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Functional redundancy of glucose acquisition mechanisms in the hindgut of Pacific hagfish (*Eptatretus stoutii*)

Alyssa M. Weinrauch[a,b,*], Alexander M. Clifford[a,b,1], Greg G. Goss[a,b]

[a] Department of Biological Sciences, University of Alberta, 116 St. and 85 Ave., Edmonton, Alberta T6G 2R3, Canada
[b] Bamfield Marine Sciences Centre, 100 Pachena Rd., Bamfield, British Columbia V0R 1B0, Canada

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ABSTRACT

This study examined the mechanisms of glucose acquisition in the hindgut of Pacific hagfish (*Eptatretus stoutii*) using *in vitro* gut sac techniques. The intestine was determined to have the capacity to digest maltose into glucose along the entirety of the tract, including the foregut. Glucose uptake was biphasic and consisted of a high-affinity, low-capacity concentration-dependent component conforming to Michaelis-Menten kinetics (K_m 0.37 mM, J_{max} 8.48 nmol/cm^2/h) as well as a diffusive component. There was no observed difference in glucose flux rate along the length of the intestine, similar to other nutrients investigated in the hagfish intestine. A reduced sodium (< 1 mM) environment did not result in a change in glucose uptake rates, likely due to a functional redundancy of glucose transporters. There was no observed effect of phloretin, yet the sodium glucose-linked transporter (SGLT)-specific inhibitor phlorizin significantly reduced glucose uptake at all concentrations tested (0.0001–1 mM). Additionally, the glucose transporter (GLUT) inhibitor cytochalasin b significantly reduced glucose transport rates. The effects of these pharmacological inhibition experiments suggest the presence of multiple types of glucose transport proteins. This study clarifies the uptake strategies used by hagfish to acquire glucose at the intestine and provides insight into the evolution of such transport systems in early-diverging vertebrates.

1. Introduction

The hagfish diverged from the vertebrate phylogeny ~300–500 million years ago (Bardack, 1998; Sugahara et al., 2016) and so are representatives of the evolution of early vertebrates. Hagfish are thus of great interest as evolutionary models and are of particular use in the study of nutrient transport system evolution owing to their unique feeding environment. Occupying a distinct trophic niche, hagfish consume both living (Zintzen et al., 2011) and dead prey items, ranging from polychaetes to cetaceans, and may become fully immersed within a carcass (Martini, 1998). Interestingly, it has been noted that hagfish preferentially consume the glycogen-rich liver (Weinrauch et al., 2017), which conforms with the favoured use of carbohydrates for fuel in particular tissues (Sidell et al., 1984). Further evidence for the importance of glucose has been demonstrated in multiple hagfish species. Glucose loading induces immediate hyperglycaemia lasting two days in *Myxine glutinosa* (Falkmer and Matty, 1966), while a one-month starvation period in *Eptatretus stoutii* incites decreased basal levels of glucose and insulin (Emdin, 1982). Additionally, the archinephric duct demonstrates glucose resorption as only 12–18% of an injected glucose load is recovered in the urine (Falkmer and Matty, 1966). Hormonal glucose regulation is also demonstrable in hagfish, albeit with variable results between species. Large quantities of exogenous insulin (1000–3000 IU/kg) were required to incite hypoglycaemia 2–3 days post-injection in *Myxine glutinosa* (Falkmer and Matty, 1966), while *Eptatretus stoutii* had sensitivities akin to mammals, wherein 0.5 IU/kg of bovine insulin produced a pronounced hypoglycaemia within 12–24 h that was rectified in the span of 5 days (Inui and Gorbman, 1977). Hormonal regulation of glucose suggests that it is an important molecule for hagfish and glucose uptake will therefore be regulated by transport systems.

Glucose is an energetically important molecule that is a primary energy source for eukaryotes (Bell et al., 1990). The plasma membrane is impermeable to large polar molecules (Bell et al., 1990), and so the uptake of glucose necessitates membrane-associated proteins such as the sodium-dependent and phlorizin-sensitive sodium glucose-linked transporters (SGLT; SLC5A family), as well as cytochalasin-B and phloretin-sensitive glucose transporters (GLUT; SLC2 family). SGLT acquires glucose against a concentration gradient due to the electrochemical gradient of sodium established by the ubiquitous basolateral

* Corresponding author at: Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2R3, Canada.
E-mail address: weinrauc@ualberta.ca (A.M. Weinrauch).
[1] Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, BC, V6T 1Z4, Canada.

