IV inhibitor, only a 10% increase in DA-DKP production was observed over 24 h at 60 °C.

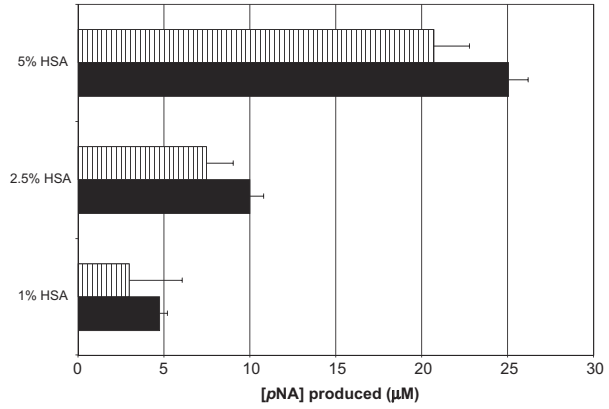

Fig.2. Effect of temperature on DPP-IV activity in a solution of 5% cHSA. DPP-IV activity ($n = 3$) is represented as the total amount of *p*NA (in μM) produced during a 2-h incubation at 37 °C (solid bars) and 60 °C (vertical lines).

Discussion

HSA is the most abundant circulating protein with ligand binding and transport properties, antioxidant functions, and enzymatic activities [7]. Because HSA is important for the regulation of blood volume and osmotic pressure in the critically ill, it is produced in mass quantities by the pharmaceutical industry. The preferred manufacturing technique of cHSA is based on the method of Cohn and coworkers, which isolates HSA using a cold ethanol fractionation process [16]. Commercial preparations of 5% HSA usually contain the stabilizers *N*-acetyl-tryptophan (NAT) and sodium caprylate at a concentration of 0.08 mmol/g HSA. The shelf life for commercial solutions of HSA is commonly 3 years. Most likely due to the production of reactive oxygen species (ROS), some age-related changes in the solution properties have been observed, including color changes, protein oxidation, proteolysis, aggregation, and precipitation [6].

Because the Cohn fractionation process is not specific for HSA, some proteins and peptides are copurified with HSA and, therefore, are present in commercial solutions [17]. In addition, because HSA has the unique ability to bind multiple ligands, other peptides and proteins with known biological activity have been identified in commercial solutions of HSA using proteomic techniques [14,17]. These copurified or bound proteins include proteases (kallikrein, cathepsin, carboxypeptidases, and dipeptidases), protease inhibitors (kininogen), cell surface adhesion proteins (selectin, cadherins, and ICAMs), and proteins involved in immunity (immunoglobulin chains and components of the complement system) [14]. Recently, a unique intrinsic proteolytic activity of the HSA molecule under reducing conditions was documented [18]. Therefore, due to its heterogeneous nature, the administration of HSA could introduce potentially unwarranted side effects to critically ill patients.

In addition to proteins, commercial solutions of HSA contain a small immunosuppressive molecule derived from the first two N-terminal amino acids of HSA, DA-DKP [9]. DA-DKP is thought to modulate T-cell cytokine production by increasing Rap1 (Ras-related protein 1, a small GTPase) activity and decreasing activation factors relevant to the T-cell receptor signal transduction pathway [19]. The mechanism of formation of DA-DKP in commercial solutions of HSA is currently unknown, with one theory suggesting the auto-degradation of the N terminus of HSA and

