Evidence that brief stress may induce the acute phase response in rats

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Deak, Terrence, Jennifer L. Meriwether, Monika Fleshner, Robert L. Spencer, Amer Abouhamze, Lyle L. Moldawer, Ruth E. Grahn, Linda R. Watkins, and Steven F. Maier. Evidence that brief stress may induce the acute phase response in rats. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1998–R2004, 1997.—Exposing rats to a single session of inescapable tail shock (IS) reduces corticosteroid binding globulin (CBG) 24 h later (Fleshner et al., Endocrinology 136: 5336–5342, 1995). The present experiments examined whether reductions in CBG are differentially affected by controllable vs. identical uncontrollable tail shock, are mediated by IS-induced glucocorticoid elevation, or reflect IS-induced activation of the acute phase response and whether IS produces fever. The results demonstrate that 1) equivalent reductions in CBG are observed in response to escapable tail shock or yoked IS, 2) IS-induced CBG reduction is not blocked by adrenalectomy in rats that receive basal corticosteroid replacement or by pretreatment with RU-38486, and 3) IS appears to activate the acute phase response, since IS reduces serum levels of an acute-phase negative reactant (CBG), increases serum levels of acute-phase positive reactants (haptoglobin and α1-acid glycoprotein), and increases core body temperature 20–24 h later.

corticosteroid binding globulin; corticosterone; adrenalectomy; haptoglobin; α1-acid glycoprotein; fever

THE PHYSIOLOGICAL CONSEQUENCES of glucocorticoid (GC) release as a result of hypothalamic-pituitary-adrenal (HPA) activation have been an intense area of inquiry for many years. In fact, changes in circulating GC levels play a role in nearly all physiological processes necessary for survival, such as release of the immune system, gestation and parturition, neuronal firing, and responses to stressors (16, 24, 25). However, few researchers take into account that, under basal conditions, ~90% of circulating GCs are bound to the carrier protein corticosteroid binding globulin (CBG), also referred to as transcortin (38). Because GCs cannot bind to their receptors while bound to CBG (38), their biological activity is regulated at least in part by circulating levels of CBG. For example, when circulating levels of CBG are diminished, the physiological consequences of elevated GCs would be much greater than normal, since a greater percentage of the GCs would be in the “free,” or unbound, state. Thus, to fully understand the impact of elevated GCs, it is necessary to take into account circulating levels of CBG as well as factors that could simultaneously alter circulating levels of CBG and GCs.

Circulating levels of CBG are dynamically regulated by GCs. Prolonged administration (typically 2–3 days) of exogenous GCs is known to suppress plasma levels of CBG (38), whereas acute elevations of GCs fail to alter CBG. Furthermore, GCs appear to suppress circulating CBG by acting at type II GC receptors but not type I GC receptors (Spencer, unpublished observations). Conversely, the removal of endogenous GCs by adrenalectomy (ADX) has been shown to increase plasma CBG levels (38). The effects of ADX are corticosterone (Cort) dependent, since ADX with basal Cort replacement normalizes CBG levels (38).

In addition to regulation by GCs, CBG is regulated by immune activation associated with the acute phase response (29). The acute phase response collectively refers to a constellation of physiological changes that are initiated immediately subsequent to pathogen infection or tissue trauma. These changes include a shift in liver metabolism such that synthesis of normal carrier proteins is inhibited, whereas production of positive acute-phase proteins is initiated. Other changes associated with acute phase activation are elevated core body temperature (CBT; fever), leukocytosis, changes in plasma ion concentrations, and HPA activation (see Ref. 2 for a recent review). Together these changes reduce the capacity of pathogens to replicate while simultaneously maximizing the host’s ability to recover from the precipitating insult. Acute phase activation is mediated by proinflammatory cytokines [interleukin (IL)-1, IL-6, and tumor necrosis factor-α], which are secreted by activated immune cells as well as by other nonimmune tissues.

Interestingly, decreases in circulating levels of CBG have been reported to occur in rats injected with turpentine, a peripheral inflammatory agent that is known to induce the acute phase response (8, 9, 29, 37). Other studies have demonstrated that human serum is depleted of CBG as early as 24 h after the onset of septic shock (30) and that adjuvant-induced arthritis also suppresses circulating levels of CBG (28). These findings have led to the classification of CBG as an acute-phase negative protein (29), i.e., a circulating protein whose concentration decreases by at least 25% during the acute phase response (39).

