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Cortisol and CBG – Getting cortisol to the right place at the right time

Names of Authors:

David Henley[1,2]

Stafford Lightman[3]

Robin Carrell[4]

Affiliations:

[1]Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, WA, Australia

[2]Faculty of Medicine, Dentistry and Health Sciences, School of Medicine and Pharmacology, University of Western Australia, Crawley, WA, Australia

[3]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, UK

[4]Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Correspondence:

Professor Robin Carrell

Trinity College, Trinity Street, Cambridge, CB2 1TQ

United Kingdom

Tel: +44 (0)1223 312970

Email: rwc1000@cam.ac.uk

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Abstract

Cortisol is transported in the blood by corticosteroid-binding globulin (CBG), a non-inhibitory member of the serpin family of serine protease inhibitors. Recent structural advances reveal how CBG acts as a releasing-agent as well as a carrier of cortisol. Taken together, the structures of the various forms of CBG and of the closely related thyroxine binding-globulin, show how the inherent conformational mechanism of the serpins has been adapted to modulate hormone release to the tissues by changes in binding affinities. A deduction from this, of the temperature dependence of hormone binding, is remarkably borne out with CBG, with a doubling in plasma free cortisol as the body temperature rises to 39ºC. Another insight, against a dogma in the corticosteroid field, is that the proteolytic cleavage of CBG in inflammation results in a partial and not a complete loss of cortisol binding. This becomes of medical importance in conjunction with recent evidence of a pool of the circulating cleaved-form of CBG. It is now evident that tissue levels of free cortisol are buffered by two responsive plasma pools, intact CBG with a high binding-affinity and, particularly in inflammation and sepsis, a further pool of cleaved-CBG with a ten-fold lower affinity. The new molecular understandings, as well as providing insights into the differential release of circulating hormones, also open prospects for therapeutic interventions and draw attention to the potential of CBG and TBG as vehicles for the targeted delivery of drugs.

Abreviations

CBG – corticosteroid-binding globulin, TBG – thyroxine-binding globulin

Key Words

CBG, cortisol, serpin, TBG, body temperature, inflammation.

1. Introduction

Of the total cortisol in the blood, only some 5% is present in its active free form with the other 95% being inertly bound to plasma proteins, primarily to its specific transport protein, corticosteroid-binding globulin (CBG) (Hammond, 1990; Lewis, et al., 2005; Westphal, 1986). The CBG-bound cortisol acts as a circulating store but critically it is the equilibrated release of cortisol from CBG that buffers and regulates the plasma concentration of free cortisol. Cortisol is carried by CBG in a 1:1 ratio and its release of free cortisol will follow the law of mass action, being determined by two factors, the percentage saturation of the CBG and by its cortisol-binding affinity. The percentage saturation will vary with the adrenal secretion of cortisol, which at peak times can exceed the binding capacity of CBG resulting in disproportionately high levels of free cortisol (Henley & Lightman, 2011; Young, et al., 2004). For the most part though, a steady state concentration of cortisol throughout the tissues is maintained with the binding capacity of CBG being only partially saturated. For example in a healthy adult a typical free cortisol concentration of 13nM (Ho, et al., 2006) will result from a 29% saturation of CBG. This then, till recently, has been the accepted role of CBG as a simple carrier of cortisol that gives an equilibrated release of the hormone to maintain uniform free cortisol concentrations throughout the tissues. The assumption has been that the cortisol-binding affinity of CBG remains a constant throughout.

This assumption of a constant binding-affinity has been overturned, as we describe here, by recent findings and particularly from structure-based studies of CBG and of the closely related carrier of thyroxine, thyroxine-binding globulin (TBG) (Gardill, et al., 2012; Klieber, et al., 2007; Qi, et al., 2011; Zhou, et al., 2006; Zhou, et al., 2008). Both CBG and TBG have virtually identical hormone binding sites and hormone release mechanisms. The surprise conclusion from structural studies of both, is that hormone binding and release is not an on-off event but rather represents an equilibrated change in binding avidity that can be allosterically modulated. Put simply, the cortisol binding-affinity of circulating CBG, and hence the concentration of free cortisol, is not fixed and can be modified by

external factors. This immediately makes good biological and physiological sense. A human is a complex assemblage of organs, each of which will have at times different hormone requirements. We give two examples here of the way CBG can respond to such varying needs by a change in its cortisol-binding affinity: locally in inflammation and systemically in fevers. Although these are at present the only proven examples of the modulatory role of CBG, there is good reason to predict that such responsive changes in binding-affinity will more widely occur by direct interaction with tissue membranes and ligands.

