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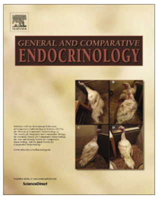

Research paper

Regulation of plasma glucose and sulfate excretion in Pacific hagfish, *Eptatretus stoutii* is not mediated by 11-deoxycortisol

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The goal of this study was to identify whether Pacific hagfish (*Eptatretus stoutii*) possess glucocorticoid and mineralocorticoid responses and to examine the potential role(s) of four key steroids in these responses. Pacific hagfish were injected with varying amounts of cortisol, corticosterone or 11-deoxycorticosterone (DOC) using coconut oil implants and plasma glucose and gill total-ATPase activity were monitored as indices of glucocorticoid and mineralocorticoid responses. Furthermore, we also monitored plasma glucose and 11-deoxycortisol (11-DOC) levels following exhaustive stress (30 min of agitation) or following repeated infusion with SO_4^{2-}. There were no changes in gill total-ATPase following implantation with any steroid, with only very small statistical increases in plasma glucose noted in hagfish implanted with either DOC (at 20 and 200 mg kg^{-1} at 7 and 4 days post-injection, respectively) or corticosterone (at 100 mg kg^{-1} at 7 days post-injection). Following exhaustive stress, hagfish displayed a large and sustained increase in plasma glucose. Repeated infusion of SO_4^{2-} into hagfish caused increases in both plasma glucose levels and SO_4^{2-} excretion rate suggesting a regulated glucocorticoid and mineralocorticoid response. However, animals under either condition did not show any significant increases in plasma 11-DOC concentrations. Our results suggest that while there are active glucocorticoid and mineralocorticoid responses in hagfish, 11-DOC does not appear to be involved and the identity and primary function of the steroid in hagfish remains to be elucidated.

1. Introduction

There has long been debate on the true phylogenetic relationship between the two extant groups of cyclostomes, the lamprey and hagfish (Janvier, 2010). Past morphology-based approaches suggested paraphyly with the lamprey being more closely related to the jawed vertebrates, gnathostomes, than the hagfish (Janvier, 2010). Contrarily, current molecular evidence provides support for monophyly with both groups forming a single clade separating from the vertebrate lineage evolution (Heimberg et al., 2010) although, the strength of these assumptions have recently been questioned (Thomson et al., 2014). A monophyletic cyclostome clade would indicate that the ancestral vertebrates were more complex than originally thought with the lamprey retaining a number of features that are present in gnathostomes and the hagfish undergoing an unprecedented loss of vertebrate features. Alternatively, the lamprey would have had to develop key physiological traits in a convergent evolutionary context with the gnathostomes, but also possess the genetic features at divergence to allow for this parallel evolution, suggesting that hagfish and lamprey diverged early on following the emergence of the cyclostome clade around 525MYA (Near, 2009).

Two examples of physiological and endocrinological processes that have either been lost, or appear more primitive in hagfish are ionoregulation and the hypothalamic-pituitary axis (HPA). When comparing ionoregulatory strategies of lamprey and hagfish, lamprey are euryhaline with blood ion concentrations similar to the rest of the vertebrate lineage at $\sim{}^{1}/_{3}$ seawater (Smith, 1932), while hagfish are strictly marine in their evolutionary history

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(Bardack, 1991). Hagfish are particularly unique within the vertebrates as their blood plasma is similar to seawater with respect to Na^+ and Cl^- concentrations (Bellamy and Jones, 1961). Furthermore, hagfish are stenohaline and thus cannot regulate Na^+ and Cl^- when placed in media differing in salt composition from seawater (Robertson, 1954; Sardella et al., 2009). Hagfish do, however, regulate the divalent ions sulfate (SO_4^{2-}), magnesium (Mg^{2+}) and calcium (Ca^{2+}), giving support to the hypothesis that these divalent cations were among the first ions to be actively regulated in vertebrates (Bellamy and Jones, 1961; Clifford et al., 2015a). It has been proposed that the early steroids and receptors used for this mineral regulation have been adopted for regulation of the monovalent ions (Na^+, Cl^-, K^+; Sardella et al., 2009)

The emergence of the hypothalamic-pituitary axis (HPA) is specific to vertebrates and is a key event in the evolution of divergent physiological processes (e.g. reproduction, growth, metabolism, stress response and osmoregulation; Bury et al., 2015). Current evidence suggests that hagfish possess a far less morphologically distinct HPA compared to lamprey (Uchida et al., 2010) in that there is no apparent partitioning of the pars distalis and pars intermedia of the pituitary gland as observed for lamprey and all the gnathostomes (*e.g.* Nozaki et al., 2007). A number of the corticotropins, melanotropins and gonadotropins that are released from the pituitary to stimulate steroid hormones synthesis have been described in lamprey (Sower et al., 2006; Takahashi et al., 1995) but have yet to be definitively identified in hagfishes (Uchida et al., 2010).

