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Corticosteroid-binding globulins: Lessons from biomedical research

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ABSTRACT

Glucocorticoids (GCs) circulate in the plasma bound to corticosteroid-binding globulin (CBG). Plasma CBG may limit access of glucocorticoids to tissues (acting as a sponge: the free hormone hypothesis), or may solely serve as a transport molecule, releasing GCs to tissues as the plasma moves through capillaries (the total hormone hypothesis). Both biomedical (focused on human health) and comparative (focused on ecological and evolutionary relevance) studies have worked to incorporate CBG in glucocorticoid physiology, and to understand whether free or total hormone is the biologically active plasma fraction. The biomedical field, however, has been well ahead of the comparative physiologists, and have produced results that can inform comparative research when considering the import of total vs. free plasma hormone. In fact, biomedical studies have made impressive strides regarding the function of CBG in tissues as well as plasma; we, however, focus solely on the plasma functions in this review as this is the primary area of disagreement amongst comparative physiologists. Here we present 5 sets of biomedical studies across genomics, pharmacology, cell culture, whole animal research, and human medicine that strongly support a role for CBG limiting hormone access to tissue. We also discuss three areas of concern across comparative researchers. In contrast to former publications, we are not suggesting that all comparative studies in glucocorticoid physiology must measure CBG, or that only free corticosterone levels are valid. However, we propose that comparative physiologists be aware of biomedical results as they investigate glucocorticoids and interpret how total hormone may or may not impact behavior and physiology of free-living vertebrates.

Definitions

Glucocorticoids (GCs): Adrenal steroids that regulate metabolism, immune function, and stress-related physiology and behavior. Cortisol is the primary GC in most mammals and fishes; corticosterone is the primary GC across birds, reptiles, amphibians, and many rodents (although both cortisol and corticosterone co-occur in most vertebrates).

Corticosteroid-Binding Globulin (CBG): large glycoprotein with a hydrophobic pocket containing a high-affinity binding site for glucocorticoids. CBG can also bind several other steroids with similar or lower affinity. Also known as transcortin, historically.

Capacity: the concentration of binding globulin molecules in the plasma, *i.e.*, the extent of the binding globulin to carry hormone.

Affinity: the strength of connection between the ligand (hormone) and the receptor (here the binding globulin). Defined as: the amount of hormone required to bind half of the receptors present. A greater value (higher number) means more hormone is required to bind half the receptors present, and so represents a weaker bond between ligand and receptor.

Law of Mass Action: Originally applied to chemical reactions stating that the rate of reaction is dependent on the concentration of reagents; in pharmacology: L + R < – > LR where the concentration of ligand (L) and the capacity of the receptor (R), as well as the affinity of ligand for the receptor (represented by the bidirectional arrow) will determine the amount of ligand bound to receptor (Paton and Payne, 1969). For binding globulins this represents the idea that more CBG will mean more GCs bound to CBG, even without a change in GC level or affinity of GC for CBG. Similarly, it represents that a change in affinity of GCs for CBG will change the amount of GC bound, even with no change in GC titers or CBG capacity.

Total Hormone: The entire amount of hormone in the blood, including bound to CBG, bound nonspecifically to albumin, and directly dissolved in plasma.

Free Hormone: The fraction of plasma hormone not bound to

Abbreviations: GC, Glucocorticoid; CORT, Corticosterone or cortisol; CBG, Corticosteroid-Binding Globulin; GR, Glucocorticoid Receptor; ECF, Extracellular Fluid

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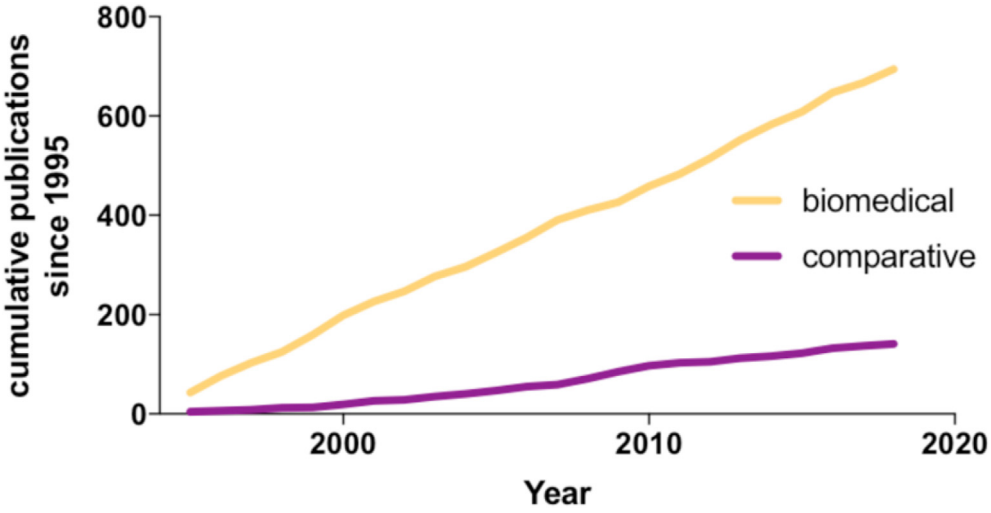

Fig. 1. Cumulative CBG/transcortin publications across fields. Biomedical studies of CBG have increased 4-fold faster than comparative studies. Data compiled from Web of Science since 1994, searching for 'corticosteroid-binding globulin' or 'transcortin', and sorting results by hand into biomedical or comparative fields.

CBG.

