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Corticosteroid-binding globulin cleavage is paradoxically reduced in alpha-1 antitrypsin deficiency: Implications for cortisol homeostasis

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ABSTRACT

High-affinity corticosteroid-binding globulin (haCBG) is cleaved by neutrophil elastase (NE) resulting in permanent transition to the low cortisol-binding affinity form (laCBG), thereby increasing cortisol availability at inflammatory sites. Alpha-1 antitrypsin (AAT) is the major inhibitor of NE. AAT deficiency (AATD) predisposes patients to early-onset emphysema due to increased proteolytic destruction from the inherent proteinase–antiproteinase imbalance. We hypothesized that AATD may result in increased CBG cleavage *in vivo*. We collected demographic data and blood samples from 10 patients with AATD and 28 healthy controls measuring total CBG and haCBG levels by parallel in-house ELISAs, as well as AAT, total and free cortisol levels. haCBG was higher (median [range]); 329 [210–551] *vs.* 250 [175–365] nmol/L; $P < 0.005$, and laCBG lower; 174 [68–229] *vs.* 220 [119–348] nmol/L; $P = 0.016$ in the AATD group, compared with controls. The ratio of haCBG:total CBG was also higher in AATD; 72 [53–83] *vs.* 54 [41–72] %; $P = 0.0001$). There was a negative correlation between haCBG:total CBG and AAT levels ($P < 0.05$, $R = -0.64$). Paradoxically, proteolytic cleavage of CBG was reduced in AATD, despite the recognized increase in NE activity. This implies that NE activity is not the mechanism for systemic CBG cleavage in basal, low inflammatory conditions. Relatively low levels of laCBG may have implications for cortisol action in AATD.

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1. Introduction

Corticosteroid-binding globulin (CBG) is a large, highly glycosylated serine proteinase inhibitor (SERPIN) protein [1] that has no inhibitory capacity. It is the major carrier of the anti-inflammatory hormone cortisol in the circulation, regulating the distribution of free cortisol to tissues [2]. CBG binds cortisol at its single binding site when in its native, or stressed, high-affinity conformation (haCBG) [3]. Neutrophil elastase (NE), a destructive serine proteinase, provides the known mechanism for CBG cleavage as it binds and cleaves the exposed reactive centre loop (RCL) of haCBG between the valine^{344} and threonine^{345} residues resulting in a conformational transition that disrupts the cortisol-binding site and decreases CBG's affinity for cortisol by 9–10 fold, forming low affinity CBG (laCBG) and effectively releasing cortisol at inflammatory sites [3–7].

Alpha-1 antitrypsin (AAT), also a SERPIN protein, is the major inhibitor of NE. AAT is produced by hepatocytes in large quantities such that NE is saturated in the general circulation. AAT is also secreted locally by macrophages, monocytes and epithelial cells in response to inflammatory cytokines and lipopolysaccharide [8,9]. When NE is released in concentrated bursts from activated neutrophils at sites of inflammation, a process described as "quantum proteolysis", it momentarily overwhelms inhibitory AAT [10,11], allowing proteolysis, including CBG cleavage, at sites of inflammation [3,7]. Patients with AAT deficiency (AATD) have increased NE activity, which leads to a recognized proteinase–antiproteinase imbalance [10,12,13]. At localized inflammatory sites in AATD, the area and duration of tissue exposed to attack by NE is increased 10 fold due to the lack of inhibiting AAT [10]. The resulting AATD phenotype is early onset basal emphysema, with increased proteolysis of lung parenchymal elastin due to this inherent proteinase–antiproteinase imbalance and specifically NE excess [14]. This theory is the basis for the administration of AAT augmentation therapy, which has been shown to decrease markers of elastin degradation due to NE in AATD [15].

It was previously thought that NE-mediated CBG cleavage did not occur systemically due to the presence of saturating levels of AAT [7]. However, using novel parallel monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs), we have shown that haCBG and laCBG coexist and can be measured in the circulation, with sometimes up to 30% of CBG in apparently healthy individuals being of the

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cleaved laCBG affinity form [16,17]. Given that these two conformations have identical half-lives of 10 h under physiologic conditions [18], systemic cleavage of CBG may be occurring continuously in the general circulation, rather than exclusively at inflammatory sites. It may be that a sufficient reservoir of haCBG is advantageous in providing a pool of cortisol for immune modulation at inflammatory sites. It is not known if systemic CBG cleavage is mediated by NE under low-inflammatory conditions.

In vitro, incubation of human CBG with activated leukocytes results in protein cleavage, a process that is blocked when cells are pre-incubated with an excess of AAT [7] and, *in vivo*, sepsis and septic shock, which are conditions associated with elevated NE [19], demonstrate increased CBG cleavage [20]. It is not known, however, whether AAT deficiency is associated with increased CBG cleavage in patients without active acute inflammation. We hypothesized that the uninhibited neutrophil elastase activity in AATD would result in increased cleavage of CBG, as shown by reduced levels of circulating haCBG and elevated laCBG. Hence, we undertook a prospective observational study using our novel monoclonal antibody ELISAs to determine the levels of haCBG and laCBG in patients with AATD *in vivo*.

