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Characterization and quantification of corticosteroid-binding globulin in a southern toad, *Bufo terrestris*, exposed to coal-combustion-waste

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Abstract

Corticosteroid-binding globulin (CBG) is a plasma protein that binds corticosterone and may regulate access of hormone to tissues. The role of CBG during a stress response is not clear. At least two hypotheses have been proposed: 1) CBG levels may increase in response to a stressor, thereby decreasing the amount of circulating free corticosterone, or 2) CBG levels may decline, making corticosterone available for its role in increased metabolic needs during stress. In this study, southern toads, *Bufo terrestris*, were exposed to a chronic pollutant (coal-combustion-waste), to determine changes in CBG and free corticosterone levels. Since toads exposed to chronic pollutants in previous studies did not exhibit the predicted changes in metabolic rate and mass, but did experience a significant elevation in total corticosterone, we hypothesized that CBG would likewise increase and thus, mitigate the effects of a chronic (i.e. 2 months) pollutant stressor. To conduct this study, we first characterized the properties of CBG in southern toads. Toad CBG has a $K_d = 20.6 \pm 1.0$ nM and a $B_{max} = 332.2 \pm 5.1$ nmol/L plasma. The rank order potencies for steroid inhibition of tritiated corticosterone are: dihydrotestosterone > corticosterone $\gg$ progesterone = testosterone $\ggg$ estrogen = dexamethasone. After characterization, we monitored the changes in CBG, total corticosterone, and free corticosterone in male toads that were exposed to either coal-combustion-waste or control conditions. CBG increased in all groups throughout the experiment. Total corticosterone, on the other hand, was only significantly elevated at four weeks of exposure to coal-combustion-waste. The increase in CBG did not parallel the increase in total corticosterone; as a result, free corticosterone levels were not buffered by CBG, but showed a peak at four weeks similar to total corticosterone. This finding indicates that, in this species, CBG may not provide a protective mechanism during long-term pollution exposure.

Keywords: Amphibian; Corticosteroid-binding globulin; Corticosterone; Stress; Metal

1. Introduction

Corticosteroid-binding globulins (CBG) are plasma proteins that affect the activity, transport, and availability of glucocorticoids in the body. The free hormone hypothesis posits that while a hormone is bound to a binding globulin, it is not biologically active (Breuner et al., 2006; Mendel, 1989). However, there is evidence that CBG can deliver corticosterone to specific tissues (Mendel, 1989), or have its own biological activity (reviewed in Rosner, 1990). Levels of CBG cycle seasonally and parallel changes in corticosterone in animals not confronting a stressor (reviewed in Breuner and Orchinik, 2000). Thus, as the concentration of corticosterone in the blood rises, the concentration of CBG also rises, binding excess corticosterone and maintaining the level of unbound (free) corticosterone relatively constant. When an organism is exposed to a stressor, however, the concentrations of CBG and corticosterone diverge and the level of free corticosterone rises above baseline

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(Breuner and Orchinik, 2000). Although a potentially important component in the stress response, the extent of corticosterone–CBG interaction has not been fully documented and the exact function of CBG in the regulation of corticosterone is still not understood (reviewed in Breuner and Orchinik, 2000).

The characteristics of CBG have been widely studied in humans, rats, and birds (e.g. Vogeser et al., 1999; Tinnikov et al., 1996; Armario et al., 1994; Tinnikov, 1993a; Deviche et al., 2001; Lynn et al., 2003, respectively). However, very little information exists about CBG characteristics and function in lower vertebrates, such as amphibians. Martin and Ozon (1975) have examined limited binding properties of numerous amphibians and Orchinik et al. (2000) have thoroughly described corticosteroid-binding in plasma of the tiger salamander (*Ambystoma tigrinum*). There are only three studies that have characterized CBGs in anurans from four families (Jolivet-Jaudet et al., 1984; Jolivet-Jaudet and Leloup-Hatey, 1996; Martin and Ozon, 1975). The actual role of CBG in glucocorticoid regulation in lower vertebrates, to our knowledge, has not been studied.

