Cortisol and corticosterone in the songbird immune and nervous systems: local vs. systemic levels during development

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Submitted 2 January 2008; accepted in final form 15 March 2008

Schmidt KL, Soma KK. Cortisol and corticosterone in the songbird immune and nervous systems: local vs. systemic levels during development. Am J Physiol Regul Integr Comp Physiol 295: R103–R110, 2008. First published March 19, 2008; doi:10.1152/ajpregu.00002.2008.—Glucocorticoids (GCs) have profound effects on the immune and nervous systems during development. However, circulating GC levels are low neonatally and show little response to stressors. This paradox could be resolved if immune and neural tissues locally synthesize GCs. Here, we measured baseline cortisol and cortisol levels in plasma, immune organs, and brain regions of developing zebra finches. Steroids were extracted using solid phase-extraction and quantified using specific immunoassays. As expected, corticosterone was the predominant GC in plasma and increased with age. In contrast, cortisol was the predominant GC in immune tissues (bursa of Fabricius, thymus, spleen) and decreased with age. Cortisol levels in immune tissues were higher than cortisol levels in plasma. In the brain, corticosterone and cortisol levels were similarly low, providing little evidence for local synthesis of GCs in the brain. This is the first study to measure 1) cortisol in the plasma of songbirds, 2) corticosterone or cortisol in the brain of songbirds, and 3) corticosterone or cortisol in the immune system of any species. Despite the prevailing dogma that corticosterone is the primary GC in birds, these results indicate that cortisol is the predominant GC in the immune system of developing zebra finches. These results raise the hypothesis that cortisol is synthesized de novo from cholesterol in the immune system as an “immunostroid,” analogous to neurosteroids synthesized in the brain. Local production of GCs in immune tissues may allow GCs to regulate lymphocyte selection while avoiding the costs of high systemic GCs during development.

adrenal; altricial; avian; bird; bone marrow; brain; bursa of fabricius; chicken; glucocorticoid; hypothalamus-pituitary-adrenal axis; hydrocortisone; immunostroid; neurosteroid; ontogeny; spleen; stress; stress hyporesponsive period; thymus; zebra finch

GLUCOCORTICOIDS (GCs) affect many systems during development, including the immune and nervous systems. For example, GCs regulate apoptosis and selection of T cells in the thymus and B cells in bone marrow (16, 21, 26). Although high GC concentrations decrease thymocyte survival, intermediate GC levels can antagonize T cell receptor-signalizing and increase thymocyte survival (71). In addition, GCs have important and long-lasting effects on the developing brain, such as regulation of neurotransmitter levels (36) and neurogenesis in the hippocampus (29, 42).

Despite the profound effects of GCs and stress on the immune system and brain during development, circulating GC levels are low and show only a slight, if any, response to stress neonatally. This has been coined the stress hyporesponsive period (SHRP). In rats, the SHRP lasts from postnatal day (PN) 3 to about PN12 (19, 38, 56). In mice, the SHRP lasts from PN1 to about PN12 (60). In birds, plasma corticosterone levels are low during the first few days of life and show little or no increase after restraint stress (61, 64, 73).

It remains a paradox that GCs have profound effects on the immune and nervous systems during development, even though circulating GC levels are low. One hypothesis that may explain this discrepancy is that GCs are produced locally in immune organs and/or brain tissue early in development. The capacity for the developing murine thymus to synthesize GCs in vitro has been well-established. In culture, developing murine thymic non-T cells produce pregnenolone and deoxycorticosterone after incubation with 22R-hydroxysterol (72). GC production in the thymus declines with age, but GC production in the adrenals increases with age (72). Furthermore, steroidogenic enzymes, including P450scc, 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), P450c21, and P450c11, are expressed in murine thymus (34, 72).

In chickens, in vitro studies suggest that the thymus and bursa of Fabricius (hereafter bursa) express all the enzymes necessary to synthesize cortisol from cholesterol, in contrast to the adrenal glands, which synthesize corticosterone (33). The bursa is a primary immune organ in birds that produces B lymphocytes and is functionally equivalent to bone marrow in mammals (1, 9). The suggestion that the chicken immune system synthesizes cortisol, not corticosterone, is surprising. Traditionally, the primary adrenal GC in birds is thought to be corticosterone, and cortisol is typically not measured in birds. Importantly, no studies have measured endogenous GC levels in immune tissues, in any species. This is a major gap in our knowledge.

