Corticosteroid-binding globulin is a biomarker of inflammation onset and severity in female rats

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Abstract

Plasma corticosteroid-binding globulin (CBG) plays a critical role in regulating glucocorticoid bioavailability and is an acute phase 'negative' protein during inflammation. In an adjuvant-induced arthritis model, plasma CBG levels decrease in rats that develop severe inflammation, and we have now determined when and how these reductions in CBG occur. After administering complete Freund's adjuvant or saline intra-dermally at the tail base, blood samples were taken periodically for 16 days. In adjuvant-treated rats, decreases in plasma CBG levels matched the severity of inflammation, and decreases were observed 4 days before any clinical signs of inflammation. Decreases in CBG levels coincided with an ~5 kDa reduction in its apparent size, consistent with proteolytic cleavage, and cleaved CBG lacked steroid-binding activity. At the termination of the experimental period, hepatic *Cbg* mRNA levels were decreased in rats with severe inflammation. While plasma TNF-α increased in all adjuvant-treated rats, increases in Il-4, IL-6, IL-10, IL-13 and IFN-γ were only observed in rats with cleaved CBG. Rats with cleaved CBG also exhibited increased spleen weights, and strong negative correlations were observed among CBG, IL-6 and spleen weights, respectively. However, there were no differences in hepatic *Cbg* mRNA levels in relation to the apparent proteolysis of CBG, suggesting that CBG cleavage occurs before changes in hepatic *Cbg* expression. Our results indicate that the levels and integrity of plasma CBG are biomarkers of the onset and severity of inflammation. Dynamic changes in the levels and function of CBG likely modulate the tissue availability of corticosterone during inflammation.

Key Words

- corticosterone
- cytokines
- spleen weight
- proteolysis
- serine protease inhibitor

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Introduction

Produced primarily by the liver, corticosteroid-binding globulin (CBG) is a plasma glycoprotein that binds ~90% of circulating glucocorticoids, and regulates their bioavailability in target tissues (Lin *et al.* 2010). Plasma CBG shares structural similarities with clade A serine protease inhibitor (SERPINA) family members (Lin *et al.* 2010). However, CBG is not a protease inhibitor, but rather serves as a reservoir for glucocorticoids that are released when its reactive centre loop (RCL) is cleaved by proteases, including neutrophil elastase (Hammond *et al.* 1990), chymotrypsin (Lewis & Elder 2014) or the bacterial proteinase, LasB (Simard *et al.* 2014).

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During inflammation, CBG acts as an acute phase 'negative' protein, and reductions in its plasma levels can be attributed to both proteolytic cleavage (Hammond *et al.* 1990) and downregulation of its production by the liver (Smith & Hammond 1992, Emptoz-Bonneton *et al.* 1997, Bernier *et al.* 1998). In humans, proteolysis of the RCL of CBG by neutrophil elastase appears to be an early event during inflammatory reactions, rendering CBG non-functional and promoting the localized release of CBG-bound glucocorticoids at sites of inflammation (Hammond 1990, Perogamvros *et al.* 2012). Enhanced exposure of tissues to anti-inflammatory glucocorticoids represses cytokine production and activity (Brattsand & Linden 1996), thereby limiting cytokine-mediated tissue damage (Simon 2003). At the same time, plasma glucocorticoid concentrations increase acutely as a result of hypothalamic–pituitary–adrenal axis activation in response to stress (Sapolsky *et al.* 2000), and likely act in synergy with elevations in inflammatory cytokines, such as interleukin-6 (IL-6), to further reduce plasma CBG production, further amplifying free glucocorticoid exposures (Bartalena *et al.* 1993, Emptoz-Bonneton *et al.* 1997, Bernier *et al.* 1998). During recovery from inflammation, normalization of CBG levels likely plays a role in determining when, and to what extent, glucocorticoids act to restore the normal homeostatic balance.

Several studies in CBG-deficient animals support the idea that CBG plays a vital role during the inflammatory process. For instance, a genetic study revealed that C57BL/6 mice are more sensitive to an acute challenge with TNF-α than DBA/2 mice, and this trait was mapped to the *Cbg* (*SerpinA6*) locus (Libert *et al.* 1999). Recently, we have found that Harlan Sprague–Dawley (SD) rats are deficient in plasma CBG (50% lower levels), when compared with Charles River SD rats (Bodnar *et al.* 2015). Harlan SD rats are also more sensitive to inflammatory challenges than Charles River SD rats in an adjuvant-induced arthritis model (Bodnar *et al.* 2015) or after treatment with lipopolysaccharide (Turnbull & Rivier 1999). A key role for CBG in these differential responses is further supported by studies in $Cbg^{-/-}$ mice in which survival rates after an acute inflammatory challenge are compromised (Petersen *et al.* 2006).

Low plasma cortisol levels in patients have led to the identification of several individuals with CBG deficiencies (Gagliardi *et al.* 2010). Genome-wide sequencing of human populations has also identified numerous other single-nucleotide polymorphisms that cause decreased CBG production or defects in steroid binding, some of which are enriched in specific ethnic groups (Simard *et al.* 2015). Although patients with CBG deficiencies have been reported to suffer from a variety of symptoms including chronic pain, fatigue, depression, hypotension and excess body weight (Gagliardi *et al.* 2010), it remains to be determined how well they cope with severe, acute inflammation. To explore this, we have determined when changes in plasma CBG levels occur during the course of acute inflammation in rats and how this relates to the severity of the inflammatory response.

