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Expression and immunohistochemical detection of Nesfatin-1 in the gastrointestinal tract of Casertana pig

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ABSTRACT

In this study, we report nesfatin-1 immunoreactivity in the gastrointestinal tract of Casertana breed pig. The newly discovered anorexigenic peptide nesfatin-1 has been shown to possess physiological relevance in regulating food intake and energy homeostasis at a central level, although evidence has been accumulating that it may also play important functions at a more local gastroenteric level. Nesfatin-1 immunoreactive endocrine cells have been detected in the gastric fundus and ileocecal valve. Nesfatin-1 immunopositive neurons and nerve fibers have been observed mainly in the enteric plexuses. Western blot analysis confirmed the immunohistochemical observations, showing immunoreactive bands in all analyzed gastrointestinal tracts with the exception of the rectum. Nesfatin-1 immunodetection in the swine digestive system reinforces the importance of the role played by nesfatin-1 at the gastrointestinal level and sustains the necessity to study the role of this peptide in the regulation of food intake in farm species for which weight gain is essential for optimizing production.

Introduction

Nesfatin-1 is an 82 amino acid polypeptide, identified in 2006 by Oh-I et al. as the amino-terminal fragment of nucleobindin2 (NUCB2), a protein of 396 amino acids highly conserved in humans, mice, and rats. The protein is proteolytically processed by the pro-hormone convertase to at least three peptides: nesfatin-1 (1–82), nesfatin-2 (85–163), and nesfatin-3 (166–396). An initial report indicates that nesfatin-1 (named as acronym for NEFA/nucleobindin2-encoded satiety- and fat-influencing protein) may have physiological relevance in regulating food intake. This was based on convergent observations that nesfatin-1 injected into the third brain ventricle reduced food consumption occurring during the dark phase, whereas nesfatin-2 or nesfatin-3 had no effect (Oh-I et al., 2006). Thus, it seems that nesfatin-2 and nesfatin-3 are not involved in feeding regulation, but possess archetypal motifs, namely possible calcium-binding sites (Barkinol-Watanabe et al., 1994; Karabinos et al., 1996). Nesfatin-1 is localized in the periventricular nucleus of the hypothalamus, as well as in a large number of regions outside the hypothalamus, including the Edinger-Westphal nucleus, dorsal motor nucleus of vagus, caudal raphe nuclei, locus coeruleus, thalamic parasympathetic neuronal groups, among others (Brailoiu et al., 2007; Foo et al., 2008; Goebel et al., 2009). Such a wide pattern of distribution of nesfatin-1, with prominent expression, not only in hypothalamic nuclei, but also in other brainstem areas and autonomic centers, strongly suggests its potential function as an integral regulator of energy homeostasis, in particular the regulation of feeding behavior and body weight (Garcìa-Galiano et al., 2010).

The mechanism whereby nesfatin-1 conducts such feeding-suppressive actions seems to be independent of leptin signaling, as nesfatin-1 is capable of inhibiting food intake in rodents bearing inactivating mutations of leptin receptor (Oh-I et al., 2006; Shimizu et al., 2009) and in conditions of leptin resistance, such as diet-induced obesity (Shimizu et al., 2009). Cells that express nesfatin-1 are often found to express other bioactive substances. A large sub-population of hypothalamic nesfatin-1 immunoreactive neurons also expresses immunoreactivity for melanin-concentrating hormone (MCH), cocaine and amphetamine-regulated transcript (CART), alpha-melanocyte-stimulating hormone (αMSH), pro-opiomelanocortin (POMC), vasopressin, oxytocin, growth hormone releasing hormone (GHRH), corticotropin-releasing factor (CRF), thyrotropin-releasing hormone (TRH), somatostatin or neurotensin (Brailoiu et al., 2007; Kohno et al., 2008; Majiema et al., 2009). These neuroanatomical studies suggest a broader role for nesfatin-1 that may encompass, in addition to the regulation of feeding behavior

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and body weight, the central regulation of gastric emptying, stress response, glucose metabolism and puberty (Garcìa-Galiano et al., 2010).

