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Responsiveness of the hypothalamo–pituitary–interrenal axis in an amphibian (*Bufo terrestris*) exposed to coal combustion wastes

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Abstract

To assess the responsiveness of the interrenal axis to stress, we injected toads exposed to coal combustion wastes and toads from an unpolluted reference site with adrenocorticotropic hormone (ACTH), as well as the vehicle alone (saline). Initial circulating levels of corticosterone in toads captured at the polluted area were significantly higher than levels in toads from the reference site. Corticosterone levels in toads from the polluted site remained high even after 2 weeks of laboratory acclimation and injection with saline. The results may suggest disruption of hepatic enzymes responsible for the metabolic clearance of steroid hormones. Injection of toads from the polluted site with ACTH had no effect on plasma corticosterone levels, whereas a similar treatment of toads from the reference site stimulated a marked increase in corticosterone. Our study provides evidence that toads exposed to coal combustion wastes may be less efficient at responding to additional environmental stressors.

Keywords: Amphibians; Toads; Pollutants; Coal combustion wastes; Hormones; Stress; Corticosterone; Adrenocorticotropic hormone

1. Introduction

Chronic exposure to sublethal concentrations of xenobiotics can have a profound impact on the physiology of organisms [15,23,26,31,33]. In recent years, a great deal of effort has focused on the development of biochemical indicators that could be used to monitor the health of organisms chronically exposed to pollutants (for review, see [33]). Given the status of many vertebrate populations today, it is critical that nondestructive diagnostic techniques are developed [13]. One proposed nonlethal biomarker is the interrenal stress response exhibited by organisms following exposure to xenobiotics [10,13].

Elevated corticosteroid levels appear to be a potentially useful biomarker for acutely exposed organisms [4]. It is well known that acute exposure to a wide variety of chemical contaminants can trigger the release of cortisol from the interrenal tissue of fish [5,7,8,11,19,27]. More environmentally realistic situations, however, are those in which organisms are exposed to noxious agents for longer periods of time. Under chronic exposure to xenobiotics, circulating levels of corticosteroids often decrease and are therefore less useful biomarkers of stress [1,5]. As a result, alternative biomarkers are necessary to detect the effects of chronic exposure. It has been suggested that prolonged exposure to toxic substances may actually lead to exhaustion of the hypothalamo–pituitary–interrenal axis [9,10,12,13]. Therefore, a more effective technique for assessing interrenal responses in chronically exposed organisms may be to determine the organisms' ability to hormonally respond to additional acute stressors

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[32]. Recent studies indicate that organisms chronically exposed to contaminants are less efficient in elevating corticosteroid levels in the presence of additional stressors [10,12–14,28].

Despite the potentially threatened status of many amphibian populations today, only two studies to date have examined the hormonal responses of adult amphibians to pollutants [10,15]. One of the studies [10] determined that mudpuppies, *Necturus maculosus*, chronically exposed to organochlorines were unable to respond as effectively to an adrenocorticotropic hormone (ACTH) injection when compared to the response stimulated in conspecifics from unpolluted reference sites. The reduced responsiveness of organochlorine-exposed mudpuppies shares similarities with the impaired responses previously observed in organochlorine and mercury-exposed fish, e.g. [12]. The present study intended to determine if an entirely different mixture of pollutants produced by the combustion of coal (characterized by high concentrations of trace elements including As, Cd, Cu, and Se) would affect the interrenal responsiveness of southern toads, *Bufo terrestris*. The study was conducted as part of a larger project investigating trace element uptake and the hormonal responses in southern toads, *Bufo terrestris*, exposed to coal combustion wastes [15,16]. We determined the ability of toads from a coal ash-contaminated site and a reference site to respond to exogenous adrenocorticotropic hormone (ACTH challenge; [10]). In addition, we transplanted toads to the contaminated habitat to determine if a relatively short period of exposure would effect their ability to respond to an ACTH challenge.

2. Materials and methods

2.1. Animal collection

Hormone levels of both free-ranging toads and transplanted toads were examined in this study. Free-ranging adult male *Bufo terrestris* were collected at a coal-ash polluted site and at a nearby unpolluted reference site between 1 and 11 August 1997 ($N = 10$ site^{-1}) (for site descriptions see [2,6,15,16,30]). All individuals were captured by hand between 20:00 and 24:00 h, and the time of capture was recorded. All toads were bled (see below) before being weighed on an Ohaus balance (to nearest 0.1 g) and toe clipped for identification. Toads were then transported to the laboratory where they were acclimated at 25°C for 2 weeks in plastic containers with moist paper towels. All toads were fed crickets 1 week prior to the ACTH challenge.

