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Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii:* Evidence for the involvement of Rh and UT proteins

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ABSTRACT

The nature of ammonia and urea excretion was examined in the Pacific hagfish (*Eptatretus stoutii*), which, under resting conditions, excreted similar quantities of nitrogen as either ammonia or urea. In the presence of high external ammonia (HEA) concentrations, ammonia was taken up at high rates and then excreted at similarly high rates upon return to normal water. However, although elevated by HEA, plasma ammonia levels were maintained at approximately 1–4 μmol $N g^{-1}$, reflecting time-dependent decreases in the rates of ammonia uptake, the possible conversion of ammonia to urea, and the potential active excretion of ammonia against a gradient. Internal injections of NH_4Cl caused marked increases in the rate of ammonia excretion and a delayed increase in urea excretion that may have resulted from increasing urea levels in the plasma. Conversely, when the rate of urea excretion was reduced in the presence of 0.1 mM phloretin, ammonia excretion was significantly elevated. Rates of urea excretion were initially increased by approximately 1000-fold following internal urea injections while the presence of high external urea levels (5–100 mM final concentration) resulted in associated linear increases in plasma urea levels. Using hagfish skin mounted in Ussing chambers, the rate of diffusion of ammonia across the skin exceeded that of urea by approximately four times when equivalent gradients were imposed. Based on western blotting and immunocytochemistry, hagfish gill appears to possess Rh proteins (Rhag, Rhbg and Rhcg1) and urea transporter proteins. Despite the tolerance of hagfish to high levels of ammonia and urea, it is suggested that the presence of ammonia and urea transporter proteins may be required during the period of time hagfish spend in burrows or while feeding, when conditions of high ammonia and/or urea might be encountered.

1. Introduction

With the exceptions of the ureotelic (excreting mainly urea) elasmobranchs and a few specialized teleosts [e.g. Gulf toadfish, *Opsanus beta* (Walsh et al., 1990) and Lake Magadi tilapia, *Oreochromis alcalicus graham* (Randall et al., 1989)], fish in aquatic environments typically excrete the majority of their nitrogenous waste as ammonia [ammonotely; see review by Wood (2001)]. While ammonia and urea were once thought to move passively through tissues, it is now known that both urea and ammonia require transporters for efficient transcellular movement; urea via urea transporter (UT) proteins (Levine et al., 1973a; Levine et al., 1973b; You et al., 1993) and ammonia via Rh proteins (Marini et al., 1997). In adult teleosts including zebrafish (*Danio rerio*; Braun et al., 2009a; Braun et al., 2009b), rainbow trout (*Oncorhynchus mykiss*; Hung et al., 2008; Tsui et al., 2009), pufferfish (*Takifugu rubripes*; Nakada et al., 2007b), Japanese eel (*Anguilla japonica*; Mistry et al., 2001) and toadfish (*O. beta*; McDonald et al., 2009), both UT and Rh proteins are concentrated on the gill epithelium, the primary site of ionic and osmotic regulation in fish.

The regulation of branchial urea and ammonia excretion, although extensively studied for years (e.g. Walsh and Henry, 1991; Wood, 1993; Wright, 1995; Wilkie, 2002), is now being re-evaluated in the light of the discovery of UT and Rh proteins (Wright and Wood, 2009; Weihrauch et al., 2009). For both urea and ammonia, the rates of excretion are thought to reflect, at least in part, the numbers or availability of transporters/channels as well as modifying neurohumoral influences (e.g. the stimulatory effect of serotonin or arginine vasotocin (AVT) on urea excretion in toadfish (Perry et al., 1998; McDonald and Walsh, 2004)) or accessory transport processes. For example, in zebrafish, the rate of ammonia excretion across the branchial apical membrane through Rhcg1 is thought to be linked to acidification of the boundary layer water (Wright et al., 1989) via the associated H^+-VATPase (Shih et al., 2008; Braun et al., 2009b).

The jawless hagfish are the most primitive of extant craniates and are thought to share features with ancient vertebrate ancestors. However, despite the potential insights they might provide into evolution of vertebrate physiology, their isolated habitats often at

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great depths and unpleasant defence mechanisms (sliming) make them relatively poorly studied. Indeed, some of the most basic of physiological mechanisms such as control of breathing have only recently been described (Perry et al., 2009b). While a detailed life history of most hagfish species is not particularly well known, their existence as burrowing scavengers in the deep ocean suggests a physiology that is both exceptionally tolerant and adaptable.

Currently, most of the processes underlying excretion in hagfish are unknown. While various measurements of excretory processes in hagfish have been recorded for decades, the results of these studies varied widely in parameters such as plasma levels of ammonia and urea (Borei, 1935; Robertson, 1954; McFarland and Munz, 1958; Robertson, 1966; McDonald et al., 1991). Recent work appears to show that they, like most fish, excrete the majority of their nitrogenous waste as ammonia, with only trace amounts of urea (Walsh et al., 2001). However, the branchial epithelium in hagfish is relatively thin (Mallatt and Paulsen, 1986) and the plasma is nearly isoosmotic with seawater (Robertson, 1954; McFarland and Munz, 1958), suggesting that the barriers to the movement of solutes, including ammonia and urea, may be small, therefore decreasing the requirements for transporters. In fact, while a search for Rh proteins has not yet been undertaken, a search for UT mRNA in hagfish proved fruitless (Walsh et al., 2001).