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sodium-potassium ATPase (NKA), while GLUT functions to move glucose down its concentration gradient and is perpetually expressed on the basolateral membrane, but may be inserted apically for enhanced uptake. Such transporters have been identified in hagfish tissues with sodium-dependent, phlorizin sensitive (SGLT) glucose uptake identified in *M. glutinosa* archinephric duct (Flöge et al., 1984) and cytochalasin b sensitive (GLUT) glucose uptake observed and characterized in *E. stoutii* erythrocytes (Ingermann et al., 1984; Young et al., 1994). The objective of this study was to characterize the mechanisms of glucose acquisition in the hindgut of the Pacific hagfish (*E. stoutii*) owing to its clear importance as an energy source for this organism.

The agastric hagfish have distinct morphological differences along the intestinal tract, with mucous cells restricted to the foregut and digestive/absorptive cells along the length of the hindgut (Adam, 1963; Weinrauch et al., 2015). As such, previous histochemical and histological studies suggest that digestive activity (including amylases, lipases, and proteolytic enzymes) is restricted to, and consistent along the hindgut (Adam, 1963). This study sought to examine whether digestive enzymes for maltose (presumably maltase) were present along the length of the intestine as a means to digest complex carbohydrates into the simpler molecule glucose, over an extended surface area. Using *in vitro* gut fluxes, we sought to characterize the kinetics, sodium-dependence and effect of pharmacological inhibitors (phloretin, phlorizin and cytochalasin b) on intestinal glucose acquisition.

2. Materials and methods

Pacific hagfish (*E. stoutii*) were collected from the Trevor channel, Vancouver Island, BC, Canada using bottom-dwelling traps (2014–2017). Fish were immediately transported to the Bamfield Marine Sciences Centre and housed in aerated, darkened 20 m^3 tanks with flow-through water. Hagfish were starved at least one week prior to experimentation. All animals were used under the licenses of the Department of Fisheries and Oceans Canada (collection permits XR-192-2014; XR-310-2015; XR-202-2016; XR-136-2017) and approved by the Bamfield Marine Science Centre (RS-14-13 (2014), RS-15-31 (2015), RS16-19 (2016), RS17-03 (2017)), and University of Alberta Animal Care (No. AUP00001126; 2014–2017). Unless noted, all chemical compounds, reagents and enzymes were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO).

2.1. Solutions

Glucose-free hagfish saline (HF saline) was used for all serosal fluid. It contained: NaCl, 490 mM; KCl 8.0 mM; $CaCl_2$ $2H_2O$ 5.0 mM; $MgSO_4$ $7H_2O$ 3.0 mM; $MgCl_2$ $6H_2O$ 9.0 mM; Na_2PO_4 2.06 mM; $NaHCO_3$ 8.0 mM; HEPES 20 mM; pH 7.6. To test for sodium-dependent glucose uptake, modifications were made to reduce the sodium concentration in the HF saline, replacing it instead with choline chloride as per previous sodium-free studies: [$C_5H_{14}NOCl$ 480 mM; $CaCl_2$ $2H_2O$ 5.0 mM; $MgSO_4$ $7H_2O$ 3.0 mM; $MgCl_2$ $6H_2O$ 9.0 mM; KH_2PO_4 2.06 mM; $KHCO_3$ 8.0 mM; HEPES 20 mM; pH 7.6] (Schultz et al., 2014). Matched mucosal salines containing all above mentioned chemicals were used for either sodium-containing or sodium-free experiments and osmotically balanced with mannitol with measurement using a Vapro vapor pressure osmometer (Model 5520; Wescor, Logan, UT). The mucosal saline also contained 0–10 mM glucose for concentration dependent experiments and 5 mM glucose in all other solutions (Bucking et al., 2011). An atomic absorption spectrophotometer (Thermo Scientific model iCE 3300) was utilized to ensure solutions had reduced sodium concentrations (< 1 mM) prior to experimentation.

