Journal of Insect Physiology 91–92 (2016) 76–83

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

A novel arrangement of midgut epithelium and hepatic cells implies a novel regulation of the insulin signaling pathway in long-lived millipedes

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 29 May 2016
Accepted 29 June 2016
Available online 30 June 2016

Keywords:
Midgut epithelium
Hepatic cells
Insulin signaling
Lifespan
Exosomes
Diplopoda

ABSTRACT

Nutrients absorbed by the epithelial cells of the millipede midgut are channeled to a contiguous population of hepatic cells where sugars are stored as glycogen. In insects and other arthropods, however, nutrients absorbed by midgut epithelia are first passed across the epithelial basal surface to the hemolymph before storage in fat body. The inter-digitation of cellular processes at the interface of hepatic and midgut epithelial cells offers a vast surface area for exchange of nutrients. At this interface, numerous small vesicles with the dimensions of exosomes (~30 nm) may represent the mediators of nutrient exchange. Longevity and the developmental arrest of diapause are associated with reduced insulin signaling. The long lifespans for which millipedes are known may be attributable to a novel pathway with reduced insulin signaling represented by the novel arrangement of hepatic storage cells and midgut epithelial absorbing cells.

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1. Introduction

Millipedes are unusual among the arthropods in being particularly long-lived. Although there are only a few well-documented studies on the lifespans of millipedes, members of this class of arthropods whose life cycles have been closely followed are known to live for not weeks or months like most arthropods, but for years. These millipedes mature over a period of several years, and the lifespan of the pill millipede *Glomeris marginata* has been recorded as having a duration of at least 11 years. The exceptional longevity of these arthropods has been attributed to the poor quality of food that they consume (Hopkin and Read, 1992). Can millipede longevity be attributed to the organization and physiology of cells and tissues that process this food?

An association between the hepatic cells and midgut epithelia observed in millipedes is not observed in insects or even in their fellow myriapods the centipedes (Rost-Roszkowska et al., 2015; Nardi and Bee, 2012; Snodgrass, 1935). In insects the sources of digestive sugars and sinks of stored sugars are disjunct, with sugars being transferred via hemolymph from midgut to storage in the fat body (Wigglesworth, 1972). In millipedes, sugars from the midgut epithelium are directly transferred to storage in contiguous hepatic cells without their traversing a blood space. This intimate association of nutrient source (midgut epithelium) and nutrient storage cells (hepatic cells) is a novel feature of these organisms that feed on nutrient-poor detritus.

Does a connection exist between millipede longevity and this intimate association of midgut epithelium and hepatic mesenchyme cells? This novel structural feature of the millipede alimentary canal channels sugars destined for storage directly from digestive cells to contiguous hepatic cells rather than being typically transported through the hemolymph to non-contiguous fat body and/or muscle for storage as they are in other arthropods (Wigglesworth, 1972). An association of nutrient-absorbing and nutrient-storing cells channeling sugars directly to storage cells and bypassing transit through the hemolymph represents a novel insulin signaling pathway for this arthropod. Altered insulin-like signaling pathways are known to prolong lifetime in other invertebrates and vertebrates (Giannakou et al., 2004; Hwangbo et al., 2004). At present information on the number, functions, and sources of insulin-like peptides in millipedes is not available. The novel structural features of the millipede midgut epithelium-hepatic cell interface, however, implies a novel organization for its insulin signaling pathway.

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http://dx.doi.org/10.1016/j.jinsphys.2016.06.011
0022-1910/Published by Elsevier Ltd.

2. Materials and methods

2.1. Sources of millipede tissues

Specimens of the common julid millipede *Cylindroiulus caeruleocinctus* (order Julida) were collected from an organic garden in Urbana, Illinois. These millipedes provided all images but two (Figs. 3c and d) for this study.

Midgut epithelia was also obtained from specimens of the cave millipede *Cambala speobia* that had been collected in Texas for a study of hindgut microbes (Nardi et al., submitted for publication). Specimens were sectioned to confirm the occurrence of the hepatic cell-midgut epithelium association in this other order of millipedes (order Spirostreptida).

2.2. Preparation of millipede gut for microscopy

Prior to dissection, millipedes were placed on ice for 15 min and then briefly dipped in 70% ethanol, quickly air-dried in a laminar flow hood, and submerged in sterile Grace's insect medium (Invitrogen) whose pH had been adjusted to 6.5. Dissection was carried out in petri dishes (35 × 10 mm) to which black Sylgard (Dow Corning) had been added as a substrate. The head and last three abdominal segments of each millipede were first removed with iridectomy scissors. With two fine forceps pulling in opposite directions, the integument was systematically removed at both anterior and posterior ends of each specimen, in this manner removing integument from the underlying intact gut several segments at a time. To the silicone surface of the dissecting dish, gut tissues were pinned with stainless steel minutien pins (0.1 mm diameter). After dissection, tissues were either processed for (1) sectioning or for (2) preparation of whole mounts.

