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A Rapid Release of Corticosteroid-Binding Globulin from the Liver Restrains the Glucocorticoid Hormone Response to Acute Stress

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A strict control of glucocorticoid hormone responses to stress is essential for health. In blood, glucocorticoid hormones are for the largest part bound to corticosteroid-binding globulin (CBG), and just a minor fraction of hormone is free. Only free glucocorticoid hormone is able to exert biological effects, but little is known about its regulation during stress. We found, using a dual-probe *in vivo* microdialysis method, that in rats, the forced-swim stress-induced rise in free corticosterone (its major glucocorticoid hormone) is strikingly similar in the blood and in target compartments such as the subcutaneous tissue and the brain. However, in all compartments, the free corticosterone response was delayed by 20–30 min as compared with the total corticosterone response in the blood. We discovered that CBG is the key player in this delay. Swim stress evoked a fast (within 5 min) and profound rise in CBG protein and binding capacity in the blood through a release of the protein from the liver. Thus, the increase in circulating CBG levels after stress restrains the rise in free corticosterone concentrations for approximately 20 min in the face of mounting total hormone levels in the circulation. The stress-induced increase in CBG seems to be specific for moderate and strong stressors. Both restraint stress and forced swimming caused an increase in circulating CBG, whereas its levels were not affected by mild novelty stress. Our data uncover a new, highly dynamic role for CBG in the regulation of glucocorticoid hormone physiology after acute stress. **(*Endocrinology* 152: 3738–3748, 2011)**

Appropriate physiological and behavioral responses to stressful events are vital for health (1, 2). One essential physiological response is the secretion of glucocorticoid hormone from the adrenal gland. Glucocorticoid hormones, cortisol in humans and corticosterone in rats and mice, regulate many processes in the body including the mobilization of energy stores and immune function (3). Furthermore, glucocorticoids play a key role in the consolidation of memories of a stressful event so the subject can respond more effectively should the event reoccur in the future (4, 5). In view of their multiple roles, it is not surprising that glucocorticoid hormone dysfunction is involved in many metabolic, autoimmune, and psychiatric diseases (6).

The majority of circulating glucocorticoid hormone in the blood is bound to a transport protein called corticosteroid-binding globulin (CBG; also called transcortin) and to a lesser extent to albumin (7–9). Consequently, only a minor fraction (<10%) of the circulating glucocorticoid hormone concentration is actually free and thus available to enter the tissue and bind glucocorticoid receptors (8, 10). Therefore, a tight regulation of the free hormone concentration and hence the concentration of circulating CBG seems to be very important for glucocorticoid physiology.

CBG is a glycoprotein of the serpin family of serine protease inhibitors (11, 12). It is mainly synthesized and stored in the liver (13). Subjects carrying certain polymorphisms within the *cbg* gene or presenting *cbg* gene deletions show aberrant baseline glucocorticoid levels and responses to stress and may suffer from chronic fatigue, hy-

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Abbreviations: AUC, Area under the curve; CBG, corticosteroid-binding globulin; CBG-ir, CBG-immunoreactive; MR, mineralocorticoid receptor.

potension, and obesity (14–17). It has been thought for many years that the circulating concentrations of CBG are rather stable with slow changes, if evoked, taking place over several hours. Therefore, after a stressful challenge, it would be expected that changes in free glucocorticoid concentration would closely reflect changes in levels of total glucocorticoid hormone. However, recently, we made a remarkable discovery in rats combining blood sampling with microdialysis in the brain (to determine total and free glucocorticoid levels, respectively). We found that after a stressful challenge, *i.e.* forced swimming, the free corticosterone response in the brain was approximately 20 min delayed compared with the rise in total hormone in the blood (18). Thus, apparently total and free glucocorticoid hormone responses do not necessarily show parallel changes. Such delayed response of free hormone may have significant implications for the timing and impact of glucocorticoid-responsive processes in response to stress. We postulated that the delay is either brain specific and depends on processes at the blood-brain barrier or is ubiquitous in the body caused by a rapid release of CBG from the liver. We found that the delayed free hormone response is indeed ubiquitous because it occurs not only in the brain but also in the blood and the subcutaneous tissue. Furthermore, we report that forced-swim stress evokes the release of CBG from the liver resulting in an increase in CBG blood concentrations within 5 min. Both the timing and the magnitude of this event suggest that the rapid increase in circulating CBG levels after stress stabilizes for approximately 20 min the concentration of free glucocorticoid hormone in the face of rising total hormone levels. These findings uncover a new, highly dynamic role for CBG in controlling free glucocorticoid hormone concentrations after acute stress and thus in glucocorticoid physiology in general.

Materials and Methods

Subjects

Male Wistar rats (Harlan, Loughborough, UK) were handled daily (~5 min/rat) starting 1 wk before any surgical or other experimental manipulation. At the time of surgery, rats weighed approximately 250 g. Rats were housed three per cage, under standard lighting (0500–1900 h lights on) and temperature (21–22 C) conditions with food and water available *ad libitum*. All surgical procedures were performed under isoflurane (Merial Animal Health Ltd., Harlow, UK) anesthesia and carprofen (Rimadyl, 4 mg/kg, sc; Pfizer, Sandwich, UK) was given for postoperative pain relief. All procedures were carried out in accordance with United Kingdom Home Office regulations.

