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The half-lives of intact and elastase cleaved human corticosteroid-binding globulin (CBG) are identical in the rabbit

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ABSTRACT

Corticosteroid-binding globulin (CBG) is a non-inhibitory member of the serpin superfamily of serine protease inhibitors and carries the majority of cortisol in circulation. It can be cleaved by neutrophil elastase at its exposed reactive centre loop which decreases its affinity for cortisol allowing the release of most of the cortisol at sites of inflammation. Intact and elastase cleaved CBG can be distinguished from each other and can coexist in circulation but with unknown half-lives. Here we treated a portion of purified human CBG with elastase, terminated the digestion and then combined this portion with intact human CBG and measured their respective half-lives in rabbits by ELISA. This investigation shows for the first time that the half-lives of intact and elastase cleaved CBG are identical (~10 h). This is an important finding as it implies that in conditions such as sepsis and septic shock where levels of intact CBG are low and the proportion of cleaved CBG is high that this is likely sustained which may affect the CBG mediated targeted delivery of cortisol to sites of inflammation. Furthermore the residual binding of cortisol to cleaved CBG may alter the overall buffering capacity of CBG for cortisol resetting the baseline concentration of free cortisol.

1. Introduction

Corticosteroid-binding globulin (CBG) is a monomeric circulating glycoprotein that binds cortisol with high affinity and plays a major role in determining plasma cortisol levels and the distribution of free cortisol to tissues [1,2]. As a non-inhibitory member of the serine protease inhibitor superfamily (SERPINS) it possesses an exposed reactive centre loop (RCL) which is susceptible to cleavage by elastase released by activated neutrophils whereupon CBG adopts a relaxed conformation lowering its affinity for cortisol allowing the release of the majority of cortisol at the inflammatory site [3,4]. The RCL of human CBG can also be cleaved by chymotrypsin [5] and the virulence factor protease LasB from *Pseudomonas aeruginosa* [6] which also disrupt cortisol binding to CBG. We recently raised a monoclonal antibody to the intact RCL of human CBG [7] and the epitope STGVTLNL spans the elastase cleavage site between Val344 and Thr345 as well as the 2 chymotrypsin sites at Leu346 and Leu348. We used this in concert with another monoclonal antibody to CBG to develop an ELISA which shows both intact and elastase cleaved CBG can coexist in circulation [8].

Circulating CBG is markedly reduced in acute inflammatory conditions such as sepsis or septic shock [9] with the proportion of cleaved or low affinity CBG to total or high affinity CBG rising and corresponding to disease severity which may potentially limit the availability of cortisol to inflammatory sites thereby perpetuating the inflammatory process [10]. However, nothing is known about the relative half-life of elastase cleaved CBG compared to intact CBG. When CBG is viewed simply as a carrier for cortisol the preferential clearance of cleaved CBG over intact CBG implies that with more intact CBG–cortisol complex more cortisol would be available for targeted enzyme mediated release at inflammatory sites. On the other hand similar half-lives would likely compromise enzyme mediated cortisol available for release from CBG. Importantly however, in addition to its transport role, CBG also has a buffering role in stabilizing free cortisol concentrations which is dependent on the binding affinity of intact CBG as well as the much reduced binding affinity of cleaved CBG [11,12]. Hence in conditions where there is a relative high concentration of cleaved CBG knowledge of relative half-lives assumes importance. Here we treated purified human CBG with human sputum elastase, terminated the digestion and combined this with purified intact human CBG which was injected into the ear vein of 2 rabbits and serial sampling carried out to determine their respective half-lives. We show for the first time that the half-lives of elastase cleaved human CBG and intact human CBG

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are identical which suggests that in conditions where CBG is low and the relative proportion of cleaved CBG is high that targeted cortisol release at inflammatory sites may be jeopardised. In addition this would likely reset the buffered baseline of free cortisol.

2. Material and methods

2.1. Animals

Two New Zealand white rabbits (A9 and A10) were housed in individual cages and fed rabbit pellets (Western Milling, 120 g/day) and hay and water ad libitum and both weighed 3.4 kg. Thirty minutes prior to experimentation animals were sedated subcutaneously with Acezine (0.1 mg/kg) and subsequently restrained in a Tecniplast Rabbit restrainer device and a catheter inserted into the ear vein for a bolus injection over 30 s of 0.8 mL of a stable mixture of intact and elastase cleaved human CBG in Tris–HCL buffer, pH 7.4. An in-dwelling catheter was then inserted into the opposite ear for timed blood collection which was removed after the 7 h sample. Following injection of CBG, blood samples (1 mL) were collected in plain tubes at 0.25, 0.5, 1, 2, 4, 7, 24, 48 h and either 72 h or 96 h for the last sample. The serum was separated and stored at −20 °C until analysed. The study was approved by the Animal Ethics Committee of the Christchurch Campus of the University of Otago (AEC No. C17/13).

2.2. CBG and elastase

Purified human CBG (90%) 5 mg was purchased from Affiland.com, Belgium, and supplied lyophilized in 5 mL of Tris–HCL pH 7.4 and reconstituted in 2.5 mL of distilled water. Human sputum elastase was purchased from Elastin Products Co., Inc., MI, USA and reconstituted in 0.05 M acetate buffer, pH 5.0 containing 0.1 M NaCl and 50% glycerol and stored as a stock solution at −20 °C (875 units/mL, 100 μg/mL).

