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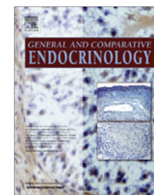

Corticosterone metabolism by chicken follicle cells does not affect ovarian reproductive hormone synthesis *in vitro*

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ABSTRACT

Glucocorticoids affect reproductive hormone production in many species. In chickens, elevated plasma corticosterone down-regulates testosterone and progesterone concentrations in plasma, but also in egg yolk. This suppression could be mediated via the hypothalamic-pituitary system but also via local inhibition of gonadal activity by glucocorticoids. As the latter has not been tested in birds yet, we tested if corticosterone directly inhibits ovarian steroid synthesis under *in vitro* conditions. We hypothesized that degradation of corticosterone by follicular cells impairs their ability to synthesize reproductive hormones due to either inhibition of enzymes or competition for common co-factors. Therefore, we first established whether follicles degrade corticosterone. Follicular tissue was harvested from freshly euthanized laying hens and incubated with radiolabelled corticosterone. Radioactive metabolites were visualized and quantified by autoradiography. Follicles converted corticosterone in a time-dependent manner into metabolites with a higher polarity than corticosterone. The predominant metabolite co-eluted with 20β-dihydrocorticosterone. Other chicken tissues mostly formed the same metabolite when incubated with corticosterone. In a second experiment, follicles were incubated with either progesterone or dehydroepiandrosterone. Corticosterone was added in increasing dosages up to 1000 ng per ml medium. Corticosterone did not inhibit the conversion of progesterone and dehydroepiandrosterone into a number of different metabolites, including 17α-hydroxyprogesterone, androstenedione and testosterone. In conclusion, avian tissues degrade corticosterone mostly to 20β-dihydrocorticosterone and even high corticosterone dosages do not affect follicular hormone production under *in vitro* conditions.

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1. Introduction

Ample research on hormone content in birds' eggs has shown that environmental conditions experienced by the avian mother affect reproductive hormone concentrations in her eggs, which in turn influence the phenotype of the developing chick (Gil, 2003; von Engelhardt and Groothuis, 2011). These hormone- mediated maternal effects have been suggested to be an epigenetic mechanism to maximize reproductive success (Groothuis et al., 2005). However, the physiological mechanism allowing the female to modulate hormone content of her eggs is still elusive (Groothuis and Schwabl, 2008). In mammals, glucocorticoids exert suppressive effects on reproductive steroid hormone production of the gonads (Hardy et al., 2005; Moberg, 1991; Rivier and Rivest, 1991; Tilbrook et al., 2000). Accordingly, stressful conditions experienced by a female bird seem to change the hormone content of her eggs (Bertin et al., 2008; Henriksen et al., 2011b; Janczak et al., 2009; Okuliarova et al., 2010). In a recent experiment, Henriksen et al. Henriksen et al. (2011a) showed that elevated concentrations of circulating corticosterone lead to a decrease of reproductive hormones both in plasma and egg: Laying hens with corticosterone-releasing implants not only had lower plasma testosterone and progesterone levels than placebo implanted control females, they also produced eggs that contained less yolk testosterone and progesterone. It was thus concluded that corticosterone suppresses ovarian steroid hormone synthesis in chickens. However, the exact mode of action of glucocorticoids on the ovary's hormone production was not addressed to date in avian species.

Based on mammalian research, glucocorticoids can affect gonadal function at multiple levels (Whirledge and Cidlowski, 2010). Glucocorticoids decrease synthesis and release of gonadotropin-releasing hormone (GnRH) from the hypothalamus by disrupting the GnRH pulse frequency (Bambino and Hsueh, 1981; Oakley

Abbreviations: DHEA, dehydroeipandrosterone; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; HSD, hydroxysteroid dehydrogenase; LH, luteinizing hormone; P4, progesterone.

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et al., 2009), but can also modulate circulating levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by inhibiting pituitary responsiveness to GnRH (Breen and Karsch, 2006; Matsuwaki et al., 2006; Saketos et al., 1993). Glucocorticoids can also exert direct action on the gonads themselves (Michael and Cooke, 1994; Tetsuka, 2007). This local effect of glucocorticoids on gonadal steroidogenesis is most likely receptor-mediated as effects can partly be prevented by blocking the glucocorticoid receptor with an antagonist (Dong et al., 2004; Orr and Mann, 1992). Corticosterone inhibits the enzymes 3β- and 17β-hydroxysteroid dehydrogenase (HSD) in Leydig cells (Orr et al., 1994; Sankar et al., 2000b). On the molecular level it was found that excess corticosterone suppresses mRNA expression of 3β-HSD1 and 17β-HSD3 enzymes (Badrinarayanan et al., 2006). This local suppressive effect of glucocorticoids on enzyme activity or availability greatly depends on the ability of the cells to modulate or regulate the amounts of glucocorticoids present. Most tissues convert glucocorticoids into their inactive 11-oxo-forms (Seckl and Walker, 2004; Tetsuka et al., 1999). For this oxidation, the presence of a co-factor that can be reduced is necessary. High amounts of glucocorticoids might thus lead to a shortage of co-factors which are also necessary for sex steroid synthesis, thereby decreasing enzymatic activity (Whirledge and Cidlowski, 2010). Latif et al. Latif et al. (2011) suggested that 11β-HSD1 is enzymatically coupled to 17β-HSD3, utilizing NADPH and NADP in intermeshed regeneration systems. Kavitha et al. Kavitha et al. (2006) showed that the inhibitory effect of corticosterone on Leydig cell steroidogenesis is mediated through defective co-factor generation, resulting in NADPH shortage caused by the involvement of corticosterone on glucose oxidation.