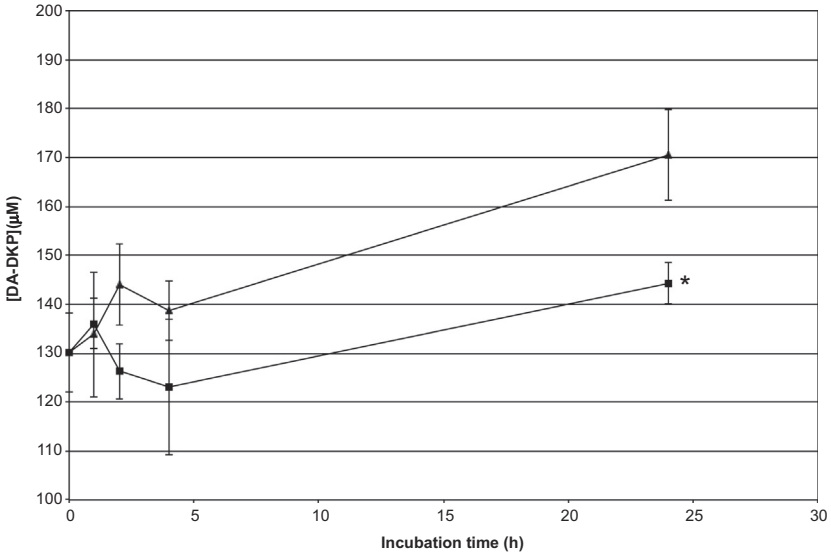

Fig.4. DA-DKP production in 5% cHSA heated at 60 °C. DA-DKP production (n = 3) was measured at different time points in a 5% cHSA solution heated at 60 °C in the absence (▲) or presence (■) of a DPP-IV inhibitor. An asterisk (*) represents statistical significance (P < 0.05) versus neat 5% HSA.

subsequent formation of DA-DKP due to the unique redox characteristics of the N terminus [5]. The theory explored in our study is based on the existence of proteases in commercial solutions of HSA, specifically DPP-IV [14]. Using a known chromogenic assay, DPP-IV activity was indeed measured in three commercial solutions of HSA. In addition, this activity was abolished by the use of a known in vivo inhibitor of DPP-IV, diprotin A [20]. Therefore, in addition to the presence of the DPP-IV protein, DPP-IV activity is also present in commercial solutions of HSA. This activity was not present in an rHSA, suggesting that DPP-IV activity in cHSA is not removed during the Cohn fractionation process. Other proteases that have enzymatic activity on DPP-IV substrates, such as DPP-8 and DPP-9, were not included in this study because they have not been positively identified in cHSA. This is probably due to the fact that they are not secreted proteins (UniProt search) and, therefore, are unlikely to be present in a plasma-derived solution such as cHSA.

During the production of cHSA, the product is pasteurized for 10 to 11 h by heating at 60 °C. Interestingly, optimal DPP-IV activity has been reported between 50 and 60 °C in serum, recombinant, and seminal DPP-IV, with a gradual loss in activity at 65 °C [12]. This unique characteristic of DPP-IV makes it a plausible candidate for the production of DA-DKP in cHSA solutions. In addition, during the Cohn fractionation process, the low-molecular-weight components (other than those bound to HSA) are most likely removed prior to the pasteurization step. Therefore, the majority of DA-DKP measured in commercial solutions of HSA is produced de novo from the pasteurization step onward. In the cHSA solutions used for this study, significant DPP-IV activity was measured at 60 °C. However, the total activity was only 70 to 80% of the activity present in the 37 °C incubations. The amount of DA-DKP produced at 60 °C in this study has been shown to cause significant immunosuppression in activated human PBMCs [9].

The production of DA-DKP in cHSA at 60 °C was examined using an LCMS method for detecting DA-DKP. In the neat solutions of cHSA, DA-DKP was produced in significant quantities over 24 h at 60 °C. When the DPP-IV inhibitor, diprotin A, was added to the cHSA solutions, the amount of DA-DKP produced at 60 °C decreased approximately 3-fold over the 24-h period. Therefore, this finding suggests that DPP-IV is partially responsible for the formation of DA-DKP in commercial solutions of HSA. Diprotin A did not completely abolish DA-DKP formation at 60 °C. Diprotin A is trapped as a tetrahedral intermediate covalently bound to Ser630 inside the active site of DPP-IV [21]. Interestingly, diprotin A (Ile-Pro-Ile) is a substrate of DPP-IV, with a low turnover leading to an apparent competitive inhibition [22]. It is possible that diprotin A is hydrolyzed to a sufficient degree after a 24-h incubation at 60 °C to allow other DPP-IV substrates into the active site such as the N terminus of HSA. In combination with the enzymatic formation of DA-DKP, we cannot completely discount the formation of DA-DKP via the auto-degradation of the N terminus of HSA [5].