Studies of the hagfish gill over the past few decades have revealed that the gills are more complex than previously thought, possessing an intricate morphology (Mallatt and Paulsen, 1986) composed of a wide suite of specialized cells housing a variety of ionoregulatory pumps and transporters including Na^+/H^+ exchanger isoform 2 (NHE2), Na^+K^+-ATPase (NKA), V-type H^+-ATPase and Cl^-/HCO_3^- exchangers (Edwards et al., 2001; Choe et al., 2002; Tresguerres et al., 2006). In fact, the number of ionocytes in the gills of Pacific hagfish (*Eptatretus stoutii*) is comparable to the gills of teleosts (Mallatt and Paulsen, 1986). This, of course, begs the question, why do animals which are isoionic for monovalent ions need so many ionocytes? While it may be true that hagfish do not actively regulate plasma levels of Na^+ and Cl^-, it is well known that they modulate acid/base disturbances (Evans, 1984; McDonald et al., 1991) and actively maintain divalent ions at significantly different levels from those in seawater.

In teleosts, Rh proteins are found within specific tissue layers of the gills to form a route for net ammonia movement across the gill (Nakada et al., 2007b; Braun et al., 2009b). For example, in the pufferfish (Nakada et al., 2007b) and zebrafish (Braun et al., 2009b) gill, ammonia movement from the blood to water is facilitated by the sequential passage of ammonia from Rhag to Rhbg to Rhcg1/Rhcg2; a similar pattern is found in the mammalian kidney (Eladari et al., 2002; Quentin et al., 2003; Verlander et al., 2003). However, hagfish gills, while ultrastructurally similar to teleost gills, exhibit many gross anatomical differences (Mallatt and Paulsen, 1986), and thus may not possess a similar pattern of Rh and UT protein localizations.

With this work, we have attempted to add to the basic knowledge of nitrogen excretion in hagfish. The goal was to monitor ammonia and urea excretion as well as plasma levels during insults aimed at replicating the elevated ammonia and urea levels under conditions meant to mimic those that hagfish might experience within the carcasses they are feeding on. Using heterologous antibodies, we performed an initial immunocytochemical examination of ammonia and urea transporters in the gills. Given the ability of hagfish to maintain relatively constant levels of plasma ammonia when faced with elevated external ammonia, a separate series of experiments was conducted *in vitro* to assess a possible role for the skin in contributing to their ammonia tolerance.

2. Materials and methods

2.1. Experimental animals

Pacific hagfish (*Eptatretus stoutii*; 50–165 g) collected in Barkley Sound (BC, Canada) were kept at Bamfield Marine Sciences Centre (Bamfield, BC, Canada) in a large outdoor tank of running seawater at 11 °C. Fish were used within 2 weeks of collection and were fasted for the duration of the study.

2.2. Phloretin, ammonia and urea exposures

Hagfish were placed in aerated 1 L plastic containers with continuously flowing seawater for at least 8 h to allow them to adjust to their environment and prevent sliming. Following this period, water flow was stopped and either stock solutions of NH_4Cl (dissolved in seawater) or phloretin (dissolved in 95% ethanol) were added to the experimental boxes to yield final concentrations of 10, 20 and 100 mM NH_4Cl and 0.1 mM phloretin. The addition of ethanol (20 mL per L of water) had no noticeable effect on fish in pilot studies. The phloretin levels were chosen based on preliminary experiments and previous reports (Pilley and Wright, 2000; Braun et al., 2009b). The animals were exposed for 9 h after which the boxes were flushed with seawater for 15 min and their recovery was followed for another 3 h. Samples were taken at 0, 3, 6 and 9 h of the experimental exposure and in some experiments at 0 and 3 h of the recovery. For all experiments, a chamber was left empty to serve as a blank and control for background production of ammonia and urea in seawater. Following each experiment, the animals were weighed.

In a separate series of experiments, hagfish were exposed to various levels of ammonia (10, 20, 40, 100 mM NH_4Cl) or urea (5, 10, 20, 40, and 100 mM urea). The exposure protocol was identical to that described previously, however, the animals were only exposed for 2 h after which plasma samples were taken for measurements of ammonia and urea levels.

2.3. Tissue sampling

For blood samples, hagfish were lightly anaesthetized with ethyl-*p*-aminobenzoate (1 g L^{-1}) to allow handling, after which blood was withdrawn from the posterior sinus using an 18-gauge needle and heparinized syringe. Red blood cells and plasma were separated by centrifugation (12,000 *g* for 1 min) and the plasma was frozen in liquid N_2 and stored at −80 °C for later analysis. After sampling, hagfish were euthanized by returning them to the anaesthetic solution.

Before dissecting out gills and livers from euthanized animals, as much blood as possible was removed from the posterior sinus after which the ventral aorta was cannulated and perfused with 50 mL of refrigerated isoosmotic saline (565 mM NaCl) using a 60 mL syringe and polyethylene tubing (PE 160; Clay Adams). This procedure was employed in an attempt to minimize the contamination of tissue samples with urea transporters or Rh proteins originating from red blood cells. Tissues used for western blots were frozen in liquid N_2. For immunocytochemistry, tissues were initially incubated in 4% paraformaldehyde [PFA; prepared in phosphate buffered saline (PBS), pH 7.4] for 20 min at 4 °C followed by several hours in a 4 °C solution of 15% sucrose/PBS before being stored in a 30% sucrose/PBS solution at 4 °C.

2.4. Ammonia and urea assays

Nitrogen excretion was measured by collecting and immediately freezing (−20 °C) 1 mL water samples for later analysis of ammonia and urea levels using the colorimetric assays of Verdouw et al. (1978) and Rahmatullah and Boyde (1980) as described previously (Braun et al., 2009a).

For measuring plasma levels of ammonia and urea, previously frozen samples were ground to a fine powder in liquid N_2 and deproteinized in 2 volumes of ice-cold 6% perchloric acid and centrifuged at 16000 *g* for 15 min at 4 °C. The supernatant was neutralized with ice-cold 2 M K_2CO_3 and centrifuged again at 16000 *g*