Postnatal development of NADPH-d neurons in the enteric nervous system of the goat

Yunfang Liu,1,2 Liujun Jia,1,3 Yaoxing Chen,1 Zixu Wang1
1College of Veterinary Medicine, China Agricultural University, Beijing, China
2College of Animal Science and Technology, Shihezi University, Xinjiang, China
3Fuwai Hospital and Cardiovascular Institute, Peking Union Medical College, Beijing, China

Abstract

The morphology and distribution of nitric oxide synthase (NOS)-expressing neurons in the myenteric plexus of small intestine of goat at different ages (2 weeks, and 2, 4, 6 and 12 months) were studied by using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, which could stain NOS-expressing neurons as immunohistochemistry. The results showed that the NADPH-d neurons and their fibres formed a clear tertiary meshwork structure in the myenteric plexus. The shapes of stained neurons were diverse, and many neurons gathered to form numerous ganglia in variable size. During development, the density of NADPH-d neurons in the myenteric plexus of goat small intestine decreased from 27.07/mm² at 2 weeks to 16.18/mm² at 12 months. The total number calculated referred to the total serosal surface of small intestine, however, increased from 1.86×10⁶ at 2 weeks to 5.84×10⁶ at 6 months, before decreasing to 4.41×10⁵ at 12 months. At different ages, the density of NADPH-d neurons was lowest, but the total number was highest, in the jejunum with respect to duodenum and ileum. The mean area of the perikaryon or nucleus was enlarged at first and then diminished as the goats growing. The nuclear:cytoplasmic size ratio in NADPH-d neurons at 2 weeks was much higher than that at 2, 4, 6 or 12 months (P<0.05), while no differences could be found among different ages (P>0.05).

Introduction

Nitric oxide (NO), a nonadrenergic/noncholinergic neurotransmitter, which has been described for decades as a major inhibitory mediator in the gut musculature (Cortesini et al., 1995), works on target cells as a neurotransmitter at releasing of other neurotransmitters within the same nerve ending, or as a neurotransmitter by acting on enteric neurons or smooth muscle cells of the gastrointestinal (GI) tract (Allescher et al., 1996). Other than in inhibitory descending neurons, NO has also been detected in interneurons, secretomotor neurons, intestinofugal neurons (Schemann and Neunlist, 2004). NO is synthesized by nitric oxide synthase (NOS) from the amino acid L-arginine, with NADPH-d as a co-factor. It has been found that the NOS-immunoreactive perikaryon was dispersed in the myenteric and submucosal nerve plexus of the alimentary tract in many types of mammals: guinea pigs (Costa et al., 1992; Nichols et al., 1992), rats (Nichols et al., 1993), mandarin voles (An et al., 2003), pigs (Van Ginneken et al., 1998; Van Ginneken et al., 2001) and sheep (Lalatta-Costerbosa et al., 2007). Using histochemical and immunohistochemical methods, Brookes (1993) revealed the spatial density of NOS-expressing neurons in the GI myenteric plexus was higher than that in the submucous plexus. In the alimentary canal of mandarin voles, the density of NOS-expressing neurons increases gradually from the duodenum to the rectum, with highest density in the colon (An et al., 2003). Moreover, Van Ginneken et al. (1998) showed that the number of nNOS-immunoreactive neurons changes significantly with age in the pig duodenum. Other experimental results also indicated a relationship between expression of NOS and development (Kalb and Agostini, 1993; Wets and Vaughn, 1993; Siou et al., 1994; Tomić et al., 1994; Ward et al., 1994). There are, however, insufficient data regarding NOS-containing neurons in the enteric nervous system (ENS) of the goat. The development and maturation of NOS-expressing neurons in the developing goat small intestine may affect the functional maturation of small intestine such as nutrient digestion and absorption.

NADPH-d was widely used as a histochemical marker for NOS, which allowing the precise anatomical localisation of cells generating NO (Bredt et al., 1991; Blottner et al., 1995). Extensive overlap between histochemical and immunohistochemical methods to reveal NOS neurons has been demonstrated in many mammals, including ruminants (Young et al., 1992; Palmnuchke et al., 2003; Lalatta-Costerbosa et al., 2007; Munnich et al., 2008). Thus, the present study, basing on NADPH diaphorase histochemistry, has been designed to provide a detailed qualitative and quantitative evaluation of nNOS-expressing neurons in the myenteric plexus of the goat small intestine, in relation to developmental stage and location.

Materials and methods

Animal and sampling

The goats were purchased from local farms and had access to food (standard pellet diet) and water ad libitum. For each age (2 weeks, and 2, 4, 6 and 12 months), four goats were used. All procedures pertaining to the care and use of live animals were conducted in accordance with a protocol approved by the China Agriculture University.