Reservoir: Hormone bound by CBG (the bound fraction), unavailable for direct movement into tissues, but available as temperature increases (lowering the affinity GCs for CBG), or as CBG is cleaved at sites of inflammation.

1. Introduction

Glucocorticoid hormones (GCs) are secreted from the adrenal or interenal glands of vertebrates, and regulate suites of traits across morphology, physiology, and behavior. GCs fluctuate at low levels on a diel cycle, increasing to 'stress-induced' levels in response to perceived or actual challenges to homeostasis. Glucocorticoids circulate in the plasma bound to corticosteroid-binding globulin (CBG). CBG is a member of the Serine Protease Superfamily, synthesized primarily in the liver (Meyer et al., 2016), although numerous extrahepatic sites of synthesis exist (e.g., Khan et al., 1984; Zhao et al., 1997). Glucocorticoids and other steroid hormones bind to CBG reversibly, with high affinity and specificity in a hydrophobic pocket (Klieber et al., 2008; Siiteri et al., 1982; Westphal, 1971; Zhou et al., 2008). CBG and its ligands bind and unbind freely at equilibrium, and additional ligand can be released as temperatures increase (lowering the affinity of CBG for ligands, (Zhou et al., 2008), or as CBG is cleaved (Hammond, 1990; Lin et al., 2010; Pemberton et al., 1988).

Activity of corticosteroid-binding globulins in the plasma has been considered in comparative stress physiology since the 1980s (Wingfield et al., 1984, although agricultural consideration of plasma CBG started in the 1970s, *e.g.* Gould and Siegel, 1978). Initially they were only considered as transport molecules; as long as there was more CBG than GCs in the plasma, they were not considered further (Wingfield et al., 1984). However, about 20 years ago, comparative fields began incorporating the law of mass action into our consideration of plasma binding globulins (L + R < – > LR; see Box 1; Boonstra and Boag, 1992; Deviche et al., 2001; Klukowski et al., 1997), as was commonly done in biomedical studies. The law of mass action recognizes that the amount of free or bound hormone in the plasma can be affected by the amount of hormone (L), the amount of binding globulin (R), or the affinity of one for the other (bi-directional arrow; see definition box). At this point disagreements arose as to the import of plasma binding globulins in determining hormone access to tissues. Are binding globulins just transport molecules, giving up hormone to tissues as they pass through capillaries (the total hormone hypothesis); or, are they a sponge, holding hormone through the capillary space so that only the unbound, free fraction of hormone enters tissues and effects physiology (the free hormone hypothesis). For decades the biomedical literature has stated that only free, unbound hormone enters tissues (e.g., Siiteri et al., 1982; Westphal, 1983), although most references are without citation to support the assertion. Over the last decade, however, biomedical research has produced several key studies that warrant serious consideration by comparative physiolgists when considering the biological relevance of total vs free hormone in the plasma.

Binding globulins are not only active in the plasma. There is recent, compelling evidence for CBG regulating the tissue activity of glucocorticoids in pituitary, brain, heart, lung, adrenal, and testis (Gulfo Cabrales, 2016; Gulfo et al., 2019; Schäfer et al., 2015; Sivukhina and Jirikowski, 2014). The exact role of CBG in these tissues is hotly debated. In this review, however, we are choosing to focus on the role of CBG in the plasma, as that is the focus of discussion across comparative researchers.

Biomedical investigations are targeted towards human health, while comparative studies aim to understand the ecological and evolutionary repercussions of physiological mechanisms. However, the two fields need not be so separate. Given the incredible level of funding for–and implications for human health in–biomedical studies, there has been a much greater level of investigation into CBG's role in glucocorticoid physiology across humans and rodent model systems in biomedical research than in comparative studies (Fig. 1). We as comparative physiologists should learn from these studies and apply findings as appropriate to our more ecologically-relevant models.

In this review, we first lay out the competing hypotheses (total vs free vs reservoir hypotheses–see below); we then review several recent lines of biomedical evidence, including investigations using genomic, binding assay, cell culture, organismal, and medical data. We consider how these studies support the total vs free hormone hypotheses, and how the reservoir hypothesis fits in. We finish with a discussion of current issues in binding globulin research relevant to comparative studies: calculation of free hormones, lab measurement techniques to optimize accuracy, and a brief discussion of albumin.

2. Competing hypotheses

There are three classic hypotheses regarding the effect of binding globulins on hormone availability: Total, Free, and Bound Hormone Hypotheses. More recently Malisch and Breuner (2010) introduced the Reservoir Hypothesis as a modification of the Free Hormone Hypothesis. These hypotheses are illustrated in Fig. 2.