Materials and methods

Animals and treatments

Female Sprague–Dawley rats (n=24, 51–52 days old) were received from Charles River Laboratories International, Inc. (St. Constant, Canada), and maintained as described previously (Bodnar *et al.* 2015). Following an acclimatization period of 5 days, pre-treatment (baseline) blood samples were taken from the tail vein, under light isofluorane anaesthesia, between 11:00 and 13:00 h for the preparation of serum, which was stored at −80°C until analysed. Three days later, rats were anaesthetized with isoflurane, and injected with 0.6 mg complete Freund's adjuvant (n=20) prepared as described previously (Zhang *et al.* 2012) or with physiological saline (control; n=4). Rats were then housed with CareFRESH (Healthy Pet, Ferndale, USA) bedding to minimize discomfort, and monitored for signs of pain, discomfort or infection, and for general signs of health including activity, coat quality and ability to rear.

Post-treatment, rats were split into two groups (n=12 per group; 2 controls and 10 adjuvant-treated rats). Blood samples (~50 µL) from the two groups were alternatively collected between 11:00 and 13:00 h under light isoflurane anaesthesia from the tail vein over a 16-day experimental period. Serum was prepared and stored at −80°C until assayed. At the time of blood sampling, rats were weighed and clinical signs of inflammation were scored and recorded. To calculate clinical scores, each of the four paws was given a score of 0–4, where 0=no signs of inflammation, 1=single focus of redness or swelling, 2=two or more foci of redness or swelling, 3=confluent but not global swelling, 4=severe global swelling (Zhang *et al.* 2012). Rats achieving an overall clinical score ≥8 (out of a possible 16) at any point during the study were classified as developing severe inflammation, whereas rats with a clinical score <8 were classified as developing mild-moderate inflammation. One animal with severe inflammation was killed on experimental day 14 for humane reasons, while all other animals were killed

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on experimental day 16. On the day of termination (08:00–10:00 h), rats were removed from the colony room and decapitated (<1 min). Trunk blood was collected in tubes containing EDTA, and plasma was stored at −80°C until analysed. In addition, livers and spleens were removed, flash frozen and stored at −80°C.

The animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the University of British Columbia Animal Care Committee.

Plasma CBG analysis

Plasma corticosterone-binding capacity of CBG was measured using an established ligand-saturation assay (Hammond & Lahteenmaki 1983). Briefly, samples were diluted (1:1000–1:3000) in phosphate-buffered saline and stripped of endogenous steroids by incubation with dextran-coated charcoal (DCC) for 30 min at room temperature followed by centrifugation. Samples were then incubated with ~10 nmol L^{-1} [^{3}H]-corticosterone (PerkinElmer Life Sciences) in the absence or presence of excess corticosterone to monitor non-specific binding. After separation of free [^{3}H]-corticosterone by adsorption with DCC for 10 min and centrifugation at 0°C, CBG-bound [^{3}H]-corticosterone in the supernatants was determined in a scintillation spectrophotometer.

To assess CBG integrity, diluted (1:200) plasma samples were subjected to SDS-PAGE and transferred to PVDF membranes using a Trans-Blot turbo transfer system (BioRad). Blots were blocked with 5% milk-PBST for 2 h at room temperature and incubated overnight at 4°C with polyclonal rabbit anti-mouse CBG antiserum diluted 1:4000 in the 5% milk-PBST (Scrocchi *et al.* 1993), followed by a horseradish peroxidase-labelled goat anti-rabbit IgG antibody (1:10,000; Sigma-Aldrich) for 1 h at room temperature. Immunoreactive CBG was then detected with ECL reagent using an ImageQuant LAS4000 (GE Health Care). Total protein concentrations in plasma samples were measured using a Micro BCAProtein Assay Kit (Thermo Fisher Scientific).

Separation and characterization of functional and non-functional CBG in plasma samples

To characterize CBG in plasma samples from rats with severe inflammation, plasma from two rats were pooled, treated with DCC to remove endogenous steroids and then applied to an 11β-hydroxy-andros-4-en-3-oxo-17β-carboxylic acid (HACA)-Sepharose affinity column (Seralini *et al.* 1989). After washing the column with 100 mmol L^{-1} Tris–NaCl, steroid-bound CBG was eluted with 1 μmol L^{-1} cortisol in the same buffer. The flow-through, wash and eluent fractions were analysed by corticosterone-binding capacity assay and Western blotting, as described above.

Cbg mRNA measurements

Total RNA was extracted from liver using Trizol reagent (Thermo Fisher Scientific), followed by purification using an RNeasy kit (Qiagen), as per the manufacturer's instructions. To obtain cDNA, 1 μg RNA was reversed transcribed using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). Real-time quantitative PCR (qRT-PCR) was completed using a pre-validated rat *Serpina6* (*Cbg*) PrimeTime Std qPCR Assay (Integrated DNA Technologies (IDT), Coralville, USA: Assay ID# Rn.PT.58.02619945) and 5 ng cDNA per reaction. The qRT-PCR reactions were run in duplicate on the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). Hepatic *Cbg* mRNA levels were normalized to those of rat *Gapdh* (IDT: Assay ID# Rn.PT.58.35727291) using the delta-delta Ct method.

