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Rh protein expression in branchial neuroepithelial cells, and the role of ammonia in ventilatory control in fish ☆

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ABSTRACT

Bill Milsom has made seminal contributions to our understanding of ventilatory control in a wide range of vertebrates. Teleosts are particularly interesting, because they produce a 3rd, potentially toxic respiratory gas (ammonia) in large amounts. Fish are well known to hyperventilate under high environmental ammonia (HEA), but only recently has the potential role of ammonia in normal ventilatory control been investigated. It is now clear that ammonia can act directly as a ventilatory stimulant in trout, independent of its effects on acid–base balance. Even in ureotelic dogfish sharks, acute elevations in ammonia cause increases in ventilation. Peripherally, the detection of elevated ammonia resides in gill arches I and II in trout, and *in vitro*, neuroepithelial cells (NECs) from these arches are sensitive to ammonia, responding with elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Centrally, hyperventilatory responses to ammonia correlate more closely with concentrations of ammonia in the brain than in plasma or CSF. After chronic HEA exposure, ventilatory responsiveness to ammonia is lost, associated with both an attenuation of the $[Ca^{2+}]_i$ response in NECs, and the absence of elevation in brain ammonia concentration. Chronic exposure to HEA also causes increases in the mRNA expression of several Rh proteins (ammonia-conductive channels) in both brain and gills. "Single cell" PCR techniques have been used to isolate the individual responses of NECs versus other gill cell types. We suggest several circumstances (post-feeding, post-exercise) where the role of ammonia as a ventilatory stimulant may have adaptive benefits for O_2 uptake in fish.

1. Introduction

In contrast to most other vertebrates, ammoniotelic teleost fish must regulate three respiratory gases — oxygen, carbon dioxide, and ammonia (Randall and Ip, 2006). This third gas is excreted at rates (M_{Amm}) about 10–20% those of oxygen consumption (M_{O2}) or carbon dioxide production (M_{CO2}). Ammonia is particularly interesting because it exists in physical solution in two forms — the dissolved gas (NH_3) and the protonated ammonium cation (NH_4^+). In this article we use NH_3 and NH_4^+ to refer to the gas and the cation respectively, and the term total ammonia (T_{Amm}) to refer to the sum of the two. NH_3 and NH_4^+ are interconvertible with a pK of approximately 9.5, such that the latter dominates quantitatively (>95%) at physiological pHs. In this regard, ammonia is analogous to carbon dioxide which exists in solution as the dissolved

Abbreviations: 5-HT, 5-hydroxytryptamine = serotonin; $[Ca^{2+}]_i$, intracellular calcium ion concentration; $CaCO_3$, calcium carbonate; CO_2, carbon dioxide; CO6a, cytochrome c oxidase subunit VIa; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; GS, glutamine synthetase; H-ATP, v-type proton adenosine triphosphatase; $[HCO_3^-]a$, arterial plasma bicarbonate ion concentration; $[HCO_3^-]v$, venous plasma bicarbonate ion concentration; HEA, high environmental ammonia; K^+, potassium ion; K_{2P}, two-pore domain potassium channel; M_{Amm}, total ammonia excretion rate; M_{CO2}, carbon dioxide excretion rate; M_{O2}, oxygen consumption rate; Mg^{2+}, magnesium ion; MSOX, methionine sulfoxamine; MRC, mitochondria-rich cell; mRNA, messenger ribonucleic acid; MS-222, tricaine methane sulfonate; Na^+, sodium ion; NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate; NH_3, ammonia gas; NH_4^+, ammonium ion; NH_4Cl, ammonium chloride; NH_4HCO_3, ammonium bicarbonate; NH_4OH, ammonium hydroxide; $(NH_4)_2SO_4$, ammonium sulfate; NHE2, sodium hydrogen exchanger-2; NKA, sodium potassium adenosine triphosphatase; NEC, neuroepithelial cell; O_2, oxygen; $PaCO_2$, arterial carbon dioxide partial pressure; $PvCO_2$, venous carbon dioxide partial pressure; PaO_2, arterial oxygen partial pressure; PvO_2, venous oxygen partial pressure; P_{NH3}, partial pressure of ammonia; PBS, phosphate buffered saline; pHa, arterial pH; pHv, venous pH; pK, negative base 10 logarithm of the dissociation constant; PVC, pavement cell; qPCR, quantitative real time polymerase chain reaction analysis; Rh, Rhesus; SDA, specific dynamic action; SEM, standard error of the mean; $[SO_4^{2-}]$, sulfate ion concentration; ST, serotonin transporter; T_{Amm}, total ammonia, the sum of NH_3 and NH_4^+; TASK-1, TWIK-related acid-sensitive potassium channel-1.

