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Research paper

Exploring binding characteristics and the related competition of different protein-bound uremic toxins

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ABSTRACT

Little is known about potential differences in binding characteristics of protein-bound uremic toxins (PBUTs) in patients with chronic kidney disease (CKD) versus healthy controls. The question arises whether eventual differences are attributed to (i) the elevated levels of competing uremic toxins, and/or (ii) post-translational modifications of albumin.

We evaluated the binding characteristics of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS), and *p*-cresylsulfate (*p*CS) by deriving a binding curve in three distinct conditions: (i) serum from healthy controls (healthy serum), (ii) blank serum from hemodialysis patients (blank HD serum; i.e. cleared from uremic toxins), and (iii) non-treated serum from HD patients (HD serum). Additionally, the mutual binding competition of these uremic toxins was studied in blank HD in pairs. In both experiments, equilibrium dialysis (37 °C, 5 h) was used to separate the free and bound fractions of each PBUT. Free and total PBUT concentrations were quantified by an ultra-high performance liquid chromatography method with tandem mass spectrometer detection and the percentage protein binding (%PB) of each PBUT was calculated.

For all four compounds, the binding capacity of healthy serum was higher than blank HD serum, which was comparable to non-treated HD serum, except for HA. The competition experiments revealed that at high uremic concentrations, mutual competition was observed for the strongly bound PBUTs IS and *p*CS. The %PB of the weakly bound HA and IAA was lower (trend) only for the addition to blank HD serum containing the strongly bound IS or pCS.

There is an intrinsic impact on protein binding in uremia, revealing a lower binding capacity, as compared to healthy controls. Competitive binding is only relevant for the strongly bound PBUTs at high uremic concentrations. In addition, at least part of the effect on binding capacity can be attributed to post-translational modifications of albumin.

1. Introduction

Chronic kidney disease (CKD) is characterized by the retention of a large number of compounds that, under normal conditions, are excreted by the healthy kidneys. Numerous retention solutes have a negative effect on many biological functions and are therefore called uremic toxins [1–9]. Among them, the protein-bound uremic toxins (PBUTs) [10,11] mainly bind to albumin and this in different degrees [12,13]. The range in percentage protein binding (%PB) implies a differential removal of these toxins during hemodialysis,

Abbreviations: CKD, chronic kidney disease; PBUT(s), protein-bound uremic toxin(s); HD, hemodialysis; PTM(s), post-translational modification(s); HA, hippuric acid; IAA, indole-3-acetic acid; IS, indoxyl sulfate; *p*CS, *p*-cresylsulfate; HSA, human serum albumin; PBS, phosphate buffered saline; ED, equilibrium dialysis; AIC, Akaike information criterion.

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since only the free fraction can pass conventional dialyzer membranes [14].

Several studies reported a decreased %PB for some of these PBUTs in uremic serum or plasma, compared to normal serum or plasma [13–17]. In these studies, it was suggested that the decrease in %PB was attributed to either (i) the elevated PBUT concentrations in CKD and hemodialysis (HD) patients, resulting in a competition among the PBUTs to bind to albumin, or (ii) post-translational modifications (PTMs) of albumin in CKD and HD patients by e.g. oxidation [18–20], glycation [21–23], carbamylation [24–26], or guanidinylation [27] of lysine residues. However, to date, it is not clear whether the observed decrease in %PB of PBUTs in CKD and HD patients is attributed to (i) the competition between them, depending on their degree of binding (i.e. %PB) or binding affinity, (ii) the modification of albumin, or (iii) the combination of both.

In the past, a substantial number of binding studies were performed to unravel the binding characteristics of the studied PBUTs [13,17,28–34], but only few of them discussed their mutual competitive binding behavior in detail [13,31,33,34]. In most cases, the interaction between indoxyl sulfate (IS) and *p*-cresylsulfate (*p*CS) was discussed, whereas – to the best of our knowledge – none of these articles discussed the competition with other, less bound UTs such as hippuric acid (HA) or indole-3-acetic acid (IAA). Furthermore, most *in vitro* binding experiments were conducted in solutions prepared from commercially available human serum albumin powder (HSA) and did not take into account the PTMs affecting albumin as observed in CKD and HD patients [12,27,35], and their possible effect on the %PB of PBUTs [27].

In this work, we intended to gain more insight into the binding of HA, IAA, IS, and *p*CS by evaluating their binding characteristics in three distinct conditions: (i) serum from healthy controls, (ii) serum from HD patients that was cleared from uremic toxins (i.e. blank HD serum), and (iii) non-treated serum from HD patients. Additionally, the mutual competitive binding of these uremic toxins was studied by spiking blank HD serum with two PBUTs in pairs, having one PBUT in a varying concentration.