The known substrates of DPP-IV include several chemokines, cytokines, neuropeptides, circulating hormones, and bioactive peptides [23]. One of the most studied DPP-IV substrates is glucagon-like peptide 1 (GLP-1), which regulates circulating plasma glucose levels and, therefore, is important in the etiology of type 2 diabetes [21]. All of the known DPP-IV substrates are polypeptides, with the N terminus of HSA never being described as a substrate until now. Access of the N terminus of HSA to the DPP-IV active site is unlikely to occur with HSA in its native confirmation due to steric hindrance. However, a significant portion of the HSA N terminus needs to be accessible to the DPP-IV active site in order to form DA-DKP. In our study, we used the HSA N-terminal peptide DAHK as a DPP-IV substrate, but we detected only the formation of the straight chain dipeptide (i.e., Asp-Ala) instead of DA-DKP (data not shown).

There are at least two plausible ways in which the N terminus of HSA can be presented to the active site of DPP-IV. First, the oxidation of HSA in commercial solutions during storage could cause the cleavage of HSA, resulting in the production of N-terminal peptides that are better substrates for the DPP-IV-active site. Redox-active metals, such as iron and copper, are found in significant quantities in solutions of cHSA [24]. Indeed, the N terminus of HSA binds copper, which could result in the in situ production of ROS, possibly leading to the cleavage of HSA N-terminal peptides [8]. Second, slow denaturation of HSA could result in the unfolding of the N terminus, making it a more accessible DPP-IV substrate. This is partially supported by the fact that at 60 °C HSA is in a reversible unfolded form, possibly exposing the N terminus [25]. Is it possible that this reversible unfolded form becomes more common during the prolonged storage of solutions of HSA, leading to the increased production of DA-DKP?

The immunosuppressive capabilities of administered HSA are well documented. In a rat model of hemorrhagic shock, HSA reduced lung permeability and neutrophil sequestration in a dose-

dependent fashion [26]. In a similar rat model of shock, administered HSA significantly down-regulated the expression of integrins and ICAM-1, factors involved in the adhesion of immune cells to the endothelium [27]. HSA also suppressed the respiratory burst of neutrophils in response to TNFα or complement exposure, resulting in the selective and reversible inhibition of neutrophil spreading [28]. Finally, HSA was found to be the least pro-inflammatory of the resuscitation fluids used in a hemorrhagic shock model [29]. Based on our previous immunological studies, DA-DKP appears to be partially responsible for the immunosuppressive capabilities of HSA.

The heterogeneity of commercial solutions of HSA could cause many beneficial or detrimental effects in critically ill patients, depending on the immunological state of the patients. Some of the compounds recently identified in commercial solutions of HSA are involved in immune regulation and function [14]. In addition, the stabilizer NAT is a well-known antagonist of the neurokinin-1 receptor, an important mediator of the immune and inflammatory response as well as vascular permeability [30]. In our study, we focused on the mechanism of formation of the anti-inflammatory DA-DKP, which is found in micromolar concentrations in commercial solutions of HSA. Commercial solutions of HSA contain significant levels of DPP-IV activity, which is inhibited by diprotin A. In addition, DPP-IV activity is unique to the cHSA solutions due to the Cohn manufacturing process, which isolates other plasma components such as DPP-IV. Finally, the de novo formation of DA-DKP in cHSA solutions heated at 37 °C was observed with a corresponding inhibition of formation in the presence of diprotin A. Therefore, in commercial solutions of HSA, the peptidase DPP-IV appears to be involved in the formation of DA-DKP, a known anti-inflammatory compound.

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