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Transcriptome responses in the rectal gland of fed and fasted spiny dogfish shark (*Squalus acanthias*) determined by suppression subtractive hybridization

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ABSTRACT

Prior studies of the elasmobranch rectal gland have demonstrated that feeding induces profound and rapid up regulation of the gland's ability to secrete concentrated NaCl solutions and the metabolic capacity to support this highly ATP consuming process. We undertook the current study to attempt to determine the degree to which up regulation of mRNA transcription was involved in the gland's activation. cDNA libraries were created from mRNA isolated from rectal glands of fasted (7 days post-feeding) and fed (6 h and 22 h post-feeding) spiny dogfish sharks (*Squalus acanthias*), and the libraries were subjected to suppression subtractive hybridization (SSH) analysis. Quantitative real time PCR (qPCR) was also used to ascertain the mRNA expression of several genes revealed by the SSH analysis. In total the treatments changed the abundance of 170 transcripts, with 103 up regulated by feeding, and 67 up regulated by fasting. While many of the changes took place in 'expected' Gene Ontology (GO) categories (e.g., metabolism, transport, structural proteins, DNA and RNA turnover, etc.), KEGG analysis revealed a number of categories which identify oxidative stress as a topic of interest for the gland. GO analysis also revealed that branched chain essential amino acids (e.g., valine, leucine, isoleucine) are potential metabolic fuels for the rectal gland. In addition, up regulation of transcripts for many genes in the anticipated GO categories did not agree (i.e., fasting down regulated in feeding treatments) with previously observed increases in their respective proteins/enzyme activities. These results suggest an 'anticipatory' storage of selected mRNAs which presumably supports the rapid translation of proteins upon feeding activation of the gland.

1. Introduction

The elasmobranch rectal gland has fascinated physiologists for decades. Capable of secreting a nearly 0.5 M NaCl solution to its lumen (for eventual voiding via the lower intestine), it plays an important role in osmoregulation and has served as a key model for understanding trans-epithelial transport of ions. Especially in the spiny dogfish shark (*Squalus acanthias*), its utility has no doubt been aided by the relatively simple architecture of the gland and the ease with which both afferent and efferent blood vessels, and the gland's duct itself, can be cannulated for in vitro experimental manipulation. Indeed, studies have mapped out a now classic model where several plasma membrane-bound proteins, including Na^+K^+ ATPase, the 'CFTR' Cl^- channel, and $Na^+K^+2Cl^-$ transporters, are coordinated to excrete salt (Silva et al., 1997; Olson, 1999; Anderson et al., 2007). Previous findings also included the demonstration of several potential natural and artificial signals for activation of the gland (e.g., C-type natriuretic peptide (CNP); vasoactive intestinal peptide (VIP), forskolin, etc.) (Epstein et al., 1983; Schofield et al., 1991). While originally it was believed that the gland functioned continuously to aid in overall osmoregulatory balance, more recently it has been shown that the gland is relatively inactive during frequent bouts of fasting, but becomes highly active very shortly after feeding; notably dogfish feed opportunistically, and therefore sporadically, in the wild (see references contained within Wood et al., 2010 for review).

The activation of the gland by feeding has been studied from a number of perspectives: morphological, physiological, metabolic, biochemical, and proteomic (for review, see Wood et al., 2010). These studies lead to an integrated view that: (i) feeding and digestion trigger activation of the gland, with at least one of the key signals being the increase in plasma pH associated with acid-secretion to the stomach

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(the so-called 'alkaline tide') (Wood et al., 2005; Shuttleworth et al., 2006; Wood et al., 2007a,b), while another may be the plasma volume expansion that accompanies the ingestion of salty food and accompanying seawater (Solomon et al., 1984; Silva et al., 1997); (ii) morphologically, the gland transitions from a 'dormant' (somewhat apoptotic) state, to a highly active secretory morphology (Matey et al., 2009), accompanied by increases in amounts of key cytoskeletal/muscular proteins (e.g., transgelin, tropomyosin) (Dowd et al., 2008); (iii) likewise, amounts and activities of key proteins of the ion transport pathway are up regulated, including Na^+K^+-ATPase and voltage-dependent anion channels (MacKenzie et al., 2002; Walsh et al., 2006; Dowd et al., 2008); (iv) the metabolic rate of the gland increases markedly, with a relatively complex pattern of metabolic pathway dependence (Walsh et al., 2006; Dowd et al., 2008). While glucose appears to be the main fuel source, evidence exists that β-hydroxybutyrate can serve as an auxiliary fuel (at least in the early stages of activation). Furthermore, although the gland appears to be a highly aerobic tissue with high densities of mitochondria, and high activities of tricarboxylic acid (TCA) cycle and energy production enzymes (e.g., isocitrate dehyrogenase, citrate synthase, ATP synthase) that increase in activity upon feeding, it is likely that at least some of its energy comes from anaerobic glycolysis as evidenced by large increases in lactate dehydrogenase activity when the gland is activated. The inability of lactate to fuel the gland at least in vitro (Walsh et al., 2006) argues against the increase in LDH being for lactate oxidation.