Free corticosterone in the blood and the subcutaneous tissue

Surgical and microdialysis procedures

Dual-probe microdialysis in freely behaving rats was used to measure free corticosterone levels in the blood and the subcutaneous tissue simultaneously. A microdialysis probe designed for microdialysis in blood vessels and peripheral tissue [CMA20 Elite with polyarylethersulfone membrane, 20-kDa cutoff, 10-mm length, and 0.5-mm membrane diameter; *in vitro* recovery of free corticosterone is 51.5 ± 2.2% (n = 6) at 37 C; CMA Microdialysis, Stockholm, Sweden] was inserted into the jugular vein by using a probe introducer and split tubing. The inlet and outlet tubing of the probe was tunneled under the skin to the neck region and secured. During the same surgery, a second microdialysis probe (CMA20 Elite, specifications as above) was inserted under the skin of the neck region and secured. Dental cement and anchor screws were used to fix a small metal peg (for later connection to a liquid swivel) to the skull. The skin was closed with surgical silk (4.0; Ethicon, Somerville, NJ). After surgery, animals were housed individually in Plexiglas cages (length, width, and height = 27, 27, and 35 cm, respectively) with food and water *ad libitum*. They were connected to a motorized five-channel swivel (with four microdialysis channels) and counterbalance arm system (MCS/5A; Instech Laboratories Inc., Plymouth Meeting, PA) via the peg. This system allows animals to move freely in all directions, including full rearing and sleeping in a curled-up position. Probes were perfused with sterile, pyrogen-free Ringer solution (147 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2$; Delta Pharma, Pfüllingen, Germany) at 2.05 μl/min using a microinfusion-pump (KD Scientific, Holliston, MA). Fluorethylene polymer tubing (Microbiotech AB, Stockholm, Sweden) with a dead volume of 1.2 μl/100 mm length was used for all connections. Dead volumes were accounted for. Microdialysis samples were collected in cooled vials using automated refrigerated sample collectors (CMA 470; CMA Microdialysis). Samples were stored at −80 C for later determination of the concentrations of free corticosterone. Experiments were performed on six animals simultaneously.

Experimental design

Collection of baseline (prestress) microdialysis samples at 10-min intervals was started 3 d after insertion of the microdialysis probes, at 0900 h. At 1100 h, animals were forced to swim individually in a glass beaker from which they could not escape (15 min, water at 25 C, as described in detail previously) (19). After the stressful challenge, rats were carefully dried and returned to their home cage and left undisturbed for the remainder of the experiment while sampling continued until 1530 h. Between 1100 h (start of stressor) and 1300 h, a sampling interval of 5 min was used.

Free corticosterone in the blood and the hippocampus

Surgical and microdialysis procedures

Simultaneous measurement of free corticosterone levels in the blood and in hippocampal brain tissue was conducted by dual-probe microdialysis in freely behaving rats. Ten days before the start of the experiment, a guide cannula (CMA12; CMA Microdialysis) was implanted just entering the hippocampus at the dorsal site as described before (20), and a peg was adhered to the skull (see above). Animals were housed as described above. After 7 d of recovery, a microdialysis probe [CMA12 Elite with polyarylethersulfone membrane, 20-kDa cutoff, 4-mm length, and 0.5-mm membrane diameter; *in vitro* recovery of free corticosterone is 27.0 ± 1.7% (n = 6) at 37 C; CMA Microdialysis] was

inserted through the guide cannula into the hippocampus and a peripheral microdialysis probe [CMA20 Elite (see above) with 4 mm length; *in vitro* recovery of free corticosterone is 27.0 ± 2.8% (n = 6) at 37 C; CMA Microdialysis] was inserted into the jugular vein as described above. Note that the membranes of the peripheral and brain microdialysis probes are made from the same material and have similar recoveries for corticosterone. Animals were connected to a swivel and counterbalance arm system, and microdialysis was performed as described above.

Experimental design

Collection of baseline (prestress) microdialysis samples at 10-min intervals was started 3 d after insertion of the microdialysis probes, at 0900 h. At 1100 h animals were subjected to forced swimming as described above or exposed to a novel environment. Exposure to a novel environment comprised of placing the rats individually in a clean, empty novel cage and increasing the light intensity in the room from approximately 200 lux to 500 lux for 30 min after which the animals were returned to their home cage. After the termination of the stress procedures, rats were left undisturbed for the remainder of the experiment while sampling continued until 1530 h. A sampling interval of 5 min was used between 1100 h (start of stressor) and 1300 h.

Plasma total corticosterone and CBG

Rats were forced to swim as described above and killed by decapitation under quick isoflurane anesthesia (<15 sec in a glass jar containing isoflurane vapor) at 0 (baseline, nonstressed controls), 5, 15, 30, 60, 120, or 480 min or 24 h after the start of the stressor. The trunk blood was immediately collected in chilled tubes containing EDTA (Sigma Chemical Co., St. Louis, MO) and Trasylol (Bayer, Newbury, UK) and centrifuged at 2500 rpm for 30 min (4 C). Plasma was split and aliquots stored at −80 C for later determination of total corticosterone (RIA) and CBG (radioligand binding and Western blot analysis) concentrations.

To assess the stressor specificity of the effects of stress on plasma total corticosterone and CBG levels, separate groups of rats were subjected to novel-environment stress (as described above) or restraint stress (30 min in a Plexiglas rat restrainer with ventilation holes) and killed at 0 (baseline controls) or 30 min (*i.e.* at the time point of the maximum effect of swim stress on plasma total corticosterone and CBG as found in the preceding experiments) after the start of the stressor. Plasma total corticosterone was measured by RIA and CBG concentrations by radioligand binding assay.

Tissue collection for CBG immunohistochemistry in the liver

Rats were forced to swim and were killed at 0, 15, or 30 min or 24 h (n = 6–12). Rats were quickly anesthetized (with isoflurane, as above) and received a terminal overdose of sodium pentobarbital (Euthetal, 0.3 ml/100 g body weight, ip; Merial), after which they were perfused via the heart with 100 ml 0.9% saline and 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The whole liver was collected and stored in 0.1 M phosphate buffer at 4 C for later immunohistochemistry.

Measurement of corticosterone by RIA

Plasma and dialysate corticosterone concentrations were measured using a commercial rat RIA (MP Biomedicals, Solon, OH) as described before (18).