Recently, it has been shown that human nesfatin-1 centrally administered suppresses feed intake in pigs, and stimulates LH secretion in prepuberal gilts, indicating that nesfatin-1 actions are not strictly limited to the control of energy homeostasis (Lents et al., 2013). Moreover, evidence has mounted that nesfatin-1 is also produced in peripheral metabolic tissues, such as the pancreas, adipose tissue and gastrointestinal tract. Indeed, in peripheral tissues, nesfatin-1 was identified in the rat stomach and also in the endocrine pancreas, pituitary gland and testis (Stengel et al., 2009, 2013). Nesfatin-1 immunosignal is mainly localized in mucosal endocrine X/A-like cells within a distinct sub-population of vesicles different from those containing the orexigenic hormone ghrelin (Stengel et al., 2009), suggesting differential regulation and release of ghrelin and nesfatin-1. In the endocrine pancreas of mammals, nesfatin-1 immunostaining is restricted to insulin positive ß-cells with a sub-cellular cytoplasmic localization distinct from that of insulin (Gonzalez et al., 2009; Foo et al., 2010). Collectively, these data provide support for a peripheral role played by nesfatin-1, which may impact on the regulation of hormone secretion, food intake and glycemic control in concert with other hormones such as ghrelin and insulin.

In this framework, we have carried out a study with the aim of identifying nesfatin-1 immunoreactivity in the gastrointestinal tract of Casertana breed pig. The immunolocalization of nesfatin-1 in the swine digestive system is useful to study the role of this peptide in the regulation of food intake in farm species for which weight gain is essential for optimizing production. We chose the Casertana breed pig owing to the commercial interest borne by this species and for the peculiar type of semi-wild breeding.

Materials and methods

Animals and tissue preparation

The gastrointestinal tracts from five healthy adult male pigs of Casertana breed were collected in the local slaughterhouse (Bonea, Benevento, Italy). Upon sacrifice, different portions of the gastrointestinal (GI) tract of each pig were promptly removed and divided into two parts: one part was immersion fixed in Bouin's fluid for 12–24 h at room temperature (RT) and was subsequently processed for wax embedding in Paraplast Plus (McCormick Scientific, Leica Biosystems, Richmond, IL, USA) following routine tissue preparation procedures; the other part was frozen in liquid nitrogen, until use. Specimens were collected from the gastric fundus, duodenum, jejunum, ileum, ileocecal valve, cecum, colon and rectum.

Immunohistochemistry

Sections (7 μm-thick) were cut by microtome, collected on slides, and stained with the avidin–biotin immunohistochemical technique. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide for 30 min at RT, sections were rinsed in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 15 min. Then, sections were incubated for 20 min at RT with normal goat serum (contained in Vectastain Elite ABC kit; PK-6101; Vector Laboratories, Burlingame, CA, USA), and then with primary antibody raised against NUCB2/nesfatin-1 (H-003-22; Phoenix Pharmaceuticals, Burlingame, CA, USA). The antibody was diluted 1:500 and applied on sections overnight at 4 °C.

The following day, sections were rinsed in PBS for 15 min and incubated for 30 min at RT with biotinylated goat anti rabbit IgG (contained in Vectastain Elite ABC kit; PK-6101). Subsequently, the sections were rinsed in PBS for 15 min and then incubated with ABC reagent (contained in Vectastain Elite ABC kit; PK-6101).The final staining was performed using a solution of 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, St. Louis, MO, USA) in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2.

The preparations were observed at a Nikon E 600 light microscope, and microphotographs were taken using a Coolpix 8400 Nikon digital camera.

Controls

Negative controls were obtained by substituting the primary antiserum with PBS or normal serum in the specific step, or alternatively, by absorbing primary antiserum with an excess of the relative peptide (100 mg of peptide/ml of diluted antiserum) (003-22A, Phoenix Pharmaceuticals). Archival samples of male Wistar rat stomach (Harlan, Italy), known to be positive to nesfatin-1, were used as positive reference controls.

Western blot analysis

Fragments of 1 g from each pig gastrointestinal tract and rat stomach were extracted in 6 ml of RIPA (Radio-Immunoprecipitation Assay) buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA), to which protein inhibitors 2 mM PMSF and protein inhibitor cocktail (P8340; Sigma–Aldrich) were added. Samples were homogenized with Ultra-Turrax T25 (IKA-Labortechnik, Staufer, Germany) at 13,500 rpm. Homogenates were then spun at 10,000 rpm for 20 min at 4 °C and supernatants were separately collected and their protein concentration was determined with Bio-Rad dye protein assay. 25 μg of protein for each sample were boiled at 98 °C for 10 min in 25 μg of loading buffer 2× (50 mM Tris–HCl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% blue bromophenol, 10% glycerol). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis with 4% stacking gel in 1% Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a Bio-Rad mini-protean cell (Bio-Rad Laboratories, Hemel Hempstead, UK) at 130 V for 2 h. The separated proteins were electrotransferred onto a nitrocellulose membrane with transfer buffer (39 mM Tris base, 0.2 M glycine, and 20% methanol (pH 8.5) in a minitransfer cell (Bio-Rad) at 100 V at 4 °C for 2 h. Membranes were incubated at 4 °C for 1 h in blocking buffer containing 1% PBS and 0.05% Tween 20 with 5% dried non-fat milk and then probed with rabbit polyclonal antibodies raised against NUCB2/nesfatin-1 (H-003-22; Phoenix Pharmaceuticals) and tubulin (used as internal marker; T3526, Sigma–Aldrich, St. Louis, MO, USA) diluted 1:1000 in blocking buffer, overnight at 4 °C. This step was followed by incubation with secondary anti rabbit IgG (1:5000) for 1 h at RT. Signals were detected by chemiluminescence with the Immobilon Western Chemiluminescent HRP substrate Kit (Millipore, Billerica, MA, USA). Prestained molecular-weight ladder (Novex Sharp protein standard, LC5800, Invitrogen, Hilden, Germany) was used to determine protein size.