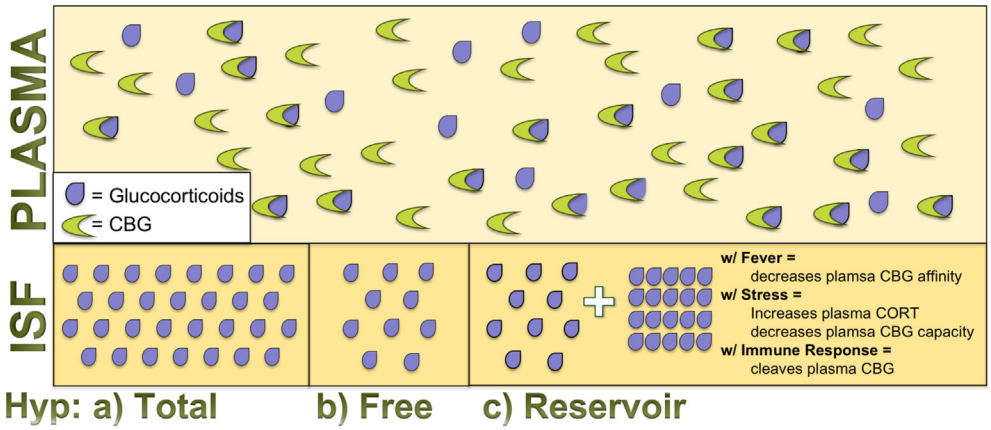

Fig. 2. Illustration of Total vs. Free vs. Reservoir Hypotheses. Given the amount of CBG and GCs in the plasma (upper panel), how much hormone will enter the interstitial fluid (ISF: lower panel)? Under the Total Hormone Hypothesis (a), all of the GCs in the upper panel are available to enter tissues. Under the Free Hormone Hypothesis (b), only the GCs not bound by CBG in the upper panel are available to enter tissues. Under the Reservoir Hypothesis (c), free plasma hormone can enter tissues (dispersed molecules in ISF), but the hormone bound to CBG (packed molecules in ISF) can become available to enter tissues as, for example, CBG levels decline, CBG affinity is lowered, or hormone levels increase.

- Total Hormone Hypothesis (Fig. 2a): This hypothesis predicts that all hormone present in the plasma freely enters tissues and binds receptors. Hence, 100% of plasma steroid has biological activity. Steroids are lipophilic, and so cannot exist in high concentrations in aqueous solution. As such, binding globulins may have evolved to allow for greater transport of steroids through the plasma. That may be the only function they serve, to carry hormone in the plasma and release it to tissues as blood enters capillaries. There is myriad support for the total hormone hypothesis in comparative systems. Measures of GCs in the blood predict behavioral, physiological, and morphological patterns across vertebrate classes (for reviews see: Breuner, 2010; Schoech et al., 2011) In cases where total and free GC levels are measured or estimated, total hormone can be a better predictor of the dependent variable than free hormone levels (e.g., Patterson et al., 2014).
- Free Hormone Hypothesis (Fig. 2b): This hypothesis predicts that only unbound, free hormone in the plasma is biologically active. Corticosteroid-binding globulins are usually 50–60 kD in size (Meyer et al., 2016). Under normal homeostatic conditions, the gaps between endothelial cells lining the capillary walls are too small for CBG to pass through. Therefore, any hormone bound to CBG will be unavailable to move into the interstitial fluid (ISF). CBG is also hydrophilic, meaning it cannot diffuse across membranes into cells. Consequently, CBG remains in the plasma, potentially limiting access of hormones to tissues. There are a number of studies across comparative systems that support the free hormone hypothesis. In European starlings free hormone levels better predict nest abandonment (Love et al., 2004). In white-crowned sparrows, free hormone levels reflect a suppressed stress response in populations with less time to raise young, whereas total hormone levels do not (Breuner et al., 2003). Also in white-crowned sparrows, food removal reduces CBG and increases free hormone over the entire 24 h of fasting, while total hormone only appears elevated over the first 6 h (Lynn et al., 2003; several studies, however, also demonstrate an extended increase in total CORT with fasting: Fokidis et al., 2011; Krause et al., 2017; Lendvai et al., 2014; Lynn et al., 2010). In white-crowned sparrows and Japanese quail, capture and handling stress does not affect total hormone levels 24 h later, but CBG is lower, causing a significant increase in free hormone titers in both species (Malisch et al., 2010, 2016). In tree lizards, increased binding globulins likely protect testosterone clearance during stress in territorial morphs–a mechanism that wouldn't be supported under the total hormone hypothesis (Jennings et al., 2000). In each of these studies, small changes in CBG capacity or affinity effect changes in free hormone levels that are not reflected in total hormone capacity, and so would not affect organismal change if the total hormone hypothesis were in effect.
- Reservoir Hypothesis (Fig. 2c): This hypothesis predicts that while the free fraction is biologically active, the bound fraction of hormone is biologically relevant (Malisch and Breuner, 2010); as binding globulin capacity and affinity decrease, bound hormone will be released and become biologically active. This hypothesis considers the bound hormone in the plasma as a reservoir that can be rapidly released. Systemic changes, such as fever, can decrease CBG's affinity for glucocorticoids (corticosterone or cortisol: CORT), and increase the amount of free hormone across the body. Local changes, such as inflammation, can cleave CBG in the near vasculature and increase free CORT by 5 to 10-fold at that local site. Seasonal changes in CBG provide strong evidence in support of the reservoir hypothesis. In several species, CBG levels increase during life history stages that coincide with heightened period of energetic challenge (such as during the breeding seaon, e.g., Romero, 2002; Williams et al., 2008). Interestingly, when CBG is measured to estimate free CORT, we don't see this same seasonal pattern in free (e.g., Breuner and Orchinik, 2002). This suggests that the bound fraction is being upregulated to provide an additional reservoir of available CORT.
- (Bound Hormone Hypothesis): This hypothesis predicts that hormone bound to CBG has biological activity. This has been demonstrated across several cell culture systems, and is thought to be achieved by membrane receptors and transporters that target CBG-bound CORT (e.g., Hryb et al., 1986) This hypothesis, however, is less relevant when considering which fraction of hormone passes from plasma into tissues (hence the parentheses). This would only be relevant if these membrane transporters were found in the vasculature, and functioned to increase CBG-bound hormone in the extracellular space because direct CBG diffusion is limited by its size. To our knowledge there is no evidence of this across biomedical or comparative literature, and so we do not consider this hypothesis further in this paper.