2.3. Preparation of sections and whole mounts of millipede gut

In order from outermost to innermost, a layer of hepatic cells, a layer of longitudinal muscle layers, and a layer of circular muscles cover the basal surface of the millipede midgut epithelium. For viewing the basal surfaces of the midgut and hindgut as a whole mount, the cylindrical gut epithelium with its attached muscles was cut along its anterior-posterior axis with iridectomy scissors and then spread as a planar rectangle by pinning the corners of its cuticle-lined anterior foregut and posterior hindgut ends. After addition of fixative, the pinned tissue retained its configuration. Gut tissue for whole mounts of the midgut was fixed briefly in Carnoy fixative prior to staining with periodic acid-Schiff reagent to reveal glycogen deposits. Harris' hematoxylin was added as a counterstain.

Specimens that were sectioned for light microscopy and electron microscopy were fixed at 4 °C in a primary fixative of 2.5% glutaraldehyde and 0.5% paraformaldehyde dissolved in a rinse buffer of 0.1 M cacodylate (pH 7.4) containing 0.18 mM $CaCl_2$ and 0.58 mM sucrose. After three hours in this fixative, tissues were washed three times with rinse buffer before being transferred to the secondary fixative (2% osmium tetroxide in rinse buffer). Tissues remained in this solution for 4 h in the cold and were then washed three more times with rinse buffer. Contrast of membranes was enhanced by placing rinsed tissues in filtered, saturated uranyl acetate for 30 min before being gradually dehydrated in a graded ethanol series (10%–100%).

From absolute ethanol, tissues for sectioning were transferred to propylene oxide and infiltrated with mixtures of propylene oxide and resin before being embedded in pure LX112 resin. Resin was polymerized at 60 °C for three days followed by an additional overnight treatment in an 80 °C oven.

Embedded tissues were sectioned with a diamond knife either at 0.5 μm for light microscopy or at ~0.09 μm for electron microscopy. Sections for light microscopy were mounted on glass slides and stained with a solution of 0.5% toluidine blue in 1% borax. Regions of alimentary canal chosen for ultrastructural examination were mounted on copper grids and stained briefly with saturated aqueous uranyl acetate and Luft's lead citrate to enhance contrast. Images were taken with a Hitachi 600 transmission electron microscope operating at 75 kV.

3. Results

3.1. Relative proportions of gut length devoted to midgut and hindgut in detritivores

Millipedes are a class of arthropods whose members are detritus feeders. Insects with similar diets, such as passalid beetles and termites have hindguts whose lengths greatly exceed the lengths of their midguts (Nardi et al., 2006). Although hindguts of both millipedes and insect detritivores are densely populated by microbes, the millipede hindguts are always shorter than their midguts (Fig. 1a) – just the opposite arrangement that is observed for insect detritivores.

3.2. Concentric arrangement of cells around the gut epithelium of millipedes

The cylindrical gut of arthropods is characterized by a stereotypical arrangement of cell layers that radiate centrifugally from the basal surface of the innermost gut epithelium. For insects the outermost layer of cells surrounding the gut is made up of a layer of longitudinal muscles. Nestled between this outermost layer of oriented longitudinal muscles lies another layer of circular muscles that is oriented perpendicular to the anterior-posterior axis of the outermost muscle layer (Snodgrass, 1935). This transverse layer of circular muscles lies adjacent to the basal lamina of the gut epithelium (Nardi and Bee, 2012). The two mutually perpendicular layers of muscles establish a gridwork of fibers separating midgut epithelium from the hepatic cells (Fig. 1b–d).

What is novel about the arrangement of cell layers around the midgut epithelium in millipedes is the addition of a layer of loosely coherent hepatic mesenchymal cells. Unlike the gut epithelial cells with their single basal lamina spread over the basal surface of their monolayer, each of these hepatic cells is circumscribed with its own thin basal lamina (Fig. 2a–d).

Nutrients (sugars) that are readily absorbed by the midgut epithelium are channeled into the mantle of hepatic cells surrounding the midgut epithelium as suggested by the massive stores of glycogen visible in these cells (Figs. 1b–d, 2 and 3). The cylindrical sheath of hepatic cells functions as a sink where reserves of glucose from the midgut can be stored. The glycogen stored in the hepatic cells is insoluble and unavailable for reabsorption by the midgut epithelial cells. These stored carbohydrates, however, can presumably be remobilized when conditions necessitate translocation. This direct transfer of glucose from midgut epithelia to hepatic cells rather than to the blood may eliminate the need to control the blood sugar level and its uptake by fat body cells or muscle cells. Instead glycogen stores in hepatic cells can be converted to blood glucose/trehalose on demand.

3.3. Asymmetric interaction at the interface

The electron-density of the cytoplasm within midgut epithelial cells contrasts with the lighter hepatic cell cytoplasm. This difference in density facilitates tracking the connections between the epithelial and mesenchymal populations. The basal processes of