Measurement of plasma CBG by radioligand binding assay

Plasma CBG levels were determined using a [^{3}H]corticosterone ([1,2,6,7-^{3}H(*N*)]corticosterone, specific activity 60–100 Ci/mmol; PerkinElmer, Boston, MA) binding assay, largely as published previously (21, 22). Briefly, binding of [^{3}H]corticosterone in the absence (total binding) or presence of 1000-fold excess of nonradioactive corticosterone (nonspecific binding) was determined in 1:100 diluted plasma samples at a single over 95% saturating concentration of 60 nM [^{3}H]corticosterone in duplicates. After overnight incubation at 0–2 C and subsequent separation of bound and free [^{3}H]corticosterone by gel filtration (Sephadex LH-20; GE Healthcare, Uppsala, Sweden), the radioactivity of the bound fraction was measured in a liquid scintillation β-counter. For the calculation of the concentration of plasma CBG binding sites (expressed as nanomolar), the specific activity of [^{3}H]corticosterone was corrected for the presence of endogenous corticosterone (as measured by RIA, see main text) in the plasma samples. The specific binding was calculated by subtraction of the nonspecific binding from the total binding. The nonspecific binding was less than 1% of the total binding.

Measurement of plasma CBG protein levels by Western blot analysis

Plasma samples were diluted 1:100 in 0.05 M Tris-HCl (pH 7.5) and sodium dodecyl sulfate loading buffer and heated at 90–100 C for 5 min. Next, the diluted plasma samples (20 μg total protein per well) were subjected to SDS-PAGE after which the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking nonspecific binding sites with 5% nonfat milk and 2% BSA (Sigma) in PBS-Tween 0.2% for 1 h at room temperature, the membrane was separately incubated overnight with first a rabbit antimouse transferrin (internal sample loading control) primary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) and second a rabbit antihuman CBG primary antibody (1:5000; Affiland, Ans-Liege, Belgium). Immunoreactive proteins were further detected by an antirabbit horseradish peroxidase-conjugated secondary antibody (1:10,000; Jackson Immunoresearch, Newmarket, UK) and detection system (ECL Western blotting detection kit; GE Healthcare). The quantification of CBG and transferrin levels was performed using ImageJ software (version 1.43). The CBG data were normalized with regard to the transferrin content in each sample and subsequently expressed as the percentage of the CBG levels in the baseline control animals. CBG presented as a single immunoreactive band of approximately 55 kDa, which corresponds with results of others using antimouse and antihuman CBG antibodies (15, 23, 24).

Immunohistochemistry of CBG in the liver

Whole livers were sectioned (50 μm) using a Vibratome (Leica, Nussloch, Germany), and immunohistochemistry was performed on free-floating sections. Sections were incubated in blocking solution (2% BSA, 50 mM glycine, 0.1% Triton X-100, 10% normal goat serum) for 1 h at room temperature and then

incubated in a rabbit antihuman CBG primary antibody solution (1:300; Affiland) for 72 h at 4 C. CBG-immunopositive liver cells were detected by biotinylated goat antirabbit secondary antibody (1:300; Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase (ABC, 1:300; Vector), followed by diaminobenzidine staining. Digital images were captured using a bright-field light microscope and camera (Leica) and analyzed using Leica Application Suite version 2.8.1 software. This CBG antibody has already been used before to specifically detect CBG immunoreactivity in the rat (25, 26). The antibody shows no cross-reactivity with other major binding proteins including T_4-binding globulin from the SERPIN family (0.00%), retinol-binding globulin (0.00%), SHBG (0.70%), and albumin (0.00%) (information provided by Affiland). The staining described here in the liver was similar to that found using a sheep antihuman CBG antibody (Biotrend, Köln, Germany; data not shown) and to staining with an antirat CBG antibody (27). Furthermore, similar baseline staining and depleting effects of swim stress were found in a pilot experiment using a new, commercial antirat CBG antibody that shows less than 5% cross-reactivity with the major serpins A1, A4, and A5 (R&D Systems, Minneapolis, MN).

Calculations and statistical analyses

Figure 1 depicts free corticosterone concentrations as measured in dialysates expressed as micrograms per deciliter without correction for membrane recovery. The time courses were analyzed by repeated-measures ANOVA (within-subject factors were time and compartment). To reduce the probability of type 1 errors, the factor time consisted of 14 levels, *i.e.* baseline and eight 15-min periods followed by five 30-min periods. The time-course data were further used to calculate the maximum concentration of free corticosterone after stress, the time point of the maximum effect, and the area under the curve (AUC) (GraphPad Prism version 5.0) and analyzed by unpaired or paired *t* test with Bonferroni correction as appropriate. To directly compare the free corticosterone concentrations with the plasma concentrations of total corticosterone and CBG, dialysate levels were subsequently calculated in nanomolar and corrected for membrane recovery using recovery data determined at 37 C *in vitro*, as described above (Fig. 2).

The effect of swim stress on plasma CBG concentrations as measured by radioligand binding was analyzed by one-way ANOVA (between-subject factor was time after stress) and Dunnett *post hoc* analysis. The effects of different stressors (novel-environment stress, restraint stress, and forced swimming) on plasma total corticosterone and CBG levels were analyzed by one-way ANOVA (between-subject factor was stressor) and *post hoc* Bonferroni comparisons. The effect of swim stress on CBG levels as measured by Western blot analysis was analyzed by one-sample *t* test.

Statistical analyses were performed using SPSS (version 16.0) with $P < 0.05$ as the level of significance.