It has also been demonstrated that exposure to chronic stressors can lead to reductions in CBG as well as elevated basal levels of GCs for several days after termination of the chronic stressor (1, 5, 18, 26, 33). More recently, however, such changes have also been...
reported after exposure to an acute stressor, a single session of inescapable shock (11). Fleshner et al. (11) exposed rats to 100 intermittent inescapable tail shocks across a 2-h session. Serum CBG was reduced 24 h later, and this reduction persisted for another 24 h. The finding that a relatively brief exposure to a stressor can reduce CBG for a prolonged period of time raises issues concerning the mechanism(s) by which stressors reduce CBG.

The present experiments address four issues. First, although many sequelae of exposure to stressors depend on the degree of behavioral control that the organism is permitted to exert over the stressor (21), the adrenocorticotropic hormone (ACTH) and GC response to the shock stressor used by Fleshner et al. (11) and in the present experiments do not. That is, a single session of both inescapable shock (IS) and equal amounts of escapable shock (ES) produce the same ACTH and GC response (22). However, it is possible that the CBG reduction is specific to IS, thereby resulting in a higher free level of GC after IS than after ES. Thus experiment 1 determined whether IS and ES lead to different subsequent levels of serum CBG.

As noted above, endogenous GCs have been argued to mediate alterations in serum CBG. A second purpose of the experiments reported was to determine whether GC increases produced by IS are responsible for the IS-induced downregulation of CBG. We used two approaches to evaluate this question. First, we evaluated whether ADX would prevent or reduce the decrease in CBG produced by IS. Basal Cort was replaced by adding it to the drinking water (15), thereby eliminating potential confounding due to ADX-associated increases in CBG (38). Second, because the effects of GCs on CBG are mediated by the type II GC receptor (Spencer, unpublished observations), we pretreated rats with RU-38486 before IS exposure. This drug was chosen due to its long half-life (estimated to be 20–48 h; Ref. 14) and selectivity for the type II GC receptor.

Finally, because carrier proteins like CBG are reduced during an acute phase response such as that induced by infection (39), the CBG reduction may then simply be part of an acute phase response triggered by IS. The possibility that a brief stressor such as that used here can activate a full acute phase response has not previously been investigated. To assess this possibility, changes in other circulating proteins known to be responsive to acute phase activation were measured after IS (expt 3).

Furthermore, if IS exposure is sufficient to activate the acute phase response, then it would also be expected to produce sustained elevations in CBT (fever). We examined this possibility in experiment 4.

**METHODS**

Animals. Adult male viral-free Sprague-Dawley rats (350–400 g; Harlan Laboratories) were individually housed in suspended wire cages (24.5 × 19 × 17.5 cm) with food and water available ad libitum. Colony conditions were maintained at 22°C on a 12:12-h light-dark cycle (lights on 0600–1800). Rats were given at least 2 wk to habituate to the colonies before experimentation. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Blood sampling and protein assay protocols. Blood samples (300 μl) were obtained from a lateral tail vein within 2 min of contacting each rat’s cage. The serum was extracted after samples dotted at room temperature and was stored at −20°C until assayed.

Serum CBG levels were assessed using a competitive binding assay adapted from (38). The samples were initially diluted 1:200 in buffer (pH 8.0) consisting of 10 mM Trizma base, 10 mM EDTA, 10% glycerol (vol/vol), and 1.0 mM dithiothreitol. The diluted sample was then mixed with [3H]Cort (15 nM) or unlabeled Cort (10 μM) at a final dilution of 1:600 and allowed to incubate overnight at 4°C. Bound and unbound steroids were separated using activated charcoal (executed in duplicate). The bound fraction was mixed with scintillation cocktail and counted with a liquid scintillation counter (TriCarb 1600TR, Packard, Meriden, CT). Data were expressed as nanomoles of specific [3H]Cort binding per liter of serum. Because one Cort molecule binds per CBG, this is equivalent to CBG per liter of serum.