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gas (CO_2) and the hydrated bicarbonate anion (HCO_3^-) which are interconvertible with an effective pK of about 6.1, such that the latter dominates quantitatively at physiological pHs. Ammonia is also similar to carbon dioxide in that both are quite toxic, both affect physiological pH, and both play intimate roles in ionoregulation such that blood plasma concentrations and body stores must be tightly regulated. In most situations, the goal of the regulatory systems appears to be to match the rate of excretion across the gills with the rate of production by the tissues, while maintaining blood plasma levels of T_{Amm} in an optimal concentration range. This appears to be about 50–300 μmol L^{-1} T_{Amm} in resting, non-fed teleosts of various species (reviewed by Wood, 1993), though levels in the 1000–2500 μmol L^{-1} range have been measured in salmonids which are actively feeding and/or surviving in high environmental ammonia (Tsui et al., 2009; Zimmer et al., 2010).

There has been a vast amount of research on ammonia as a toxicant in fish (reviewed by Randall and Tsui, 2002; Eddy, 2005; Ip et al., 2001), as well as on its mechanisms of branchial excretion and role(s) in ionoregulation (reviewed by Wright and Wood, 2009, 2012; Weihrauch et al., 2009). Perhaps the most important finding of the last decade in these areas is that ammonia movement through the branchial epithelium is facilitated by specific channels, the Rh glycoproteins (Nakada et al., 2007; Nawata et al., 2007). While some ammonia may pass by simple diffusion through the lipoprotein cell membranes of the gills, a significant fraction moves by channel-mediated facilitated diffusion. Even though NH_4^+ dominates at physiological pH, and is the moiety which binds at the channel gate, the actual form moving through the Rh channel appears to be NH_3 (Nawata et al., 2010b), so the H^+ removed from NH_4^+ must be shuttled by another mechanism (Na^+/H^+ exchanger or v-type H^+-ATPase linked to a Na^+-selective channel) if the fish is to excrete NH_4^+ on a net basis. Therefore, these Rh channels appear to play an integral role in a "Na^+/NH_4^+ exchange complex" consisting of several transporters working together as a metabolon which provides a loose coupling of Na^+ uptake with branchial ammonia excretion under normal circumstances (Wright and Wood, 2009; Ito et al., 2013). This modern model is rather close to the original ideas of August Krogh (1938) for linkage of Na^+ uptake with NH_4^+ excretion at the gills of aquatic animals, yet again illustrating the prescience of the father of comparative physiology!

Under external ammonia loading, elements of the metabolon may be involved in the active excretion of ammonia against a gradient, energized by Na^+,K^+-ATPase (NKA — e.g. Hung et al., 2007; Nawata et al., 2007, 2010a; Tsui et al., 2009; Braun et al., 2009; Zimmer et al., 2010; Wood and Nawata, 2011; Wood et al., 2013; Sinha et al., 2013), with indications that NH_4^+ can effectively substitute for K^+ on Na^+,K^+-ATPase in at least some species (Mallery, 1983; Balm et al., 1988; Randall et al., 1999; Nawata et al., 2010a; Wood et al., 2013). Additionally, elevated ammonia excretion through the metabolon is now thought to drive active Na^+ uptake in fish chronically exposed to low pH and/or ion-poor water (Kumai and Perry, 2011; Shih et al., 2012; Lin et al., 2012), circumstances in which earlier models predicted that Na^+ uptake would become impossible (Avella and Bornancin, 1989: Randall et al., 1996; Parks et al., 2008). mRNA expression data indicate that the system is also activated in response to internal loading by ammonia infusion (Nawata and Wood, 2009), exhaustive exercise (endogenous ammonia production by adenylate breakdown; Mommsen and Hochachka, 1988; Wood, 1988; Wang et al., 1994; cf. Fig. 1A) and feeding (endogenous ammonia production by deamination of amino acids; Wicks and Randall, 2002a,b; Bucking and Wood, 2008; Zimmer et al., 2010; cf. Fig. 1B).