2.3. Elastase treatment

One millilitre of CBG solution was treated with 40 μL of elastase for 2 h at room temperature and a 10 μL aliquot removed and 1 μL PMSF added to stop further digestion. This aliquot was used for western blotting and CBG determinations by ELISA to confirm total digestion. The remainder was promptly frozen to terminate digestion and stored at −20 °C awaiting western blotting and quantitative CBG analyses. Following confirmation of digestion 100 μL of rabbit serum was added to the digest which served to completely inhibit the added elastase. Prior to this we carried out a small pilot titration experiment in order to determine the volume of normal rabbit serum required to inhibit the elastase. This strategy avoided the use of toxic PMSF, the usual elastase inhibitor, and ensured that a stable mixture of elastase cleaved CBG and intact CBG was injected into rabbits. One millilitre of CBG solution with 40 μL of acetate buffer served as a non elastase control with a similar aliquot removed for western blotting and CBG determinations. For injection 1 mL of elastase treated CBG and 0.75 mL of intact CBG were combined and assayed for total and intact CBG prior to the rabbit half-life studies with the remaining untreated CBG set aside for polyclonal antibody production.

2.4. CBG ELISAs

Total and intact human CBG were measured, in duplicate, by ELISA using parallel microtitre plates coated with an “in house” rabbit polyclonal antibody to human CBG as described previously [8]. Briefly, following “blocking” in assay buffer, phosphate buffered saline (PBS) containing 0.1% Tween 20 (v/v) and 0.1% gelatin (w/v), 150 μL/well), the wells were emptied by inversion and either 100 μL of standard (1:1000 in assay buffer) or 100 μL of dilutions of elastase and non-elastase treated CBG (1:8000 to 1:32000), appropriate controls or dilutions of timed rabbit serum samples (1:50) added for 30 min incubation at 20 °C. The plates were washed and CBG monoclonal antibody supernatant from clone 12G2 (1:20 in assay buffer, 100 μL/well) was added to one plate, which measures total CBG and to the other the supernatant from the RCL specific CBG monoclonal antibody 9G12, which measures intact CBG [7], (1:20 in assay buffer, 100 μL/well) for a 30 min incubation at 20 °C. The plates were again washed and either antimouse IgG1-peroxidase (1:2000 in assay buffer, 100 μL/well) was added to the plate for total CBG determination or antimouse IgG2a-peroxidase (1:1000 in assay buffer, 100 μL/well) was added to the plate for intact CBG determination. Both plates were incubated a further 15 min at 20 °C. The plates were finally washed and tetramethybenzidine substrate was added (100 μL/well). Colour development was terminated by the addition of 1 M HCL (100 μL/well) and the absorbance was read at 450 nm. Monoclonal antibody 12G2 does not recognise the RCL of human CBG and hence can be used to measure total CBG whereas monoclonal antibody 9G12 recognises amino acid residues 341–348 which span the intact elastase cleavage site of the RCL region of human CBG. Therefore, the CBG ELISAs performed in parallel provide measures of total and intact or uncleaved CBG [7].

2.5. SDS-PAGE-western blotting

Vertical SDS-PAGE of purified CBG treated with or without elastase (1% w/w at 10 min and 100 min at room temperature) was carried out in 10% polyacrylamide gels in the presence of mercaptoethanol [13]. Following transfer nitrocellulose was “blocked” using 5% skim milk powder (overnight) and further blocked in Tris-buffered saline (TBS; 0.015 M Tris, 0.15 M NaCl, pH 7.4) containing 0.1% Tween-20 (v/v) and 1% BSA (w/v). After washing in TBS containing Tween-20 nitrocellulose was incubated with RCL monoclonal antibody 9G12 (1:100) or diluted CBG polyclonal antibody (1:2500) for 60 min at 20 °C followed by either antimouse IgG2a-peroxidase or antirabbit IgG-peroxidase (1:5000 in TBS, containing Tween-20 and BSA). Immunoconjugates were visualised using TMB precipitating substrate. Portions of enzyme treated CBG were assayed for total and intact CBG using antibodies 12G2 and 9G12, respectively, to confirm RCL cleavage.

2.6. Serum cortisol ELISA

Rabbit serum cortisol was measured by ELISA following extraction with dichloromethane. Briefly serum (200 μL) was extracted with 1.0 mL dichloromethane and 500 μL evaporated to dryness. The dried extract was reconstituted with 200 μL of assay buffer and 50 μL aliquots used in duplicate for cortisol ELISA as previously described [14].

2.7. Endogenous cleavage of the RCL by rabbit serum

The possibility of endogenous cleavage of human CBG by the rabbit serum samples was investigated by using a peptide that spanned the antibody recognition site of the intact RCL sequence of human CBG containing an additional threonine and 4 residue proline leash with a cysteine C-terminus. This peptide STGCTLNLTPPPPC was coupled to bovine serum albumin and used to coat microtitre plates overnight (100 μL/well) containing 0.25 μg/well in 6 M aqueous guanidine hydrochloride. The following day the plate was washed and endogenous elastase cleavage by the rabbit serum samples tested by adding each of the