There is also strong evidence that steroids are produced de novo from cholesterol in the developing and adult brain (neurosteroids). Studies in rats suggest that dehydroepiandrosterone (DHEA) and progesterone are synthesized in the brain (3, 52, 67). Neural DHEA synthesis declines during development (8). There is also evidence that estrogens are neurosteroids in songbirds, with declining synthesis during development (22, 39). There is little evidence thus far that GCs are neurosteroids. However, GC synthetic enzymes are expressed in the adult rat brain (43). Aldosterone may be synthesized in the adult rat brain (17). Because corticosterone is a precursor to aldosterone, these data indirectly suggest neural corticosterone synthesis.

Here, we measured endogenous GCs in plasma, immune tissues, and brain of a developing songbird, the zebra finch...
(Taeniopygia guttata). Songbirds are excellent animal models for studying local steroid synthesis in the immune and nervous systems. The avian immune system is well-characterized (9, 12), and the thymus and bursa can both be readily examined. Moreover, the avian brain robustly expresses steroidogenic enzymes (18, 20, 50, 58).

We measured corticosterone and cortisol at the day of hatch (P0), P3, and P30. First, we predicted that GC levels in plasma would be low early in development, but GC levels in immune organs and brain would be high because of the local synthesis of GCs. This result would indicate that GCs can have important effects on immune system and brain development, even though systemic GC levels are low. Second, we predicted that GC levels in plasma would increase with age as the SHRP ended, whereas local GC levels would decline with age. Third, we predicted that the primary GC in plasma and adrenal glands would be corticosterone, and the primary GC in the immune organs would be cortisol. This result would raise the possibility that corticosterone and cortisol have different functions, despite the prevailing belief that the two GCs are interchangeable.

MATERIALS AND METHODS

Subjects. Research was carried out under a University of British Columbia Animal Care permit (A06-0408), and procedures were approved by the Canadian Council on Animal Care. Breeding pairs of adult zebra finches were housed separately and given millet seeds, water, grit, and cuttlefish bone ad libitum. Breeding pairs were also given a food supplement consisting of boiled chicken eggs, cornmeal, and bread daily. The light cycle provided 14 h of light and 10 h of dark (lights on at 8:00 A.M.). Temperature was held constant at 23°C and relative humidity at ~50%.

Subjects were developing male and female zebra finches at P0 (n = 16), P3 (n = 16), or P30 (n = 8) (n = 40 subjects total). Zebra finches are altricial. At P0 and P3, the chicks’ eyes were closed, and chicks could not thermoregulate well (14, 78). At P30, chicks had fledged from the nest but remained in the same cage as their parents. At P0 and P3, samples were pooled from two birds of the same sex and age. In total, 136 plasma and tissue samples were analyzed for both corticosterone and cortisol.

Sample collection. All subjects were sampled between 10:00 and 11:00 A.M. to control for possible diel changes in GCs. A blood sample was collected within 3 min [2.35 ± 0.10 (SE) min] of opening the cage. GC levels are generally at baseline if animals are sampled within 3 min of disturbance (53, 77). At P30, blood samples were taken from the brachial vein by puncturing the vein with a 26-gauge needle and collecting blood into heparinized microhematocrit tubes. At P0 and P3, because of the difficulty of collecting a sufficient quantity of blood from the brachial vein, blood was collected via cardiac puncture with heparinized 0.5-ml syringes with 26-gauge fixed needles. Blood was centrifuged at 10,000 rpm for 10 min. Plasma was collected with a Hamilton syringe and stored at −20°C until analysis.

Immediately after blood collection, birds were rapidly decapitated. The period from opening the cage to death was 2.52 ± 0.11 min. The brains and bodies were immediately chilled at −20°C. After 2 min, brains were dissected. Brain regions collected include the rostral telencephalon (rTel), caudal telencephalon (cTel), and cerebellum. First, the cerebellum was collected. Next, the telencephalon was bisected midway to separate the rostral and caudal sections. The rTel and cTel were then separated into the left and right hemispheres. Next, the body was dissected. The bursa was collected at all three ages. The thymus and spleen were collected only at P30 because of the difficulty of collecting these organs in the younger animals. Adrenal glands and breast muscle were also collected at P30 (n = 4), as positive and negative controls, respectively. Tissues were immediately frozen on dry ice and stored at −80°C.