2. Materials and methods

2.1. Sample collection

Blood samples from twelve healthy controls and pre-dialysis blood samples from sixty-seven stable HD patients were collected in Venosafe serum tubes (Terumo Europe, Leuven, Belgium). After clotting, these tubes were centrifuged [Beckman Coulter X-15R centrifuge, 3000 rpm (range: 784 *g*–2095 *g*), 10 min] and aliquots were stored at −80 °C. After thawing, four different serum pools were prepared according to the requirements of the four experiments, i.e. the construction of a binding curve in (i) healthy serum, (ii) blank HD serum, and (iii) non-treated HD serum and the competition experiments in (iv) blank HD serum. The blank HD sera were cleared from uremic toxins by a 24 h *in vitro* dialysis procedure, followed by the addition of activated charcoal, as recently described by de Loor et al. [36]. Each pool was stored at −80 °C until the experiments were performed.

This study was performed according to the Declarations of Helsinki, was approved by the local Ethical Committee (EC 2010/033 for healthy serum, 2015/0571 for non-treated HD serum, and 2015/0932 for blank HD serum), and all patients gave their written informed consent.

2.2. Chemicals

HA, IAA, IS, and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *p*CS from TCI Chemicals (Zwijndrecht, Belgium). Water (HPLC grade) was purchased from Acros Organics (Thermo Scientific, Geel, Belgium). All stock solutions were prepared in PBS buffer and stored at −20 °C.

2.3. Binding characteristics and mutual competition

Binding curves were constructed, in a single run, for the four PBUTs by spiking healthy serum, blank HD serum, and non-treated HD serum samples (190 μL) with eight concentrations (10 μL, 20x stock solutions of the final concentration) of either HA, IAA, IS, or *p*CS. For each compound, the theoretical final concentrations were 100, 250, 500, 750, 1000, 2500, 5000, and 10000 μM, corresponding to normal physiological [11] over uremic [11] up to supra-physiological concentrations to ensure binding saturation. Finally, 150 μL of the spiked serum was used in equilibrium dialysis.

In a second series of experiments, the mutual binding competition of all four studied PBUTs was investigated by spiking blank HD serum (1980 μL) with a concentration (20 μL, 100x stock solution of the final concentration) of one PBUT to reach a final uremic concentration of 100 μM for *p*CS and IS, 10 μM for IAA, and 400 μM for HA [11], followed by an incubation step (37 °C, 30 min). Subsequently, the spiked blank HD serum was divided into aliquots of 190 μL, and the so-called competing PBUT was added to each aliquot in different concentrations (10 μL, 20x stock solutions of the final concentration), up to high uremic concentrations [11], followed by a second incubation step (37 °C, 30 min). Finally, 150 μL of the serum was used in equilibrium dialysis. These experiments were performed in triplicate.

2.4. Equilibrium dialysis

In both series of experiments, equilibrium dialysis (ED) was used to separate the free fractions using a HTDialysis 96b system (HTDialysis, Connecticut, USA). The dialysis membranes, consisting of regenerated cellulose with a molecular weight cut-off of 3.5 kDa, were hydrated and used according to the manufacturer's guidelines. The spiked serum (150 μL) was dialyzed against a PBS solution (150 μL, pH = 7.4, ionic strength = 0.15 M) for 5 h at 37 °C on a reciprocating shaker and an adhesive film was used to seal the wells. After 5 h, equilibrium was reached (determined in a pilot experiment, data not shown) and samples were taken from both sides of the dialysis chamber and stored at −80 °C until bulk analysis.

2.5. Analyses

Total and free PBUT concentrations were determined by an Acquity H Class (Waters, Zellik, Belgium) ultra-high performance liquid chromatography system. Chromatographic separation was performed on an Acquity CSH Fluoro Phenyl column (50 × 2.5 mm, 1.7 μm, Waters, Zellik, Belgium) with an Acquity CSH Fluoro Phenyl VanGuard pre-column (10 × 2.5 mm, 1.7 μm, Waters, Zellik, Belgium) and PBUTs were detected using a Xevo TQS tandem mass spectrometer (Waters, Zellik, Belgium). The sample preparation and chromatographic analysis method are described in more detail by de Loor et al. [36].

Total protein and albumin analyses were performed in the routine laboratory of Ghent University Hospital on a Cobas 8000 c701 (total protein) and c502 (albumin) analyzer (Roche Diagnostics, Mannheim, Germany) using the biuret and bromocresol green method, respectively.

2.6. Calculations

Total protein and albumin concentrations were measured in all