The above picture has been developed using several tools, and leads to a relatively 'straightforward' view of the gland's activation involving changes in morphology, ion secretion, and supporting metabolic pathways. The time course of changes (especially protein concentrations and enzyme activities), many of which were elevated after only 6 h post-feeding, was consistent with either a coordinated increase in both transcription and translation for appropriate genes, or rapid translation of pre-existing message (Dowd et al., 2008). With this background in mind, we undertook a study of the response of the transcriptome of the gland to feeding/fasting in order to test the hypothesis that widespread transcriptomic responses occur rapidly and agree with the physiological activation of the rectal gland upon feeding. Our null hypothesis is that a transcriptomic response was not a large part of the gland's activation in the short-term. Furthermore, assuming at least some role for transcriptional activation, we wished to gain insight into aspects of the activation of the gland that might have been overlooked by prior approaches.

For this examination, we used the approach of suppression subtractive hybridization (SSH), in which the mRNA (converted to cDNA) population of a 'tester' sample (e.g., rectal gland tissue from a fed dogfish) is hybridized with an excess of mRNA from a 'driver' sample (e.g., rectal gland tissue from a fasted dogfish), such that the mRNA transcripts in common are subtracted from the tester, enriching the tester sample for its unique transcripts. The SSH approach (Diatchenko et al., 1996), although not without its biases, has proven to be a useful tool in complementary studies to traditional physiological and metabolic biochemistry approaches, including many studies in comparative biology (Gracey et al., 2001; Fiol et al., 2006).

2. Materials and methods

2.1. Animals and feeding protocols

Males of the spiny dogfish shark (*Squalus acanthias suckleyi*) were captured by angling or trawl in Barkley Sound, BC (Canada) offshore from Bamfield Marine Sciences Centre in June, 2007 and 2009 and August 2012 under various permits from the Canadian Department of Fisheries and Oceans. Note that Ebert et al. (2010) have recently proposed that these north-east Pacific dogfish may be a separate species (*Squalus suckleyi*) rather than a subspecies of *S. acanthias*. Consistent with our prior feeding experiments (see Wood et al., 2010), fish were maintained in a 155,000 L circular tank supplied with running seawater and aeration. After acclimation to the tank, dogfish fed voraciously on a diet of frozen hake supplied every 4th day to a ration size of approximately 2.5% body weight. At 6 h, 22 h (48 h for qPCR only), and 7 days post-feeding (this latter group was removed to a separate tank to ensure that additional feeding did not take place, and is referred to as 'Fasted' below), dogfish were sacrificed by overdose of MS-222 (1 g/L) and the rectal glands removed, frozen in liquid nitrogen and stored at −80 °C. Feeding status was confirmed by examining the gut for the presence (or absence) of food. All experiments were conducted in accordance with guidelines of the Canadian Council of Animal Care, and under approvals from the Animal Care Committees of the University of Ottawa, McMaster University and the Bamfield Marine Sciences Centre.

2.2. Isolation of RNA and construction of a normalized cDNA library

A normalized cDNA library from dogfish rectal gland was created in order to form a potential basis for comparison to the distribution of genes identified (e.g., Gene Ontology (GO) analysis categorization) from more specific treatments by the SSH approach. Total RNA was isolated by standard Trizol (Invitrogen, Grand Island, NY, USA) extraction methods from individual rectal gland samples from 6 fish each from the 6 h, 22 h and 'Fasted' treatments and the RNA integrity was verified with a BioAnalyzer 2100 (Agilent, Mississauga, ON, Canada). Equal amounts of total RNA from these 18 fish were pooled to yield a single sample from which polyA mRNA was further purified by a magnetic bead-based method (Ambion Poly(A)Purist MAG, Ambion, Austin, TX, USA). A normalized cDNA library was created using the Creator SMART cDNA Construction Kit (Clontech, Mountain View, CA, USA) and CDS-3M Adaptors in the Trimmer Direct cDNA Normalization Kit (Evrogen, Moscow, Russia); following normalization, *Sfi*I digestion, and size fractionation, cDNA was directionally inserted into a pTB vector, and transformed into *Escherichia coli* via electroporation. Bacteria were grown on LB agar plates with carbenicillin (50 μg/mL), and individual colonies were hand-picked at random and grown overnight at 37 °C in LB medium with carbenicillin. PCR was performed directly on 1 μL aliquots of this broth (using primers against the arms of the pTB vector) and PCR products cleaned and sequenced on an ABI 3730xl sequencer (using slight modifications of the methods from Oleksiak et al., 2001).

2.3. Construction of SSH libraries

Equal quantities of total RNA from the above samples of 18 glands were pooled (6 fish per pool) into three samples each representing 6 h, 22 h and 'Fasted' treatments and mRNA was purified as above. Tester and Driver cDNA was synthesized and adaptor-ligated, and subtractions performed as per instructions in the PCR-Select cDNA Subtraction Kit (Clontech). Six libraries were created representing forward and reverse subtractions for the three treatments (letters correspond to those in tables in Results and discussion): (A) fasted minus 6 h; (C) 6 h minus fasted; (E) fasted minus 22 h; (G) 22 h minus fasted; (I) 6 h minus 22 h; and (K) 22 h minus 6 h. PCR products from second amplifications were cloned into pCR2.1 TOPO vector (Invitrogen), transformed into *E. coli*, and colonies were hand-picked, grown in liquid broth overnight at 37 °C and PCR and sequencing was performed as above, except that primers were to the M13R and M13F(-20) sites of this vector.

2.4. Sequence analysis and annotation

Much of the analysis was similar to that conducted in a prior study by our group on transcriptome variation in *Aplysia* sp. (Fiedler et al., 2010). We have defined quality ESTs as sequence reads with length ≥100 bp after trimming of low quality (phred score < 20) ends, and vector sequence removal using cross_match (Ewing and Green, 1998;