Multiplex cytokine immunoassays

Plasma cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α) were measured in plasma samples collected at termination using the Proinflammatory Panel 2 (rat) V-PLEX kit from Meso Scale Discovery (MSD, Rockville, MD, USA; catalog #: K15059D-1). The cytokine assays were performed according to the established MSD protocol. The assay plate was read using a MESO QuickPlex SQ 120 and data were analysed using the MSD Discovery Workbench software v. 4.0. Lower limits of detection for cytokines (pg/mL) are as follows: IL-4 (0.16), IL-5 (6.89), IL-6 (7.18), IL-10 (6.18), IL-13 (0.45), TNF-α (1.04), IFN-γ (1.48).

Statistical analyses

Data (expressed as mean ± S.D. or S.E.M., as indicated) were analysed using t-tests or analysis of variance (ANOVA), with repeated measures as required, followed by Fisher or Tukey's *post hoc* tests to examine significant main effects. Differences were considered significant at $P \leq 0.05$, and trends ($0.06 > P > 0.05$) were examined, as appropriate. *t*-test, ANOVA and *post hoc* p values are shown in results; ANOVA F statistics are included in the figure legends.

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Results

Body weights and clinical scores

The body weights of all animals increased over the course of the experiment (i.e. 242.0±8.7 g to 285.0±10.4 g), but there were no differences in body weight between groups. Five of the 20 adjuvant-treated animals (25%) developed inflammation, as indicated by their clinical scores (Fig. 1 in parenthesis), which generally increased over time. The mean time of inflammation onset (i.e. the first day of a clinical score >0) was 12.8±0.8 days.

Time course of changes in plasma CBG levels

Adjuvant-treated rats had a 30–50% reduction in plasma CBG levels 24-h post-injection, irrespective of whether or not they developed inflammation, and this decrease resolved by day 3 post-injection (Fig. 1). This was not seen in the saline-treated control rats. When compared with baseline values, 40–80% decreases in CBG-corticosterone-binding capacity were subsequently observed over the experimental period in rats that developed inflammation (Fig. 1), but mean plasma CBG levels were unchanged in saline-treated rats (not shown) or in adjuvant-treated rats that did not develop inflammation (Fig. 1).

Overall, the magnitude of decline in plasma CBG levels matched the severity of inflammation, with animals that developed severe inflammation showing the largest decreases in CBG levels (Fig. 1). In two severely inflamed rats, 50% reductions in plasma CBG were seen as early as day 8 post-injection, which was 4 days before any clinical signs of inflammation were evident. In these animals, CBG levels reached as low as 20% of baseline by 12–13 days post-injection. The two rats with mild-moderate inflammation also had significant reductions (35% and 55% of baseline values at 13–14 days post-injection) in CBG levels. Total protein levels in plasma samples did not differ in rats over the course of the study in relation to the severity of inflammation (data not shown).

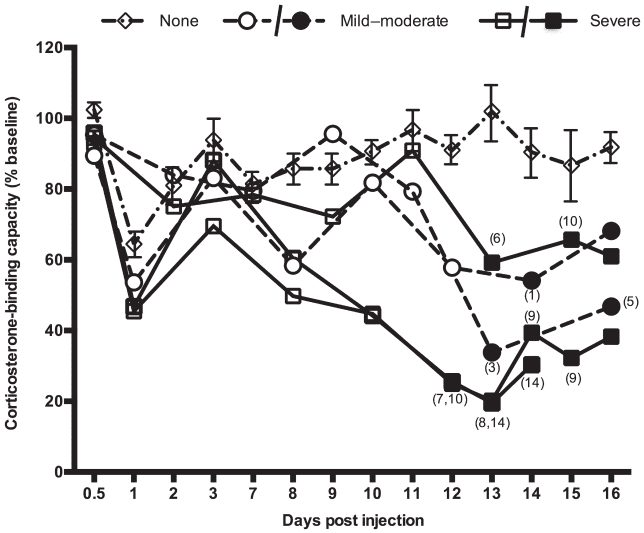

Figure 1
Time course of plasma CBG levels during inflammatory responses. Blood samples were collected from each animal before treatment (baseline values) and after complete Freund's adjuvant injection, as indicated. A reduction in the corticosterone-binding capacity of serum CBG was observed 24-h post-injection in all adjuvant-treated animals. Following this, rats that developed inflammation had large decreases in CBG levels, whereas the mean CBG levels in animals without clinical signs of inflammation (none, diamonds) were unchanged. Rats with mild-moderate inflammation (circles) displayed reduced CBG levels 12–13 days post-injection. Two of three rats with severe inflammation (squares) had reduced CBG levels as early as day 8, several days before clinical signs of inflammation were evident. Solid symbols denote the presence of clinical symptoms with the corresponding clinical score given in parentheses, while open symbols denote the absence of clinical signs of inflammation. Data for rats (n=15) without inflammation are presented as mean±S.E.M. for comparison.

Plasma CBG and hepatic *Cbg* mRNA levels in relation to clinical score

Plasma CBG levels at termination declined in relation to clinical score (main effect of inflammation severity, P=0.0001; Fig. 2A). Rats with severe inflammation had significantly lower CBG levels when compared with saline-treated controls or adjuvant-treated rats that did not develop inflammation (P<0.001). Significant differences were also seen in *Cbg* mRNA levels in livers collected on the day of termination (main effect of inflammation severity, P<0.02; Fig. 2B). When compared with saline-treated rats or adjuvant-treated rats that did not develop inflammation, *post hoc* tests revealed that animals with severe inflammation had lower *Cbg* mRNA levels (P<0.05).

