Comparative Biochemistry and Physiology, Part D 22 (2017) 32–38

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Metabolic responses to intestine regeneration in sea cucumbers *Apostichopus japonicus*

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ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form 27 January 2017
Accepted 2 February 2017
Available online 04 February 2017

Keywords:
Sea cucumber
Metabolic responses
Gene expression
Intestine regeneration

ABSTRACT

Sea cucumbers are excellent models for studying organ regeneration due to their striking capacity to regenerate most of their viscera after evisceration. In this study, we applied NMR-based metabolomics to determine the metabolite changes that occur during the process of intestine regeneration in sea cucumbers. Partial least-squares discriminant analysis showed that there was significant differences in metabolism between regenerative intestines at 3, 7, and 14 days post evisceration (dpe) and normal intestines. Changes in the concentration of 13 metabolites related to regeneration were observed and analyzed. These metabolites included leucine, isoleucine, valine, arginine, glutamate, hypotaurine, dimethylamine, *N*,*N*-dimethylglycine, betaine, taurine, inosine, homarine, and histidine. Three important genes (betaine-aldehyde dehydrogenase, betaine-homocysteine *S*-methyltransferase 1, and dimethylglycine dehydrogenase) were differentially expressed to regulate the levels of betaine and *N*,*N*-dimethylglycine during intestine regeneration. These results provide an important basis for studying regenerative mechanisms and developing regenerative matrixes.

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1. Introduction

Sea cucumbers have become a representative species for studying tissue regeneration (García-Arrarás et al., 1998; Sun et al., 2011). These animals are capable of regenerating their intestine, respiratory tree, and body wall after evisceration or injury. In recent years, visceral regeneration has gained much attention in regeneration and development research (Ba et al., 2015; Mashanov et al., 2013, 2014, 2015a,b). Intestine regeneration has especially raised the interest of many scholars. Intestinal regeneration in sea cucumbers is a complex combination of morphogenetic events involving cell migration, cell division, cell death, cell dedifferentiation, and extracellular matrix remodeling (García-Arrarás et al., 1998, 2006, 2011; Quinones et al., 2002; San Miguel-Ruiz and García-Arrarás, 2007). Because of their complexity, the regulatory mechanisms involved in sea cucumber regeneration remain unclear.

Due to the limitation of molecular biotechnology prior to 2000 year, a series of studies have been performed to investigate the mechanisms of intestine regeneration in sea cucumbers in the last 15 years (García-Arrarás et al., 2011; Mashanov and García-Arrarás, 2011; Mashanov et al., 2010, 2011, 2014, 2015a; Sun et al., 2013a). During this time, major progress has been made in understanding the cellular and molecular events involved in this process. The nature and possible sources of the cells involved in tissue formation of the regenerative intestine, as well as the role of cell proliferation, migration, and death, have been previously reported and discussed (Mashanov and García-Arrarás, 2011). With the development of cellular event studies, more efforts were needed to characterize the genetic basis of the regulatory mechanisms of intestine regeneration. The original strategy to characterize the genetic basis of these regulatory mechanisms was to analyze the genes associated with development or regeneration in other species by conducting analyses gene by gene. Examples of genes analyzed using this strategy include *ependymin* (Suarez-Castillo, 2004), *wnt6*, *Hox6* (Sun et al., 2013b), *Wnt9*, and *Bmp1* (Mashanov et al., 2011), among others. Fortunately, the emerging high-throughput sequencing technology now provides a powerful tool to construct global gene expression profiles, which allow for a more comprehensive view of intestine regeneration in sea cucumbers (Pablo et al., 2009; Sun et al., 2011, 2013a).