The HPA controls the synthesis of a number of steroid hormones that circulate the body and bind to nuclear hormone receptors to act as ligand inducible transcription factors, examples of which are the sex [estrogen (ER), androgen (AR) and progesterone receptors (PR)] and adrenal hormone receptors [the two corticoid receptors (CR); glucocorticoid (GR) and mineralocorticoid receptors (MR)] of the gnathostomes. The origins of these groups of receptors can be traced back to the estrogen “like” receptor (ERR) present in some groups of Protostomes, e.g. the molluscs and annelids (Keay et al., 2006; Keay and Thornton, 2009). These early ERR receptors are constitutively active and their mode of regulation remains unclear (Keay et al., 2006; Keay and Thornton, 2009). In the cephalochordate *Branchiostoma floridae*, an ERR (Paris et al., 2008), ER and steroid receptor (SR) have been reported (Bertrand et al., 2011). The SR is expressed in the gonads (Callard et al., 2011) and is activated by estrogens (Katsu et al., 2013), and negatively regulated by the ER (Callard et al., 2011), this suggests that the ancestral role of the SR may be to regulate reproduction.

Whole genome duplication (WGD) events in the early vertebrate lineage has lead to the emergence of 3 distinct hormone receptors the CR, ER and PR in cyclostomes (Bridgham et al., 2006; Rossier et al., 2015). The cloning of these receptors and *in vitro* assays has identified the receptors hormone binding and transactivation profiles. The CRs of lamprey and Atlantic hagfish are promiscuous, being activated by a number of different corticoids including cortisol, 11-deoxycorticosterone (DOC), 11-deoxycortisol (11-DOC) and corticosterone (Bridgham et al., 2006). However, even though these *in vitro* assays have proven invaluable in identifying potential mechanisms of protein evolution at a molecular level (Bridgham et al., 2009) they do not provide information on the actual active hormones *in vivo* or the evolution of their physiological role (Close et al., 2010). For example, even though the lamprey CR is activated by a wide range of corticoids *in vitro*, Close et al. (2010), recently identified that only 11-DOC is elevated in the plasma of lamprey and that this hormone performs both a classical GR role, controlling gluconeogenesis, and a MR role, regulating ion homeostasis.

The clear divergence in ionoregulatory strategies between these two agnathan taxa and the knowledge that steroid hormones regulate ion balance in lamprey and more derived vertebrates provides the impetus to reevaluate steroid function in hagfish. In this study, we aim to identify the presence of classical corticosteroid hormone responses in the Pacific hagfish (*Eptatretus stoutii*) through either *in vivo* perturbations (handling or elevation of plasma sulfate *via* injection) or *via* hormonal implants followed by monitoring of classical glucocortoicoid (plasma glucose) and mineralocorticoid (gill ATPase activity and plasma sulfate regulation) responses. We utilized administration of cortisol, DOC, 11-DOC and corticosterone via coconut oil implants in order to investigate their potential to either directly cause a corticosteroid response or induce a response following steroidal biotransformation. Plasma 11-DOC levels were measured following *in vivo* perturbations. Furthermore, analysis of a hagfish transcriptome allowed us to identify the presence of specific enzymatic elements of the corticosteroid biosynthesis pathway.

2. Materials and methods

2.1. Experimental animals and holding

Pacific hagfish (*Eptatretus stoutii;* 65–227 g) were captured near Bamfield, BC, Canada and held at Bamfield Marine Sciences Centre (BMSC) as previously described (Clifford et al., 2015b; Schultz et al., 2014). Fish remained unfed during captivity and were used for experimentation within 2 weeks of capture. All animals were used under the licenses of Department of Fisheries and Ocean Canada collection permits XR 214 2007, XR 214 2010, XR 214 2011 and XR 214 2013 and Bamfield Marine Science Centre Animal Care protocol numbers BMSC RS 10-42, RS 11-26, and RS-13-24.

2.2. Chemicals

Unless noted, reagents and enzymes were supplied by Sigma-Aldrich (St. Louis, MO). Cortisol, 11-DOC, DOC, and corticosterone were obtained from commercial suppliers (Stereloids, USA). Coconut oil was purchased from a local health food store. Tricaine methanesulfonate (TMS) was obtained from Syndel laboratories (Nanaimo, BC, Canada).

2.3. Experiment 1 – exogenous elevation of plasma hormone concentrations

To induce elevated plasma hormone concentrations, hagfish were administered molten coconut oil (kept at 27 °C prior to injection) impregnated with cortisol, corticosterone or DOC at dose of 20, 100 or 200 mg kg^{-1} hagfish. Briefly, animals were lightly anaesthetized in seawater containing TMS (0.75 g L^{-1}) buffered with 1.5 g L^{-1} sodium bicarbonate for 3–5 min. The animals were then removed from the water and were held vertically causing pooling of blood in the caudal subcutaneous sinus within 20 s. A 200 µL control blood sample was removed from the sinus with a heparinized 21G needle and 1 mL disposable syringe, centrifuged briefly (30 s, 14,000*g*) and the plasma was removed and rapidly frozen on liquid nitrogen (-80 °C) for later analysis. The animals were then laid on a flat surface and steroidal implants were placed in the body cavity approximately 3 in. caudal to the last branchial pore using a warm (27 °C) 18*g* needle and 3 mL syringe. All implant concentrations were administered at a dose equivalent to 2% of body mass. Coconut oil injections without steroid served as a control for all experiments. Blood samples were also removed 4 and 7 days post-implantation. Confirmation of successful placement of the implant was evaluated visually upon termination and dissection. Plasma cortisol and glucose concentrations were measured 4 and 7-days post-implantation, whereas total gill ATPase activities

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