Excised intestine was rinsed and soaked in sodium-free, aerating hagfish saline for 30 min prior to the preparation of individual gut sacs in order to remove free endogenous sodium. Serosal fluid was again measured using the atomic absorption spectrophotometer following experimentation and no significant changes in sodium concentration were detected. Radiolabelled D-[1,^{14}C] glucose (Perkin Elmer, Boston, MA, USA) was added at a specific activity of 0.05 μCi/mL. Glucose uptake was measured with and without the presence of pharmacological inhibitors. Phloretin, a weak inhibitor of SGLT and GLUT, was used at concentrations of 0, 10, 50, 100, 500, and 1000 μM. Phlorizin, an inhibitor of SGLT, was used at concentrations of 0, 0.1, 1, 10, 100, and 1000 μM. Cytochalasin-B, an inhibitor of GLUT, was utilized at a concentration of 200 μM. Each inhibitor was dissolved in DMSO and added to solutions at a final concentration of 0.1%. A 0.1% DMSO control was used for each inhibitor experiment.

2.2. Intestinal fluxing protocols

Hagfish (N = 56; 81.4 ± 4.1 g; mean ± SEM [standard error of the mean]) were euthanized using 4 g/L tricaine methanesulfonate (TMS; Syndel Laboratories Ltd., Vancouver, BC, Canada) neutralized with 1.2 g/L sodium hydroxide. Weight measurements were obtained prior to dissection where the intestine was removed in two pieces; a region of the pharyngocutaneous duct anterior to the first gill pouches, and the hindgut from the biliary duct to the cloaca, which was then further divided into three pieces. Additional tissue was flash frozen and stored at − 80° until enzymatic analysis (see below). Mesentery and gonads (if present) were removed and the intestinal tube was flushed multiple times with glucose-free HF saline. Gut sacs were prepared as reported previously (Glover et al., 2011b; Schultz et al., 2014). Briefly, each intestinal piece had one end tied off with suture thread and a flared piece of polyethylene (PE)-90 tubing sutured on the other side for sample insertion/removal. The sacs were filled with glucose-containing mucosal HF saline and placed in aerating serosal saline solution for the flux period. A 200 μL sample was removed at both the beginning and end of the flux period and the sacs weighed to determine flux volume. Two 50 μL subsamples were taken and combined with ACS (aqueous counting scintillant; Amersham Bioscience, Baie d'Urfe, Quebec, Canada) and radioactivity measured on a Beckman Coulter LS6500. The remaining sample was stored at − 80 °C until further analysis of glucose content within a 2 week period. Preliminary studies demonstrated no glucose in the serosal fluid following a flux period (data not shown). Following a flux period, the sacs were cut open and spread on graph paper where surface area was determined using Image J Software (National Institute of Health). Glucose concentration was determined using the following procedure. Briefly, 20 μL of sample was added to 200 μL of glucose cocktail (50 mL Triethylamine-hydrochloride pH 7.53; 0.22 g $MgCl_2$; 0.050 g ATP; 0.050 g NAD; 2.4 U/mL glucose-6-phosphate dehydrogenase) and absorbance (A340 nm) was recorded using a spectramax 190 (Molecular Devices, Sunnyvale, CA, USA). A final A340 measurement was conducted after 15 min of incubation following the additional of hexokinase (5 U/sample) and the difference between initial and final absorbance represents the concentration of glucose when corrected to μmol/L using a standard curve (Bergmeyer, 1983). The disappearance of glucose from the mucosal saline was then calculated using the following equation:

$$J_{glucose}\ (nmol\ cm^{-2}h^{-1}) = \frac{\Delta CPM/SAct}{SA/t}$$

where ΔCPM represents the decrease of 1-^{14}C-D-glucose radioactivity inside the gut sac, SAct is the specific activity (CPM $nmol^{-1}$), SA is the surface area (cm^{-2}) and t is the flux time (h). Of note, the data for Figs. 2, 5a, and b were collected in 2014 and Figs. 3, 4 and 5c were collected in 2017 (see discussion).

2.3. Presence of maltase in the hagfish intestine

Maltase presence was measured indirectly by homogenizing different intestinal segments in a homogenization buffer (PBS 23 mM, Tris-HCl 5 mM, EDTA 1 mM, pH 7.0) modified from Cox and Secor (2008). The homogenate (50 μL) was added in a 1:1 (v:v) ratio to