Steroid extraction. Steroids were extracted from tissue and plasma using solid phase extraction with C18 columns as previously described (45). This extraction procedure results in high and consistent steroid recoveries and effectively removes interfering substances from lipiddrich samples (45). Briefly, tissue samples were homogenized in ice-cold deionized water (dH2O). Then HPLC-grade methanol was immediately added, and samples were incubated overnight at 4°C. C18 columns were primed with 5 ml HPLC-grade ethanol and equilibrated with dH2O. Tissue samples were centrifuged at 3,000 g, the supernatant was brought up to 10 ml with dH2O, and samples were loaded on C18 columns. Plasma samples were also brought up to 10 ml with dH2O and loaded on C18 columns. Columns were then washed with dH2O, and steroids were eluted with 5 ml of 90% HPLC-grade methanol. Samples were evaporated under N2 at −40°C. Dried extracts were resuspended in 70 µl assay buffer (phosphate-buffered diluent provided with the cortisol assay). We used absolute ethanol (5% of resuspension volume) to aid in resuspension of steroids (45).

Steroid recovery was determined by spiking plasma and tissue pools with known amounts of radioinert corticosterone and cortisol and comparing spiked samples with unspiked samples (n = 3 pairs for each of plasma, immune tissue, and brain tissue). Recovery of corticosterone was 108 ± 4.23% in plasma, 92 ± 4.62% in immune tissue, and 95 ± 3.14% in brain tissue. Recovery of cortisol was 110 ± 1.90% in plasma, 84 ± 3.35% in immune tissue, and 110 ± 2.68% in brain tissue. Samples were corrected for recovery where applicable.

Corticosterone radioimmunoassay. Levels of corticosterone were determined using a sensitive and specific double-antibody 125I RIA (07-120103; MP Biomedicals) that has been validated for songbird plasma and tissue samples (45, 46, 74). The detection limit was 3.12 pg corticosterone/tube. The corticosterone antibody was highly specific (Table 1). The cross-reactivities of the corticosterone antibody to the metabolites and precursors of cortisol and corticosterone were determined by us, if the information was not provided by the manufacturer. Specifically, we determined the cross-reactivity of the corticosterone antibody to cortisone and dehydrocorticosterone (Table 1).

Of the 70 µl resuspension, 20 µl were used for the corticosterone assay (29% of the sample). These 20 µl were brought up to 100 µl with the phosphate-buffered diluent provided with the corticosterone assay, and 50 µl were assayed in duplicate for corticosterone. Average coefficient of variation between duplicates was 1.8%.

Because samples were resuspended in 5% ethanol, we ensured that the ethanol had no effect on the RIA by comparing a 50-pg standard containing 5% ethanol with a 50-pg standard without ethanol (n = 3 pairs). The standard with ethanol was similar to the standard without ethanol (t = 1.35, P = 0.25). Water blanks and known standards were analyzed in each assay. All extracted water blanks (n = 10) were nondetectable for corticosterone (<3.12 pg corticosterone). For corticosterone, a 30-pg control was analyzed in each corticosterone assay (n = 4). We obtained on average 3.4 ± 2.15 pg. Interassay coefficient of variation was 12%.

Cortisol enzyme immunoassay. Levels of cortisol were determined using a sensitive and specific enzyme immunoassay (EIA) (Salimetrics, 1-3012). Several cortisol assays were compared, and this assay

Table 1. Specificity of immunoassay antibodies

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Corticosterone Antibody, %</th>
<th>Cortisol Antibody, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>0.34</td>
<td>2.24</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>Dehydrocorticosterone</td>
<td>0.50</td>
<td>2.03</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.50</td>
<td>0.13</td>
</tr>
</tbody>
</table>

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was the most sensitive and highly specific. The cortisol assay was similar to the corticosterone assay in several respects. Both assays were highly specific, had similar detection limits, and had similar intra- and interassay variation. The detection limit was 3 pg cortisol/well. The antibody was highly specific for cortisol (Table 1). The cross-reactivities of the cortisol antibody to the metabolites and precursors of cortisol and corticosterone were determined by us, if the information was not provided by the manufacturer. Specifically, we determined the cross-reactivity of the cortisol antibody to 11-deoxy-corticosterone and dehydrocorticosterone (Table 1). For the cortisol antibody, the manufacturer reported a low cross-reactivity to corticosterone (0.20%). Nonetheless, we verified this by testing, in duplicate, 1,000, 100, and 10 pg of corticosterone in the cortisol EIA, all of which were nondetectable. These results verify that cross-reactivity of the cortisol antibody to corticosterone was <0.30%.

