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Analysis of the morphology and distribution of argentaffin, argyrophil and insulin-immunoreactive endocrine cells in the small intestine of the adult opossum *Didelphis aurita* (Wied-Neuwied, 1826)

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ABSTRACT

The aim of this study was to identify and quantify the argyrophil, argentaffin and insulin-immunoreactive cells (IIC) in the small intestine of the opossum *Didelphis aurita*. Seven adult male specimens of opossums were investigated. The animals were captured, and their blood insulin levels were determined. After euthanasia, fragments of the small intestine were processed for light microscopy and transmission electron microscopy, and submitted to histochemistry and immunohistochemistry for identification of argyrophil and argentaffin endocrine cells, and IIC. Argyrophil and argentaffin cells were identified in the intestinal villi and Liberkühn crypts, whereas IIC were present exclusively in the crypts. Ultrastructure of the IIC revealed cytoplasmic granules of different sizes and electron densities. The numbers of IIC per mm^2 in the duodenum and jejunum were higher than in the ileum ($p < 0.05$). The animals had low levels of blood insulin (2.8 ± 0.78 μIU/ml). There was no correlation between insulin levels and the number of IIC in the small intestine. The IIC presented secretory granules, elongated and variable morphology. It is believed that insulin secretion by the IIC may influence the proliferation of cells in the Liberkühn crypts, and local glucose homeostasis, primarily in animals with low serum insulin levels, such as the opossum.

1. Introduction

Coordination of the activities of the various components of the gastroenteropancreatic endocrine system is directly related to the activities of all the endocrine cells scattered throughout the gut and pancreas. Peptide hormones control the secretion, absorption, motility, and proliferation of these cells in a synchronized manner. Some of these hormones are called "candidates" because their functions have not yet been fully established (Freitas-Ribeiro et al., 2011).

Drucker (2007) reported that the gastrointestinal tract plays a crucial role in controlling nutrient intake and absorption. This author added that enteroendocrine cells control important regulatory mechanisms, synthesizing hormones, and activating and inhibiting neural circuits. Further, gastrointestinal hormones are secreted in response to specific stimuli through a diffuse system of specialized cells that can be found scattered throughout the digestive tract mucosa (Santos and Zucoloto, 1996).

Located inside the stomach and intestines of vertebrates, enteroendocrine cells constitute approximately 1% of the total intestinal epithelium, and represent the largest population of hormone-producing cells in the body (Rehfeld, 1998). These cells show immunoreactivities to serotonin, somatostatin, cholecystokinin, gastrin, secretin, glucagon, motilin, histamine, and gastric inhibitory peptide (Krause et al., 1989). The hormone insulin is produced primarily by the pancreas. However, in studies using immunohistochemical techniques, it has also been detected in the prostate (Stahler et al., 1988), nephron (Coutinho et al., 1985), central nervous system (Devaskar et al., 2002), retina (Meimaridis et al., 2003), and intestines of several mammals (Bendayan and Park, 1991; Kendzierski et al., 2000).

As part of the pancreas, β cells located in the intestinal mucosa have specialized secretory granules that are involved in the synthesis, maturation, and storage of insulin with potential paracrine activity (Bendayan and Park, 1991). In a previous study using a porcine model, Kendzierski et al. (2000) found insulin immunoreactivity in superficial epithelial cells of the colon, and glandular cells of the intestine. According to these authors, there is no doubt glucagon and other pancreatic peptides are synthesized in the gut; however, confirmation of intestinal insulin production still needs further investigation. Although researchers have principally investigated the effects of insulin on carbohydrate metabolism, insulin influences the speed of other processes independent of its role in

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glucose metabolism. According to Kendzierski et al. (2000), insulin has important intestinal paracrine or even autocrine functions in controlling cell division and peptide secretion, absorption, and motility.

In studying the endocrine system, *didelphidae* opossums are viewed as a good model due to the simultaneous differentiation of the digestive system and endocrine glands when the animal is still in the intra-marsupial period (Krause et al., 1989; Fonseca et al., 2002). In addition, the conservation of the histological organization of the opossum's digestive system throughout its evolution adds to knowledge about the evolution of this physiological system, and the development of comparative studies with other animal species which have experienced different levels of morphofunctional adaptation and organization of organs and tissues (Coutinho et al., 1985; Paiva et al., 1992).

Given the relevance of studying endocrine cells that are diffusely present in the intestines of mammals, there are few immunohistochemical studies of IIC that address their morphology in the digestive tract of marsupials (Takagi et al., 1990; Fonseca et al., 1998, 2002; Christensen, 2003). To support additional studies on this subject, there are still doubts about the location and distribution of intestinal IIC, primarily due to differences in the findings of Kendzierski et al. (2000) as compared to Coutinho et al. (1984), Ito et al. (1988), and Bendayan and Park (1991). Thus, in an attempt to fill in gaps in the available information about the morphology and distribution of IIC compared to argentaffin and argyrophil endocrine cells in the small intestine, this study was designed to identify and quantify these cells in the duodenum, jejune, and ileum of the adult opossum (*Didelphis aurita*).