Total serum protein levels were measured using a microtiter plate version of the Coomassie blue protein assay adapted from Ref. 4. To measure serum albumin, samples and bovine serum albumin standards were diluted 1:20 and mixed with BCG reagent (Sigma lot 631–2), and absorbances were read at 630 nm using a microtiter plate reader. Serum levels of α2-acid glycoprotein were determined using a modification of the seromucoid assay previously reported (27).

Haptoglobin was measured by "rocket" immunoelectrophoresis as previously described (23). Briefly, samples were diluted with an equal volume of barbital (Veronal) buffer and added to a 0.8% agarose gel containing 0.96% rabbit antimurine haptoglobin polyclonal antibody (DAKO code A0030). Gels were electrophoresed at 200 V (15–25 mA) for 18 h at 4°C. The rockets were visualized by staining for 30 min with 0.1% Coomassie brilliant blue 30:10:60 methanol-glacial acetic acid-water. Concentrations were estimated by measuring the height of the rockets and comparing them with a standard curve.

**Experiment 1. Effects of stressor controllability on serum CBG.** Thirty-eight rats (n = 8–10/group) were randomly assigned to treatment groups of either IS, ES, restraint, or home cage controls (HCC). Baseline blood samples and stress protocols were always administered between 0800 and 1100. After a baseline blood sample was taken, rats received ES, IS, a comparable time of restraint, or were returned to their home cages. The ES procedure was identical to that previously reported (31). Briefly, rats were shocked in Plexiglas wheel turn chambers. Each rat’s tail was loosely fastened with adhesive tape to a Plexiglas post protruding from the rear of the chamber. Modified fuse-clip electrodes coated with electrode paste were then fastened loosely to each rat’s tail. Each rat received 100 (1.6 mA) tail shocks approximately once per minute on a variable intertrial interval ranging from 30 to 90 s. Each shock could be terminated by the rat turning a small wheel in the front wall of the chamber as previously described (31) in assessing the effects of controllability on social interaction. Rats in the IS treatment group were paired (yoked) with a rat in the ES group and received the same intensity, duration, and pattern of shock. After termination of the stressor, rats were returned to their home cages. A second blood sample was taken 24 h later.
Experiments 2a and 2b. Effects of ADX and RU-38486 on IS-induced reduction in CBG. In experiment 2a, 27 rats (n = 6–7/group) were randomly assigned to ADX-HCC, ADX-IS, Sham-IS, or Sham-HCC groups. Bilateral ADXs were aseptically performed with animals under halothane anesthesia (Halocarbon Laboratories lot 39419). All tissue removed from the animal was examined immediately to ensure complete removal of the adrenal gland. Sham-operated animals received the identical procedure except that the adrenal glands were gently manipulated with forces but not removed. Steroid replacement for ADX animals began immediately after surgery. ADX animals received Cort replacement in their drinking water, since this method has been shown to mimic the normal circadian pattern of Cort secretion (15). Cort was initially dissolved in ethyl alcohol (EtOH) and diluted to a final concentration of 25 µg/ml in 0.2% EtOH-0.5% saline. Sham-operated animals received drinking water containing 0.2% EtOH. After 10 days of recovery, blood samples were taken before and 24 h after either IS or HCC treatment. IS rats were each placed in a Plexiglas restraining tube (15 × 7 cm) and given 100 1.6-mA tail shocks (5 s, variable intertrial interval 60 s; range 30–90 s).

In experiment 2b, 24 rats (n = 6/group) were injected subcutaneously at the nape of the neck with either vehicle (propylene glycol) or 10 mg/kg RU-38486 (mifepristone, RBI lot GS-493A). This dose was chosen because it has been shown to block the effect of IS on in vivo antibody levels (10) and produces maximal receptor blockade (Spencer, unpublished observations). Thirty minutes after injection, half of each group of rats remained in their home cages and the other half received IS. Thus the design was a 2 × 2 factorial. A second blood sample was taken 24 h after baseline.

Experiment 3. Effects of IS on acute-phase reactants. After a baseline blood sample was taken, rats (n = 7/group) were either returned to their home cages or received IS as in experiment 2. Subsequent blood samples were taken at 6 and 24 h postshock or control treatment. Serum was assayed for positive and negative reactants of the acute phase response as previously described.