The remaining 50 μl of resuspension (71% of the sample) were brought up to 70 μl with the phosphate-buffered diluent provided with the cortisol EIA, and 25 μl were assayed in duplicate for cortisol. Average coefficient of variation between duplicates was 1.6%. A plate washer was used to ensure that wells were washed consistently (Tecan Columbus washer: I 109011). Absorbance was measured using a plate reader (Sunrise remote: f039300) at 450 nm, with a 620-nm background correction.

Because samples were resuspended in 5% ethanol, we ensured that the ethanol had no effect on the EIA by comparing a 100-pg standard containing 5% ethanol with a 100-pg standard without ethanol (n = 3 pairs). The standard with ethanol was similar to the standard without ethanol (t = 0.94, P = 0.40). Water blanks and known standards were analyzed in each assay. For cortisol, 8 of 10 water blanks were nondetectable (<3 pg cortisol). The average amount of cortisol detected in the water blanks was 0.65 ± 0.44 pg. For corticosterone, a 25-pg control was analyzed in each cortisol EIA (n = 5). We obtained on average 24.18 ± 1.33 pg. Interassay coefficient of variation was 12%.

Statistics. Nondetectable samples (below the lowest standard on the standard curve) were conservatively set to zero for both assays. Data were analyzed in SPSS (version 11 for Mac OS X).

Data were analyzed with mixed-design three-way ANOVA tests on each tissue and plasma, with age and sex as between-subjects variables and steroid (corticosterone vs. cortisol) as a within-subjects variable. The sex factor was not significant (main effects or interactions with another factor) in any of the analyses, and thus data from males and females were combined. The lack of sex differences is consistent with data on other songbird species (73 and K. Schmidt, E. Chin, K. Soma, unpublished observations). Where applicable, significant interactions between age and steroid were broken down into simple main effects using paired t-tests. If the interaction was not significant, significant main effects of age were broken down using Tukey’s honestly significant difference test.

Steroid levels in tissues were compared with steroid levels in plasma (as in Refs. 6, 10, and 17). Note that 1 ml of songbird plasma weighs 1.005 ± 0.17 g (n = 3; unpublished observation). Mixed-design two-way ANOVA tests were performed to compare levels of GCs in plasma and tissue using age as a between-subjects variable and sample (tissue vs. plasma) as a within-subjects variable. In addition, within individuals, we subtracted plasma GC levels from tissue GC levels.

For tissues that were only collected in P30 animals, levels of corticosterone and cortisol were compared using paired t-tests, and plasma levels were compared with tissue levels using paired t-tests. Tests were two-tailed. Test results were considered significant for P < 0.05. Results are presented as means ± SE.

RESULTS

GC levels in plasma. In the plasma, levels of corticosterone were lowest at P0 and increased with age (Fig. 1). Cortisol levels showed the opposite pattern and were highest at P0 and decreased with age (Fig. 1). The interaction between age and steroid was significant [F(2,20) = 9.22, P = 0.002]. A breakdown of the interaction into simple main effects revealed that corticosterone was the predominant GC in plasma at P3 (t1 = 2.73, P = 0.03) and P30 (t1 = 6.06, P < 0.001); however, corticosterone and cortisol were present at similar levels in P0 plasma (t1 = 0.24, P = 0.82).