2. Corticosteroid binding globulin

2.1 Plasma CBG

Corticosteroid-binding globulin (CBG), or transcortin, has become well documented as the 50-60 kDa high affinity plasma transport glycoprotein for the glucocorticoid hormones, principally cortisol (Westphal, 1986). It can also bind progesterone with relatively high affinity, however the amounts bound to CBG in the peripheral circulation are relatively low compared with glucocorticoids (Cameron, et al., 2010; Rosner, 1990). CBG is secreted principally from hepatocytes (Khan, et al., 1984), as a 383-residue polypeptide after cleavage from a 22-amino-acid signal peptide. Functionally, CBG was known to be monomeric, to have a single steroid binding site per molecule (Mickelson, et al., 1982; Westphal, 1986), to circulate at concentrations of 175-365 nmol/L (Nenke, et al, 2016) and in keeping with this, to become saturated at plasma cortisol levels above 400-500 nmol/L (Ballard, 1979).

Although all this was known, a central puzzle remained. The rate of production of cortisol and its plasma concentration are poorly correlated, with CBG apparently being involved in determining the kinetics of cortisol transport from plasma to tissue (Bright, 1995; Bright & Darmaun, 1995). The question facing the field was how does CBG play this active role in the release of cortisol as well as in its transport? The answer came in a most satisfying way with the determination, at molecular level, of the way CBG binds cortisol and of the structural changes that influence its release (Klieber, et al., 2007; Zhou, et al., 2008). Not only is CBG seen to act as a circulating glucocorticoid reserve readily available in case of

an emergency (Moisan, 2013) but also, importantly, to have an inbuilt mechanism that modulates its glucocorticoid release (Chan, et al, 2013; Lin, et al., 2010; Qi, et al., 2011; Zhou, et al., 2008).

2.2 What structures tell us about hormone carriage and release

Advances over the last two decades have seen a transformation of fundamental concepts in endocrinology, from a descriptive to a precise molecular basis. What has made this possible has been the realisation that proteins, even with quite different functions, exist as members of a discrete number of families, with each family sharing the same overall structure. Thus knowledge of the structure and function of any one member of a protein family is of relevance to all the members. This is well borne out by the carriers of thyroxine and cortisol in the blood, TBG and CBG. Both are members of the serpin family of proteins (Flink, et al., 1986; Hammond, et al., 1987), having almost identical structures and molecular mechanisms. Furthermore, the archetype of the serpin family and a coding neighbour to CBG on chromosome 14, was another plasma protein, of known crystallographic structure, alpha1-antitrypsin. Unlike TBG and CBG, which have both lost their inhibitory activity, alpha1-antitrypsin has retained its eponymous serpin function as a serine protease inhibitor. The predominance of the serpins as protease inhibitors in the blood is due to their unique spring-like action that has been realistically likened to that of a molecular mousetrap. The active inhibitor circulates in a stressed S-form, which readily springs back into a stable relaxed R-form to give the entrapment of the target protease (Carrell & Owen, 1985; Huntington, et al., 2000); the entrapment being triggered by the cleavage of a large exposed peptide loop by the protease, as indicated in Figure1.

The confirmation from their amino acid sequences that TBG and CBG were serpins allowed the ready modelling of their molecular structure on the known template of alpha1-antitrypsin (Huber & Carrell, 1989) and subsequently of the hormone binding site in CBG (Edgar & Stein, 1995). TBG and CBG have both lost any function as protease inhibitors but have clearly retained the overall serpin conformational mechanism, with both hormone carriers undergoing the S-to-R conformational change on exposure to inflammatory proteases (Pemberton, et al, 1988). This transition was seen to be accompanied by a loss of hormone binding affinity, though even in these early findings it was noted

that the loss of binding affinity in CBG was incomplete. Further studies confirmed the deduction that the release of cortisol in response to cleavage by neutrophil proteases represented a mechanism for the delivery of the hormone to inflammatory loci (Hammond, et al., 1990).