The second portion of this study involved transplanting toads from the uncontaminated sites to enclosures located in the polluted site or in another uncontaminated (reference) site (2 enclosures site^{-1}) (for enclosure descriptions, see [15,16]). 32 adult males captured between 6 and 17 July 1996 were placed in the enclosures (8 toads enclosure^{-1}) within 3 h of being captured and bled in the field. Toads remained in the enclosures at each site for 7 and 12 weeks. Following the exposures, all individuals were returned to the laboratory where they were weighed and then allowed to acclimate for 2 weeks in the same manner as free-ranging toads.

2.2. Blood collection

Toads were bled within 3 min of capture or removal from the enclosures via cardiac puncture. Approximately 100 μl of blood was collected from each individual in heparinized 1-cc syringes and placed on ice for transport to the laboratory. Blood was also collected 10 h after injection with ACTH (see below). Blood collected from toads was centrifuged for 10 min at 3000 rpm. Separated plasma was pipetted off, frozen, and stored at -20°C for later analyses.

2.3. ACTH challenge

Numerous studies have indicated that mammalian ACTH triggers the release of interrenal hormones in amphibians [22]; for review, see [29]. After a 2-week laboratory acclimation, six toads collected from the polluted site and the reference site received an intraperitoneal injection of 1000 ng mammalian ACTH$^{1-39}$ (Sigma, St. Louis, MO, USA) dissolved in 0.9% saline. Concurrently, four toads from each site received an injection of the vehicle alone (0.9% saline). Injections were administered at 11:30 h within a 30-min time period. The total volume of the injections was 0.1 ml and injections were administered within 1 min of handling each individual. All toads were immediately returned to their respective holding containers and remained undisturbed for 10 h. At 21:30 h toads were bled via cardiac puncture. All bleeds were completed within 1 h. For the transplant experiments, toads were challenged with ACTH using the same protocol. In the toads exposed for 7 weeks, three toads from each site received the saline injection, while ACTH was administered to four and five toads from the reference site and polluted site, respectively. In the experiment where toads were exposed for 12 weeks, three toads from each site received the saline injection while ACTH was administered to five toads from the reference site and three toads from the polluted site.

2.4. Plasma extraction and radioimmunoassay

Extraction and radioimmunoassay procedures follow protocols detailed in recent studies [15,25]. After extrac-

tion with ether, duplicate aliquots of resuspended samples were incubated overnight at 4°C with the tritiated hormone (Dupont NEN Corticosterone NET-399) and antibody (Endocrine Sciences, Calabasas, CA. Corticosterone B21–42). A third aliquot was used to determine percent extraction efficiency. After being corrected for the plasma volume and percent extraction efficiency, plasma steroid content was expressed as nanograms per milliliter of plasma. Percent extraction efficiency averaged 92%. Interassay and intraassay variation averaged 12.2 and 5.4%. Sensitivity of the assay was 10 pg ml^{-1}.

2.5. Statistics

Plasma hormone concentrations from free-ranging and transplanted toads were tested for normality and homoscedasticity using the Shapiro–Wilk and Hartley's test, respectively. Data were log transformed. One-way analysis of variance was used to compare resting corticosterone levels of field-captured toads from both sites. Two-way repeated measures analysis of variance was used to determine whether the corticosterone responses of toads to ACTH or saline differed between sites. One-way repeated measures analysis was used to determine the effect of ACTH versus saline injection on corticosterone levels within a site.

3. Results

At initial capture, plasma corticosterone levels from toads at the polluted site were significantly elevated compared to toads captured at the reference site ($F_{1,18} = 115.1$; $P < 0.0001$; Fig. 1). After a 2-week acclimation period under laboratory conditions, toads from the polluted site exhibited a significantly different response to injections than the reference toads (Wilks' $\lambda = 0.74$, $P = 0.03$). Toads from the reference site exhibited a significantly different interrenal response to the saline and ACTH injections (Wilks' $\lambda = 0.44$, $P = 0.01$; Fig. 1). Saline injections had no detectable effect on the corticosterone levels of toads from the reference site, whereas ACTH injection produced a marked increase in corticosterone levels. In contrast, toads from the polluted site did not exhibit a significantly different response to the injection of ACTH or saline (Wilks' $\lambda = 0.93$, $P = 0.45$; Fig. 1). Rather, corticosterone levels were consistently elevated in toads from the polluted site regardless of their treatment (Fig. 1).