GC levels in the immune system. Local GC levels in the immune system were high at P0 and decreased with age (Fig. 2). Furthermore, cortisol, not corticosterone, was the predominant GC in the immune system (Fig. 2). In the bursa, there was a significant main effect of steroid that revealed that cortisol levels were significantly higher than corticosterone levels at all three ages [F(1,20) = 13.92, P = 0.002]. Furthermore, there was a significant main effect of age [F(2,20) = 8.51, P = 0.003]. Post hoc tests revealed that GC levels in the bursa were higher at P0 than at P3 and P30. GC levels in the bursa were not significantly different between P3 and P30, suggesting that there is a rapid decrease in GC levels in the bursa soon after hatch. A comparison between cortisol levels in the bursa and plasma revealed a significant main effect of sample, indicating that cortisol levels in the bursa were higher than cortisol levels in the plasma at all three ages [F(1,20) = 20.29, P < 0.001] (Table 2). In contrast, corticosterone levels in the bursa were not significantly different from corticosterone levels in the plasma [F(1,20) = 0.80, P = 0.38] (Table 2).

GC levels in the thymus and spleen were analyzed at P30 only (Fig. 2). As in the bursa, cortisol was the primary GC in the thymus and spleen. Cortisol levels were significantly higher than corticosterone levels in the thymus and spleen (t1 = 2.50, P = 0.04 and t1 = 2.45, P = 0.04, respectively). Furthermore, cortisol levels in the thymus and spleen were significantly higher than corticosterone levels in the plasma (t1 = 3.77, P = 0.007 and t1 = 4.29, P = 0.003, respectively) (Table 2). In contrast, corticosterone levels in thymus and spleen were lower than corticosterone levels in the plasma (t1 = 7.57, P < 0.001 and t1 = 3.60, P = 0.008, respectively) (Table 2).

GC levels in the brain. GC levels in the brain were low, and many samples were nondetectable for corticosterone and cor-
Levels of corticosterone and cortisol in immune organs of zebra finches on P0, P3, and P30. In the bursa of Fabricius (bursa), cortisol levels were higher than corticosterone levels at all three ages. Glucocorticoid levels in the bursa were highest at P0 and decreased with age. In the thymus and spleen, cortisol was also the predominant glucocorticoid at P30. Numbers along the x-axis, below the vertical bars, indicate the percentage of detectable samples. Nondetectable samples were set to zero. Data are means ± SE. *P < 0.05.

In the rTel (Fig. 3A), there was a significant interaction between steroid and age [F(2,20) = 7.83, P = 0.004]. Decomposition of this interaction indicated that, at P30, corticosterone levels were significantly higher than cortisol levels (t7 = 3.19, P = 0.02). At P0 (t7 = 2.25, P = 0.07) and P3 (t5 = 0.36, P = 0.75), corticosterone and cortisol levels did not differ significantly in the rTel (Fig. 3A). Corticosterone levels in the rTel were significantly lower than cortisol levels in the plasma at all three ages [F(1,20) = 55.84, P < 0.001] (Table 2). However, cortisol levels in the rTel and plasma did not differ significantly at any age [F(1,20) = 0.71, P = 0.41] (Table 2).

In the cTel (Fig. 3B), there were no significant effects of age [F(2,20) = 2.59, P = 0.11] or steroid [F(1,20) = 2.54, P = 0.13], indicating that corticosterone and cortisol levels were similar and did not change with age. Although cortisol levels in the cTel at P0 appear higher than cortisol levels in the cTel at the older ages and appear to be higher than corticosterone levels in the cTel at P0, the interaction between age and steroid failed to reach significance [F(2,20) = 1.96, P = 0.17]. A post hoc power calculation revealed that power for this comparison was 0.75. It is possible that larger sample sizes would result in a significant interaction between age and steroid.

Corticosterone levels in the cTel were significantly lower than corticosterone levels in the plasma at all three ages [F(1,20) = 17.20, P = 0.001] (Table 2). Cortisol levels in the cTel and plasma did not differ significantly at any age [F(1,20) = 1.60, P = 0.22] (Table 2).

In the cerebellum (Fig. 3C), there was a significant interaction between age and steroid [F(2,20) = 4.07, P = 0.04]. Analysis of this interaction revealed that corticosterone levels in the cerebellum were significantly higher than cortisol levels at P30 (t7 = 2.59, P = 0.04) (Fig. 3C). At P0 (t7 = 1.51, P = 0.18) and P3 (t7 = 2.11, P = 0.07), corticosterone and cortisol levels did not differ significantly. Corticosterone levels in the cerebellum were significantly lower than corticosterone levels in the plasma at all three ages [F(1,20) = 56.52, P < 0.001] (Table 2). Cortisol levels in the cerebellum and plasma did not differ significantly at any age [F(1,20) = 0.39, P = 0.54].