An assumption made by many from these early studies was that the binding and release of thyroxine and cortisol from their carrier proteins was a direct on-and-off process: on with the intact S-conformation, off with the transition to the cleaved R-form. The solving however, of what is a whole series of structures of TBG and CBG, has revealed a much more subtle release mechanism. The structures show how TBG and CBG have adapted the complex inhibitory mechanism of the serpins to differentially regulate the tissue release of thyroxine and corticosteroids. X-Ray crystallography has given us single-frame snapshots of frozen structures of TBG and CBG; of the high affinity native S-forms with an exposed reactive loop and of the lowest affinity cleaved R-forms with a fully incorporated reactive loop, as indicated in Figure 1a (Klieber, et al., 2007; Zhou, et al., 2006). However, what matters physiologically are the changes in binding affinity that take place in between these two states. These changes in binding affinity in circulating CBG and TBG are induced by small movements of the intact reactive loop in and out of the body of the molecule (Qi, et al., 2011). The movements of the loop although small nevertheless influence the shape and flexibility of the adjacent hormone binding site. We can now see how these subtle shifts take place in atomic detail, based on the extensive structural studies of another serpin, antithrombin, which has developed a mechanism, closely identical to that of CBG, for the binding and release of a heparin pentasccharide (Langdown, et al., 2009).

Although the modulatory movement of the reactive loop that drives the release mechanism has been referred to as a flip-flop shift, it perhaps would have been better to call it nudge-nudge rather than flip-flop, as it primarily reflects the movement of just a single amino acid residue into and out of the body of the molecule (Figure1b). This small modulatory movement of the reactive loop is dynamic, occurring constantly and reversibly; with the change in affinity reflecting changes in the relative populations of different conformational states. So it is simplistic to expect that such subtle shifts will affect antigenicity (Lewis, et al., 2016). This is not an on-off switching mechanism but rather an equilibrated shift that affects the plasticity of the binding-site, and hence allows readily responsive changes in binding avidity.

3. Dynamics of Cortisol release

3.1 Cortisol & thyroxine

The solving of the structures of TBG and CBG revealed their shared mechanisms for hormone carriage and release but along with this came structural hints as to their different missions. The concentration of free thyroxine in the plasma is precisely controlled at picomolar levels whereas concentrations of free cortisol fluctuate in health over a wide nanomolar range. Thyroxine is bound exceptionally tightly by TBG, with only 0.03% of the total blood thyroxine being in the free form. Even small changes in the free thyroxine can have profound consequences, an increase of just 40% from the normal concentration in blood can mean thyrotoxicosis and a decrease of similar proportion can constitute hypothyroidism. The modulation of thyroxine release is consequently cautious and defined. The much more ready release of cortisol by CBG over a wide range of concentrations is reflected in its reactive loop conformation. Whereas the residue in the reactive loop that triggers the release mechanism in TBG (Figure 1b), is a threonine with a small side-chain, the residue in the same position in CBG is a bulky valine which on entry into the body of the molecule will cause a much greater triggering displacement (Klieber, et al., 2007; Zhou, et al., 2006). Metaphorically, CBG as opposed to TBG, could be described as trigger happy!

Physiologically, cortisol levels vary rapidly due to the pulsatile nature of cortisol secretion. The pattern of ultradian (pulsatile) glucocorticoid secretion can be markedly altered by the physiological and pathological state (Henley, et al., 2009; Lightman, et al., 2000), and in particular, inflammatory diseases are major modifiers of endogenous pulsatile secretion (Gibbison, et al., 2015; Windle, et al., 2001). Furthermore, the peak of these secretory pulses may exceed the saturation of CBG (Cameron, et al., 2010), increasing the height of the ultradian peaks of cortisol. This can effectively magnify the excursion of the pulses (Henley & Lightman, 2011), allowing large pulses of free hormone to occur in extravascular tissues such as the brain and subcutaneous tissue (Qi, et al., 2014). This is of relevance

because the ultradian rhythm has been shown to be important in glucocorticoid signaling and gene transcription (Stavreva, et al., 2009).

3.2 A Protein Thermocouple

A major proportion of human metabolism is directed to the precise maintenance of a body temperature of 37ºC. A prime reason for this closely defined specificity is that the structures of the proteins and enzymes controlling vital functions are flexible rather than rigid, with a flexibility that increases with temperature. Thus individual proteins have evolved so as to function optimally at the kinetic flexibility present at 37ºC. This is particularly true of the interactions of proteins with ligands and it was predictable that the avidity of binding of cortisol by CBG would decrease with rises in body temperature (Mickelson, et al., 1981). There is however a special additional factor contributing to the temperature responsiveness of both TBG and CBG. Earlier findings with another serpin, antithrombin, had highlighted the temperature dependence of its modulatory mechanism. As with TBG and CBG, the mechanism that controls the activity of antithrombin involves small 'nudge-nudge' movements of the reactive centre loop into and out of the body of the molecule. The sensitivity of these movements to changes in temperature became apparent in patients with mutations in their antithrombin that marginally facilitated the entry of the reactive loop (Beauchamp, et al., 1998). The affected individuals remain fit and well unless they develop a fever. Then even a small increase in their body temperature, as can occur with a minor infection, is sufficient to cause the inactivation of the mutant antithrombin and the consequent onset of thrombosis.