Results

Nesfatin-1 immunolocalization in the gastrointestinal tract

Various nesfatin-1 immunoreactive endocrine cells were observed in the glands of the gastric fundus (Fig. 1a–c). Rare nesfatin-1 immunoreactive endocrine cells were detected in the glands of the ileocecal valve (Fig. 1d). These cells were different in shape and were of the closed type.

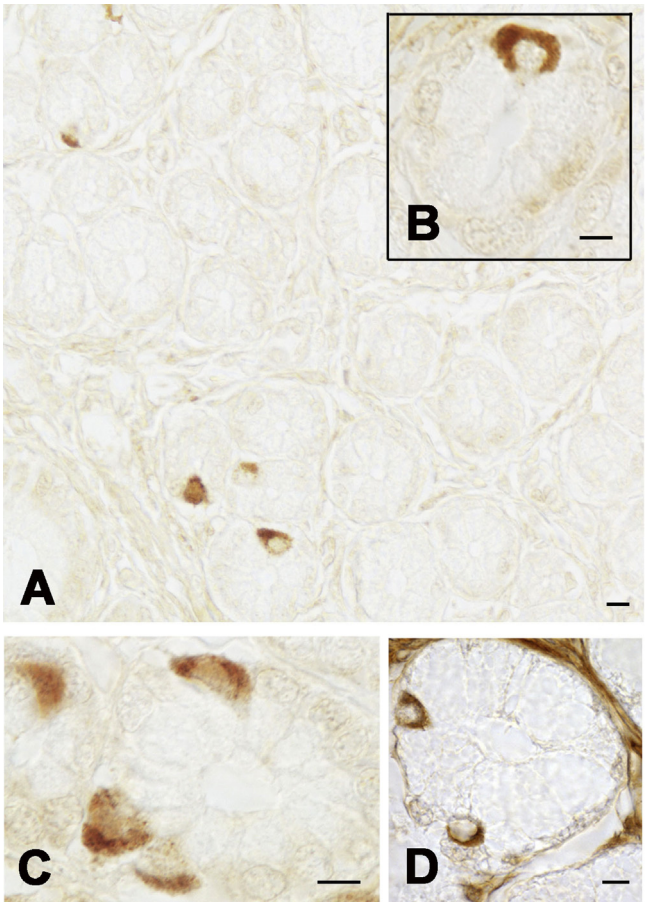

Fig. 1. Presence of some nesfatin-1 immunopositive endocrine cells in the glands of the gastric fundus (a–c). Few nesfatin-1 immunoreactive endocrine cells in a gland of the ileocecal valve (d). Scale bars = 30 μm (a) and 10 μm (b–d).

In the other examined areas of the intestinal tracts, with the exception of the rectum, nesfatin-1 immunopositive neurons and nervous fibers were observed mainly in submucous and myenteric plexuses. In particular, numerous nesfatin-1 immunopositive neurons were detected in the internal submucous plexus of the duodenum (Fig. 2a) and in the external submucous plexus of the ileum (Fig. 2b). In the colon some nesfatin-1 immunoreactive neuronal cells were identified in the myenteric plexus (Fig. 2c).

Non-specific staining was not obtained by the avidin–biotin method. Replacement of the primary or secondary antisera with PBS and normal serum gave negative staining (Fig. 2d).

Protein expression

Protein extracts of gastric fundus, duodenum, jejunum, ileum, ileocecal valve, cecum, colon and rectum were subjected to Western Blot Analysis. The results showed a specific band of ~42 kDa molecular weight and an non-specific band with a major molecular weight in all gastrointestinal tracts with the exception of the cecum and colon, where only a specific band was present and the rectum where no bands were present (Fig. 3). Rat stomach was used as positive control. The specificity of the response was confirmed by preincubation of NUCB2/nesfatin-1 antibody with its respective blocking peptide, which resulted in the absence of signal. The presence of tubulin, used as internal marker, was always detected (Fig. 3).

