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Examining urea flux across the intestine of the spiny dogfish, *Squalus acanthias*

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ABSTRACT

Recent examination of urea flux in the intestine of the spiny dogfish shark, *Squalus acanthias*, has shown that feeding significantly enhances urea uptake across the intestine, and this was significantly inhibited following mucosal addition of phloretin. The present study examined potential mechanisms of urea uptake across the dogfish intestine in starved and fed dogfish. Unidirectional flux chambers were used to examine the kinetics of urea uptake, and to determine the influence of sodium, ouabain, competitive urea analogues, and phloretin on urea uptake across the gut of fed dogfish. Intestinal epithelial preparations from starved and fed dogfish were mounted in Ussing chambers to examine the effect of phloretin on bidirectional solute transport across the intestine. In the unidirectional studies, the maximum uptake rate of urea was found to be 35.3 ± 6.9 μmol.cm^{-2}.h^{-1} and K_m was found to be 291.8 ± 9.6 mM in fed fish, and there was a mild inhibition of urea uptake following mucosal addition of competitive agonists. Addition of phloretin, Na-free Ringers and ouabain to the mucosal side of intestinal epithelia also led to a significant reduction in urea uptake in fed fish. In the Ussing chamber studies there was a net influx of urea in fed fish and a small insignificant efflux in starved fish. Addition of phloretin blocked urea uptake in fed fish when added to the mucosal side. Furthermore, phloretin had no effect on ion transport across the intestinal epithelia with the exception of the divalent cations, magnesium and calcium.

1. Introduction

Homer Smith was the first to examine the physiological consequences of the ureosmotic strategy employed by marine elasmobranch fish (Smith, 1936) where the internal osmolality is maintained similar to or slightly higher than that of the marine environment largely through the retention of high concentrations of urea. Despite the significant molecular and structural modifications to gill epithelia cells in elasmobranch fish, thought to be related to urea retention (Fines et al., 2001), it is recognised following extensive studies on the dogfish shark, *Squalus acanthias*, that not only are these fish ureosmotic but they are also ureotelic (Wood et al., 1995), with most urea being lost across the gill epithelia (Pärt et al., 1998). This incipient urea loss has been demonstrated in long term starvation studies (Cohen et al., 1958; Kajimura et al., 2008) such that it was estimated that *S. acanthias* would need to feed every 5–6 days to maintain nitrogen balance (Kajimura et al., 2006). Thus, despite the high concentration of urea in the plasma of the dogfish shark they are in fact severely nitrogen limited (Wood et al., 2005). These findings are part of a significant research effort into understanding the role of the fish intestine and how feeding results in profound changes in homeostatic regulation of acid–base balance, nitrogen balance, ion regulation and metabolism (Wood and Bucking., 2011). However, despite the obvious potential for dramatic changes in the dogfish shark intestinal tract following a single meal that may be as much as 10% body mass (Wood et al., 2007), examination of intestinal physiology in fed and starved elasmobranch fish is limited to much fewer studies (Anderson et al., 2012; Liew et al., 2013; Wood et al., 2007).

In 2007 Wood et al. reported a rapid increase in the osmolality of chyme entering the intestine from the stomach following a single meal. This was found to be largely the result of increases in urea, Na^+ and Cl^-. In more distal regions of the intestine and/or the colon the same solutes were thought to be reabsorbed. Indeed accumulation of ^{14}C-urea in the mucosa of colonic tissue from starved dogfish provided some support for this hypothesis (Anderson et al., 2012). Most recently, Liew et al. (2013) using intestinal sac preparations reported a net loss of

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urea to the intestinal lumen in starved fish similar to the result previously shown in the little skate, *Leucoraja erinacea* (Anderson et al., 2010). However, this was significantly reversed to a net urea uptake within 24–48 h following a feeding event. Furthermore, the intestine (or spiral valve) was the only region of the GI tract of the four examined (cardiac stomach, pyloric stomach, intestine, and colon) where this occurred, and the effect was significantly blocked through the addition of the general urea transport blocker, phloretin, to the mucosal side of the preparation (Liew et al., 2013). The significant uptake of urea from the isolated intestinal sacs reported by Liew et al. (2013) was in direct contrast to the assumed secretion, at least in the anterior part of the intestine in vivo, reported by Wood et al. (2007). Liew et al. (2013) suggest that the contrasting results may be due to addition of urea by elevated biliary and pancreatic secretions that would resemble a net intestinal secretion of urea post-feeding (Wood et al., 2007).

In the present study two preparations were employed, both using ^{14}C-urea to examine the effect of feeding and various known inhibitors and competitors on urea transport in isolated intestinal epithelia from the dogfish shark. *Series 1* used only tissue from fed fish and was similar to the protocol developed for examination of nutrient uptake across the skin in the Pacific Hagfish, *Eptatretus stoutii* (Glover et al., 2011). Only fed fish were examined in this first series because Liew et al. (2013) had reported that net uptake of urea meeting a key criterion of active transport (transport against the concentration gradient) occurred only under fed conditions, and not under starved conditions. Therefore, kinetic and pharmacological investigation of unidirectional urea transport in the fed preparation would likely yield the most informative results. Urea uptake was determined based on the disappearance of ^{14}C-urea from the mucosal side of the preparation. Various experiments examined the concentration-dependent kinetics of urea uptake, the influence of sodium removal and ouabain on urea uptake, as well as the effects of phloretin as an antagonist and thiourea, methylurea and acetamide as competitive agonists. In *Series 2*, we then followed up using Ussing chambers to examine the differences in bi-directional and net flux rates between fed and starved preparations. Bidirectional flux of ^{14}C-urea was examined in both fed and starved individuals under control conditions and following addition of phloretin to the mucosal and serosal sides of the preparation. These latter experiments allowed for comparison of bidirectional flux, and therefore the two components of net flux, whereas previous work with gut sac preparations measured only net flux (Liew et al., 2013).