With the precept of antithrombin in mind, an immediate proposal that arose with the solving of the structure of TBG was that its hormone release mechanism would be similarly temperature responsive (Zhou, et al., 2006). And indeed it proved to be so. Moreover, the temperature responsiveness of TBG has clearly evolved to physiological advantage. As shown in Figure 2, TBG effectively functions as a protein thermocouple: as body temperature decreases and the basal metabolic rate declines, there will be an accompanying increase in binding-affinity and an appropriately decreased release of thyroxine. Conversely, when the temperature rises above 37ºC, as in fevers, there will be a boosted release of

thyroxine, with the equilibrated concentration of free thyroxine at 39ºC increasing to near thyrotoxic levels. Evidence that this potential boost of thyroxine release in fevers is purposeful, comes from the presence in an Australian aboriginal population of an adaptive mutation that turns off the fever-induced acceleration of thyroxine release (Qi, et al., 2014). The mutation occurs within the site linking the immediate point of entry of the reactive loop into the body of the molecule with the adjacent hormone binding pocket (Figure 1b).

The concept of this thermocouple-like mechanism arose from the solving of the structure of TBG and of the S-to-R conformational change in CBG (Zhou, et al., 2008). Experimental confirmation followed with recombinant and plasma CBG (Cameron, et al., 2010; Henley & Lightman, 2011) and definitively with K_D determinations of both intact and cleaved plasma CBG over a range of temperatures (Chan, et al., 2013). Not surprisingly, the effect of changes in temperature on the much more sensitive release-mechanism of CBG is striking (Figure 2). Whereas with TBG there will be a 25% increase in free thyroxine as body temperature rises above 39ºC, with CBG there will be a threefold or more increase in free cortisol. These are changes that rapidly re-equilibrate as the temperature rises and falls. A patient in fever may have a threefold increase in free cortisol but this will revert almost immediately to a normal level as the body is cooled to 37ºC. Similarly and confusingly so, if a blood sample taken during a fever is then analysed in the laboratory at 37ºC, the result represents what the free cortisol concentration is in the blood sample at 37ºC and not what it was in vivo at the time of sampling.

3.3. Inflammation and cleavage

The finding that the cleavage of CBG by neutrophil proteases resulted in a loss of binding-affinity initially focused attention on the release of cortisol at foci of inflammation. But a realisation of the wider significance of this proteolytic transition has become apparent from a study which defined the range of changes in binding-affinity, K_D, that can take place in plasma CBG (Chan, et al., 2013). As the authors point out, the cleavage of the reactive loop in CBG (Figure 1) results in a large diminution, but not a loss of binding affinity. The transition from the intact plasma S-CBG to the cleaved R-CBG is accompanied by an increase in K_D from 32nM to 292nM, which will result in the release of most of the

bound cortisol but by no means all, with the retention by the cleaved R-CBG of a minimal binding saturation of 4.3%. The release of cortisol on cleavage of CBG will be most significant in situations where blood-flow is sluggish, as in an indurated site of inflammation. In such a compartment, in the presence of typical levels of other cortisol-binding proteins, a total cleavage of CBG would result in a 4-fold spike in free cortisol. This would be accompanied by a re-equilibration of the percentage saturation of the cleaved GBG to 16%, a level at which it could effectively buffer the raised free cortisol level. If, alternatively, the cleavage of CBG had resulted in a complete loss of cortisol affinity, free cortisol would rise to 103 nM, but it would be an unbuffered rise without the backup of a reservoir of CBG-bound cortisol.

The concept that the cleaved and apparently inactivated R-CBG might have a role systemically, as a backup buffer to that of the intact circulating S-CBG, has become a reality with the recent development of an immunoassay capable of differentiating the two forms of CBG (Lewis & Elder, 2011, 2013). With this assay, Lewis and colleagues have shown the half-life of the cleaved R-CBG in the plasma to be the same as that of intact S-CBG and also how in severe sepsis the rise in plasma levels of the cleaved form can reach near equivalence with that of the intact form (Lewis, et al., 2015; Nenke, et al., 2015). Thus, in severe systemic inflammation there will be two major buffering systems: that of intact CBG with a high cortisol affinity and that of cleaved CBG with a tenfold lower affinity. The kinetics of the buffered release of cortisol in peripheral tissues is complex. As with most biological systems there is a built-in redundancy in the hormone delivery system. CBG has the overwhelming role but it is backed by other plasma proteins with much lesser cortisol affinities, namely albumin and orosomucoid (Kerkay & Westphal, 1968). The significance of this binding of cortisol by plasma proteins becomes apparent in the congenital or induced absence of CBG, leading to a 30% rise in plasma free cortisol but with the remainder being substantially bound by albumin (Lewis, et al., 2005). The presence of cleaved CBG, which has a much greater affinity for cortisol than that of albumin, will add an intermediate buffer that will predictably play a direct as well as backup part in the release of free cortisol in the tissues.