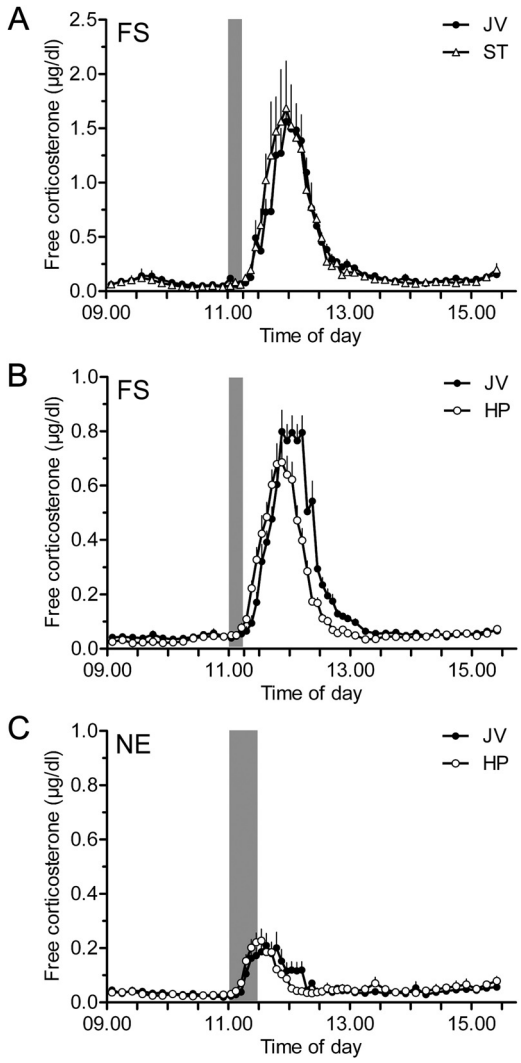

FIG. 1. Effect of stress on free corticosterone in different body compartments as assessed by simultaneous dual-probe microdialysis. A, Forced-swim stress (15 min, *gray bar*) caused a comparable increase in free corticosterone levels in the blood (jugular vein) and the subcutaneous tissue (n = 6; Table 1). B, The free corticosterone response to forced-swim stress was similar in the blood and the hippocampus during the rising phase of the response, but levels in the hippocampus returned to baseline faster than in the blood (n = 11; Table 2). C, Compared with swim stress, novel-environment stress (30 min, *gray bar*) caused a significantly smaller increase in free corticosterone in the blood and the hippocampus (n = 8; Table 2). The free corticosterone responses in the two compartments were very similar during the rising phase, but hippocampal levels returned to baseline slightly faster compared with those in the blood. *Symbols* are placed at the midpoint of the sample duration. Sample duration decreased from 10 to 5 min between 1100 and 1300 h. Free corticosterone levels (micrograms per deciliter) are not corrected for recovery by the microdialysis membrane; levels depicted in B and C are lower than those in A because of the shorter length of the dialysis membrane used in blood-brain studies. Values represent mean + SEM. FS, Forced-swim stress; HP, hippocampus; JV, jugular vein; NE, novel environment; ST, subcutaneous tissue.

Results

Effects of swim stress on free corticosterone in three body compartments

To investigate whether the time courses of stress-induced free glucocorticoid hormone differ between the blood, the subcutaneous tissue, and the brain, we made a comparison between these compartments using simultaneous dual-probe microdialysis. Figure 1A shows that forced swimming caused a profound increase in the levels of free