There has been far less research on ammonia's possible "respiratory" role. However, it is well known that fish hyperventilate in the latter two circumstances of internal ammonia loading. Increased breathing is needed to support the elevated O_2 demands of post-prandial specific dynamic action (SDA; Jobling, 1994; Secor, 2009), and to pay off the anaerobic component of exhaustive exercise (Scarabello et al., 1991). Fish also exhibit marked hyperventilation during high environmental ammonia (HEA) exposure (e.g. Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph, 1996). Is it possible that elevated internal and/or external ammonia levels provide the proximate stimulus for hyperventilation under these circumstances? This might be particularly important after feeding if ammonia could serve as a ventilatory stimulant to counteract any depression of ventilation caused by the post-prandial 'alkaline tide' (Wood et al., 2005; Bucking and Wood, 2008; Cooper and Wilson, 2008; Wright and Wood, 2012)? And if so, since rapid penetration of ammonia to the ammonia-sensing sites would presumably be needed, is it possible that Rh proteins play a role?

Indeed in mammals, it has long been known that under certain pathological conditions (e.g. liver failure), internal ammonia buildup can serve to stimulate ventilation (Roberts et al., 1956; Vanamee et al., 1956; Poppell et al., 1956; Warren, 1958; Campbell et al., 1973; Wichser and Kazemi, 1974; Felipo and Butterworth, 2002). Ammonia-induced hyperventilation (causing respiratory alkalosis) may help to offset the lactacidosis that often accompanies hepatotoxicity. Is it possible that this emergency response had its evolutionary roots in the normal ventilatory responsiveness of fish to ammonia?

Much of what we know about the control of breathing in fish, and indeed in vertebrates in general, comes from the fundamental contributions of Bill Milsom and his colleagues over the past 30 years (e.g. Jones

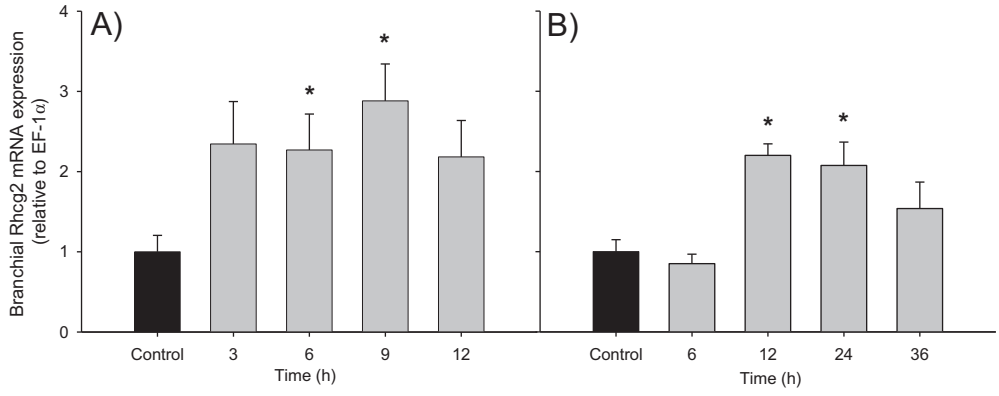

Fig. 1. Responses of Rhcg2 mRNA expression in the gills of adult rainbow trout to treatments which elevate internal ammonia levels by increased endogenous generation. (A) Exhaustive exercise. Rainbow trout (163–330 g; n = 6 for each group) were exercised to exhaustion by chasing them in a circular tank (800 L) for 6 min at 15 °C. Fish were sacrificed at 3, 6, 9, and 12 h post-exercise and gills were analyzed for Rhcg2 mRNA expression. (B) Feeding. Rainbow trout (179–209 g; n = 6 for each group) were fed to satiation at 15 °C and subsequently sacrificed at 6, 12, 24, and 36 h. The gills were analyzed for Rhcg2 mRNA expression. Means + 1 SEM. Asterisks represent a significant increase from the control values (unexercised or fasted fish respectively).
Previously unpublished data of C.M. Nawata and C.M. Wood.