2. Materials and methods

2.1. Participants and design

This was a prospective, observational cohort study, conducted between December 2014 and April 2015 at a single tertiary centre in Adelaide, South Australia. The protocol was approved by the Human Research Ethics Committee, Royal Adelaide Hospital. Participants were identified through the Clinical Trials Database, Department of Thoracic Medicine, and were mailed an invitation to participate. Control patients were recruited from the community and hospital outpatient clinics by advertising. Inclusion criteria: either no known AATD or previously diagnosed AATD of phenotypes ZZ, SZ or MZ, as confirmed by isoelectric focusing [21], and age > 18 years. Exclusion criteria: previous lung or liver transplant, known disease of the hypothalamic–pituitary–adrenal (HPA) axis, pregnancy, use of oral contraceptive or hormone replacement therapy, or active infective condition. Participants who responded to the invitation or advertising were screened and provided written informed consent prior to enrolment.

2.2. Assessments

Baseline measurements including height, weight, and blood pressure were performed. Venipuncture was performed with blood collected into one serum gel tube and one EDTA tube, put immediately on ice. Following centrifugation, serum or plasma was aliquoted and stored at −20 °C. Blood samples (median) were taken at 10:23 am in the control group and 11:37 am in the AATD group.

Serum total cortisol was measured by electrochemiluminescence immunoassay on a Roche Cobas® analyzer (Roche Diagnostics, Castle Hill, New South Wales, Australia). Fraction of free cortisol was determined using a temperature-controlled ultrafiltration/ligand-binding method [20] validated against equilibrium dialysis [22]. Free cortisol was calculated from total cortisol level and free cortisol fractions [22].

Serum AAT levels were performed on patients with known AATD by immunoturbidity using a Tina-Quant α1-antitrypsin assay (Roche Diagnostics, Castle Hill, Australia) on a Thermo KoneLab 30 Analyzer, calibrated with reference to CRM 470 protein standardization materials (reference interval 0.9–2.0 g/L). Phenotyping was performed by isoelectric focusing using a HYDRAGEL 18 A1AT ISOFOCUSSING kit.

CBG affinity forms were measured in serum using the validated technique previously described [20]. Total CBG and matching haCBG assays are performed in parallel with two controls to ensure validity. Two 96-well microplates were coated with in-house polyclonal antibody to CBG and incubated overnight. Plates were washed 4 times then non-specific binding blocked with 200 μL assay buffer per well. After emptying by inversion, 100 μL of standard or 1:1000 thawed patient serum was added to duplicate wells. Standard was prepared by serially diluting commercially available recombinant CBG (Sino Biological Inc. Beijing, Cat no. 10,998-H08H). After incubation at room temperature for 60 min plates were washed and 100 μL/well of appropriate specific in-house monoclonal antibody was added to the respective plates at dilutions of 1:20 and incubated for 90 min. Assays with antibody 12G2 gave total CBG levels, and 9G12 gave intact haCBG. After washing, 100 μL/well of diluted peroxidase-conjugated anti-mouse antibodies was added to the respective plates. After 30 min, the plates were washed and 100 μL/well of tetramethylbenzidine substrate added. Color development was blocked with 100 μL/well 0.9 M hydrochloric acid. Absorbance was read at 450 nm and concentrations determined by interpolation from standard curves. laCBG is calculated indirectly by subtraction. The intra-assay coefficients of variation for the 12G2- and 9G12-based assays were 9.3 and 3.5% respectively.

As NE is rapidly inhibited *in vivo*, direct measurement in circulating plasma is of doubtful value. Furthermore, direct and indirect markers of elastin degradation can be measured, but have not been well validated and their role in clinical trials has been questioned [23]. Hence we have not performed measures to confirm the widely accepted over-activity of NE in our AATD patients [23].

2.3. Statistical analyses

Data were analyzed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, CA). Results are presented as median (range) unless otherwise stated. Comparisons between groups were performed using the Mann–Whitney U test as data were not normally distributed. Chi-squared testing was used to determine differences between categorical variables. Comparison between three or more groups was performed by Kruskal–Wallis one-way analysis of variance of ranks. Correlations between continuous variables were assessed with Spearman's rank correlation coefficient. $P < 0.05$ was taken as statistically significant.

3. Results

Ten AATD patients responded to the invitation and were enrolled: see Table 1 for baseline characteristics. All seven women were post-menopausal. Two patients of ZZ phenotype were receiving treatment with purified human AAT protein (Zemaira®, CSL Behring LLC; terminal half-life 5.1 days) and in these cases laboratory measures were taken at the dosing trough prior to the weekly infusion. Notably, the AAT levels of these patients did not differ from those AATD PiZZ patients not on AAT infusions ($P = 0.133$). Eight out of ten patients were taking long-term inhaled corticosteroids (ICS) for chronic obstructive pulmonary disease (COPD), one was taking long-term physiologic dose prednisolone (5 mg/day) and three had used a short course of high-dose oral corticosteroids within the past month. Four patients were non-smokers, four were ex-smokers and two were current smokers. As expected, patients with the ZZ genotype had significantly lower AAT levels than those with the SZ/MZ genotypes; 0.27 (0.02–0.4) *vs.* 0.43 (0.04–0.89) g/L, $P < 0.001$ (Table 2). Twenty-eight healthy controls were also analyzed. They did not differ from AATD patients in gender, age or BMI, although none were taking long-term inhaled corticosteroids. Three healthy controls were ex-smokers and there were no current smokers.

CBG affinity form levels are detailed in Table 2. Total CBG concentrations did not vary between the patient groups. Overall, total CBG and haCBG levels fell in a right-skewed distribution. Patients with PiZZ AATD had higher haCBG ($P = 0.0004$), lower laCBG ($P = 0.01$) and a higher proportion of haCBG ($P < 0.0001$) than controls, with Pi MZ/SZ patients having intermediate values for all parameters (Fig. 1 and Table 2). These analyses remained statistically significant when the

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