2. Materials and methods

Male spiny dogfish, *S. acanthias*, were caught by rod and line or trawl by commercial fishermen in Barkley Sound British Columbia in July of 2012 ($n = 18$; mean body mass 1.42 ± 0.12 kg). Following capture, fish were transferred to a 151,000-L indoor flow-through aquarium at Bamfield Marine Sciences Centre, where water was held at ambient temperature (12 ± 0.1 °C), salinity (30 ± 2 ppt) and photoperiod. Both fed and starved free-swimming fish were used in the present study where feeding and fasting regimens followed those previously described (Liew et al., 2013). Briefly, for the fed fish the diet consisted of approximately 3% ration of frozen hake, *Merluccius productus*, delivered every 4 days. Fed fish were sacrificed between 24 and 48 h post-feeding and food was with-held from starved fish for a minimum of 7 days prior to sacrifice. All described procedures were conducted under approved animal care protocols at Bamfield Marine Sciences Centre under the guidelines of the Canadian Council for Animal Care.

Following immersion in a terminal dose of tricaine methanesulfonate (250 ppm MS-222), the intestine was removed, and a longitudinal incision was made to open the spiral valve. The anterior two intestinal folds were dissected out and set aside for mounting in the Ussing chambers and the remaining folds were dissected out and mounted in modified flux chambers as previously described (Glover et al., 2011).

2.1. Series 1: unidirectional flux measurement

As described, intestinal folds from the spiral valve were carefully removed between 24 and 48 h after the feeding event. Both sides of an intestinal fold are covered with mucosal epithelium, so in this series, the fold was kept intact, and the surface exposed to the experimental solution was considered the mucosal surface. Unidirectional uptake was measured by disappearance of ^{14}C-urea from this experimental (mucosal) solution.

Each fold was rinsed with Elasmobranch Ringers (in mM; 257 NaCl, 7 Na_2SO_4, 6 $NaHCO_3$, 0.1 Na_2HPO_4, 4 KCl, 3 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 5 glucose, 100 TMAO and 350 urea, pH 7.8) to remove any chyme or undigested material. The saline composition was identical to that of Pärt et al. (1998), except for the removal of colloids and the elevation of TMAO from 15 mM to 100 mM. A section was then cut and fitted over the opening of a 7-ml scintillation vial. An aperture of known surface area (1.13 cm^2) was present in the lid of the scintillation vial. This lid was then screwed onto the vial to seal the tissue in place. The vial contained 2 ml of the experimental (mucosal) solution (modified Elasmobranch Ringers) which had been pre-equilibrated with a specialty gas mixture of 99.7% O_2:0.3% CO_2 and labelled with 3.7 kBq·ml^{-1} (0.1 μCi·ml^{-1}) of ^{14}C-urea (original specific activity 2.04 MBq·mmol^{-1}; Perkin Elmer, Waltham, MA, USA). A sample of the experimental solution was taken for measurements of initial radioactivity. The whole preparation was then blotted gently, weighed to 1 mg accuracy, inverted, and placed into a bathing solution (serosal) containing 5 ml of Elasmobranch Ringers which was continuously bubbled with the same gas mixture. Isosmotic conditions on the two sides of the fold were maintained in all treatments, as explained below. The preparation was then allowed to incubate in a water jacketed chamber at 12 °C for the following 3 h. After the 3-h incubation period, the preparation was removed, blotted, and reweighed, and a mucosal sample was taken for measurement of final radioactivity, so as to allow calculation of uptake rates. Data from preparations showing weight changes of more than 50 mg (indicative of potential leakage) were discarded.

The concentration-dependent kinetics of urea uptake were determined based on the disappearance of ^{14}C-urea from the mucosal side. Mucosal urea concentration was adjusted to between 70 and 700 mM and the serosal urea concentration was maintained at 350 mM. The osmotic pressure of both the mucosal and serosal solutions in this series was balanced with the addition of mannitol to the Ringers solutions, as verified with a Vapro 5520 vapour pressure osmometer (Wescor, Logan, UT, USA). The effect of sodium on urea uptake was examined through; a) the removal of NaCl from the mucosal Ringers with NaCl being replaced by equimolar amounts of N-methyl-D-glucamine (NMDG) and; b) addition of the Na,K,ATPase inhibitor, ouabain (1 mM), to the mucosal Ringers solution. The effects of urea agonists on urea uptake were determined following the addition to the mucosal Ringers solution of 350 mM of one of thiourea, acetamide or N-methylurea (i.e. equimolar to urea). Again the serosal Ringers solution had 350 mM urea and any difference in osmotic pressure between the serosal and mucosal Ringers solutions was resolved with the addition of mannitol. Finally the potential involvement of specific urea transporters in urea uptake across the intestinal epithelia in fed dogfish was examined following the addition of phloretin (0.25 mM in 0.1% dimethyl sulfoxide, DMSO), or 0.1% DMSO alone, to the mucosal Ringers solution. In all experiments, a minimum of 3 preparations (i.e. 3 folds of intestinal tissue) were examined from one animal for each treatment, including the control, and the results of the replicates were averaged as $N = 1$. Actual N numbers (number of animals) are reported in the figure legends.

2.2. Series 2: bidirectional flux measurement — Ussing chambers

Intestinal tissue was removed as described in both fed and starved dogfish. The anterior one to two folds were carefully rinsed in dogfish