The goats were euthanased under deep anaesthesia induced by 846 Compound Anaesthetic Agent (J&K, Beijing, China). The duodenum (5 cm distal to the pyloric sphincter), jejunum (midpiece of the jejunum) and ileum (10 cm proximal to the ileocaecal orifice), each approximately 1 cm in length, were isolated immediately after death, rinsed in 0.1
M phosphate buffered saline (PBS), then fixed for 24 h in 4% freshly prepared paraformaldehyde (0.1 M, pH 7.4). The length and circumference of each intestinal segment were scaled and the total serosal surface of the duodenum, jejunum or ileum was estimated.

**Analysis of the segments of the small intestine preparations**

The whole-mount preparations were made according to Hanani et al. (1995) with some modification. In short, a lesion was made in the longitudinal muscle layer along the mesenteric attachment line. Then, the inner circular smooth muscle together with the submucous layer and the mucosa was peeled off from the outer longitudinal muscle layer. A dissecting microscope (Olympus SZH, Tokio, Japan) was used to facilitate visualization of the myenteric plexus. After being straightened by cutting the border zones, the remaining slabs containing the myenteric plexus were soaked in PBS. The submucous layer, which contained the submucous plexuses, was separated from the muscle layers and soaked in PBS.

To evaluate the cross-sectional area of neurons, the small intestine was cut into coronal frozen sections. Simply station, segments of the small intestine were cryoprotected in 30% sucrose in PBS, after embedding in OCT (optimal cutting temperature) compound, the fragments were then cut into 15 μm sections at –20°C, and mounted on slides coated with poly-L-lysine.

The whole-mount preparations or cryostat sections were incubated in a NADPH reaction solution containing 0.5 mg/mL Nitro Blue Tetrazolium (NBT, Amresco, Solon, OH, USA), 0.3% Triton X-100 and 1 mg/mL NADPH (Roche) in 0.01 M PBS (pH 7.4) at 37°C for 50 min, and the reaction was terminated by washing in 0.01 mol/L PBS (pH 7.4). Subsequently, the preparations were mounted, aired, cleared and coverslipped for analysis.

**Image analysis of the small intestine samples**

The whole mount preparations and sections were viewed and photographed on an Olympus microscope (BX51, Tokio, Japan), and the images were managed using Adobe Photoshop 7.0. In each intestinal section at a different age, 100 neurons were randomly sampled. The cross-sectional area of the cell body and nuclei (in μm²) was measured using SCNIMAGE. At a magnification of ×400, all the stained neurons in the myenteric plexus were counted in three randomly-selected whole mount preparations, in each intestinal segment. The spatial density of NADPH-d neurons was obtained by dividing the number of NADPH-d neurons counted by the total sampling area. Therefore, the total number of positive neurons in the three different intestinal segments was given by the spatial density of NADPH-d neurons for a given segment multiplied by the total serosal surface.

**Statistical analysis**

All parameters were expressed as mean±SEM. Statistical differences between different intestinal segments and/or ages were established using one-way analysis of variance (ANOVA). A P-value of less than 0.05 was considered statistically significant.

**Results**

In conjunction with the neuronal processes, the perikarya of NADPH-d neurons, which varied widely in size, were stained blue using NADPH histochemistry. Their shapes were oval, circular, fusiform, or irregular (Figure 1A, B), and we can obviously find that there significantly different among the labelling neurons (Figure 1A, F). More NADPH-d neurons were found in the myenteric nerve plexus (Figure 1C, D) than in the submucosal nerve plexus (Figure 1E). In the myenteric and submucosal nerve plexus, cell bodies of NADPH-d neurons mostly resided within ganglia, or were occasionally dispersed in the fibre bundles. The neurites of some positive soma connected with each other in the ganglia (Figure 1F).

In the duodenum and ileum, the spatial density of NADPH-d neurons in the goat myenteric plexus decreased gradually from 2 weeks to 12 months of age. In the jejunum, there was also a decrease, but it is not gradual (Table 1). There were significant differences between stained neurone densities at 2 weeks or 2 months and 4, 6 and 12 months in the duode-
num, and between 2 weeks and 2, 4, 6 or 12 months in the jejunum and ileum. At each age, the highest density of NADPH-d neurons was observed in the ileum. Overall, the spatial density of stained neurons in the myenteric nerve plexus of goat small intestine fell from 27.07±0.84/mm² at 2 weeks to 16.18±0.65/mm² at 12 months (Table 1). The differences between the densities at 2 weeks and the four other ages were prominent, as they were between 12 months and 2 weeks, 2 months, 4 months or 6 months. There was no significant difference in density among 2, 4 or 6 months.

The total number of NADPH-d neurons at 2 weeks and 2, 4 and 6 months was 1.86×10⁶, 2.74×10⁶, 4.06×10⁶ and 5.84×10⁶, with the number falling to 4.41×10⁶ at 12 months (Figure 2). However, compared with the duodenum and jejunum, the decrease of NADPH-d neurons in the ileum started as early as 4 months (Figure 2). Although the density of stained neurons in the jejunum myenteric plexus was lowest among the regions examined, the total number was highest at different stages (Table 1, Figure 2).