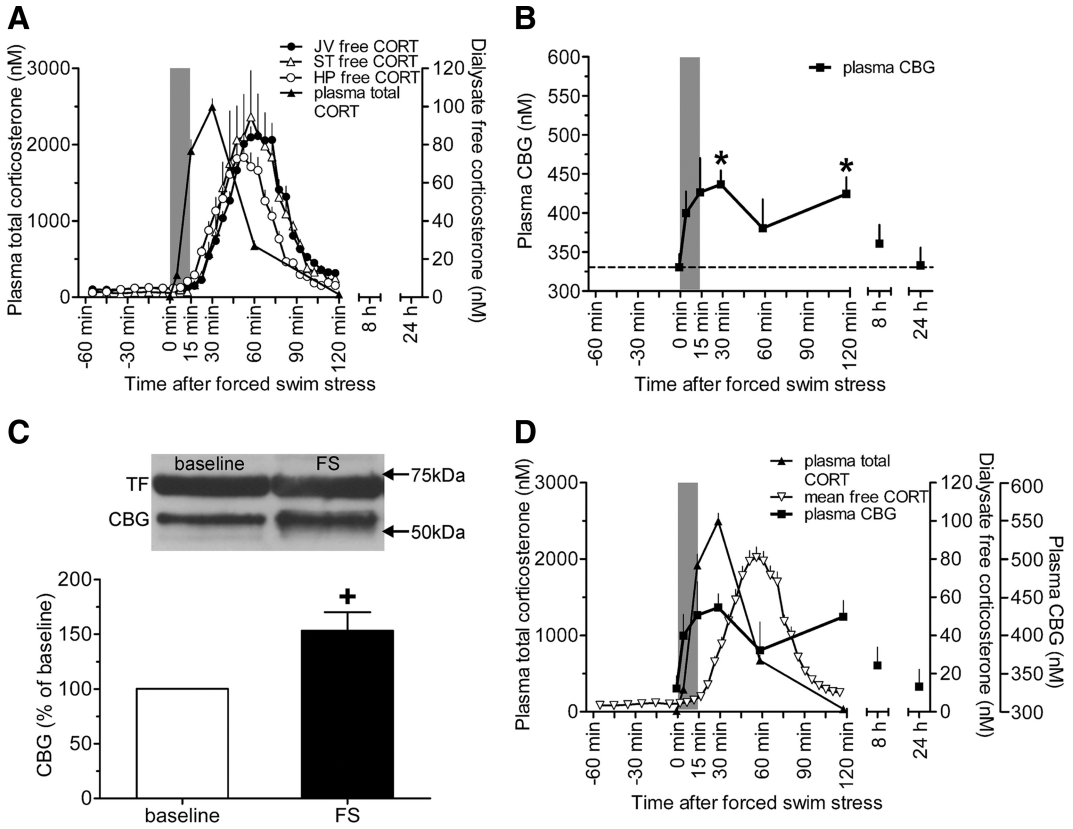

FIG. 2. Swim stress (15 min) causes a rapid increase in plasma total corticosterone and CBG levels but a delayed response in free corticosterone in blood and target tissues. A, Plasma total corticosterone levels (n = 6–12) were significantly elevated 5 min after the onset of swim stress and reached peak levels at approximately 30 min. All free corticosterone responses were significantly delayed by 20–30 min [derived from Fig. 1, A and B: jugular vein (JV) n = 17; subcutaneous tissue (ST) n = 6; hippocampus (HP) n = 11]. B, Forced-swim stress caused an increase in plasma concentrations of CBG, as measured by radioligand binding assay (n = 5–12). CBG levels increased within 5 min and reached maximum levels approximately 30 min after the onset of stress. *, $P < 0.05$ compared with baseline values (Dunnett's *post hoc* test). C, *top*, Representative Western blot of CBG (*lower bands*, 50–55 kDa) and transferrin (*upper bands*, 73–80 kDa, sample loading control) in plasma from rats killed under baseline conditions or 30 min after stress onset; *bottom*, forced-swim stress (n = 12) significantly increased plasma concentrations of CBG compared with control (n = 6). +, $P < 0.01$, one-sample *t* test. D, Free corticosterone levels in the three target compartments (see A) were calculated as one overall mean (n = 34) and plotted together with the swim-stress-induced responses in plasma total corticosterone and CBG. The graph clearly shows that the increase in plasma CBG parallels the rise in plasma total corticosterone levels and that the magnitudes of the CBG and the free corticosterone response are in the same range (85–105 nM). Values in A, B, and D are expressed as nanomolar, and free corticosterone levels are corrected for recovery by the membrane (see *Materials and Methods*). Further details are as in Fig. 1. CORT, Corticosterone; TF, transferrin.

corticosterone [repeated-measures ANOVA, effect of time $F_{(13,65)} = 24.69$; $P \leq 0.0005$] with remarkably similar time courses in the blood and the subcutaneous tissue (repeated-measures ANOVA, interaction compartment × time $F_{(13,65)} = 1.41$; $P > 0.05$]. No significant differences in the maximum responses and AUC were found between these two peripheral compartments (Table 1). The levels of free corticosterone started to rise at 5–10 min after

TABLE 1. Effect of forced-swim stress on free corticosterone levels in the blood and the subcutaneous tissue as assessed by simultaneous dual-probe microdialysis in rats

Compartment	Baseline free corticosterone (μg/dl)	Maximum stress-induced free corticosterone concentration (μg/dl)	Time point of maximum response (min after initiation of stressor)	AUC (1100–1400 h; arbitrary units)
Blood	0.08 ± 0.02	1.62 ± 0.20[a]	58.34 ± 2.10	17.25 ± 1.94
Subcutaneous tissue	0.07 ± 0.02	1.85 ± 0.37[a]	55.83 ± 3.25	17.97 ± 3.54

Baseline levels of free corticosterone were comparable between the blood and the subcutaneous tissue ($P > 0.05$, paired *t* test). Forced-swim stress (15 min at 25 C) caused a profound increase in free corticosterone in both compartments. No significant differences were found in the time point and the level of the maximum response and in the AUC between the two compartments ($P > 0.05$, paired *t* test). Free corticosterone levels are not corrected for recovery by the microdialysis membrane. Values represent mean ± SEM (n = 6) and are calculated from the time courses shown in Fig. 1A.

[a] $P < 0.01$ effect of stress as compared with baseline within the respective compartment, paired *t* test.

TABLE 2. Effect of forced-swim stress and novel-environment exposure on free corticosterone levels in the blood and the hippocampus as assessed by simultaneous dual-probe microdialysis in rats

Stressor	Compartment	Baseline free corticosterone (μg/dl)	Maximum stress-induced free corticosterone concentration (μg/dl)	Time point of maximum response (min after initiation of stressor)	AUC (1100–1400 h; arbitrary units)
Forced swimming	Blood	0.04 ± 0.01	0.85 ± 0.06[a]	55.00 ± 1.65	9.23 ± 0.55
Forced swimming	Hippocampus	0.03 ± 0.01	0.73 ± 0.07[a]	49.10 ± 1.90	7.43 ± 0.69[c]
Novel environment	Blood	0.03 ± 0.01	0.23 ± 0.04[a,b]	26.88 ± 2.49[b]	2.82 ± 0.37[b]
Novel environment	Hippocampus	0.03 ± 0.01	0.26 ± 0.04[a,b]	25.00 ± 1.64[b]	2.60 ± 0.34[b]

Baseline levels of free corticosterone were comparable between the blood and the hippocampus ($P > 0.05$, paired *t* test). Both forced-swim stress (15 min at 25 C) and exposure to a novel cage (novel environment, 30 min) caused clear increases in free corticosterone levels. However, the effect of novel environment exposure was much smaller than that of forced swim stress as indicated by significant differences in the maximum response and the AUC between the two stressors. Also, in both compartments, the time point of the maximum effect was significantly (about 25–30 min) earlier after novel-environment stress than after forced-swim stress. Although the free corticosterone responses were very similar in the blood and the hippocampus, ANOVA with repeated-measures analyses revealed a significant interaction between compartment and time for both stressors (see *Results*). This is reflected in a trend toward a significantly smaller AUC in the hippocampus as compared with the blood after forced-swim stress. Free corticosterone levels are not corrected for recovery by the microdialysis membrane. Note that free corticosterone levels are lower than those presented in Table 1 because of the shorter length of the dialysis membrane used in these blood-brain experiments. Values represent mean ± SEM (forced swimming n = 11; novelty n = 8) and are calculated from the data shown in Fig. 1, B and C.

[a] $P < 0.01$ effect of stress as compared with baseline within the respective compartment, paired *t* test.

[b] $P \leq 0.0005$ for the comparison between forced-swim and novel-environment stress within the respective compartment, unpaired *t* test.

[c] $P = 0.055$ for the comparison between the blood and the hippocampus, paired *t* test.

completion of the stress procedure and reached maximum levels at 55–60 min in both compartments (Table 1). Next, we directly compared the responses in free corticosterone between the blood and the brain (*i.e.* the hippocampus). During the rising phase of the swim stress-induced response, free hormone concentrations followed very similar kinetics in the two compartments (Fig. 1B). The free corticosterone levels peaked at the same time, and maximum levels were not significantly different (Table 2). However, free corticosterone levels in the hippocampus returned to baseline significantly faster compared with those in the blood [repeated-measures ANOVA, interaction compartment × time $F_{(13,130)} = 11.30$; $P \leq 0.0005$], resulting in a trend toward a smaller AUC in the brain tissue (Table 2). Nevertheless, the similarity in the rising phase and peak times of the responses in the two compartments suggests that these aspects of the free hormone response are not brain specific.

