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Review

Advances in conservation endocrinology: The application of molecular approaches to the conservation of endangered species

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ABSTRACT

Among the numerous societal benefits of comparative endocrinology is the application of our collective knowledge of hormone signaling towards the conservation of threatened and endangered species – conservation endocrinology. For several decades endocrinologists have used longitudinal hormone profiles to monitor reproductive status in a multitude of species. Knowledge of reproductive status among individuals has been used to assist in the management of captive and free-ranging populations. More recently, researchers have begun utilizing molecular and cell-based techniques to gain a more complete understanding of hormone signaling in wildlife species, and to identify potential causes of disrupted hormone signaling. In this review we examine various *in vitro* approaches we have used to compare estrogen receptor binding and activation by endogenous hormones and phytoestrogens in two species of rhinoceros; southern white and greater one-horned. We have found many of these techniques valuable and practical in species where access to research subjects and/or tissues is limited due to their conservation status. From cell-free, competitive binding assays to full-length receptor activation assays; each technique has strengths and weaknesses related to cost, sensitivity, complexity of the protocols, and relevance to *in vivo* signaling. We then present a novel approach, in which receptor activation assays are performed in primary cell lines derived from the species of interest, to minimize the artifacts of traditional heterologous expression systems. Finally, we speculate on the promise of next generation sequencing and transcriptome profiling as tools for characterizing hormone signaling in threatened and endangered species.

1. Introduction

Comparative endocrinology, defined here as the study of hormone signaling across a variety of taxa, provides numerous benefits to society. Most apparent among these benefits may be the insight into evolutionary history that the similarities and differences in hormone signaling mechanisms across major taxonomic groups provide. For instance, comparative studies of the protein sequences of nuclear steroid receptors has revealed numerous functional and phylogenetic relationships among extant vertebrates species (Thornton, 2001). In addition, these sequences have been used to reconstruct the ancestral steroid receptors giving rise to this broadly significant gene family and to examine the evolutionary forces driving receptor–ligand specificity (Eick et al., 2012; Thornton et al., 2003). Comparative endocrinology research also finds itself at the forefront of novel scientific discovery. One example is mPRα, the first membrane bound, non-classical steroid hormone receptor identified for any vertebrate species. First cloned from the ovary of the spotted seatrout (*Cynoscion nebulosus*), a common estuarine fish, mPRα is an intermediary in progestin induction of oocyte maturation (Zhu et al., 2003b). In other species, including humans, mPRα and related receptors appear to be involved in a wide array of physiological functions, many of which are likely of biomedical importance (Dressing et al., 2012; Thomas, 2012; Thomas and Pang, 2013; Zhu et al., 2003a).

Perhaps not as widely recognized is the contribution that comparative endocrinology has made, and continues to make, towards the conservation of threatened or endangered species. For example, hormone concentrations are used to assess the current reproductive status of an individual based on previously characterized endocrine profiles for the reproductive cycle of that species. Determination of the reproductive status of a representative sample of a population can be especially useful for resource managers attempting to model changes in population size over time. Threats to

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long-term population viability can be more readily identified when the data are compared to endocrine data collected previously or from similar populations. Furthermore, a reliable method of assessing fecundity leads to more effective utilization of limited resources – particularly in intensively managed populations, such as those found in zoos, aquariums, and some parks and reserves. The utility of using hormone concentrations to assess and/or manage populations is well demonstrated in the African elephant (*Loxodonta africana*). The female African elephant reproductive cycle has been well characterized in terms of changing hormone concentrations over the course of the ovarian cycle and gestation (Hildebrandt et al., 2011). This knowledge is routinely applied in the form of serum, urine, or fecal progestagen monitoring at zoos housing mixed-sex herds to detect reproductive activity. Recently, Benavides Valades et al. (2012) utilized our collective understanding of elephant reproductive endocrinology to evaluate immunocontraception of free-ranging cows on a South African game reserve. The efficacy of a gonadotropin-releasing hormone (GnRH) vaccine was evaluated based on fecal progestagen concentrations as an indicator of luteal activity. Interestingly, the vaccine, which has been used successfully in a wide range of mammals, failed to induce anestrus in African elephants, further underscoring the need for more comparative studies.