Experiment 4. Effects of IS on CBT. Mini-Mitter telemetry probes (model VM-FH) were aseptically implanted intraperitoneally (n = 7/group) in animals under halothane anesthesia. CBTs were measured with a miniature receiver (model RLA3000, Mini-Mitter) and frequencies read from a frequency counter (Heath model 400–101, Mini-Mitter). Frequencies were converted to temperature (in °C) using a standard curve fitted by cubic regression for each telemetry probe.

On day 1 of the experiment, baseline readings of CBT were gathered once per hour for 3 h before treatment. After the baseline readings, rats received either IS or remained in their home cages as in experiment 2. Body temperatures were then recorded for both groups after 0, 25, 50, 75, and 100 shocks. Immediately after shock, rats were returned to their home cages, and CBT was measured once per hour for 10 h. Subsequent CBT readings were taken in the morning of the next 2 days. Rats were then weighed and immediately given a sham intraperitoneal injection. CBT was then recorded every 10 min for 2 h after exposure to this mild secondary stressor.

Statistics. For protein data, baseline comparisons were made between groups to establish whether any reliable differences in baseline existed. Subsequent time points were analyzed as between group comparisons using analysis of variance. Post hoc analyses were performed using the Student-Neuman-Keuls test. CBT data were analyzed using repeated-measures design.

RESULTS

Experiment 1. Although it has previously been reported that rats exposed to ES or yoked IS do not differ in their HPA response to their respective stressors (22), serum CBG levels have not been previously assessed. If these treatments differentially affected serum CBG, then rats receiving ES vs. IS could differ markedly in the amount of Cort available to bind to receptors. Experiment 1 examined whether ES- and IS-treated rats differed in their subsequent serum levels of CBG. A restraint control group was included in this experiment because we have not previously demonstrated that the IS-induced reduction in serum CBG was a result of the shock exposure per se and not simply due to the restraint aspect of the stressor.

No differences in baseline levels of CBG were observed between groups [F(3,34) = 0.876, P > 0.05]. However, CBG levels did differ significantly at the 24-h time point [F(3,34) = 12.938, P < 0.0001]. Post hoc analysis revealed a reliable reduction in serum CBG in ES- and IS-treated rats compared with either restraint controls or HCCs (P < 0.05; Fig. 1). These data demonstrate the IS-induced reduction in CBG observed by Fleshner et al. (11) does not vary as a function of stressor controllability.

Experiments 2a and 2b. There are several reports of stress-induced reductions of serum CBG in the literature. Although most researchers have assumed that these effects are mediated by the elevated GCs occurring as a result of stressor exposure, there is no evidence to support this claim. Experiment 2 sought to determine whether the IS-induced reduction in serum CBG was mediated by the GC response to IS.

Serum Cort was measured in baseline samples as a verification of complete ADX (data not shown). Cort levels in ADX rats were undetectable. No differences between groups in baseline levels of CBG were found [F(1,26) = 0.665, P > 0.05]. IS exposure significantly reduced serum levels of CBG 24 h later [F(1,26) = 4.921, P < 0.05; Fig. 2A]. Serum CBG levels were significantly reduced in both sham-operated controls...
status of the animal. More specifically, changes in other acute-phase proteins might also be observed, indicating that acute phase activation has occurred.

No reliable baseline differences were found in any of the circulating proteins measured (P > 0.05). Similarly, no differences were found between treatment groups at the 6-h time point for any of the proteins except haptoglobin. Serum haptoglobin was significantly reduced in IS-treated rats compared with HCCs [F(1,12) = 42.341, P < 0.0001]. At the 24-h time point, CBG was significantly reduced in IS-treated rats [F(1,12) = 34.202, P < 0.0001; Fig. 3A], whereas α1-acid glycoprotein and haptoglobin were significantly increased in IS-treated rats [F(1,12) = 5.140, P < 0.05 and F(1,12) = 22.705, P < 0.001; Fig. 3, C and D, respectively]. There were no reliable effects of IS at the 24-h time point on total serum protein levels [F(1,12) = 0.195, P > 0.05; data not shown] or serum albumin [F(1,12) = 3.642, P > 0.05; Fig. 3B]. These data suggest that exposure to IS leads to activation of the acute phase response.