Discussion

In this study, we report the presence of nesfatin-1 in the gastrointestinal tract of the Casertana breed pig. Nesfatin-1 is an anorexigenic peptide involved in the balance of energy homeostasis (Stengel and Tachè, 2012). In recent studies, it has been hypothesized that nesfatin-1 may play a pivotal role in the regulation of gastric functions (Zhang et al., 2010; Geobel-Stengel and Wang, 2013), intestinal enzyme activation, nutrition, absorption, and preservation of the intestinal walls (Zhang et al., 2010). Recently, it was observed that central administration of nesfatin-1, at doses sufficient to inhibit food intake, results in the inhibition of vagally stimulated secretion of gastric acids in rats (Xia et al., 2012). Moreover, a possible action of nesfatin-1 in the appetite regulation and gonadotropic axis has been also postulated in pigs. NUCB2 is expressed in fat depots of pigs and its level of expression is sensitive to stimulation of appetite-regulating pathways in the hypothalamus (Lents et al., 2013). Thus, the presence of nesfatin-1 in Casertana pig gastrointestinal tract, let us hypothesize a possible role played by such a peptide in the regulation of gastrointestinal secretion and motility, as shown in other species.

In the Casertana pig, nesfatin-1 immunoreactive endocrine cells of closed type were observed in the glandular epithelium of the gastric fundus and in the glandular epithelium of the ileocecal valve. Endocrine cells of closed type represent a conspicuous population of cells among the oxyntic gastric endocrine cell populations in mammals (Rindi et al., 2004). However, the functions of such cells have only recently been revealed and evidence indicates that such cells express peptides capable of modulating food intake through both stimulatory and suppressive mechanisms (Stengel et al., 2009). The endocrine cells of the stomach (Stengel and Tachè, 2012) provide the main source of ghrelin, a 28 amino acids orexigenic peptide characterized by Kojima et al. (1999). Recent studies show that nesfatin-1 is co-expressed with ghrelin in the same cells, sustaining an important role for endocrine gastric cells in regulating food intake (Stengel et al., 2013). Although in this study we did not carry out ghrelin immunodetection in the gastrointestinal tract of the Casertana pig, immunolocalization of ghrelin orexigenic peptide in stomach X/A like cells of pig at different ages has been reported (Vitari et al., 2012), suggesting that a ghrelin-nesfatin-1 interplay is likely to occur also in this species and sustaining the concept that we are dealing with shared mechanisms among mammals.

As far as we are aware, this is the first report to show the presence of nesfatin-1 in the ileoecal valve. This anatomical structure regulates food transit from the small to the large intestine. The significance of nesfatin-1 immunoreactivity in such an anatomical localization in the pig remains unknown, however, the high levels of nesfatin-1 expression in this structure, suggest that nesfatin-1 may be involved in major ileocecal junction functions such as bile and short-chain fatty acids absorption (Imamura et al., 1987; Thompson et al., 1998).

In this study, we observed nesfatin-1 immunopositive neurons and nerve fibers in the internal submucous plexus of duodenum, external submucous plexus of ileum and in the colon myenteric plexus. The enteric nervous system (ENS) of pig comprises 3 ganglionic plexuses: the myenteric plexus (MP), the internal submucous plexus (ISP) and the external submucous plexus (ESP), with ISP and ESP separated by submucosal vascular arcades (Stach, 1988; Timmermans et al., 1990; Pearson, 1994; Balemba et al., 1998). Characterization of ENS neurons and ganglia with vasoactive intestinal peptide (VIP) shows that VIP-like IR neurons are abundant in the ISP, infrequent in the ESP and very scarce in the myenteric plexus (Timmermans et al., 1990; Balemba et al., 1998), supporting the notion that plexuses differ in neurochemical and neuronal contents. Among the various actions of VIP on