Historically, comparative endocrinologists have focused largely on associations between various hormone concentrations and physiological phenomena. This is especially true for those studying endangered species, for which biological samples are often limited to those that can be collected non-invasively, such as urine, feces or saliva. However, more recent work has begun to investigate other aspects of hormone signaling responsible for maintaining homeostasis and regulating essential functions of life – in particular, hormone receptors. Characterizing hormone receptors, elucidating the mechanisms regulating their expression, and understanding the cellular responses to hormone receptor–ligand interactions have shed new light on hormone-mediated processes. For instance, androgen-dependent territorial behavior in spotted antbirds (*Hylophylax naevioides*) persists year-round despite lower testosterone concentrations during the nonbreeding season. Elevated mRNA expression of androgen receptor (AR) and estrogen receptor α (ESR1) in the brain during the nonbreeding season suggests increased sensitivity to sex steroids plays a role in maintaining aggressive behavior in this species (Canoine et al., 2007). Although pioneering work on hormone binding sites in wildlife species was conducted in the 1970s (e.g., Licht and Midgley, 1976), only recently have the tools required to study hormone receptor–ligand interactions at the molecular level become widely accessible to the comparative and conservation-oriented endocrinologist. These tools include molecular cloning, recombinant protein expression, cell culture, and various measures of mRNA, protein, and/or metabolite expression. In this review we provide a survey of the application of molecular endocrinology aimed at increasing our understanding of hormone receptor signaling in wildlife species. In addition, we will present a few of the successes and potential shortcomings of various approaches that we have employed in our efforts to examine the role of dietary phytoestrogens in the poor reproductive success of captive southern white rhinoceros (SWR, *Ceratotherium simum simum*).

2. Molecular cloning and recombinant receptors

Molecular cloning technology has revolutionized our ability to biochemically and functionally characterize hormone receptors in threatened and endangered species. Traditional methods of characterizing hormone receptors began by isolating and purifying receptors from relatively large quantities of tissue. The availability of the tissue required for such methods is generally limited in species that are listed as threatened or endangered. On the other hand, small amounts of tissue expressing the mRNA of the receptor of interest can be used to isolate complete coding sequences and generate unlimited quantities of recombinant receptors. Small quantities of high quality tissues can be obtained through blood samples and tissue biopsies with minimal risk to the subject. The recombinant receptors generated from these samples can then be used to characterize ligand binding and receptor activation. This approach has been shown to be particularly useful in the arena of endocrine disruption, where inappropriate hormone signaling can be caused by the interaction of hormone receptors and exogenous ligands (Katsu et al., 2013; Naidoo et al., 2008; Tubbs et al., 2012).

2.1. Recombinant receptor binding assays

Identifying potential ligands of a receptor and determining the affinity of that receptor for those ligands provides valuable insight into the receptor's function. Competitive binding assays, in which labeled and unlabeled ligand compete for a limited quantity of recombinant receptor, can be used to partially assess the sensitivity of the receptor to various ligands relative to some standard, which is typically the putative endogenous ligand. This is particularly useful for screening exogenous compounds for species- and receptor-specific endocrine-disrupting capabilities (Rider et al., 2009). Competitive binding assays using recombinant receptors in cell-free systems were used to screen potential endocrine-disrupting chemicals for interactions with alligator and human ESR1 (Rider et al., 2010). Several xenoestrogens exhibited species-specific affinities for ESR1, with *p,p′*-dicofol displacing 50% of the estradiol tracer from alligator ESR1 at 4 μM, an order of magnitude lower than the concentration required to do the same in human ESR1.

The ability of an exogenous compound to inhibit the binding of a bona fide ligand to its receptor does not necessarily confer agonistic or antagonistic activity to that compound. However, this approach is useful for rapidly screening a large number of compounds or complex mixtures for potential endocrine-disrupting activity. If a compound is capable of inhibiting the binding of a known ligand, further investigation is required to determine if the compound stimulates or inhibits receptor-dependent activity at physiologically relevant concentrations. We have employed this approach with white rhinoceros and greater one-horned rhinoceros (*Rhinoceros unicornis*; GOHR) ESRs to quantify receptor binding by phytoestrogens and estrogenic substances in extracts of components of captive diets (i.e., commercial pellets and hays) and extracts of grasses predominantly consumed by these species in the wild (Tubbs et al., 2012) (Tubbs et al., *unpublished obs.*).

2.2. Recombinant receptor activation assays

Recombinant receptor activation studies are an emerging tool for studying the comparative endocrinology of endangered species *in vitro*. In contrast to receptor binding assays, activation assays allow one to discern agonist or antagonist capability of potential ligands. Whereas binding assays are relatively inexpensive and cell-free ligand–receptor binding reaches equilibrium in hours, activation assays are dependent upon transcription and translation occurring in cultured cell lines, adding to both their time and cost. Nevertheless, receptor activation assays are able to detect species-specific differences in receptor function that are not detectable by binding assays alone. For example, when investigating phytoestrogen interactions with rhinoceros ESRs, we found no difference in binding of coumestrol or daidzein between SWR and GOHR receptors. However, activation of SWR ESR1 by daidzein and SWR ESR1 and estrogen receptor β (ESR2) for coumestrol was significantly higher than the respective GOHR ESRs (Tubbs et al., 2012). In