3.4 CBG as a Hormone

The likelihood of the conformational mechanism of CBG being modified by interactions with tissue-specific ligands and membrane proteins arises from observations of other plasma serpins. The conformation and activity of the serpin inhibitors of coagulation are known to be modified by endothelial bound oligosaccharides, as are the serpin controllers of tissue growth by vitronectin and other tissue factors. Although such modifying interactions with CBG have not as yet been recognised, evidence for its binding to cell membrane sites dates back nearly three decades. Surface binding sites for CBG have been demonstrated in liver, endometrium, placenta, prostate, spleen and kidney (Gunaratna, et al., 2004; Hryb, et al., 1986; Maitra, et al., 1993; Singer, et al., 1988; Strel'chyonok & Avvakumov, 1991). Membrane binding sites for the plasma steroid-binding proteins share many of the general properties of receptors including: binding specificity; time and temperature dependence of binding; a dissociation constant approximately equal to the plasma concentration of ligand (Rosner, et al., 1988); and evidence for transmembrane signaling with induction of adenylate cyclase in a dose dependent manner (Nakhla, et al., 1988). The latter appears to be cortisol-bound dependent, and since cortisol, with a half-life of 60 - 90 minutes, is secreted with a diurnal and ultradian rhythmicity, CBG's activity may be rapidly regulated without changing its plasma concentration. Further indications of the interactions of CBG at a tissue level come from studies of mice with a complete genetic deficiency of CBG. Their inability to appropriately respond to the excess of free corticosterone in the absence of CBG infers an active role for CBG in the bioavailability, local delivery and/or cellular signal transduction of glucocorticoids (Petersen, et al., 2006; Willnow & Nykjaer, 2010). Genetic variants of CBG have also been associated with fatigue-pain syndromes and hypotension, suggesting a potential effect of CBG on the access of cortisol to brain glucocorticoid receptors (Henley & Lightman, 2011). Overall, these findings provide the basis for a novel concept of the mechanisms through which the body regulates access of glucocorticoids to the brain and other tissues of the body. However, a cell-surface receptor for CBG has not as yet been cloned nor has a tissue-specific ligand been identified.

4. Therapeutic Implications

The new understandings of molecular mechanisms of hormone delivery have opened concepts of direct relevance to patient management and therapy, along with insights of wider significance in medicine. With these come present day questions and challenges.

4.1 Are assays fit for purpose?

The new understandings described here require a new mindset; an ability to consider and assess corticosteroid status in molecular terms. To report the concentration of plasma cortisol in nmol/L and of CBG in pg/L is meaningless, whereas a CBG of 250 nmol/L with a plasma cortisol of 350 nmol/L immediately tells of complete binding-saturation and hence a spiking excess of free cortisol. The interpretation of plasma cortisol levels in critically ill patients and the indications for exogenous glucocorticoid therapy has been a controversial area in clinical medicine. A major problem has been a lack of understanding of the corticosteroid status in these patients. We can now see that a full assessment will need the determination of the concentrations of the main carriers of cortisol, including intact CBG, cleaved CBG, and albumin. From these values together with the total plasma cortisol and the known dissociation constants, the free cortisol will be calculable over a range of body temperatures (Chan, et al., 2013; Nguyen, et al., 2014). The direct assay of free cortisol is demanding and very dependent on the temperature at which the assay is carried out - best at a rigorously controlled 37^{o}C. Clinicians can readily convert the 37^{o}C laboratory result to the level in a pyrexic patient, by doubling the 37^{o}C result for a temperature of 38-39^{o}C, and tripling it at 40^{o}C (Figure 3). Nevertheless, while such rule of thumb estimates may suffice for everyday clinical management if corticosteroid endocrinology is to advance as a medical science a full range of carefully controlled assays will be required.