Western blotting was used to assess the integrity of CBG in plasma samples taken at termination. An ~5 kDa reduction in the apparent molecular size of CBG was evident in rats with mild-moderate and severe inflammation (Fig. 2C, lanes 4 and 5), consistent with the cleavage of its RCL (Gardill *et al.* 2012). This was also observed in some adjuvant-treated rats without clinical signs of inflammation (Fig. 2C, lane 3), suggesting the presence of an underlying inflammatory state in those animals. As expected, this evidence of CBG proteolysis coincided with decreased plasma CBG levels as

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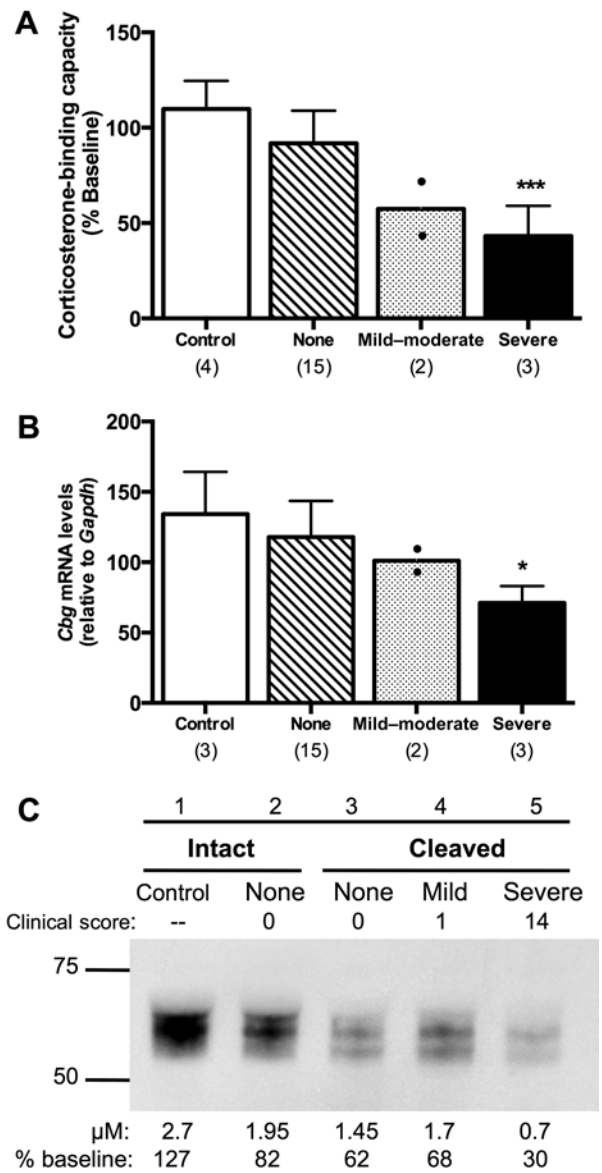

Figure 2
Plasma CBG (A) and liver *Cbg* mRNA (B) levels, and evidence of CBG proteolysis (C) in relation to clinical score (none, mild–moderate or severe). In A and B, the numbers of animals in each group are shown in parentheses, and rats ($n=2$) with mild-moderate inflammation were not included in statistical analyses. Rats with severe inflammation were compared with both saline-treated control animals and adjuvant-treated animals without clinincal signs of inflammation (none). (A) Plasma CBG levels decreased in relation to clinical score with a significant decrease in rats with severe inflammation (main effect of inflammation severity, $F(2,19)=14.85$, $P=0.0001$). (B) Liver *Cbg* mRNA levels were significantly lower in animals with severe inflammation (main effect of inflammation severity, $F(2,17)=5.59$, $P<0.02$). Data in A and B are presented as mean ± S.D. Tukey's *post hoc*: $^{*}P<0.05$; $^{***}P<0.001$, (C) Representative Western blotting illustrating the proteolysis of plasma CBG, as indicated by an ~5 kDa size reduction (lanes 3–5), in concert with reductions in CBG corticosterone-binding capacity (μM) when compared as a percentage of pre-treatment values (shown below). Note that CBG proteolysis was also observed in some animals before clinical signs of inflammation (none, lane 3).