Experiment 4. The results of experiment 3 suggest that IS exposure activates the acute phase response using changes in circulating proteins as an index of acute phase activation. However, we were interested in the generality of this phenomenon and whether this conclusion would be supported by other indexes of acute phase activation. We chose CBT because it is a highly sensitive, well-accepted, and easily measured index of acute phase activation. Four separate repeated-measures analyses were conducted for these data, each of which represented a different phase of the experiment. All data from the 1st day of experimentation (including baselines, during shock session, and for 10 h after IS termination) comprised the first analysis. In this analysis, CBT was significantly elevated in IS-treated rats compared with HCC [F(1,12) = 32.434, P < 0.0001; Fig. 4A]. The second analysis included only CBT readings from the morning after IS treatment (20–24 h). CBT was also significantly elevated in IS-treated rats the morning after IS [F(1,12) = 5.772, P < 0.05; Fig. 4B]. Analysis of data from the second morning after IS exposure (44–48 h) demonstrated that group differences in CBT were no longer significant [F(1,12) = 3.763, P > 0.05; Fig. 4B]. After exposure to the secondary stressor, a potentiated fever response was observed in rats that received IS treatment 48 h previously [F(1,12) = 9.312, P < 0.01; Fig. 4C]. These data further support the hypothesis that IS results in activation of an acute phase response.

DISCUSSION

The present experiments replicate the finding that a single session of IS reduces serum CBG 24 h later. They add the findings that this reduction follows ES as well as IS and is not blocked by ADX or pretreatment with RU-38486. In addition, IS exposure increased and ADX rats, suggesting that IS-induced depletion of serum CBG is not mediated by GCs.

The data from experiment 2b confirm the findings of experiment 2a. No differences in baseline CBG were found between IS- and HCC-treated rats nor between vehicle- and RU-38486-treated rats (Fig. 2B). However, 24-h serum CBG levels differed significantly from baseline dependent on whether the rats received IS or HCC treatment [F(1,20) = 11.522, P < 0.01]. Serum CBG levels were significantly reduced in both vehicle- and RU-38486-treated rats receiving IS, further demonstrating that the IS-induced decrease in CBG is independent of GC regulation.

Experiment 3. The IS-induced reduction in serum CBG is clearly not mediated by GCs. Because acute stressor exposure has been reported to increase plasma levels of the proinflammatory cytokine IL-6 and serum CBG levels are known to be responsive to acute phase activation, it could be that the reduction in serum CBG is but one of a constellation of changes in the immune and ADX rats, respectively.

Fig. 2. A: rats received either sham surgery or bilateral adrenalectomy (ADX) with chronic basal corticosterone replacement (25 µg/ml in drinking water). Animals were given 10 days to recover before experimentation. After recovery, a baseline blood sample was taken and half of rats of each surgical group were either returned to their home cages or received inescapable tail shock (IS; n = 6–7/group). A second blood sample was taken 24 h later. Serum levels of CBG were significantly reduced 24 h after IS exposure in both sham-operated and ADX rats (P < 0.05). B: rats were pretreated with RU-38486 (10 mg/kg sc) or equivolume vehicle. Thirty minutes later, rats (n = 6/group) were exposed to IS or remained in their home cages. Blood samples were taken before injection and 24 h after IS exposure. Serum CBG was significantly reduced in IS-treated rats whether administered vehicle or RU-38486 before IS (*P < 0.05).
serum levels of acute-phase proteins and produced a long-lasting increase in CBT. These data demonstrate that the IS-induced reduction in serum CBG occurs independently of GC regulation. Furthermore, these data suggest that the reduced serum CBG observed after IS exposure may be a part of a larger IS-induced acute phase response. Although these experiments have not elucidated the mechanism by which IS initiates the subsequent acute phase response observed here, these findings represent an important transition in our understanding of physiological responses to stressors.