A lesson in what not to do, comes from the related area of thyroxine and its release from TBG. The unsatisfactory status of assays in this field became apparent in recent studies subsequent to the determination of the K_D values for TBG over a range of temperatures (Qi, et al., 2014; Qi, et al., 2011). Perplexingly, free thyroxine (FT4) concentrations calculated from the newly-determined K_D's were consistently higher than quoted literature concentrations. Perusal of the literature also showed a disconcerting variation in the quoted 'normal ranges' of FT4 and an inconsistency in defining the

demarcations between euthyroid, hypo-, and hyperthyroid function. Enquiries to individual laboratories as to their normal ranges were met by evasive replies until obtaining an unpublicised reference-centre range (Thienpont, et al., 2010). It was significantly higher than literature ranges, and satisfyingly matched the range determined by calculation from the newly-determined TBG K_D values. What became clear on further enquiry is that FT4 concentrations have for years been assayed at differing temperatures, in many cases listed as 25ºC, a frequently used euphemism for that most unscientific of all measurements, room temperature! Laboratories and kitset manufacturers have responded to these anomalies by adjusting their methodologies, including in some cases standards and controls, to fit with what has become not a normal range but a Perceived Normal Range (Christofides & Midgley, 2009; Jonklaas, Kahric-Janicic, et al., 2009; Ross & Benraad, 1992; van der Sluijs Veer, et al., 1992). Moves are apparently now underway with manufacturers and kitset suppliers to re-calibrate FT4 analyses at 37ºC by 2018. But in the meantime, without accurately defined standards and controls, results in this field are dubiously fit for purpose - not adequate for diagnosis or for investigative medicine and certainly not for medical science.

4.2. Are fevers beneficial?

A myriad of things will be happen in fever, both to our physiology and to our cellular functions. The new understandings described here give us just a glimpse of how in fever bodily processes move into overdrive. A rise of just one or two degrees C will automatically raise the plasma FT4 to a concentration that in health would move into the hyperthyroid range. With corticosteroids the same increase in temperature will lead to a massive release from the circulating store in CBG, to give a threefold increase in plasma free cortisol. It is not difficult to see how such increases in active thyroxine and cortisol will be of benefit to the body in a crisis. Fever is clearly a universal defensive response to infection and inflammation. But it is important that physicians are aware of the accompanying changes in corticosteroid availability. The rapid boost to free cortisol levels in pyrexia results from a change in an equilibrium that will be immediately reversed on return to normal body temperature. An induced cooling of body temperature will lead to a prompt threefold fall in plasma cortisol. Is this good for the patient or should it be countered by supplementary corticosteroids? These and other points will need

consideration. A first priority in the field however will be to convince physicians of the relevance of body temperature in the assessment of corticosteroid status. A free cortisol determined in the laboratory at 37ºC has only indirect relevance to the actual level in the blood of a pyrexic patient, and a cortisol measured at room temperature is only of direct relevance when the patient is dead.

4.3 Parenteral therapy or hot poultices?

Good science has a universal applicability, to everyday life as well as to the esoteric; to a falling apple as well as to an orbiting planet. The demonstration here of the inherent temperature-dependence of hormone release is true, independent of whether the CBG or TBG is in a laboratory cuvette, or a blood sample awaiting analysis or, in vivo, in the circulation or tissues. Hence a bonus from the new understandings is that they provide a scientific rationale for the age-old use of heat for both bodily relaxation and in the treatment of localised pain and inflammation. Active cortisol and thyroxine concentrations will rise and fall with body temperature, be it localised or systemic. A hot tub soak can raise the whole body temperature to above 39ºC (Harvey, et al., 1981) and so, as with Archimedes, augment our thoughts and well-being. Focused tissue temperatures can similarly increase with the direct application of heat. So therapeutically, the doubling or tripling of localised tissue cortisol levels should be readily achievable, without pills or injections, by the application of heating pads, radiant heat or even with the hot poultices of our greatgrandmothers' days.

4.4. Is induced cortisol-release feasible?

The full shift in CBG from its high affinity S-form to the low affinity R-form, with a massive release of cortisol, takes place on cleavage of the exposed reactive loop of CBG, which becomes irreversibly inserted into the main beta-sheet of the molecule, as in Figure 1. A question arising from this is whether the conformational transition could be exogenously induced without proteolytic cleavage? The answer is encouragingly positive, based on experimental evidence from the very first days of serpin studies, when the only structure of an intact serpin was that of the egg-white protein, ovalbumin (Stein, et al., 1990). These early studies predictably defined the inherent mobility of the reactive centre loop of the serpins (Figure 3) and proposed the occurrence of the modulating partial movements of the loop, as