3. Biomedical evidence

There is a wealth of biomedical research on CBG at many levels of examination from genomes to pharmacology to cell culture to organismal research. The resounding conclusion from this research is that when considering which fraction is biologically active (total, bound or free) the free hormone hypothesis is well supported across levels of examination. Here we detail recent biomedical evidence that supports the role of the free fraction in each of these contexts. Collectively, these advancements in CBG physiological research provide compelling evidence for why CBG should continue to be an area of active research in comparative studies, and offer varied approaches for comparative physiologists to take in their studies of GC physiology.

3.1. A genomic approach: 3 CBG-related SNPs predict morning cortisol

Total Cortisol levels in humans are 30–60% heritable, but no specific genetic component had previously been identified explaining inter-individual variation in cortisol levels. To approach this question, Bolton et al. (2014), conducted a genome-wide association analysis from 12,597 Europeans. Across 2.5 million SNPs, the only major genetic signal explaining morning cortisol levels occurred at SERPINA6 (the gene for CBG) and SERPINA1 (functionally associated with CBG, explained below). Functional studies evaluating the biochemical phenotype on a subset of subjects identified how these SNPs alter CBG function.

SERPINA1 contained two SNPs significantly predicting morning cortisol. The primary SNP (SNP1) predicts higher morning cortisol, and is associated with an increase in CBG capacity (that is, greater number of total CBG molecules in circulation). The free hormone hypothesis predicts that total hormone levels in plasma should adjust to maintain free hormone levels as CBG increases. With greater CBG in SNP1, less hormone will be free to enter tissues, less will be free to have negative feedback on the HPA axis, and so total cortisol levels should increase to bring free levels into the correct range. In subjects with SNP1, total cortisol levels are higher but measured free cortisol levels are not different than free levels found in subjects with the other alleles, supporting predictions of the free hormone hypothesis. The Total Hormone Hypothesis predicts that total hormone levels should be maintained, irrespective of CBG level. That is, if all hormone can enter tissues, total hormone concentration should be maintained at normal levels by negative feedback, and free hormone should be lower (because there is more CBG present). However, this prediction is not supported by the subject data. The Reservoir Hypothesis is based on the free hormone hypothesis, so it similarly predicts that total hormone levels will increase to maintain equal free hormone levels across different SNP variants. However, it also predicts that bound hormone in reserve will be greater with SNP1 (more total hormone, but similar free hormone, will lead to a greater reservoir of CORT in the plasma). This study did not report findings on bound hormone.

The second SNP (SNP2) in SERPINA1 predicts lower morning cortisol, and is associated with a change in CBG affinity. SNP2 appears to represent a known CBG mutation called the Leuven mutation, wherein a Leu^{115}His substitution lowers CBG's affinity for cortisol by 33% (Van Baelen et al., 1982). As before, the free hormone and reservoir hypotheses predict that total hormone levels should change to keep free hormone levels close to wild type. Lower CBG affinity for cortisol means less hormone will be bound, and so more of the hormone will be free to enter tissues, increasing negative feedback on the HPA axis and therefore decreasing total hormone levels until free levels are near normal. In SNP2 subjects (as in Leuven mutation patients) total cortisol levels are significantly lower than in patients with wild type CBG, while measured free hormone levels are indistinguishable between the two (Bolton et al., 2014; Emptoz-Bonneton et al., 2000). The total hormone hypothesis predicts that total hormone levels would not vary across SNPs, but this prediction is not supported.

The third SNP (SNP3) occurs in the SERPINA1 gene. This gene encodes alpha1-antitrypsin, which can indirectly affect CBG function. As background: The GC binding pocket in the body of the CBG molecule is affected by linked movements of an exposed peptide strand, called the reactive center loop (RCL). When this loop is intact, binding affinity for GCs remains high, and CBG can carry GCs in the plasma. However, cleavage of the RCL changes the conformation of CBG, perturbing the GC binding pocket, and drastically reducing CBG's affinity for GCs. CBG's RCL can be cleaved by several types of elastase: neutrophil elastase (Hammond et al., 1990), chymotrypsin (Lewis and Elder, 2014), and LasB, an elastase produced by a common pathogenic bacteria in humans (Simard et al., 2014). Neutrophil elastase is a serine protease secreted by activated neutrophils. At sites of inflammation neutrophils secrete neutrophil elastase, which can cleave CBG in the vasculature and release GCs locally into tissues (based on the free hormone and reservoir hypotheses; see Perogamvros et al., 2012 for a clear illustration of this process). GCs have potent effects on immune function, both augmenting and inhibiting the immune system depending on timing and the specific arm of the immune system. In fact, Pemberton et al. (1988) suggest that CBG evolved as a carrier for GCs because CBG can be cleaved at sites of inflammation, thereby directly effecting immune action at that site. Hence, neutrophil elastase-directed cleavage of the RCL reduces CBG's affinity for GCs, locally increasing free GCs to affect immune function. Alpha1-antitrypsin (the gene containing SNP3 in Bolton's study) suppresses neutrophil elastase. Hence, SNP3 may alter (possibly increase) alpha1-antitrypsin expression, which would increase suppression of neutrophil elastase, resulting in less CBG being cleaved and an increase in CORT retained in the plasma. However, the authors note that this association is the weakest of the three, and are only able to postulate this relationship without good support from the functional studies.