The failure of stressor controllability to modulate the reduction in CBG produced by the tail shock stressor is consistent with prior findings that a single session of ES and IS using the same parameters as those employed here produced equal levels of Cort and ACTH both during and after the stressor as well as 24 h later in reaction to a second stressor (22). Because ES and IS have quite different behavioral and neurochemical effects, there has been the expectation that HPA activity would also differ. The prior finding (11) that IS reduces CBG suggested the possibility that perhaps ES would not do so, thereby providing a mechanism whereby IS would yield higher levels of free Cort than ES. The present results do not support such a possibility. In addition, Fleshner et al. (11) found that an injection of Cort at a dose which mimicked the levels of Cort produced by IS across time had no effect on serum levels of CBG. Thus increased GCs are neither necessary nor sufficient to produce the reduction in CBG.

Because reductions in the synthesis of carrier proteins by the liver are a part of the acute phase response produced by infection and inflammation (39), an alternative is that the reduction in CBG is simply part of an acute phase response but induced by a stressor. The proposal here is that the regulation of CBG by IS is not selective but part of a larger alteration. The first step in the evaluation of this hypothesis is the determination of whether IS does, in fact, lead to an acute phase response. Acute phase responses are characterized by a rise in liver production of acute-phase proteins, and IS did indeed increase plasma levels of α1-acid glycoprotein and haptoglobin measured 24 h later. The acute phase response is also characterized by fever. It is not surprising that CBT rose during the IS session, since the animals were restrained in tubes and the IS elicits motor movement. Indeed, if CBT had been measured only during IS, the rise in CBT could be described as behavioral hyperthermia rather than a true fever. However, CBT was significantly elevated 1 day after IS, and even 2 days later, there was still a trend in this direction. Furthermore, the IS animals reacted to handling and a sham injection with an exaggerated increase in CBT, indicating that the fever circuitry was

Fig. 3. After a baseline blood sample was taken, rats (n = 7/group) received IS or remained in their home cages. Subsequent blood samples were taken 6 and 24 h later. Serum CBG was significantly reduced 24 h after IS (A; *P < 0.05), while there was no effect on total serum protein (data not shown) or albumin (B). α1-Acid glycoprotein was significantly elevated 24 h after IS (C; *P < 0.05). IS exposure significantly reduced haptoglobin 6 h after shock while increasing it 24 h later (D; *P < 0.05).
still sensitized. Of course, it is possible that the reduction in CBG and acute phase measures are independently regulated, and experiments are required that block the acute phase response.

Nevertheless, we are currently pursuing other potential mediators of stress-induced activation of the acute phase response. It is likely that these effects could be mediated by proinflammatory cytokines such as IL-6 or IL-1, since several stress responsive centers are also known to secrete these cytokines. For example, cells of the anterior pituitary secrete IL-6 (32, 34), and the adrenal secretes IL-1 and IL-6 (13, 17). Of course, the adrenal is not a likely participant in the IS-induced responses studied here, since ADX did not reduce the effects obtained. All of the other cell types, however, are possible sources of IS-induced cytokines. In this regard, it can be noted that increased circulating levels of epinephrine have been reported to elevate plasma IL-6 (6), with some evidence suggesting endothelial cells as a likely source (36). Intriguingly, there is recent evidence that stress-related hormones may even be able to activate macrophages. Although pharmacological quantities of corticosteroids suppress macrophage function, high but physiological levels such as those observed during stressors can increase macrophage phagocytic activity (12) and IL-1β mRNA after lipopolysaccharide stimulation (19). Thus IS and other stressors might be capable of stimulating monocyte-macrophage production of cytokines via hormonal mechanisms as well as cytokines from other cell types.

There is also a quite different mechanism by which stressors could lead to monocyte-macrophage activation and consequent cytokine production. A number of reports have suggested that stressor-induced sympathetic activation can cause bacteria to translocate across the intestinal mucosa, infecting the mesenteric lymph nodes, liver, spleen, kidney, and blood (3, 7, 35). This would, of course, directly activate monocytes and/or macrophages. Clearly, these mechanisms are not incompatible and all may play a role.

Perspectives

The generality of the present results beyond IS as a stressor and their functional significance remains to be determined. However, it can be noted that exposure to a novel environment (20), restraint, foot shock, and simple exposure to conditioned stimuli that were present during foot shock (40) all produce increased levels of plasma IL-6. The initiation of an acute phase response in reaction to potential or actual threat may well represent an anticipatory defensive immune response involving cells of the “innate immune system,” promoting restriction of infection, inflammation, and injury produced by the threat.

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