2. Methods

2.1. Animals

Seven male specimens of opossum (*D. aurita*) with an average weight of 1.03 ± 0.14 kg were selected for study. The animals were considered to be adults according to their body weight and because they had full dentition, according to the classification of Macedo et al. (2006).

Hook-like traps measuring 75 cm × 31 cm × 31 cm were used to capture them. The animals remained in captivity for a period of 24 h, receiving water, banana, and an egg until the moment of their sacrifice. This diet was similar to their natural diet (Carvalho et al., 2005). The captures were authorized by IBAMA (license no.19555-1) and completed between July 2009 and October 2010 in a small forest located in the Zoology Museum, Federal University of Viçosa (UFV), Minas Gerais, Brazil. The experimental procedures involving animals were approved by the Ethics Commission, Department of Veterinary, UFV (protocol 39/2009).

2.2. Biometrical and biochemical analysis

Following capture, while still inside the trap, the animals were anesthetized with sodium thiopental 30 mg/mL at a dose of 60 mg/kg intraperitoneally. After being removed from the cages, the snout-rump length of the animals was measured from the apex of the snout to the base of the tail (Fonseca et al., 2002). Under anesthesia, the abdominal cavity of each animal was opened at its midline. In addition, 10 mL blood samples were collected from the cava and the portal veins to determine blood insulin levels using a radioimmunoassay method with high analytical sensitivity, as previously described (Souza et al., 1982). Then, the duodenum, jejunum, and ileum were identified and delimited by means of a ligature tied with strings. Euthanasia was performed by intracardiac administration of potassium chloride 0.25%, depending upon body weight. The intestines were removed and placed on a spout, allowing for real measurement by caliper with no distension of the segments (Fonseca et al., 2002).

2.3. Histochemical and immunohistochemical analysis

For the histological study, two 1 cm^2 pieces (one-third between the early and middle, and one between the middle and end) of the duodenum, jejunum, and ileum of each animal, totaling six fragments, were collected. These pieces were fixed in 10% buffered formalin, dehydrated in ethanol, diaphanized in xylene, and embedded in paraffin. Next, 4 μm thick histological sections were cut in a Leica Multicut 2045® rotary microtome (Reichert-Jung Products, Germany). To avoid repeat analysis of cells, sections were evaluated in semi-series, using one in every ten sections (Fonseca et al., 2002). The sections were mounted on histology slides and stained using the Grimelius (Grimelius and Wilander, 1980) and Masson-Fontana (Masson, 1928) methods for argyrophil and argentaffin endocrine cells, respectively. Using a direct peroxidase staining technique (Sternberger, 1979), sections also were submitted to immunohistochemistry to mark IIC, using anti-insulin monoclonal antibodies (Bethyl Laboratory, lot number A90-117p-4). Sections of pancreas from the opossums were used as positive controls, and subjected to all of the same histological processing techniques described.

The slides were examined, and the images captured using a BX-60® light microscope connected to a QColor-3® digital camera (Olympus, Toquio, Japan). The endocrine cells were investigated in ten random fields for each staining technique and intestinal segment. These cells were quantified in the intestinal mucosa using a 40× objective lens. Further, each cell type was determined in a total area of 1.41×10^6 μm^2 for each intestinal segment analyzed. The counting of positive cells was performed by unit area in μm^2 using the image analysis software Image Pro-plus 4.5® (Media Cybernetcs, Silver Spring, MD, USA). In addition, cell density was determined. The results of the cell counts were represented as average values from the two regions analyzed for each intestinal segment.

2.4. Ultrastructural analysis

The segments of the duodenum with the highest densities of IIC were transferred to a fixative solution (2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer [pH 7.2]) for 24 h at 4 °C. After rinsing with buffer, samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h. Dehydration was then carried out in a series of graded acetones, followed by embedding in Epon 812 resin. After inclusion, semithin sections were acquired and subjected to the deplastification technique of Lane and Europa (1965), followed by immunohistochemistry to mark the IIC. Then, ultrathin sections were obtained in the region of the same block containing the cells identified as IIC. The sections were delimited using the previously described trapezoidal method (Fonseca et al., 1998). Ultrathin sections were stained with 2% uranyl acetate and 0.2% lead citrate in 1 M sodium hydroxide, and observed with a Zeiss EM 109 transmission electron microscope.

2.5. Statistical analysis

The data were reported as means and standard deviations (mean ± S.D.). The normalcy of the data distribution was verified using the Shapiro–Wilk test. The Wilcoxon–Mann–Whitney test was used to compare the number of endocrine cells per mm^2 in regions 1 (between the initial third and the middle) and 2 (between the middle and the end) of each intestinal