These data demonstrate that changes in CBG can alter total hormone levels. Does this necessarily support the free hormone hypothesis? Or, if CBG is just a transport molecule, not restricting hormone access to tissues, would changes in total hormone occur with increases in CBG? It is unlikely. This would only occur if GC levels were typically higher than CBG capacity to bind GCs. GCs are lipophilic, and so don't dissolve in plasma well. With more CBG, however, more GCs could be carried in the blood, and so total hormone levels could be higher. In the vast majority of studies measuring total hormone and CBG capacity, however, CBG capacity is already large enough to hold even stress-induced levels of hormone in the blood (Charlier et al., 2009; Li et al., 2017). If CBG capacity is always higher than hormone titers, then a change in CBG capacity will only affect free hormone levels (under the law of mass action), not total hormone levels. Under the free hormone hypothesis, an increase in CBG will a) reduce the amount of free hormone entering tissues, b) thereby releasing negative feedback on the HPA axis, and c) increasing total hormone secretion. It is important to note that these arguments are all based on the plasma role of CBG. As mentioned in the introduction, there is strong evidence for extra-hepatic expression of CBG that could function to limit or deliver CORT directly to cells. One example that may affect our outcomes here: CBG present in the pituitary can buffer CORT from binding intracellular receptors, weakening negative feedback on further CORT secretion (Berdusco et al., 1995). If SNPs 1 and 2 explained here also effect tissue-specific expression of CBG, that could separately alter total hormone secretion. This cannot be ruled out as a major driver of plasma total hormone levels; however, our predictions are supported by the functional studies of CBG in the *plasma* of subjects in the Bolton study, supporting the role of CBG in regulating total hormone levels by its actions in the plasma.

Summary of the genomic study: These data support the free hormone hypothesis at a genetic level, suggesting that the primary genetic determinant of total hormone levels is CBG capacity and affinity. Other target gene studies have found as many as 15 human SERPINA6 polymorphisms that have been characterized with defects in the production or steroid-binding activity of CBG (Simard et al., 2015), and many have demonstrated effects on human health (Meyer et al., 2016; Perogamvros et al., 2011, see below). Hence, there is widespread genetic evidence for CBG regulating GC physiology.

3.2. A pharmacological approach: binding globulin capacity predicts clearance rates

One estimate of hormone movement into tissues is hormone clearance rate. If hormone is limited to the plasma it cannot enter tissues to be metabolized and cleared. Hence, if binding globulins limit hormone access to tissues (a prediction of the free and reservoir hormone hypotheses), then binding globulin capacity should relate to hormone clearance rate. If all hormone (bound and free) can enter tissues (a

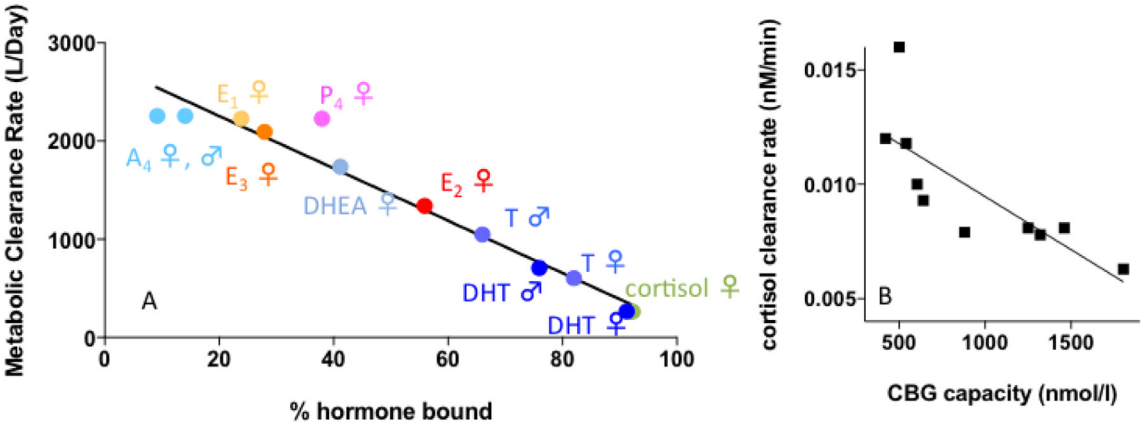

Fig. 3. Hormone clearance rate depends on binding globulin capacity. A) Metabolic clearance rate as a function of % of hormone bound to its binding globulin in human plasma (redrawn from Siiteri et al., 1982). As the amount of hormone bound to binding globulin increases, hormone clearance rate declines. B) Cortisol clearance rate as a function of CBG capacity (redrawn from Bright, 1995). As the amount of plasma CBG increases the clearance rate of cortisol declines.

prediction of the total hormone hypothesis), then binding globulin capacity should not affect clearance rates.

An early study by Siiteri et al. (1982) compared the metabolic clearance rates (MCR) of a suite of hormones against the % of hormone bound in plasma (Fig. 3a). Sitteri directly measured % of hormone bound in human plasma through measures of free hormone (using a dialysis method that separates bound from free) before and after heat inactivation of binding globulins. This allows for calculation of the % of hormone that was bound by CBG or sex-hormone binding globulin (depending on which hormone was measured). They then obtained clearance rate data from the literature. Fig. 3a demonstrates a large effect of binding globulin on metabolic clearance rates (linear regression: $F_{1,10} = 195.5$, $p < 0.0001$, $R^2 = 0.95$).

However, this study looks across hormones, and takes clearance rates from the literature instead of directly from the subjects with whom % bound was measured. Bright (1995) evaluated both cortisol clearance rate (after bolus injection of deuterated cortisol) and total CBG capacity in a cohort of healthy female subjects (humans). As with the broader study, hormone clearance rates vary as a function of binding capacity (Fig. 3b; linear regression: $F_{1,8} = 12.63$, $p < 0.00785$, $R^2 = 0.61$).