**GC levels in the adrenal glands and breast muscle.** Analysis of GC levels in adrenal glands of P30 birds revealed that, as expected, corticosterone levels in the adrenals (2,605.03 ± 747.88 ng/g, 100% of samples detectable) were significantly higher than cortisol levels in the plasma (t3 = 3.48, P = 0.04). Cortisol was also present in P30 adrenals, but at lower levels than corticosterone (t3 = 3.46, P = 0.04). Cortisol levels in the adrenals (68.07 ± 17.21 ng/g, 100% of samples detectable) were significantly higher than corticosterone levels in the plasma (t3 = 3.93, P = 0.03).

Both corticosterone and cortisol levels were low in breast muscle, and levels of the two GCs did not differ from one another (t3 = 0.49, P = 0.66). Corticosterone levels in the breast muscle (1.38 ± 0.38 ng/g, 75% of samples detectable) were significantly lower than cortisol levels in the plasma (t3 = 7.93, P = 0.004). Cortisol levels in the breast muscle (0.91 ± 0.46 ng/g, 75% of samples detectable) were not different from cortisol levels in the plasma (t3 = 1.57, P = 0.22).

**DISCUSSION**

To our knowledge, this is the first study to measure 1) cortisol in the plasma of songbirds, 2) corticosterone or cortisol in the nervous system of songbirds, and 3) endogenous corticosterone or cortisol levels in the immune system of any species. In plasma, the ratio of corticosterone to cortisol increased with age. On P0, plasma corticosterone and cortisol levels were similar; however, within 3 days, corticosterone was more abundant. In contrast, cortisol was more abundant than corticosterone in the immune system at all three ages. Furthermore,

Table 2. Plasma glucocorticoid levels subtracted from tissue glucocorticoid levels

<table>
<thead>
<tr>
<th>Age</th>
<th>Corticosterone</th>
<th>Cortisol</th>
<th>Corticosterone</th>
<th>Cortisol</th>
<th>Corticosterone</th>
<th>Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bursa</td>
<td>2.49±0.72</td>
<td>46.27±14.23†</td>
<td>−1.69±1.72</td>
<td>15.63±4.79†</td>
<td>−5.64±0.99</td>
<td>2.68±0.82†</td>
</tr>
<tr>
<td>Thymus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>−5.13±0.59†</td>
<td>2.66±0.70†</td>
</tr>
<tr>
<td>Spleen</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>−3.55±0.99‡</td>
<td>5.92±1.43†</td>
</tr>
<tr>
<td>rTel</td>
<td>−2.04±0.42‡</td>
<td>−0.95±1.10</td>
<td>−2.35±0.53‡</td>
<td>−0.02±0.35</td>
<td>−4.70±0.82‡</td>
<td>−0.10±0.14‡</td>
</tr>
<tr>
<td>cTel</td>
<td>−0.58±1.69‡</td>
<td>8.51±6.60</td>
<td>−2.79±0.79‡</td>
<td>−0.05±0.26</td>
<td>−4.61±0.77‡</td>
<td>0.93±0.20‡</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>−2.42±0.51‡</td>
<td>−1.81±1.56</td>
<td>−2.79±0.79‡</td>
<td>0.74±0.69</td>
<td>−5.11±0.853‡</td>
<td>−0.05±0.19‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. P0, P3, and P30, day of hatch and 3rd and 30th days after hatch, respectively. rTel, rostral telencephalon; cTel, caudal telencephalon. †Tissue levels were significantly higher than plasma levels. ‡Tissue levels were significantly lower than plasma levels. ND, data are not available.

AJP-Regul Integr Comp Physiol • VOL 295 • JULY 2008 • www.ajpregu.org
In all three regions studied, neural corticosterone levels were lower than plasma cortisol levels. In contrast, neural cortisol levels were similar to plasma cortisol levels. These data suggest differential metabolism of circulating GCs by neural 11β-hydroxysteroid dehydrogenase (23). Alternatively, there may be differential active transport of corticosterone and cortisol out of the brain. For example, the multidrug resistance P-glycoprotein at the blood-brain barrier transports cortisol, but not corticosterone, out of the brain in mice and humans (30).