As the majority of the performed investigations used mammalian species, in birds knowledge about physiological mechanisms is much more fragmentary. It is however likely that glucocorticoids inhibit reproductive hormone production in birds also via at least two of the pathways described above: Circulating LH concentrations decreased due to glucocorticoid elevation (Etches et al., 1984; Goutte et al., 2010) indicating suppressive effects on the hypothalamic-pituitary level. It was also found that stress downregulates reproductive hormones' concentrations in chickens without affecting plasma levels of LH and FSH (Rozenboim et al., 2007), indicating a direct modulation of ovarian function. To our knowledge, the local action of glucocorticoids on the gonads has not been addressed to date in birds. We therefore investigated if corticosterone exerts an inhibitory effect on ovarian steroid hormone synthesis in the chicken ovary under *in vitro* conditions. We hypothesize that the degradation of corticosterone by enzymes present in the follicular cells inhibits the production of reproductive hormones by either competition for common co-factors or by inhibition of specific enzymes. We performed two experiments: In the first experiment we tested if corticosterone is metabolized by chicken follicle cells, thereby confirming the presence of enzymes and co-factors necessary for degradation. We incubated follicular tissue with radiolabelled corticosterone. The formed metabolites were separated via thin-layer chromatography and visualized and quantified by autoradiography. Tissues from other organs were also incubated with radiolabelled corticosterone to assess whether the formed metabolites are tissue-specific for the ovary. In the second experiment we investigated the enzymatic conversion of radiolabelled progesterone (P4) and dehydroepiandrosterone (DHEA) by follicular tissue in the presence of corticosterone. Unlabelled corticosterone was added in increasing concentrations to the cell culture medium. We chose the steroid hormones P4 and DHEA as precursor hormones to be able to differentiate if corticosterone selectively inhibits specific enzymes (17α-hydroxylase and 17,20-lyase for conversion of P4 into androstenedione and 3β-HSD for conversion of DHEA into androstenedione). Investigations were carried out using ovarian follicles of different sizes in both experiments, because the enzymatic capacities of birds' follicles change during the course of maturation (Tilly et al., 1991).

2. Materials and methods

2.1. Animals

We used 16 female and 3 male adult laying hybrids that were offspring from chickens bred for another experiment (Henriksen et al. *in prep.*). Due to the set-up of the previous experiment all birds were cross-breeds between white Leghorn, ISA brown and Rhode Island Red laying hybrids. They were aged 37 weeks, housed in same-sex groups in open aviaries and received commercial layer mash and water *ad libitum*. All females regularly laid eggs. Average weight of the females was 2.2 ± 0.2 kg (mean ± sd). All birds were euthanized by intravenous injection of a barbiturate overdose followed by decapitation.

2.2. Experiment 1- metabolism of radiolabelled corticosterone by chicken tissues

Four females were used for experiment one. Immediately after euthanasia, the females' ovaries were harvested. Three different sized follicles with mean (±sd) diameters of 34 ± 1 mm, 26 ± 2 mm and 10 ± 2 mm (referred to as F1, F2 and F3) respectively, were collected from each female. The follicles were cleaned from connecting tissue, opened and everted. By rinsing the tissue with pre-warmed physiological saline solution, yolk basal lamina and granulosa cells were flushed out (Gilbert et al., 1977). The remaining theca layers were washed with saline solution to remove any left over yolk and divided into tissue fragments of 0.05 g. Each tissue fragment was incubated in 1 ml medium (Eagles MEM) which contained 42,000 Bq per ml of radiolabelled corticosterone (NET-399; [1267-^{3}H(N)]- corticosterone; 2830.5 GBq/mmol; obtained from Perkin Elmer, MA, USA). Samples were incubated in a water bath at 39 °C. Of every vial 0.2 ml were taken out after 1 h, 2 h, 4 h and 8 h of incubation respectively, and frozen immediately at −20 °C. Samples were transferred to the lab on dry ice. We also incubated radiolabelled corticosterone without any tissue material in medium only as control incubations, to assess a possible spontaneous degradation of corticosterone. Tissues from three adult males were incubated as described above for the follicle tissue. From each male, 0.05 g of tissue from testes, liver, lung, kidney, muscle, brain, adrenals and fat were collected and incubated in 1 ml medium, each together with the radiolabelled corticosterone. Again, 0.2 ml were taken out of each incubating vial after 1 h, 2 h and 4 h and frozen immediately.

2.3. Experiment 2- inhibitory effects of corticosterone on ovarian hormone synthesis

In total, twelve females were used for the second experiment. Again, three different sizes of follicles with mean (±sd) diameters of 33 ± 2 mm 24 ± 2 mm and 8 ± 1 mm (referred to as F1, F2 and F3) respectively, were prepared as described above. From all follicles, three tissue fragments of 0.05 g each were incubated in 1 ml cell culture medium. Unlabelled corticosterone was added to the media in three different concentrations (0 ng/ml, 100 ng/ml and 1,000 ng/ml). Subsamples of each six females were incubated with either 8,000 Bq per ml radio-labelled 4-pregnene-3,20-dione (P4) or 3β-hydroxyandrostene-17-one (DHEA), all radiolabelled hormones from Perkin Elmer, MA, USA). All samples were incubated for four hours at 39° C and frozen afterwards.

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