During the early and middle stages of intestine regeneration, sea cucumbers cease food intake before resuming the function of the regenerated intestine. Hence, various metabolic and physiological parameters change dramatically when energy and nutrients are needed to maintain cell differentiation and proliferation for the rapid formation of new intestinal tissue. Various macro-metabolic changes in *A. japonicas* that occur during viscera regeneration have been reported. For example, the average oxygen consumption of normal sea cucumbers was 25.96 ± 1.82 μg g^{-1} h^{-1}. In contrast, the oxygen consumption of

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http://dx.doi.org/10.1016/j.cbd.2017.02.003
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eviscerated sea cucumbers decreased from 6.22 ± 1.20 μg g^{-1} h^{-1} to 3.29 ± 1.39 μg g^{-1} h^{-1} at 5 days post evisceration (dpe). As the regeneration process continued, oxygen consumption increased slowly to normal levels (Tan et al., 2008). Furthermore, the total ammonia excretion rate increased gradually during intestine regeneration. At the start of the regeneration process, the ammonia excretion rate was 0.0060 ± 0.002 μg g^{-1} h^{-1}, which was only 8.67% of the average ammonia excretion rate of the control (Tan et al., 2008). It has also been demonstrated that during intestine regeneration, lipids and carbohydrates are consumed as energy materials and that the content of proteins and crude lipids also decrease (Tan et al., 2008). In our previous study, we found that gene ontology terms associated with the "primary metabolic process" were significantly enriched during intestine regeneration (Sun et al., 2013a). Moreover, the expression of genes involved in the regulation of the pathways "Glycerophospholipid metabolism", "alpha-Linolenic acid metabolism", "Starch and sucrose metabolism", "Glutathione metabolism" and "Glycine, serine, and threonine metabolism" changed during regeneration (Sun et al., 2013a). However, how micro-metabolism changes and how these pathways modulate the production of metabolites during sea cucumber intestine regeneration remain unknown.

Metabolomics is a systems biology tool that allows for the accurate and reproducible determination of the metabolic composition of a sample and the identification of metabolic changes occurring in specific physiological states. Nuclear magnetic resonance (NMR) spectroscopy-based metabolomics is a robust means to provide basic information on a large range of endogenous, low-molecular-weight metabolites in tissues or cells (Wu et al., 2008). NMR-based metabolomics has been successfully applied in research examining the metabolomic responses of sea cucumbers to thermal stresses (Shao et al., 2015), metabolic responses of Manila clams exposed to mercury and copper (Liu et al., 2011a,b; Zhang et al., 2011), biomarker identifications of liver regeneration (Bollard et al., 2010), metabolic changes of systemic lupus erythematosus (Ouyang et al., 2011), the rapid screening test for urinary tract infection (Lam et al., 2014), and nutrition research (dietary intervention, dietary biomarker and diet related disease studies) (Brennan, 2014).

Intestine regeneration of sea cucumbers can be divided into four key stages: wound healing (0–3 dpe), blastema formation (3–7 dpe), lumen formation (7–14 dpe), and intestine differentiation and growth (14 dpe) (García-Arrarás et al., 1998). In the present study, NMR-based metabolomics was used to investigate metabolic changes during intestine regeneration (at 3, 7 and 14 dpe) in *Apostichopus japonicus*. Betaine-aldehyde dehydrogenase (BADH) has been demonstrated to catalyze the last step of betaine synthesis (Ishitani et al., 1995). Betaine-homocysteine *S*-methyltransferase 1 (BHST) can convert betaine to *N*,*N*-dimethylglycine and was found to play a neuroprotective role in protecting the brain from certain cerebrovascular and neurodegenerative diseases (Zhang et al., 2013). Dimethylglycine dehydrogenase (DMDH) converts *N*,*N*-dimethylglycine to sarcosine, and subsequently, sarcosine dehydrogenase converts sarcosine to glycine (Binzak et al., 2000). Hence, the mRNA expression of key regulatory genes was investigated to analyze the reason for metabolic changes.