During stressful situations, the concentration of plasma glucocorticoids in vertebrates increases. In the short-term, this increase in glucocorticoids is adaptive and contributes to the survival of the organism by decreasing unnecessary physiological activities and increasing available metabolic energy (Bonga, 1997). However, if the stressor persists the effects of increased glucocorticoids can become detrimental. CBG is hypothesized to modulate the stress response by controlling the concentration of plasma free corticosterone. Most studies measuring changes in CBG during a stress response focus on short-term stressors (those lasting a few minutes to a few days) in mammalian (Fleshner et al., 1995; Tinnikov, 1993a; Viau et al., 1996; Vogeser et al., 1999) or avian (Breuner and Orchinik, 2000; Lynn et al., 2003; Silverin, 1986) systems with mixed results. Some studies found that CBG decreased with stress (e.g. Fleshner et al., 1995) and others found that CBG increased, mirroring total plasma corticosteroid concentrations (e.g. Breuner and Orchinik, 2000). For example CBG decreases with tail shock but increases during the stress of breeding (Fleshner et al., 1995; Breuner and Orchinik, 2000). It has been proposed that animals exposed to a chronic stress experience an increase in CBG. Two studies have shown that animals chronically exposed (1 month) to increased corticosterone levels by corticosterone implant experienced an increase in CBG that mirrored the increase in corticosterone levels (Breuner et al., 2003; Jennings et al., 2001). However, the organisms in these studies were not exposed to an actual stressor and, thus, would probably not have the same energy needs or responses as a stressed organism. Alternatively, CBG may decline due to persistent stressors. Several mammalian studies have demonstrated that stressors lasting several days actually cause a decline in CBG (see Breuner and Orchinik, 2002 for review). The role of CBG in response to a persistent (e.g. >1 month), low-level stressor is still unclear. The role of CBG in the stress response of lower vertebrates, in either acute or persistent situations, has also not been evaluated.

Given the current concern regarding amphibian decline, which some attribute to long-term environmental degradation (e.g. pollution, ultraviolet radiation, habitat destruction, Pounds and Crump, 1994; Carey and Alexander, 2003), further research on how amphibians respond to chronic stressors seems warranted. This study first characterizes the properties of corticosteroid-binding globulin (CBG) in the southern toad, *Bufo terrestris*, and then documents changes in CBG levels in control toads versus those chronically exposed (2 months) to coal combustion waste, a contaminant stressor. Toads exposed to this contaminant have been shown to have contradictory physiological responses in light of the stress response, indicating that CBG may be buffering circulating corticosterone (Ward et al., 2006). CBG levels were measured and compared to total plasma corticosterone concentrations, and then used to calculate free plasma corticosterone levels. These data will help to determine if CBG may function in a protective role, by reducing free corticosterone levels, or exacerbate stress reactivity, by buffering less of the corticosterone in plasma.

2. Materials and methods

2.1. Animal capture

Male southern toads were captured by hand at the Savannah River Site, Aiken, SC, USA, in early spring: March and April, 2001 ($n = 96$) and 2002, ($n = 168$). Toads were collected from either a coal-combustion-waste storage area, ash basin, (see Gutherie and Cherry, 1979 for description) or a control site, ~15 km from the ash basin. Mass was measured on a digital balance (0.01 g accuracy) and an initial blood sample was taken (see Section 2.3). All toads were then given an individual identification number by toe clip.

2.2. Housing and experimental design

Within two days of capture, toads were transported to Auburn University, Auburn, AL, USA, and transferred into mesocosms (208L Rubbermaid containers with screen lids). Each mesocosm contained 70% sediment (coal-combustion-waste, ash, collected from the capture site or play sand purchased from a local hardware store) and 30% water by area, 6 cm deep. Toads were placed in mesocosms with the sediment type equivalent to the type on which they were captured. There were 60 total mesocosms (30 ash and 30 sand controls) each housing three toads. Sediment was covered with pine straw, 5 cm deep, and a 10×25 cm piece of pine bark was included for shelter. All mesocosms were located outdoors under a shade-cloth tent and subject to ambient conditions.

Toads were acclimated in mesocosms containing their capture sediment for one month before being transferred to the experimental sediment. Toads were assigned into one of four groups ($n = 40$ per group). Toads acclimated to control (C) or ash (A) sediments were then transferred to mesocosms containing control ($C \rightarrow C$, $A \rightarrow C$) or ash ($C \rightarrow A$, $A \rightarrow A$) sediments. Toads were fed weekly (~10 crickets per toad *ad lib.*) with crickets raised on either a control diet of cat food or dry cat food chow contaminated with coal fly ash (50/50 mixture by volume), depending on treatment sediment. All toads were euthanized after four months by immersion in an aqueous solution of MS222 (300 ppm) (Andrews, 1993).