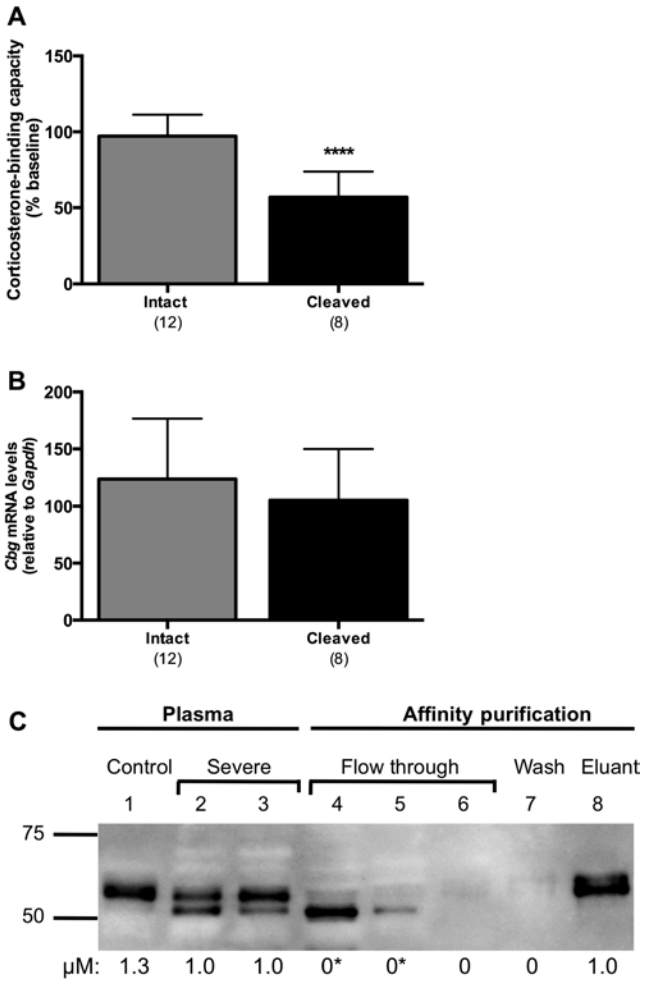

Figure 3
Proteolysis of CBG is associated with reduced plasma CBG levels without changes in liver *Cbg* mRNA levels, and evidence that cleaved CBG in plasma lacks steroid-binding activity. (A) Rats with cleaved CBG had significantly lower plasma CBG values than rats with intact CBG. (B) Liver *Cbg* mRNA levels were similar irrespective of CBG proteolysis status. In A and B, samples were grouped for analysis based on CBG integrity, as assessed by Western blotting (Fig. 2C), and classified as being either intact or cleaved. The numbers of animals in each group are shown in parentheses, and data are presented as mean ± S.D. $^{****}P<0.0001$. (C) Plasma from rats with severe inflammation (lanes 2 and 3) were pooled and purified by steroid-affinity chromatography. Cleaved CBG did not bind the steroid-affinity matrix and eluted in the flow through (lanes 4 and 5). Intact CBG bound to the steroid-affinity matrix and was eluted with buffer containing excess corticosterone (lane 8). CBG-corticosterone-binding capacity values (μM) are shown under each lane. There was no detectable CBG steroid-binding activity (*) in the flow-through fractions that contained cleaved CBG (lanes 4 and 5). Intact CBG (lane 8) exhibited full steroid-binding activity. An intact control (saline) sample is shown (lane 1) for comparison.

determined in the corticosterone-binding capacity assay (Fig. 2C). Therefore, on the day of termination, decreases in the corticosterone-binding capacity of plasma CBG in animals with severe inflammation are associated

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with decreased *Cbg* mRNA levels, as well as evidence of proteolytic cleavage of CBG.

Plasma CBG and hepatic *Cbg* mRNA levels in relation to CBG proteolysis

Plasma samples taken at termination were classified into two groups depending on the integrity of CBG as assessed by Western blotting: i.e. samples in which CBG proteolysis was either evident (cleaved) or not (intact), as illustrated in Fig. 2C. Reductions in CBG levels were found in rats with evidence of cleaved CBG (Fig. 3A), when compared with rats with intact CBG ($P<0.0001$). When classifying samples in this way, no differences were found in corresponding liver *Cbg* mRNA levels (Fig. 3B), suggesting that CBG proteolysis precedes changes in liver *Cbg* mRNA levels.

Next, we investigated whether cleaved CBG in plasma samples retained high-affinity corticosterone-binding activity. Plasma samples from rats with severe inflammation (Fig. 3C, lanes 2 and 3) were pooled and CBG was captured by HACA-Sepharose affinity column chromatography. Cleaved CBG, as indicated by an ~5 kDa size reduction, failed to bind the affinity column and eluted in the flow-through fractions (Fig. 3C, lanes 4 and 5). Moreover, the flow-through fractions containing the cleaved CBG had no detectable CBG-corticosterone binding activity. There was no immunoreactive CBG or corticosterone-binding activity in the wash fraction (Fig. 3C, lane 7). Importantly, the CBG eluting from the affinity column using excess (200 μM) corticosterone (Fig. 3C, lane 8) appeared to be intact and retained full corticosterone-binding activity.

Plasma cytokine biomarkers of inflammation and spleen weight

Pro- and anti-inflammatory cytokine levels were measured in plasma samples collected at termination (Fig. 4A, B, C, D, E, F and G). Measurements in samples from saline-treated control animals and adjuvant-treated animals were classified according to whether CBG was intact or cleaved (Fig. 2C). No significant differences were found in plasma levels of IL-5 (Fig. 4A). Trends for a main effect of CBG proteolysis were found for the anti-inflammatory cytokines IL-4 (Fig. 4B, $P=0.051$) and IL-10 (Fig. 4C, $P=0.056$), as well as the pro-inflammatory cytokine IFN-γ (Fig. 4D, $P=0.057$). Planned pairwise comparisons revealed significantly elevated IL-4, IL-10 and IFN-γ levels only in rats with cleaved CBG, when compared with saline-treated controls ($P<0.05$).