2. Materials and methods

2.1. Animals

Specimens of *A. japonicus* (100 ± 20 g) were collected from the coast of Laoshan, Shandong Province, China. All animals were cultured in seawater aquaria at 15 ± 1 °C for a week to ensure environmental adaptation. Evisceration was induced by injecting 2.5–4 mL of 0.35 M KCl into the coelom. Non-eviscerated sea cucumbers (*i.e.*, controls) were fed once a day, and the eviscerated sea cucumbers were not fed until the intestine collection. Eight replicates (*i.e.*, individual sea cucumbers) were used at each regeneration time point (3, 7 and 14 dpe) for each experimental group. Regenerative intestines were dissected on days 3, 7, and 14 post evisceration. Normal intestines of non-eviscerated sea cucumbers were dissected as control tissues. After dissection and tissue collection, the samples were immediately flash-frozen in liquid nitrogen and then stored at −80 °C for the next experiment.

2.2. Metabolite extraction

Polar metabolites of the *A. japonicus* intestine (eight biological replicates for each treatment) were extracted according to the protocol described by Wu et al. (2008). In Brief, ~80 mg of intestine was ground in liquid nitrogen and homogenized in 400 μL of methanol, 500 μL of water, and 200 μL of chloroform. The mixture was then centrifuged at 2000 × *g* for 10 min at 4 °C. The methanol/water layer was transferred to a centrifugal concentrator where it was dried. The samples were then re-dissolved in 600 μL of sodium phosphate buffer in D_2O followed by centrifugation at 2500 × *g* for 5 min at 4 °C. Approximately 500 μL of the supernatant was transferred into 5 mm NMR tubes for NMR analysis.

2.3. NMR spectroscopy, spectral pre-processing, and data analysis

Metabolite extracts from intestines were analyzed using a Bruker AV 500 NMR spectrometer set at 500.18 MHz and 25 °C as described by Zhang et al. (2011). Briefly, one-dimensional (1D) ^{1}H NMR spectra were obtained using a standard 1D NOESY pulse sequence according to the manufacturer's instructions. The NMR spectra were obtained using an 11.9 ms pulse, a 6009.6 Hz spectral width, a mixing time of 0.1 s, and a 3.0 s relaxation delay with a standard 1D NOESY pulse sequence with 128 transients collected into 16,384 data points. Datasets were zero-filled to 32,768 points, and exponential line-broadenings of 0.3 Hz were applied before transformation. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin 2.1 (Bruker). ^{1}H NMR spectra from control and regenerative intestines were converted using custom-written ProMetab software in MATLAB version 7.0 (The MathsWorks, Natick, MA, USA), processed by SIMCA-P software (version 11.0, Umetrics, Sweden), and further analyzed by orthogonal projections to latent structure discriminant analysis according to a previously described method (Liu et al., 2011a,b; Zhang et al., 2011). One-way analysis of variance (ANOVA) was conducted to perform comparisons between control intestines and intestines at different stages of regeneration, as well as to test for statistical significance ($P < 0.05$) of the separations.

2.4. Real-time PCR

To further explore the response mechanism of key metabolites during intestine regeneration, we analyzed three genes: betaine-aldehyde dehydrogenase, BADH; betaine-homocysteine *S*-methyltransferase 1, BHST; and dimethylglycine dehydrogenase, DMDH. These genes code for key enzymes that regulate the production and metabolism of betaine and *N*,*N*-dimethylglycine. Total RNA was extracted and treated with DNase using an RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Germany) according to the manufacturer's instructions. The quality and concentration of RNA were evaluated using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and gel electrophoresis. The RNA Integrity Number of all extracted RNA is ≥9.4, and the RNA ratio (28S/18S) is ≥1.8. First-strand cDNA was synthesized in a 25-μL reaction system as follows: RNA (4 μL) and oligodT18 (1 μL) were denatured at 70 °C for 5 min. Then, M-MLV reverse transcriptase (1 μL; Promega), M-MLV buffer (5 μL; 25 mmol/L KCl, 10 mmol/L Tris-HCl, 0.6 mmol/L MgCl2, and 2 nmol/L DTT, pH 8.3), dNTPs (5 μL), ribonuclease inhibitor (1 μL), and RNase-free water (8 μL) were added. This reaction mix was incubated at 42 °C for 1 h. The synthesized cDNAs were diluted with RNase-free water and stored at −80 °C for subsequent analysis by real-time PCR.

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