described here with CBG and TBG (Carrell, et al., 1991). But what was also shown is that the S-to-R conformational transition could be induced by the annealing to the body of the serpin molecule of small reactive loop peptide-sequences (Schulze, et al., 1990). In the subsequent years extensive studies have been carried out of the use of related small peptides as annealing agents to block the pathological polymerisation of another serpin, alpha1-antitrypsin (Parfrey, et al., 2004). A surprise finding was that the polymerisation of alpha1-antitrypsin was efficiently blocked not only by the annealing of reactive loop peptides but also by a range of smaller peptides, totally unrelated in sequence (Carrell, et al., 2008). Pertinently, these peptides included a four- and a six-residue cholecystokinin neuropeptide present in the circulation. X-Ray crystallography showed how the annealing of the cholecystokinin tetrapeptide fixes the serpin in its R-conformation: the low-affinity conformation of CBG and TBG (Zhou, et al., 2004).

The findings have immediate relevancy to the serpin hormone-carriers. A lesson learnt from the serpins as a whole is that evolution has adapted to advantage every facet of their remarkable ability to change their shape. A challenge for future research therefore will be to see whether, as seems likely, CBG has utilised the potential to interact with small peptides to give a reversible transition to the low-affinity R-conformation. Conversely these observations also open pharmacological opportunities. Therapeutically there is a need to bolster circulatory corticosteroids and the most direct way of achieving this is by bolstering the release of cortisol from the large pool bound inertly to CBG. This is achieved naturally in inflammation by the proteolytically-induced transition of CGB to its low affinity R-form. The possibility of inducing this transition with exogenous small molecules and peptides has been extensively explored elsewhere, with the aim of preventing the polymerisation of alpha1-antitrypsin that underlies its common genetic deficiency. The challenge with alpha1-antitrypsin is daunting as its polymerisation occurs intracellularly. By comparison the possibility of inducing the conformational transition in CBG, which is readily accessible in the blood, seems encouragingly achievable.

5. Targeting drug delivery

A culmination of twenty-five years of studies of individual members of the family has revealed how the serpins can uniquely change their shape in order to modulate their function. With this understanding comes a challenge. Can the workings of the serpins be adapted for good purpose? Specifically, can the ability of the binding-serpins to transport and deliver hormones be tamed to provide a molecular mule: a biological made-to-order delivery service? An obvious application is for the tissue-specific delivery of drugs, such as cytotoxics, that need to be protectively encapsulated during transport until their release in a targeted neoplasm. The feasibility of achieving this has been demonstrated with CBG (Chan, et al., 2014). Chan and colleagues have shown in principle how recombinant CBG can be engineered to provide the preferential binding of a drug and how the modulatory mechanism can be tuned to allow the augmented release of the carried drug in target tissues.

5.1 Engineered binding

As seen in Figures 1 and 4, cortisol and thyroxine are carried as ligands bound in homologous pockets situated between two helices on the surface of their carrier proteins. The pockets have a degree of flexibility so the affinity of binding is in part dependent on the fit between the ligand and the carrier pocket. Notably however, the side chain of one amino acid in the pocket is critical in contributing to the specificity of binding. An arginine in TBG (R381) interacts with the orbiting pi electrons of the aromatic rings of thyroxine, while in the same position in CBG the pi orbitals of a tryptophan (W371) stack in parallel with the steroid rings of either cortisol or progesterone (Gardill, et al., 2012). The significance of the contribution of this single amino acid in the determination of binding specificity has been convincingly demonstrated by Chan and colleagues (Chan, et al., 2014) with the engineered replacement of the tryptophan in CBG by an arginine (W371R) resulting in a complete loss of affinity for cortisol matched with a gained affinity for thyroxine. The mutation effectively converts CBG into a TBG!

Although this single amino acid has a key part in defining the selectivity of binding, the readiness of the binding-pocket to independently accommodate a range of small organics and heterocyclic drugs has been well demonstrated with TBG (Qi, et al., 2011). Crystallographic structures of the complexes of a

series of drugs with TBG illustrate the flexibility of the binding-site, which is seen to contract and adjust to embrace the liganded drugs. The observed flexibility of the binding-site and the ready readjustment of the primarily hydrophobic bonding that takes place with varied ligands, give confidence as to the feasibility of engineering the specificity as well as the affinity of ligand-binding.