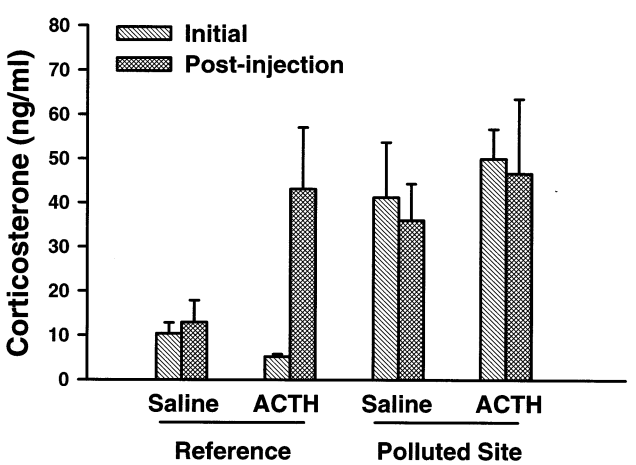

Fig. 1. Circulating corticosterone levels in field-captured toads, *Bufo terrestris*, from a reference site and a polluted site before and after injection with saline ($N = 4$ site^{-1}) or ACTH ($N = 6$ site^{-1}). Values are expressed as mean ± 1 S.E.

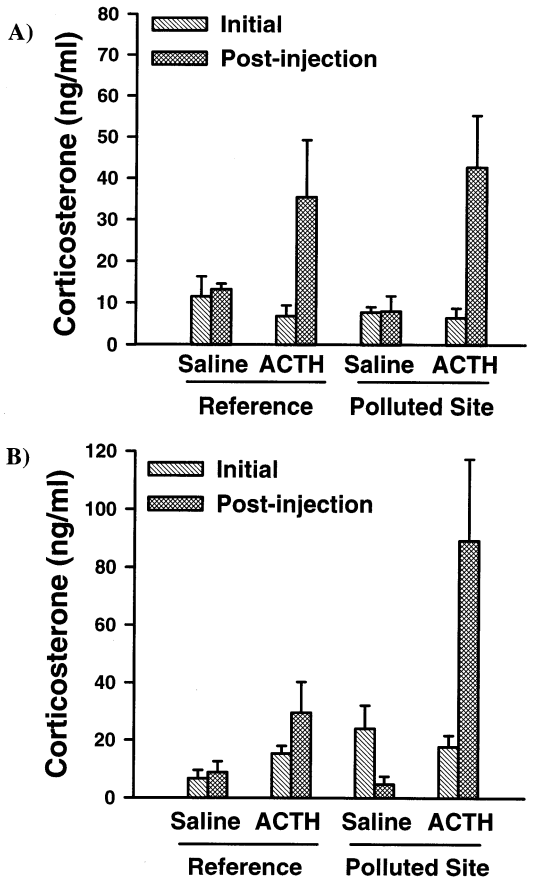

Fig. 2. (A) Circulating corticosterone levels in toads, *Bufo terrestris*, transplanted from a reference site to another reference site and to the polluted site for 7 weeks before and after injection of saline ($N = 3$ site^{-1}) or ACTH (Reference: $N = 4$; Polluted site: $N = 5$). Values are expressed as mean ± 1 S.E. (B) Circulating corticosterone levels in toads, *Bufo terrestris*, transplanted from a reference site to another reference site and to the polluted site for 12 weeks before and after injection of saline ($N = 3$ site^{-1}) or ACTH (Reference: $N = 5$; Polluted site: $N = 3$). Values are expressed as mean ± 1 S.E.

There was no significant difference in response to injections between toads transplanted to the polluted site and toads transplanted to the reference site for 7 weeks (Wilks' $\lambda = 0.97$, $P = 0.60$; Fig. 2(A)). Toads transplanted to the polluted site for 12 weeks, however,

had a significantly different response to injections than reference toads after 12 weeks (Wilks' $\lambda = 0.48$, $P < 0.01$). Toads at the reference site for 12 weeks exhibited similar responses to the injections of ACTH and saline (Wilks' $\lambda = 0.99$, $P = 0.90$). Toads at the polluted site, however, responded to the ACTH injection with significantly higher circulating corticosterone levels than after saline injection (Wilks' $\lambda = 0.23$, $P = 0.02$; Fig. 2(B)).

4. Discussion

We recently determined that toads inhabiting coal ash settling basins had elevated tissue concentrations of a number of toxic trace elements including arsenic and selenium [16]. Toads that accumulated these pollutants exhibited elevated circulating levels of corticosterone and testosterone regardless of month of capture or behavioral state [15]. In addition, toads transplanted to the polluted site for a relatively short period of time accumulated trace elements in their tissues and exhibited an interrenal stress response [15,16]. The current study demonstrates that toads naturally inhabiting the polluted site also exhibit a different hormonal profile when confronted with an additional stressor (the ACTH challenge) than the response exhibited by conspecifics from the reference site.