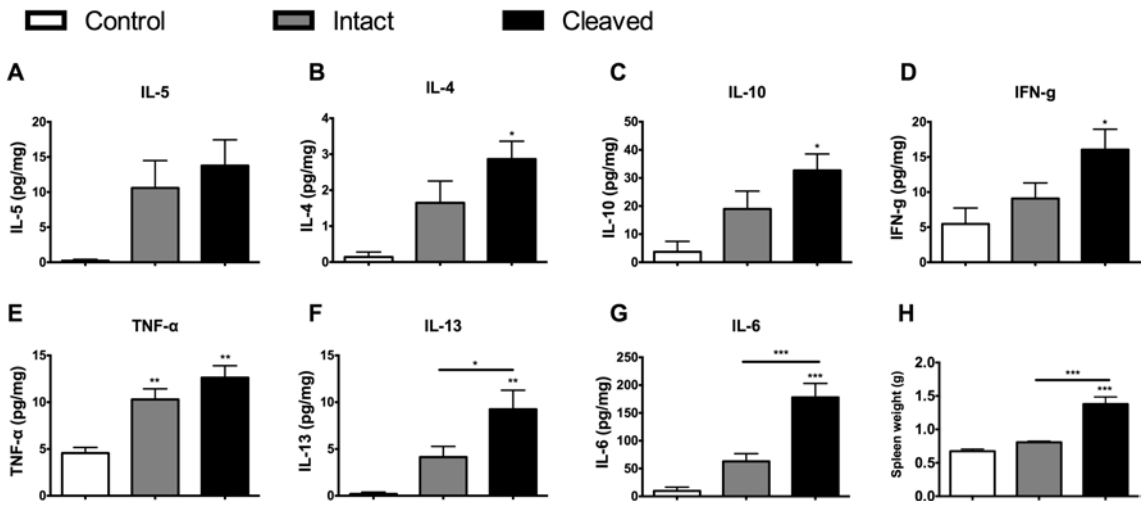

Figure 4

Associations among CBG proteolysis, plasma cytokine levels (A, B, C, D, E, F and G) and spleen weights (H). As in Fig. 3, results from adjuvant-treated rats were grouped based on CBG proteolysis status (i.e. cleaved vs intact CBG). When compared with control (saline-treated) rats, all plasma cytokines, apart from IL-5 (A), were significantly increased in rats with evidence of CBG proteolysis, as was spleen weight (H). TNF-α (E, main effect of CBG proteolysis, $F(2,23)=7.30$, $P<0.004$) showed a treatment effect with significantly increased levels in adjuvant-treated animals (intact and cleaved CBG) when compared with controls. Plasma levels of IL-13 (F, main effect of CBG proteolysis, $F(2,23)=6.36$, $P<0.007$), IL-6 (G, main effect of CBG proteolysis, $F(2,23)=16.50$, $P<0.001$) and spleen weight (H, main effect of CBG proteolysis, $F(2,23)=30.47$, $P<0.001$) were significantly increased in rats with cleaved CBG vs intact CBG. Cytokine levels were Blom transformed for statistical analysis; untransformed data (pg/mL) are presented as mean ± S.E.M. Control, $n=4$; Intact, $n=12$; Cleaved, $n=8$. Fischer's *post hoc*: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

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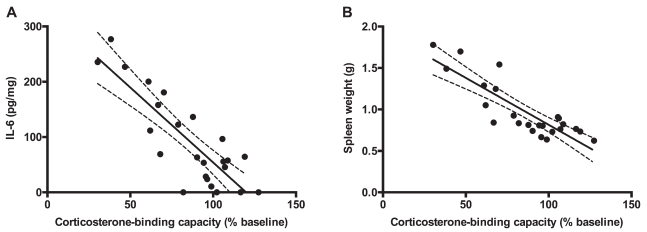

Figure 5
Strong negative correlations between plasma CBG and IL-6 levels and CBG and spleen weights. Data were analysed using linear regression, with plasma CBG levels expressed as a % of pre-treatment values. (A) Plasma CBG and IL-6 levels (r^2=0.71) and (B) plasma CBG and spleen weights (r^2=0.73), were negatively correlated. The best-fit linear regression line (solid) and 95% confidence interval (dotted) are also shown.

A significant main effect of CBG proteolysis was found for TNF-α (P<0.004), IL-13 (P=0.007) and IL-6 (P<0.001), respectively. A treatment effect was found for the pro-inflammatory cytokine TNF-α, with *post hoc* tests revealing that adjuvant-treated animals (both intact and cleaved CBG) had higher cytokine levels compared with control animals (Fig. 4E, P<0.01). A different pattern was observed for the anti-inflammatory cytokine IL-13 for which *post hoc* tests indicated that plasma levels were significantly increased in rats with cleaved CBG, when compared with saline-treated controls (P<0.001) or rats with intact CBG (P<0.05), respectively (Fig. 4F). In terms of changes in plasma cytokine levels, the largest increases were seen for IL-6 (Fig. 4G), with *post hoc* tests indicating significant increases in rats with cleaved CBG, as compared with the saline-treated controls or rats with intact CBG (P<0.001). Similar to IL-6, spleen weight (Fig. 4H) was very significantly associated with CBG proteolysis (main effect of CBG proteolysis, P<0.001). *Post hoc* tests also indicated that rats with cleaved CBG had significantly elevated spleen weights, when compared with the saline-treated controls or adjuvant-treated rats with intact CBG (P<0.001). Furthermore, there were strong negative correlations between plasma CBG and IL-6 levels (Fig. 5A, r^2=0.71) and between plasma CBG levels and spleen weights (Fig. 5B, r^2=0.73) but not between plasma CBG and IL-13 levels (r^2=0.22).