5.2 Targeting and augmented release

Approaches to the tissue targeting of a carried drug and the preferential triggering of its release are summarised in Figure 4. A relaxation of the reactive loop '2', will allow (as shown in '1') the ready entry of the side chain of the critical P14 amino acid, with the displacement of an underlying tyrosine and a consequent diminution of the flexibility and affinity of the adjacent binding-site. This release mechanism is triggered and maximised in vivo when the reactive loop is cleaved at sites of inflammation by neutrophil proteases. Chan and colleagues have adapted this proteolytic triggering to provide a tissue targeting of ligand release. To achieve this they altered the sequence at the proteolytic cleavage site ('2'), to change the susceptibility of cleavage from that of neutrophil elastases to that of tissue-specific proteases, specifically to the protease associated with prostatic neoplasms, and to the causative protease in thrombotic crises.

The engineering of CBG by Chan and colleagues also shows how increasing the bulk of the side-chain of the triggering amino acid (P14,'3' in figure 4) can augment the release of the carried drug or ligand at a target-site. This modification of the triggering mechanism is just one approach to boosting the release mechanism. Other quite different strategies, proven in vivo, come from the finding of adaptive polymorphisms in human populations that in some instances enhance and in others diminish the temperature-induced release of ligands.

5.3 Proof-of-concept to reality

The pilot demonstration of the adaptation of CBG as a drug delivery vehicle is at proof-of-concept level but there is confidence that if required this will be achievable in reality. The serpin framework is robust and the manipulated regions are unlikely to give rise to antigenicity, as the binding-site is buried and the reactive loop is unstructured. Moreover, tailoring the specificity of binding should be readily

possible by random mutation and selection, with the added advantage that crystallographic checking of binding can be simply confirmed by crystal soaking (Qi, et al., 2011). There is precedent too for parenteral administration, with for a decade or more, thousands of people safely receiving regular monthly infusions of the closely related serpin, alpha1-antitrypsin (Stocks, et al., 2010). So reality is achievable but till a need arises engineered CBG remains a concept-in-waiting.

6. Overall conclusions

In reviewing the new structural understandings it is useful to look beyond the mechanistic details to the broader significance of the findings. What has been revealed with CBG and TBG is yet another layer in the processes that control bodily hormone levels. Here though, the regulation is at tissue level and responsively so. The differential secretion of hormones at tissue level has been met by the selection of serpins as the carriers of cortisol and of thyroxine. The ready modulation of the conformational flexibility of the serpins allows dynamic changes in the plasticity of the hormone-binding site and hence of hormone release. Gross examples of this are discussed; there will literally, as well as metaphorically, be differences in the free hormone levels between cold-feet and hot-heads! Of greater physiological significance however will be the existence of as yet unrecognised tissue receptors and ligands that will interact locally to influence the conformational flexibility and hormone avidity of CBG and TBG.

Overall the understandings we now have of the mechanisms of hormone carriage and release, at atomic definition, place corticosteroid- and thyroxine-endocrinology at a forefront of molecular medicine. But with this status comes expectations. The quality of science at this level is dependent on the assays that underpin the field; these are currently somewhat inadequate for the cortisol-CBG axis and woefully so for thyroxine and TBG.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure1. Hormone carriage and release. a. CBG: showing the cortisol binding site and (right) the transition from the S- to R-forms on cleavage of the reactive loop in yellow. The cleaved loop moves into a stabilising central-strand position in the main beta-sheet of CBG, in red. b. TBG: Indicating the small nudging movement of the reactive loop that fine-tunes the binding avidity of the underlying thyroxine-binding pocket. Encircled in black on left is the site of the mutation in the Australian Aboriginal that abolishes the boost in thyroxine release that otherwise takes place with fever temperatures (see Fig 2b).

Figure 2. Temperature responsive hormone release. Plots of the change in ratios of the binding affinities ($K_D/K_{D(37^oC)}$) with temperature. a. With CBG there will be a near fivefold increase in free

cortisol with a rise in body temperature to 42ºC; b. with TBG the increase in released thyroxine will be much less but its purposeful occurrence is evidenced, dashed line, by a mutation (Fig 1) that negates the boosted increase at temperatures above 37ºC.

Figure 3. Potential therapy? Serpin conformations as predicted in 1991 from the earliest serpin structures. The conformations in a., b. & c. are now seen to be directly relevant to CBG and TBG, and d., the induction of the low affinity R-form, by peptide annealing (binary complexing BC) opens the potential of the exogenous induction of cortisol release. (Ov. ovalbumin). *Reproduced from Carrell, et al. (1991), Nature 353:576-579.*

Figure 4. **Targeting drug delivery.** The focal regions for engineered change are shown on the TBG framework. 1. The binding site with thyroxine depicted in sticks. 2. The protease specific cleavage sequence in the reactive loop (red). 3. The triggering insertion of P14 as also shown in 1.