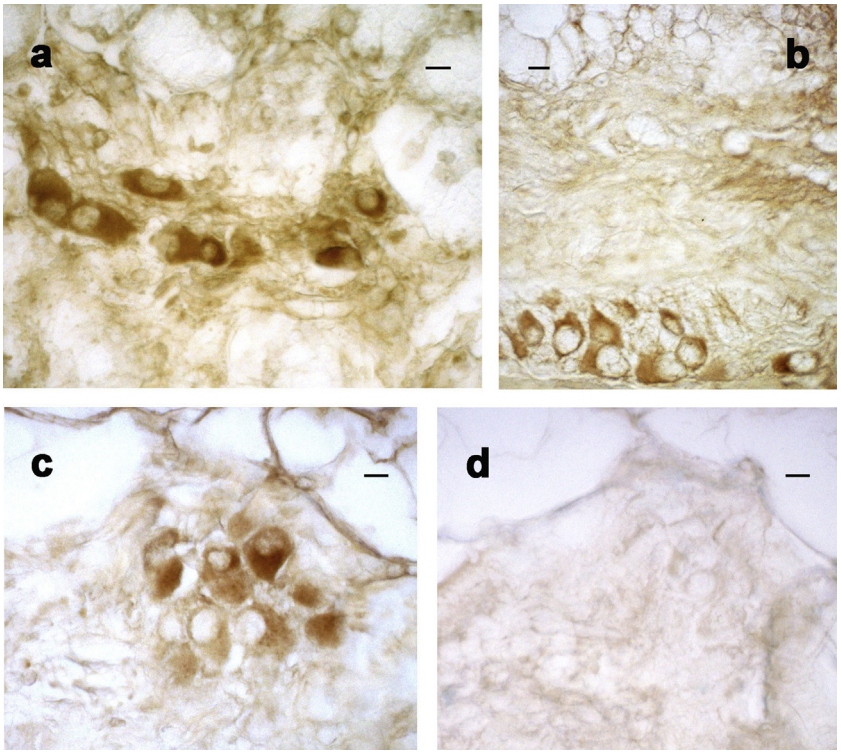

Fig. 2. Nesfatin-1 immunoreactive neurons in the duodenum internal (a) and ileum external (b) submucous plexuses and in the colon myenteric plexus (c). Negative control of colon section (d). Scale bars = 10 μm (a–d).

gastrointestinal tract, the most important is the inhibition of migrating motor complex (MMC) (Ljung and Hellström, 1999). VIP acts on MMC directly or by mediation of L-arginine/nitric oxide or orexin A/orexin 1 receptor pathways (Hellström and Ljung, 1996; Näslund et al., 2002). Thus, the presence of nesfatin-1 immunopositive neurons in pig ENS let us hypothesize that nesfatin-1 may be implicated in the modulation of intestinal motility as described above for the orexigenic peptide orexin A. Further ultrastructural and physiological studies will be needed to confirm this hypothesis. Conversely, in a recent paper, Gonkowski et al. (2012) reported the presence of nesfatin-1 immunoreactive cells in the mucosal layer of the canine duodenum, jejunum and colon, other than the stomach, but not in the enteric neurons.

The findings regarding the local distribution of nesfatin-1 in the gastrointestinal tract of Casertana pig were confirmed by protein expression. Nesfatin-1 is the N-terminal fragment of nucleobindin-2 (NUCB2). The antibody against nesfatin-1 recognizes both full length of NUCB2 and nesfatin-1, thus the immunolabeling represents NUCB2/nesfatin-1. In fact, Western Blot analysis demonstrated the presence of prepronesfatin (predicted size 42 kDa), in the protein extracts of gastric fundus, duodenum, jejunum, ileum, ileocecal valve, cecum and colon. Furthermore, an additional immunoreactive band of approximately 50 kDa was identified in the gastric fundus, duodenum, jejunum, ileum and ileocecal valve. This additional band was also described in rat testis and stomach (Garcìa-Galiano et al., 2012), but its identity is yet to be defined.

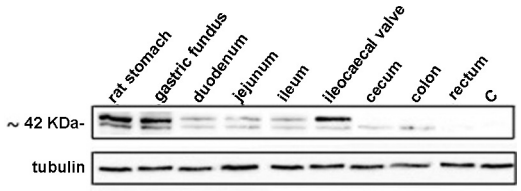

Fig. 3. Western blot for NUBC2/nesfatin-1 (upper blot) and tubulin (lower blot) in rat stomach, gastric fundus, duodenum, jejunum, ileum, ileocecal valve, cecum, colon and rectum. Rat stomach was used as positive control. Negative control line obtained by preadsorption with blocking peptide (C).

In conclusion, in this study, we have extended our knowledge concerning the distribution and the potential role of this anorexigenic peptide in the digestive system of a farmed species. Our results show that nesfatin-1 immunoreactive endocrine cells of closed type were present in the glandular epithelium of the gastric fundus and in the glandular epithelium of the ileocecal valve, other than in the submucous and myenteric plexuses. Immunohistochemical observations were confirmed by Western blot analysis. The immunolocalization of nesfatin-1 in the swine digestive system reinforces the importance of the role played by nesfatin-1 at the gastrointestinal level and sustains the necessity to study the role of this peptide in the regulation of food intake in farm species for which the weight gain is essential for optimizing the production.

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