Discussion

Changes in plasma CBG levels occur during inflammation in humans, with very low levels reported during sepsis (Ho *et al.* 2006), septic shock (Pugeat *et al.* 1989, Ho *et al.* 2006, Bendel *et al.* 2008), burn injuries (Bernier *et al.* 1998) and after open heart surgery (Tinnikov *et al.* 1996). In addition, decreased CBG levels have been reported after thermal injuries to mice and rats (Garrel *et al.* 1993, D'Elia *et al.* 2005) and in pigs treated with lipopolysaccharide (Carroll *et al.* 2003). These latter studies model acute, all-or-none inflammation, while the adjuvant-induced inflammation model we have used allows those rats that developed inflammation to be compared with those that did not. In doing so, it was possible to monitor temporal changes in the plasma levels of CBG and inflammatory markers, as inflammation developed at different rates and degrees of severity.

In adjuvant-treated rats, the corticosterone-binding capacity of plasma CBG decreased 30–50% at 24-h post-injection, irrespective of whether or not rats eventually developed inflammation. Based on previous reports (Billiau & Matthys 2001), we suspect that an initial inflammatory response to the complete Freund's adjuvant is responsible for decreases in plasma CBG levels because this did not occur in saline-treated controls. However, this initial response was short-lived and CBG levels in adjuvant-treated animals returned to baseline by day 3 post-injection. Over the subsequent experimental period, we found consistent decreases in plasma CBG levels of 40–80% in those animals that developed inflammation, and the magnitude of this decline aligned with the severity of inflammation, as indexed by clinical scores. Notably, marked (50%) decreases in plasma CBG levels occurred 4 days before any clinical symptoms of inflammation were evident. Such dynamic changes in CBG levels before or during inflammation are expected to modulate the availability of corticosterone to its target cells, thereby affecting the inflammatory reaction as well as the healing process. When considered together, these results suggest that CBG may be a useful biomarker of inflammation onset and severity.

As noted previously (Bodnar *et al.* 2015), significant decreases in plasma CBG levels occurred in adjuvant-treated rats that developed severe inflammation, and we have now defined the mechanisms responsible for this. At day 14 or 16 post-adjuvant injection (termination), rats with severe inflammation had reduced plasma CBG levels and this was associated with an ~5 kDa reduction in CBG molecular size by Western blotting. Changes in the carbohydrate composition of plasma proteins have been reported to occur during acute inflammation, including decreases in core fucosylation (Rombouts *et al.* 2016). However, small changes in the composition of the six N-linked oligosaccharides associated with rat CBG will not result in detectable differences in molecular size by Western blotting. It is also known that the complete loss

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of *N*-glycosylation of human CBG at Asn238 causes a loss of steroid-binding activity (Avvakumov *et al.* 1993); however, there is no evidence that compositional changes in the N-linked glycosylation of CBG, in any species, adversely effect its steroid-binding activity. Differences in N-linked oligosaccharide composition will not account for the substantial reduction in the molecular size of CBG, or the complete loss of steroid-binding activity associated with immune-reactive CBG that does not interact with the steroid-affinity chromatography matrix, and we, therefore, conclude that these observations are the result of proteolytic cleavage.

It has been reported that cleaved CBG can be detected in human blood samples using ELISAs with highly specific monoclonal antibodies that discriminated between CBG with an intact vs cleaved RCL (Lewis & Elder 2011), but direct evidence that RCL cleavage of CBG actually occurred in these samples is lacking. A size reduction in CBG consistent with RCL cleavage has never been observed in human blood samples, and it has been postulated that human CBG is rapidly removed from the blood circulation after RCL cleavage (Mast *et al.* 1991). In rats, this does not seem to be the case and it appears that cleaved CBG is cleared more slowly from the circulation. However, the site of RCL cleavage and the protease responsible remain to be identified.

The steroid-binding activity of CBG is undetectable in rat plasma samples in which CBG appears to have undergone proteolysis, and this is in line with a marked loss in cortisol-binding affinity observed when the RCL of human CBG is cleaved by neutrophil elastase (Hammond *et al.* 1990), chymotrypsin (Simard *et al.* 2015) or the bacterial proteinase, LasB (Simard *et al.* 2014). However, our observations of *in vivo* rat CBG proteolysis under pathophysiological conditions contrasts with a previous report that *E. coli*-produced rat CBG, mutated to allow for cleavage by human neutrophil elastase, only undergoes a two-fold reduction in binding affinity (Gardill *et al.* 2012). We attribute this discrepancy to the fact that *E. coli*-expressed CBG is not glycosylated and has about a 10-fold lower affinity for corticosterone when compared with native CBG in rat blood samples (Gardill *et al.* 2012). It is known that the *N*-glycosylation of human CBG is critically important for the formation of a high-affinity binding site (Avvakumov *et al.* 1993, Avvakumov & Hammond 1994), and the RCLs of human and rat CBGs both contain *N*-glycosylation sites, but in different locations (Hammond *et al.* 1991). It is possible that *N*-glycosylation within the RCL of rat CBG influences how, and in what location, it is cleaved by a protease that allows for RCL insertion and the subsequent protein conformational rearrangement that disrupts the high-affinity steroid-binding properties of CBG (Lin *et al.* 2010). Unexpectedly, plasma CBG proteolysis was also evident in rats that developed mild-moderate inflammation, as well as in a subset of rats that did not display clinical signs of inflammation. However, significant reductions in plasma CBG were evident in rats where CBG proteolysis appears to have occurred, despite the fact that this did not coincide with a reduction in liver *Cbg* mRNA levels, which suggests that plasma CBG proteolysis occurs before changes in liver *Cbg* mRNA levels.