Stressor specificity of the free corticosterone response

To investigate the stressor specificity of the free corticosterone response, we challenged a separate group of rats with a mild form of psychological stress, *i.e.* exposure to a novel environment, and measured free corticosterone in the blood and the hippocampus simultaneously. Novelty stress induced a rise in free corticosterone levels between 5 and 15 min after the start of the stressor with maximum levels reached near the end of the 30-min stressful challenge. No differences between the two compartments were observed in the rising phase of the free corticosterone response (Fig. 1C and Table 2). However, similar to the response to forced swimming, after novelty stress, free corticosterone levels returned to baseline faster in the hippocampus than in the blood [repeated-measures ANOVA, interaction compartment × time $F_{(13,91)} = 3.26$; $P \leq 0.0005$]. The free corticosterone response to novelty was significantly smaller than the response to forced-swim stress in both compartments, which further underlines the high degree of similarity in free corticosterone responses in different compartments [repeated-measures ANOVA, interaction time × stressor $F_{(13,221)} = 56.66$; $P \leq 0.0005$].

The delayed response of free corticosterone is not compartment specific

The free corticosterone responses in the three compartments were strikingly similar with a maximum found between 50 and 60 min after the onset of swim stress. To compare these responses in free corticosterone with the response in plasma total corticosterone, rats were killed at different time points after forced swimming, and the plasma total corticosterone concentration was measured (Fig. 2A). The plasma total hormone concentration rose very rapidly, attaining already significantly increased levels at 5 min after stress onset [one-way ANOVA, effect of stress $F_{(5,51)} = 216.91$; $P \leq 0.0005$; Dunnett *post hoc* analysis, 5-min time point *vs.* baseline $P < 0.05$], and reached its maximum at 30 min. A comparison of the changes in total corticosterone with those in free hormone clearly demonstrates that a delay of 20–30 min in the free hormone response is not a brain-specific phenomenon but a ubiquitous finding among all investigated compartments (Fig. 2A). Moreover, because the delay is observed in the blood, it is unrelated to any tissue penetration. These observations indicate that there exists a mechanism transiently restraining the rise of the free hormone fraction after stress.

Rapid increase in plasma CBG after swim stress

We hypothesized that after swim stress, an increased concentration of circulating CBG would absorb the initial rise in corticosterone levels consequently resulting in a delayed increase in free corticosterone levels. Therefore, using a radioligand binding assay, we determined the concentrations of CBG in the plasma samples obtained from rats killed at different time points after swim stress. Baseline plasma CBG levels were 330.5 ± 16.6 nM (n = 11). After challenging rats with forced swimming, CBG levels rose within 5 min and reached maximum levels of 436.6 ± 18.1 nM (n = 12) at 30 min after stress onset (Fig. 2B). CBG levels were still elevated after 2 h and returned to baseline after 2–8 h [one-way ANOVA, effect of stress $F_{(7,62)} = 2.38$; $P < 0.05$]. These findings were further substantiated by Western blot analyses demonstrating that the concentration of plasma CBG protein was significantly elevated by 53.2 ± 16.9% (n = 12) at 30 min after swim stress (Fig. 2C). Thus, acute forced-swim stress resulted in increased CBG binding capacity of corticosterone in plasma through an increase in the circulating CBG protein concentration. The stress-induced increases in plasma total corticosterone and CBG levels evolved in parallel (Fig. 2D). Furthermore, the magnitude of the CBG rise (~105 nM) corresponded very well with the response in free corticosterone levels, which reached maximum levels of approximately 85 nM. Together, the data strongly indicate that the increase in plasma CBG is responsible for the delay in the rise in free corticosterone levels after acute stress.

Stressor specificity of the CBG response

The response in free corticosterone to novelty stress peaked at 25–30 min, which is similar to that known for the maximum response in plasma total hormone. Thus, regarding novelty, there seemed to be no delay in the free hormone response. We postulated that novelty stress had not mobilized any CBG and, in general, that a rise in CBG levels after stress depends on the intensity of the stressor. Therefore, we measured plasma levels of total corticosterone and CBG 30 min after the onset of three stressors of different intensity, *i.e.* exposure to novelty (mild), restraint (intermediate), and swim stress (strong). As expected, all stressors increased plasma total corticosterone levels, but the increase was highly dependent on the severity of the stressor [Fig. 3A; one-way ANOVA, effect of stress $F_{(3,55)} = 311.16$; $P \leq 0.0005$]. Moreover, the stress-induced rise in plasma concentrations of CBG depended greatly on the severity of the stressor [Fig. 3B; one-way ANOVA, effect of stress $F_{(3,53)} = 9.04$; $P \leq 0.0005$] with novelty stress exerting no significant effects.

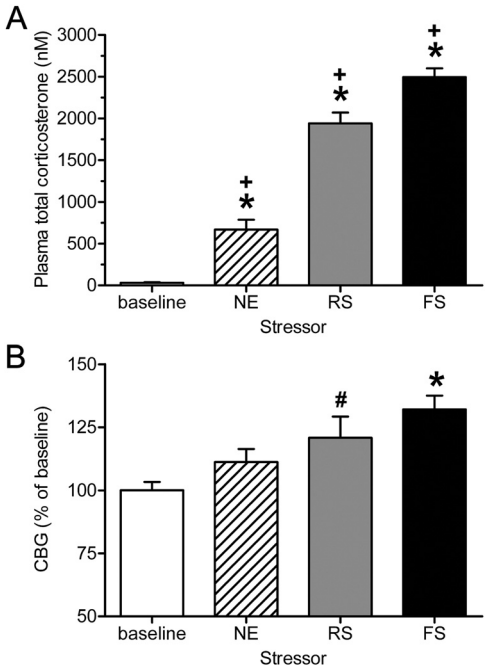

FIG. 3. Effects of stress on plasma levels of total corticosterone and CBG are stressor dependent. A and B, Plasma total corticosterone (nanomolar) (A) and CBG levels (percentage of baseline) (B) in response to novel-environment stress, restraint stress, or forced-swim stress. Rats were killed under baseline conditions (n = 29) or 30 min after stress onset (n = 8–12). #, $P < 0.05$; *, $P \leq 0.0005$ compared with baseline; +, $P \leq 0.0005$ compared with other stressors (Bonferroni *post hoc* test). Values represent mean + SEM. FS, Forced-swim stress; NE, novel environment; RS, restraint stress.