Circulating corticosterone levels were relatively low (< 10 ng ml^{-1}) in toads captured at the reference site in August. Following injection with saline, corticosterone levels remained low indicating that toads from the reference site were not stressed by the experimental conditions or handling (Fig. 1). After injection with ACTH, however, toads from the reference site experienced more than an 8-fold increase in circulating corticosterone levels (Fig. 1). In contrast, toads inhabiting the polluted ash basin habitat exhibited a completely different corticosterone profile. Toads initially collected from the polluted site in August had significantly higher circulating corticosterone levels than conspecifics collected at the reference site (Fig. 1). In fact, circulating corticosterone levels in toads from the polluted site were almost six times higher than the levels found in toads from the reference site. After 2 weeks of laboratory acclimation and subsequent injection with saline, toads from the polluted site continued to exhibit significantly higher corticosterone levels than the reference toads (Fig. 1). Moreover, when the toads from the polluted site were injected with ACTH, corticosterone levels did not increase as they did in the reference animals. Corticosterone levels in these toads are apparently elevated to a degree that further stimulation of the interrenal axis with the ACTH injections could not produce a greater response.

Exposure to toxicants in the polluted site appear to prevent interrenal steroids from returning to basal levels following an acclimation period. It is possible that ash basin pollutants interfere with the metabolic clearance of circulating corticosterone. High levels of trace elements incorporated in toad tissues may interfere with hepatic enzymes and prevent efficient clearance of circulating steroid hormones [15]. A number of elements including cadmium, lead, and mercury have been shown to inactivate enzymatic activity [21]. Other pollutants, such as the organochlorine *o,p*-DDD, have also been shown to interfere with enzymatic mechanisms involved with steroid clearance [18]. The interrenal tissue of tilapia superfused with *o,p*-DDD produced higher resting levels of cortisol than controls and was unable to respond to additional stimulation with ACTH [17]. The investigators found that the time needed to metabolize cortisol was 370% longer for organochlorine-exposed fish than for controls [18].

Possibilities other than altered metabolism exist that may explain why the ACTH challenge was unable to provoke an additional release of corticosterone in toads from the polluted site. Gendron et al. [10] found that organochlorine-exposed mudpuppies exhibited elevated resting levels of corticosterone and decreased responsiveness to additional stressors; findings that share similarities with what we currently document in toads. The authors suggested several potential mechanisms that may also apply to coal ash-exposed toads. One possibility may be a functional disruption along the hypothalamo–pituitary–interrenal axis downstream from the pituitary corticotropes [10]. It may be that due to chronic stimulation, the steroidogenic cells of the interrenal gland are already maximizing corticosterone production and are therefore incapable of responding to further stimulation. This appears plausible since circulating corticosterone levels in field-captured, saline injected, and ACTH-injected toads from the polluted site are all comparable to the corticosterone levels of ACTH-stimulated toads from the reference site (Fig. 1). Another possibility, however, is that in conjunction with altered steroid metabolism or maximized corticosterone production, continuous stimulation of the interrenal axis by pollutant-exposure may have resulted in reduced responsiveness of interrenal ACTH receptors to endogenous, as well as exogenous, ACTH [10]. Indeed, desensitization (downregulation) of receptors is an important regulatory response to the hyper-secretion of specific hormones [24].

Although toads transplanted to the polluted site exhibited increased tissue concentrations of trace elements [16], they did not exhibit hormonal responses consistent with what was observed in field-captured toads from the polluted site (present study; [15]). In contrast to the toads collected from the population inhabiting the polluted site, toads transplanted to the polluted site for up to 12 weeks did not exhibit a lack of response to the ACTH challenge (Fig. 2). It is important to note that

the levels of certain trace elements in toads transplanted to the polluted habitat were considerably lower than the levels found in field-captured toads from the polluted site [16]. Apparently, more prolonged exposures are necessary to accumulate higher levels of pollutants and to induce the hormonal responses observed in field-captured toads.

Although the mechanisms involved remain uncertain, it is clear that toads naturally exposed to coal combustion wastes exhibit chronically high circulating levels of corticosterone and reduced responsiveness to an ACTH challenge. Chronic elevations in glucocorticoids can be detrimental to the health of organisms since prolonged stimulation of the interrenal axis is known to adversely effect reproduction, osmoregulation, and the immune response [3–5,11,20]. Our study, in conjunction with others, indicates that diverse pollutants can interfere with the ability of the interrenal axis to respond to additional stressors in both fish and amphibians [10,12,13,28]. To assess the utility of altered adrenal responses as indicators of vertebrate health, it is critical that future efforts focus on a variety of other species exposed to diverse environmental contaminants.

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