Plasma CBG production by the liver is down-regulated by glucocorticoids (Smith & Hammond 1992) and cytokines (Emptoz-Bonneton *et al.* 1997). Although plasma corticosterone levels were not measured in this study, they were increased in our previous study using the same experimental protocol, with the highest levels found in animals with severe inflammation (Bodnar *et al.* 2015). In this model, increased levels of IL-6, as seen here, and corticosterone, as seen previously (Bodnar *et al.* 2015), are likely contributors to the significant decreases in liver *Cbg* mRNA levels that we have now observed in rats with severe inflammation. This is not entirely surprising given the presence of a *cis*-regulatory element for IL-6 in the rat *Cbg* promoter (Underhill & Hammond 1995), and the established role of IL-6 in the acute phase response during inflammation (Fonseca *et al.* 2009). This is also in line with inverse relationships between IL-6 and plasma CBG levels in humans (Bernier *et al.* 1998, Tsigos *et al.* 1998), and studies in human hepatoblastoma-derived (HepG2) cells, where an IL-6-induced reduction in CBG production (Emptoz-Bonneton *et al.* 1997) was associated with decreased *Cbg* mRNA stability (Bartalena *et al.* 1993). Moreover, the strong negative correlation we observed between plasma CBG and IL-6 levels further supports the proposition that IL-6 inhibits CBG production during inflammation. Together, our results suggest that the mechanisms responsible for decreases in plasma CBG levels during inflammation are multi-factorial and occur in a sequential manner. First, the RCL of CBG undergoes proteolysis rendering it essentially non-functional as a steroid-binding protein, thereby amplifying free plasma corticosterone levels. Further decreases to plasma CBG levels are then caused by decreases in liver *Cbg* mRNA levels, mediated by increased IL-6 and corticosterone levels.

Activated immune cells produce a wide variety of cytokines, and cytokine levels increase in the circulation during inflammation (Choy & Panayi 2001,

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Ramadori & Armbrust 2001). We have now found that animals that showed evidence of CBG proteolysis also had increased cytokine (IFN-γ, TFNα, IL-4, IL-6, IL-10 and IL-13) levels, in association with large increases in spleen weight, which is a recognized marker of inflammation. These changes in plasma cytokine levels are expected to act together with increases in corticosterone levels and bioavailability to repress the production of cytokines in an attempt to alleviate cytokine-mediated tissue damage in rats with cleaved CBG. Increases in the plasma levels of positive acute phase plasma proteins, including other SERPINA family members, such as alpha-1 antitrypsin (SERPINA1) that inhibits the activities of neutrophil elastase in humans, may have occurred in animals that developed inflammation. Although this may afford some protection to CBG proteolysis, there is little information about the roles of alpha-1 antitrypsin and other related SERPINAs, or their target proteases, in rats in relation to inflammation.

The fact that both plasma IL-6 and IL-13 levels are markedly elevated in animals with cleaved CBG, while only IL-6 levels were inversely correlated with plasma CBG levels, suggests that these cytokines function in different ways. In contrast to IL-6, the lack of any relationship between plasma CBG and IL-13 levels suggests that IL-13 does not influence CBG production or proteolysis. Moreover, the coincidence of increased cytokine levels and spleen weights in rats with cleaved CBG indicates an underlying inflammatory response, even in the absence of clinical symptoms. Although some rats with cleaved CBG did not display overt signs of inflammation over the experimental period, it is possible that these may have developed if the study had been extended. Nevertheless, these data indicate that evidence of CBG proteolysis in plasma samples is a potential pre-symptomatic biomarker of inflammation.

In conclusion, our time course study of CBG changes during inflammation demonstrates that significant decreases in the corticosterone-binding capacity of plasma CBG occur in rats that developed inflammation, with the magnitude of the decline matching the clinical severity. Notably, large decreases in plasma CBG levels occur before clinical signs of inflammation. At termination, significant decreases in the corticosterone-binding capacity of CBG appear to coincide with CBG proteolysis that causes a loss of its steroid-binding activity. Significant increases in pro- and anti-inflammatory plasma cytokine levels, as well as increased spleen weights, were all associated with evidence of CBG proteolysis. These novel findings suggest that CBG proteolysis is a marker of active inflammation and, perhaps even more importantly, is a prognostic indicator of inflammation onset. In addition, the fact that rats with evidence of plasma CBG proteolysis had similar liver *Cbg* mRNA levels to rats with intact CBG suggests that cleavage of plasma CBG occurs before any reductions in hepatic CBG production occur. Overall, our data suggest that changes in CBG, particularly CBG proteolysis, are an early, pre-symptomatic marker of inflammation and a useful biomarker of inflammation onset and severity.

Declaration of interest
The authors have declared that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L A H and T S B carried out the experiments and analysed the data. L A H and G L H wrote the manuscript. All authors designed the study, had final approval of the submitted version of the manuscript and have read and agreed with the manuscript written.

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