These results provide little evidence for local GC synthesis in the developing brain. However, there is some evidence that GC synthetic enzymes are expressed in the developing and adult rat brain (17, 43), so more studies are needed before this possibility can be excluded. For example, it is possible that the brain synthesizes GCs only after stress or only in specific regions.

**GC levels in the immune system.** In the immune system, cortisol levels in the bursa are higher than corticosterone levels in the bursa at all three ages. In addition, cortisol levels in the bursa are higher than cortisol levels in the plasma at all three ages. Very similar results were obtained for the thymus and spleen at P30. With age, cortisol levels in the bursa decreased, whereas corticosterone levels in the plasma increased, suggesting that local cortisol synthesis in the immune system declines as systemic corticosterone levels increase. The abundance of cortisol in avian immune tissues is quite surprising because traditionally the primary GC in birds has been thought to be corticosterone (24, 44, 77). Nonetheless, in birds, corticosterone binding globulin binds both corticosterone and cortisol with high affinity (76), and an intracellular glucocorticoid receptor (GR) binds both corticosterone and cortisol with high affinity (68 and C. Breuner, personal communication).

These data suggest local cortisol synthesis in the developing immune system. The present results are consistent with in vitro data demonstrating that the chicken bursa and thymus contain all the steroidogenic enzymes, including P450c17, to synthesize cortisol de novo from cholesterol (33). Also, studies in mice have measured steroidogenic enzyme proteins and activities in the thymus (34, 72). Although these previous studies examined enzyme activities in vitro or cultured thymic cells (34, 72), the present study fills an important gap by measuring endogenous corticosterone and cortisol levels in uncultured tissue. Taken together, the data raise the hypothesis that cortisol is synthesized de novo in the avian immune system as an “immunosteroid” (41), analogous to neurosteroids synthesized in the nervous system (10).

It is possible that high local cortisol levels are due to the sequestration of cortisol in these organs, rather than local synthesis. However, previous studies that demonstrate steroidogenic enzymes in the bursa and thymus support the hypothesis that high levels are due to synthesis and not just sequestration (33, 34, 72). Also, in preliminary studies, we have performed short-term (24 h) incubations of bursa and thymus in vitro and measured GCs in conditioned media. We detected high levels of cortisol, but low levels of corticosterone, in the conditioned media (K. Schmidt, Y. Yu, K. Soma, unpublished observations).

The functional significance of local GC synthesis in the immune system is an important issue. GCs play an important role in the regulation of lymphocyte development. The mutual antagonism model postulates that signaling through either the...
GR or the T cell receptor alone induces thymocyte apoptosis. However, when both receptors are simultaneously stimulated, their signals oppose one another, leading to positive selection and thymocyte survival (21, 70). In this model, GC levels that are either too high or too low can induce lymphocyte apoptosis (26). Interestingly, when transgenic mice that overexpress GRs specifically in thymocytes are adrenalectomized, GR overexpression still leads to a decrease in thymocyte number, even though systemic GCs have been removed (49). These data suggest a role for local GCs in thymocyte selection.

Because high levels of GCs can induce lymphocyte apoptosis, one possibility is that high local levels of GCs in the zebra finch immune system suppress the development of the costly adaptive immune system, thus shunting resources to rapid body growth during early development. Zebra finches exhibit a very fast “pace of life” and reach reproductive maturity at an early age (~P90; see Ref. 78). This life history strategy may favor reduced allocation of resources to the development of costly adaptive immune defenses (35). Thus a critical question is whether local GC production results in “high” or “intermediate” local GC levels in zebra finches. Comparative studies (35, 40) and experimental hormone manipulations (15) will be useful for resolving this issue.

Roles of corticosterone and cortisol? Corticosterone and cortisol are often viewed as interchangeable, and a common belief is that a species has just one predominant GC, corticosterone or cortisol. However, there are species that have similar levels of the two GCs in circulation, for example, New Zealand White rabbits (69). Another study of rabbits found that, at baseline, corticosterone was the predominant adrenal GC, but, after chronic ACTH treatment, cortisol became the predominant adrenal GC (31). Thus the primary circulating GC may be context-specific.