While not recent additions to the biomedical literature, these studies provide strong support for the free and reservoir hormone hypotheses. If hormone bound to binding globulins could enter tissues, binding globulin capacity, or the % of hormone bound by binding globulins would not affect clearance rates of that hormone.

3.3. A tissue culture approach: CBG limits GC access to cells

Evidence presented so far includes genomic and pharmacological evidence supporting the free hormone hypothesis. However, neither example evaluates glucocorticoid action as CBG levels change. If CBG regulates GC access to tissues, then alterations in CBG level should alter GC activity at the cellular level. Perogamvros et al. (2011) tested whether altering CBG capacity in cell culture media could alter glucocorticoid receptor (GR) activation. They evaluated 'glucocorticoid bioactivity' by bathing HeLa cells (transfected with human GR-alpha and the MMTV-Luc reporter gene) in human serum that had been stripped of endogenous hormone and spiked with cortisol. Cells were bathed in 10, 20, 50, or 100% serum, each spiked with the same amount of cortisol. Hence, the same amount of total cortisol was available outside the cells, but CBG capacity varied by 10 fold. Perogamvros measured free cortisol levels in both the 100% and 10% sera, demonstrating a much lower level of free hormone in the 100% serum (Fig. 4, light green bars). The total hormone hypothesis predicts that altering CBG capacity would have no effect on GC entrance into cells

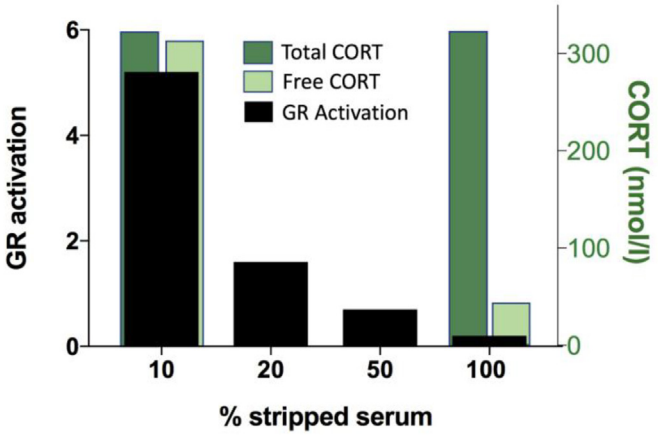

Fig. 4. Increasing CBG capacity decreases glucocorticoid receptor activation, matching serum free CORT levels. Glucocorticoid receptors transfected into HeLa cells were less active (black bars) as cell culture media contained more CBG, but the same amount of cortisol across treatments. Total and Free CORT levels (green bars) were measured in the 10% and 100% serum. Redrawn from Perogamvros et al. (2011). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and therefore GR activation. The free hormone and reservoir hypotheses predict that increased CBG capacity would limit hormone access to cells and reduce GR activation. When human serum was spiked with approximately 300 nM cortisol, more concentrated serum (with greater CBG capacity) had lower free CORT (Fig. 4, light green bars) and lower glucocorticoid receptor activation (black bars). These data suggest that increasing CBG capacity decreases glucocorticoid bioactivity, supporting the free hormone hypothesis. There is, though, a complication here. Throughout this review we have focused on the plasma effects of CBG, setting tissue-specific actions of CBG and CORT-bound CBG aside. There is strong evidence that myriad tissues have CBG present in the extra-cellular fluid or inside the cells, or CBG receptors present in cell membranes (see introduction). There is no evidence for HeLa cells having membrane CBG receptors, but if they do, then conclusions drawn from this study may be more complex.

3.4. An organismal approach: Tissue hormone level matches free, not total plasma hormone in awake, behaving animals

There is evidence suggesting that only free hormone activates GR in tissue culture cells, but this may not represent what is happening in the whole animal. One of the clearest tests of the free vs total hormone

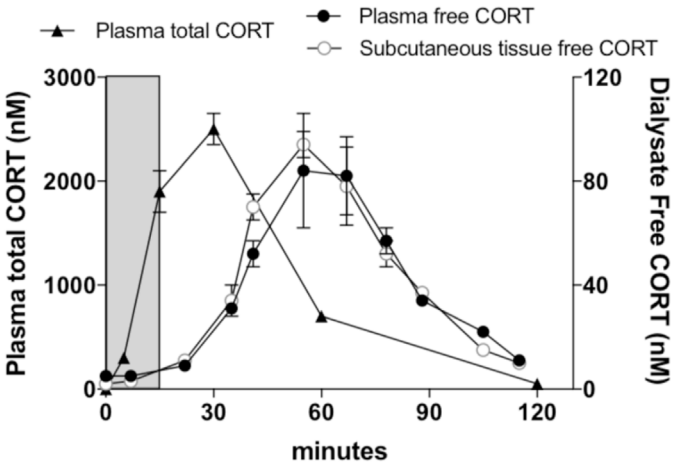

Fig. 5. Timing of tissue CORT level after stress mirrors plasma free CORT, not plasma total CORT. Rats were sampled through a 15 min forced swim test (gray shading), dried, and returned to their home cages. Trunk blood from different subjects was used to measure total CORT, while free plasma and tissue CORT were measured every ~10 min from dialysis across the same individuals. Redrawn from Qian etal. (2012).

hypotheses would be to measure total and free hormone in the plasma while also measuring hormone in the tissues. The Total Hormone hypothesis predicts tissue levels of hormone would mirror total hormone levels, while the Free Hormone and Reservoir Hypotheses predict tissue hormone would mirror free hormone levels. Qian et al. (2011) used dialysis probes to measure jugular (plasma) and subcutaneous (tissue) hormone levels in rats over a forced-swim test trial, continuing measures over the subsequent 24 h. Microdialysis only measures free hormone levels (CBG is too large to pass into the probe), so total plasma hormone levels were collected in a separate cohort of rats subject to the same test.