CBG is released from the liver

After the swim challenge, plasma CBG levels, as measured by radioligand binding assay, rose by 35% within 30 min, which is both substantial and rapid given that the protein is over 50 kDa. Calculations indicate that within this short time frame, approximately 50 μg CBG is released into the circulation (based on a total plasma volume of ~8 ml in a rat of 250 g body weight, and an increase in CBG of ~105 nM over baseline levels). To determine the origin of this considerable amount of protein, we studied CBG immunostaining in the liver as the main site of CBG production and storage. CBG-immunoreactive (CBG-ir) cells were primarily found surrounding the sinusoids of the liver. Immunostaining of CBG was, in accordance with previous observations (27), observed only in the cytoplasm. Under nonstressed conditions, CBG-ir cells presented mainly as a pattern of diffusely distributed individual immunostained cells (Fig. 4, A and E) or as a pattern of clustered immunostained cells (Fig. 4, B and F). Remarkably, at 30 min after swim stress, thus at the peak of plasma CBG levels, no CBG-ir cells were detectable in the liver (Fig. 4, C and G). Liver tissue collected immediately at the end of the forced-swim session (*i.e.* at 15 min) also presented no detectable levels of CBG-ir (data not shown), indicating that the stressor causes a rapid depletion of CBG from its

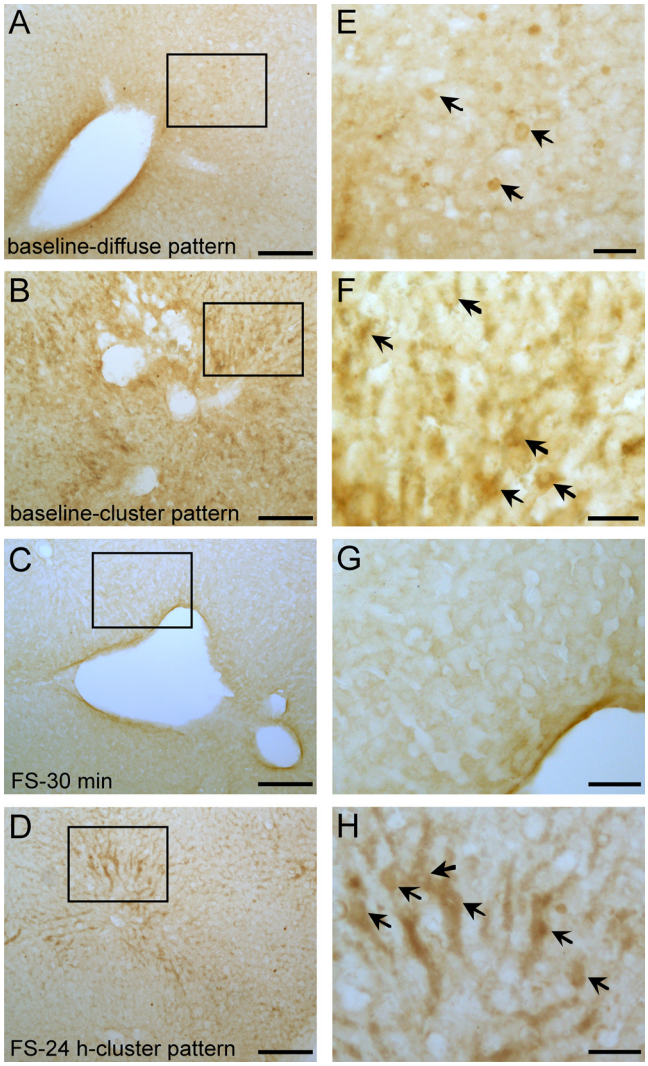

FIG. 4. The forced-swim stress-induced increase in plasma CBG levels is caused by release of the protein from the liver. Presented are examples of CBG immunoreactivity patterns in the rat liver under baseline and swim stress conditions. A and B, Diffuse (A) and cluster (B) patterns of CBG-ir liver cells with condensed staining were found surrounding the sinusoids of the liver under baseline conditions. C, Neither the diffuse nor the cluster pattern of CBG-ir cells was found 30 min after the onset of 15-min swim stress. D, CBG-ir liver cells were again found 24 h after stress in both the cluster pattern (shown here) and the diffuse pattern (data not shown). E–H, Magnifications of the *boxed areas* in A–D, respectively. CBG-ir cells are marked with *arrows*. *Scale bars*, 100 μm (A–D); 25 μm (E–H). FS, Forced-swim stress.

stores. Prestress levels of liver CBG immunostaining [as both diffusely distributed patterns (data not shown) and as clustered patterns (Fig. 4, D and H)] were reestablished within 24 h after the stress event, which corresponds with the CBG binding data (Fig. 2B).

Discussion

We investigated whether under stressful conditions free corticosterone levels are differentially regulated in different compartments of the body. Therefore, we established a dual-probe microdialysis method to measure free corticosterone simultaneously in blood and target tissue. As expected, we found that the effects of stress on free corticosterone are stressor specific with respect to both the magnitude and the duration of the response. This observation is in good agreement with the stressor specificity known for the total corticosterone response to swim and novelty stress (28) and our previous work on free corticosterone in rats and mice (18, 29, 30). Importantly, however, we demonstrated here for the first time that the time courses and the magnitudes of the free corticosterone responses to stress are very similar in the blood, the subcutaneous tissue, and the brain. This observation leads to two key conclusions: 1) the free corticosterone response in target tissues is not affected by tissue penetration because no significant differences between blood and tissues were found during the rising phase of the response, and 2) processes at the blood-brain barrier do not impede the entrance of free corticosterone into the rat brain. There has been some debate as to whether corticosterone is a ligand for efflux pumps, such as P-glycoprotein, located at the blood-brain barrier (31–33). However, the similar rising phases of free corticosterone in the blood and the brain strongly support the notion that corticosterone is not transported by P-glycoprotein. Given that cortisol is a substrate for P-glycoprotein (31, 32), further research is needed to clarify the situation in humans. Free corticosterone levels showed a slightly accelerated decline in the hippocampus compared with the blood and the subcutaneous tissue after stress. The reason for this difference is unclear. An involvement of P-glycoprotein in the clearance of corticosterone from the brain tissue is unlikely given that the conformational structure of this protein makes it inaccessible for substrates from the extracellular space (34). Whether specific changes in the metabolism of corticosterone in the brain are involved is unknown. Our data clearly show that free corticosterone levels are very similar in different compartments of the body. However, other intracellular factors, such as the presence of CBG-like molecules in certain tissues [*e.g.* the anterior pituitary (35, 36) and the hypothalamus (25)], and the regeneration or inactivation of corticosterone by 11-β-hydroxysteroid dehydrogenase-1 and -2, respectively (37), may influence the impact of glucocorticoid hormones in target tissues.