The present results suggest that the primary GC can also be organ-specific and age-specific. Our finding that cortisol levels are highest in early development is consistent with studies showing that the adrenal glands in embryonic and newly hatched chickens produce corticosterone and cortisol at similar levels, but cortisol production by the adrenals declines soon after hatch (25, 27, 28). Our results suggest that cortisol may have unique effects during early immune system development. Both cortisol and corticosterone administration decrease the weight of the bursa in young chickens, but cortisol causes more vacuolar spaces within the lymph follicle of the bursa, suggesting that cortisol has a stronger effect on bursa histology than corticosterone (13). Interestingly, in young chickens, GRs in the bursa have a higher affinity for cortisol than corticosterone (68).

At the molecular level, steroid transporters and receptors can differentiate between corticosterone and cortisol. In mice and humans, P-glycoprotein at the blood-brain barrier transports cortisol, but not corticosterone, out of the brain (30). Moreover, in rainbow trout, two GRs are present, and both GRs bind cortisol, the primary circulating GC in fish. However, only one GR (rainbow trout GR 2) binds 11-deoxycorticosterone and corticosterone, indicating that multiple GRs with differential affinities to corticosterone and cortisol can exist within a single organism (5).

Local vs. systemic signals. Prolonged high levels of GCs in the circulation can suppress body growth (20, 55), bone deposition, digestion, immune function (57), and feather growth (54). Low systemic GC levels in developing animals could be beneficial by minimizing the negative effects of high circulating GCs (37, 56, 60). Local GC production may provide GCs where they are needed, while avoiding the “costs” of high circulating GCs.

A similar hypothesis states that circulating testosterone levels are low in songbirds during the nonbreeding season to avoid the costs of high circulating testosterone (65, 75). In nonbreeding songbirds, sex steroids can be locally synthesized in the brain, to support the expression of aggression for defense of winter territories (66).

Extra-adrenal GC production. In addition to the immune system, evidence suggests extra-adrenal production of GCs in many organ systems (11). The genes for GC synthetic enzymes are expressed in the intestinal mucosa (7), heart (32), fetal lung (51), and skin (62).

Interestingly, corticotropin-releasing hormone (CRH) and ACTH are also locally expressed in the immune system, skin, and other tissues, potentially providing a mechanism for regulating local GC synthesis. In the immune system, ACTH is expressed in lymphocytes (4), and injection of a virus into hypophysectomized mice increases ACTH-immunoreactive lymphocytes in the spleen (63). CRH is also expressed in the thymus and spleen (2, 47). In the skin, there is evidence for local expression of CRH, CRH receptor, ACTH, ACTH receptor, and all the GC synthetic enzymes. This has been described as a “miniature hypothalamic-pituitary-adrenal axis” or “hypothalamic-pituitary-adrenal axis homolog” in the skin (48, 62, 79). Future studies will examine CRH and ACTH expression in bursa, thymus, and spleen and the effects of immune challenges.

Perspectives and Significance

The present data indicate that cortisol is present in the general circulation, immune system, and nervous system of developing zebra finches. Cortisol concentrations can be equal to or greater than corticosterone concentrations. Furthermore, circulating GC levels may not be indicative of local GC levels. Last, the ratio of corticosterone to cortisol is organ-specific and age-specific. Our results support the hypothesis that cortisol is an immunostereoid synthesized de novo from cholesterol in the developing avian immune system. Importantly, our results suggest that the identities of locally produced steroids may differ from the identities of systemic steroids. Future studies should measure mRNA for enzymes involved in GC synthesis in the developing songbird immune system and confirm high cortisol levels in the immune system using liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry.

ACKNOWLEDGMENTS

We thank L. Sheldon, A. Shah, and A. Newman for assistance and D. Pradhan, A. Newman, and Dr. L. Martin for comments on the manuscript. We thank Drs. G. Miller, E. Chen, and J. Weinberg for the use of their equipment and Dr. K. Wynne-Edwards for technical advice.

GRANTS

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Michael Smith Foundation for Health Research, and the Canada Foundation for Innovation to K. K. Soma and a NSERC Canada Graduate Scholarship to K. L. Schmidt.
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