Total hormone levels in the plasma increased significantly within 5 min of starting the forced swim stress, peaked at 30 min (15 min after the trial ended), and returned to baseline after 2 h (Fig. 5, triangles). Free hormone levels, surprisingly, did not follow the same temporal pattern. Qian et al. identified a surge in plasma CBG within 5 min of the forced swim test, buffering and delaying the increase in plasma free CORT. Plasma free CORT did not increase until between 15 and 30 min after initiation of the stressor, and peaked at 60 min post-initiation (Fig. 5, filled circles). Tissue levels of cortisol (Fig. 5, open circles) mirrored plasma free hormone in both amount and time course, and was very different from total hormone levels. Hence, direct measures of cortisol across plasma and tissue supports the free hormone hypothesis.

3.5. Human medicine approach: CBG abnormalities associated with human disease, and free hormone levels predict illness severity more closely than total levels

There are several mutations identified in human CBG that result in either 3-4-fold reduction in affinity for cortisol (Emptoz-Bonneton et al., 2000; Van Baelen et al., 1982), or a complete loss of plasma binding activity due to either disruption of the cortisol binding site (Hill et al., 2012; Perogamvros et al., 2010), or an early stop codon in the DNA that leads to a complete loss of function (Torpy et al., 2001, 2012). These mutations are associated with a variety of clincal conditions, such as chronic pain, chronic fatigue, depression, relative hypotension and obesity (see Meyer et al., 2016 for a description of all 9 mutations discovered to date with their associated clinical condition). They may affect human health through alteration of free cortisol levels, although many are associated with large decreases in total cortisol to maintain free cortisol levels compared to controls. It is more likely that the associatd morbidity arises from either a smaller reservoir of CBG-bound CORT in the plasma (thought to be active in regulation of inflammation as described in section 3A), or tissue-specific activities of CBG independent of what occurs in the plasma. Interestingly, CBG deficient pregnant women have sex ratios skewed toward female offspring (Lei et al., 2015); this may result from higher free cortisol levels, as elevated cortisol during pregnancy is also associated with greater numbers of female offspring (Navara, 2010).

More direct support for the free hormone hypothesis comes from clinical studies over the last decade evaluating total and free cortisol in ICU patients. Septic shock is the most common trauma studied, but they have also covered respiratory failure, hypertensive crisis, and multitrauma (Cohen et al., 2012; Ho et al., 2006; Poomthavorn et al., 2009). In each study, higher free cortisol (either measured directly or calculated from total cortisol and CBG) predicts patient mortality rate, where total cortisol does not. There are exceptions: free and total cortisol do not predict mortality resulting from pneumonia (Christ-Crain et al., 2007); and both free and total cortisol predict mortality in sepsis and septic shock in one study from a different group (Bendel et al., 2008).

Free cortisol is often elevated compared to total under conditions of extreme acute stress, likely resulting from reduced CBG. CBG declines during acute illness such as septic shock (Beishuizen et al., 2001), burns (Garrel, 1996), and cardiac surgery (Vogeser et al., 1999). Other stressors reduce CBG in animal models: tail shock (Fleshner et al., 1995), immobilization (Tannenbaum et al., 1997: Breuner et al., 2006), and fasting (Lynn et al., 2003; Tinnikov, 1999). Overall, high stress levels reduce CBG, increase free CORT levels compared to total, and reduce CBG-bound CORT in the plasma that may be used as a reservoir to regulate CORT access to tissues. Hence, while ICU patients will have elevated total cortisol, their free cortisol levels may be relatively much higher given reductions in CBG capacity.

4. Issues to consider from a comparative viewpoint

4.1. Estimating free hormone levels from total hormone and CBG

There is disagreement across researchers as to the utility of calculating free hormone levels in the plasma. Meyers et al. (2016) and Levine et al. (2007) recommend estimating free CORT given the labor-intensive methods (ultrafiltration, dialysis, and steady-state gel filtration) necessary to directly measure free hormones in the plasma. Additionally, in comparative studies, animals are often too small to get enough plasma to measure free levels. Hammond and co-authors (e.g., Charlier et al., 2009) tend to avoid estimation of free hormone levels; Hammond, 2016 warns of the likely misestimation of free levels due to the different CBG affinities within and across individuals: CBG affinity for CORT can vary with temperature, CBG-glycosylation, and genetic polymorphism (as described in section IIIA). To test the accuracy of free-CORT calculations, either in absolute or relative terms, one can calculate free CORT using a variety of methods, and compare that to absolute levels measured directly from the blood.