Our previous work has shown that there is a delay of approximately 20 min between the total corticosterone response in the blood and the free corticosterone response in the brain after forced-swim stress (18). However, direct comparison of the time courses of free corticosterone measured in the blood, the subcutaneous tissue, and the hippocampus with the time course of plasma total cortico-

sterone reveals that this 20- to 30-min delay is in fact omnipresent and not specific for the brain. The involvement of brain-specific mechanisms can therefore be ruled out. Given that CBG binds the majority of corticosterone in the blood, we investigated whether CBG concentrations would undergo any major changes after acute stress. Swim stress indeed evoked a profound increase in plasma CBG levels. Furthermore, CBG seems only to respond to stressors of moderate to strong intensity. Thus, both swim stress and restraint stress result in significant increases in CBG, whereas exposure to a novel environment is without effect. We propose that the rise in CBG levels is the main reason for the delay in the free corticosterone response for the following reasons. 1) The time courses of the rises in CBG and in total corticosterone are virtually identical, showing elevated levels within 5 min after stress onset and peak levels at 30 min. During the initial surge in CBG levels, the levels of free corticosterone remain low and stable and start to increase only at 20–25 min after the start of swim stress. 2) Free corticosterone and CBG levels attain very similar maximum increases of approximately 95 nM. Thus, the CBG response fits well in terms of both timing and magnitude to restrain and delay the free corticosterone response during the initial phase of the stress response.

Immunohistochemical studies demonstrated that the increase in circulating CBG is caused by a rapid release of CBG from liver cells. Plasma CBG levels had returned to baseline 24 h after swim stress, and at that time point, also CBG-ir was detectable again in liver cells. Whether this is caused by reuptake or new synthesis of CBG remains to be identified. The mechanisms underlying the fast, transient release of CBG from the liver are unknown. Given the low levels of free corticosterone in the early phase of the stress response it is unlikely that glucocorticoid receptor-mediated processes play a role. However, the sympathetic nervous system may be involved. The liver is highly innervated by the autonomic nervous system, which regulates the synthesis and secretion of, among others, glucose and lipids (38). Rapid changes in plasma levels of epinephrine and norepinephrine have been reported after various forced-swim stress protocols (39, 40). Recently, we found that plasma levels of epinephrine and norepinephrine start to rise at ~5 min and peak at ~20 min after the start of swim stress in rats (Wallinga, A. E., X. Qian, A. Collins, A. C. E. Linthorst and J. M. H. M. Reul, unpublished observations). Thus, stress-induced catecholamines may be promising mobilizers of liver CBG. In addition, rapidly released neuropeptides such as vasopressin and prolactin (for which liver cells express receptors) may be involved as well.

Because CBG levels have been regarded as relatively constant, it has received little attention in stress research. Although there are some indications in birds that CBG may rise (41) or fall (42) shortly after acute stress, in rats, however, only slow decreases in CBG have been reported after severe and/or more prolonged forms of stress. Thus, 6 and 24 h immobilization stress (43) and chronic social stress (44) were found to decrease blood CBG levels in rats. Exposure of restrained rats to a 90-min tail-shock session caused a decrease in CBG levels at 24 h after the stressor (45). In contrast, our study is the first to demonstrate that a rapid release of CBG from the liver occurs in rats after acute stress and that this release is able to restrain the levels of free corticosterone for about 20 min. The release of CBG has important implications. 1) A delayed increase in free corticosterone inherently results in a postponed glucocorticoid hormone action including a postponed negative feedback. This will lead to stress-induced, glucocorticoid-sensitive processes going on for longer than previously thought on the basis of the time course of plasma total hormone levels. Accordingly, in glucocorticoid-sensitive behavioral tests such as Morris water maze learning and the forced-swim test, glucocorticoids exert their facilitatory action well into the consolidation phase of behavioral responses (46, 47). 2) The absence of a rise in free corticosterone during the early phase of the stress response may allow the full initiation of (other) physiological defense mechanisms that are under inhibitory control of glucocorticoids (*e.g.* the immune system) (3). Interestingly, fever elevates free glucocorticoid levels by decreasing glucocorticoid hormone binding to CBG. Therefore, further complex interactions between CBG and free corticosterone are to be expected at later stages of the immune stress response (48, 49). However, because our forced-swim animals experience a decline in body temperature, this process is not expected to play a role in our studies (19). 3) During the initial phase of the stress response, glucocorticoid effects mediated by high-affinity intracellular mineralocorticoid receptors (MR) (50) will prevail in high MR-containing brain regions such as the hippocampus. Thus, for instance, rapid changes in hippocampal neurotransmitter release after swim stress (30) take place in the context of activated intracellular MR but still largely unoccupied (low-affinity) membrane MR (51) and intracellular glucocorticoid receptors (1).

In conclusion, our study has revealed that a rapid release of CBG from the liver restrains the rise in free corticosterone levels after stress signifying a novel, highly dynamic role in the regulation of glucocorticoid hormone physiology. Given that mutations within the *cbg* gene seem to be implicated in chronic fatigue, blood pressure regulation, and obesity, further research into the function and regulation of CBG release during stress is paramount.

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