There are several methods used to estimate free CORT; a few of the methods have been evaluated for accuracy against actual measures of free CORT. The Free Cortisol Index (le Roux et al., 2002) is the ratio of total plasma CORT to CBG capacity. This simple metric is commonly used in clinical studies, and has been evaluated against measured free cortisol in humans and swine (FCI predicts measured free cortisol in humans with an r of 0.9, Fig. 6a; Le Roux et al., 2002, and an r of 0.78 in swine, Fig. 6b; Adcock et al., 2006). The Coolens formula (Coolens et al., 1987) estimates an actual concentration of free CORT, instead of just providing a unitless index. The Coolens calculation predicted actual measured free CORT with an r of 0.98 and 0.90 (Coolens et al., 1987 and Ho et al., 2006, respectively). Both of these methods (FCI and the Coolens formula) ignore CBG's affinity for CORT. Other, more complicated formulas incorporate CBG affinity into the equation. Barsano and Baumann (1989), for example, present a complicated formula that accounts for CBG affinity and has been commonly used across

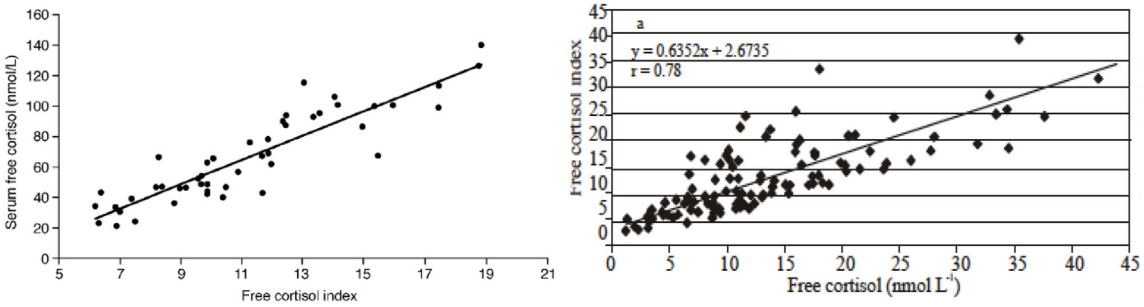

Fig 6. Free CORT estimates taken from the ratio of total plasma CORT: CBG capacity (the Free Cortisol Index) predict actual measured free CORT determined from the same samples. Data reprinted from Le Roux et al., 2002), and Adcock et al., 2006.

comparative studies (Breuner et al., 2003; Deviche et al., 2001; Patterson et al., 2014; Zysling et al., 2006). However, CBG affinity is usually measured once for a species (using an equilibrium saturation binding approach on a plasma sample pooled from several individuals), and that affinity is then plugged into the formula for all subsequent estimations. Affinity could easily vary across individuals, by sex, season, disease state, or genetic disposition (as noted in Hammond, 2016). Hence, these estimations may not be accurate. One would assume, however, that estimates that include affinity (even at a species level), should be better at estimating free hormone than those equations that don't include affinity.

4.2. Assay Temperature

There are myriad critiques regarding CBG measures from across comparative researchers; assay temperature (Schoech et al., 2013) is a valid concern that certainly warrants investigation. Historically, CBG assays measuring affinity and capacity have been done at 4 °C, to maximize specific to non-specific binding ratios. However, these measures of affinity would then be used in estimates of free CORT. Affinity of steroids for binding globulins can change significantly with temperature (Cameron et al., 2010; Lentjes and Romijn, 1999; Mickelson and Westphal, 1980; Mickelson et al., 1981), with lower affinity at higher temperatures. Hence, our estimates of free CORT from 4 °C may underestimate free CORT at 37 °C or 41 °C. We are currently testing affinity differences across traditional lab (4 °C) and organismal (41 °C in birds) temperatures to evaluate the level of error induced by this technique.

4.3. Albumin

Albumin binds steroid hormones nonspecifically with very low affinity. However, there can be over 1000-fold more albumin in the plasma than CBG (Dunn et al., 1981). There are conflicting results on whether albumin significantly affects free CORT levels (for example, see Dunn et al., 1981 vs., Ho et al., 2006, Molenaar et al., 2015, Thomas and Thomson, 2019 demonstrating very little effect). We have not discussed albumin at length here. Hammond (2016) present a thoughtful discussion on albumin, noting that it warrants further investigation.

5. What lessons can we learn from biomedical research?

Here we presented 5 different sets of biomedical studies that clearly support the free hormone hypothesis and by extension, the reservoir hypothesis. A). Genomic analysis of over 12,000 human subjects identified only 3 SNPs (of the 2.5 million evaluated) that explain variance in total morning cortisol levels; two are from the CBG gene, and the third produces a protein that protects CBG from cleavage. If CBG only transported, but did not limit hormone access to tissues, changes in CBG affinity and capacity would not alter total cortisol levels. However, if CBG limits access to tissues, alterations in CBG will change how much cortisol can return to tissues to regulate cortisol production, altering total cortisol levels. B) Pharmacological evidence suggests a strong role for binding globulin capacity in regulating hormone clearance rates. Across hormones, hormone binding capacity (the % hormone bound to binding globulin in plasma) closely predicts clearance rate of that hormone. A more detailed study within subjects shows that CBG capacity predicts cortisol clearance rates. If only free hormone can enter tissues to be metabolized and cleared, then binding globulins should regulate clearance rates. C) Experiments using cell culture altered the amount of CBG in the culture media, while keeping cortisol levels constant across treatments. Increased CBG in the media decreased activation of the glucocorticoid receptor, supporting a limiting role for CBG in cortisol access to tissues. D) Microdyalisis studies in rats demonstrate that tissue corticosterone levels mirror free, but not total hormone levels in the plasma through a forced swim test. And finally, E) Human mutations that reduce affinity or eliminate CBG entirely are associated with illness; while mortality rates are predicted by free cortisol but not total, especially in sepsis and septic shock.

In comparative endocrinology, we seek to link endocrine patterns with organismal output, understanding how environmental change links to performance through endocrinology. To best understand these links, we should incorporate measures that enable the clearest explanation of endocrine patterns. We believe that patterns of hormone action will be clearer when binding globulin levels are taken into account. We don't suggest that everyone must measure CBG, but we hope researchers recognize its importance in regulating the organismal response to stress.

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