The area of the perikarya and nuclei of NADPH-d neurons showed a prominent enlargement from 2 weeks to 2 months, and then gradually decreased until 12 months of age, except for that at 4 months (data not shown). From 2 weeks to 6 months, the average area of NADPH-d perikarya in the jejunum myenteric plexus was significant higher than that in duodenum and ileum (P<0.05), and became highest in the duodenum at 12 months. The nuclear:cytoplasmic size ratio of NADPH-d neurons at 2, 4, 6 and 12 months was markedly reduced when compared with that at 2 weeks (P<0.05) (Figure 3). No differences, however, were revealed in this ratio among the ages of 2, 4, 6 and 12 months (P>0.05).

**Discussion**

In the present study, the distribution of NADPH-d neurons was revealed in the myenteric nerve plexus of goat small intestine by NADPH diaphorase histochemistry. Variation in staining intensity of neurons expressing NOS may be attributable to the existence of different isoenzymes, as proposed by Cracco and Filogamo (1994). As in the results obtained by Jarvinen et al. (1999) in rats and Van Ginneken et al. (1998) in pigs, the tertiary meshwork structure of the myenteric plexus was apparent under the light microscope. Many NOS-containing neurons in the ileum and proximal and distal colon project anally, as do the inhibitory motor neurons (McConagogue and Furness, 1993; Messenger, 1993). Lack of NOS, associated with impaired local production of NO, may be responsible for defective relaxation in the GI tract (Takahashi, 2003). Therefore, we can deduce that this refined tertiary structure, composed of NOS-positive neurons and their neurites, plays an important role in inhibitory regulation of the goat small intestine.

Many reports have indicated that the density of NOS-expressing neurons varies prominently in different segments of the GI tract. In the present study, the lowest density of NADPH-d neurons was found in the jejunum, and was higher in the duodenum (other than animals 12 months old) and ileum at the different ages. In the GI tract, it has been demonstrated that NO is a major inhibitory, nonadrenergic, noncholinergic neurotransmitter (Bult et al.,

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Table 1. Density of NADPH-d neurons in the myenteric plexus of goat small intestine (neurones/mm²).

| Age       | Duodenum | Jejunum | Ileum | Small intestine |
|-----------|----------|---------|-------|----------------|
| 2 weeks   | 26.38±1.20A | 23.31±0.67A | 31.51±0.78A | 27.07±0.84A |
| 2 months  | 24.19±1.60A | 12.21±1.10B | 26.57±1.41A | 20.99±1.41B |
| 4 months  | 20.79±0.77B | 16.77±1.28B | 24.83±2.39B | 20.80±0.91B |
| 6 months  | 19.51±1.92B | 14.91±1.08B | 23.85±2.77B | 19.15±1.26B |
| 12 months | 12.49±0.54C | 15.23±0.65B | 20.83±0.69C | 16.18±0.65C |

A P-value less than 0.05 was considered to indicate statistical significance; A,B,C values significantly different among different gut parts at the same age.
Therefore, the lower density of NOS in the jejunum may be of benefit for the movement and evacuation of the jejunum. In sheep, the intrinsic NO plays a crucial role in the regulation of duodenal tone and maintenance of continuous secretion by the exocrine pancreas (Onaga et al., 2000). This is in accordance with the higher density of NOS in the duodenum (other than animals 12 months old). The higher density may also be related to the relaxation control of the thick smooth muscles in these segments.

Gabella (1987) and Gabella and Trigg (1984) found that the neurone size increased and the density decreased in the myenteric nerve plexus of the small intestine of mice, guinea pigs and sheep, similar to the results of the present study for neurons expressing NOS in the myenteric plexus of the goat. Yunker and Galligan (1998) showed that extrinsic denervation, especially a loss of extrinsic sensory nerves, is associated with an increase in NOS expression in the myenteric plexus, and that there was a significant increase in expression of nNOS mRNA and protein in the tissues of rats that had undergone splanchic ganglionectomy or 6-OH-dopamine treatment, but not in tissues from rats that had undergone vagotomy (Nakao et al., 1998). So, this decline in neurone density may be regulated by extrinsic innervation, which did not include the vagus nerve.

It can be deduced that the increase in NADPH-d neurons might be due to the adaptive changes related to development or feeding behaviour. For example, in ENS of lamb a high percentage of neurons transiently expressed TH (Chiocchetti et al., 2004), and TH is considered a progenitor of NOS enteric neurons (Newgreen and Young, 2002). In the sheep rumen Pfannkuche et al. (2003) described age-related changes in the neurochemical code of myenteric neurons. In ruminant, according to their different feeding types, Munnich et al. (2008) hypothesized different neurochemical code of rumen myenteric neurons.

As mentioned above, besides in inhibitory descending neurons, NO was also expressed in interneurons, secretomotor neurons, intestino-foetal neurons. In the present study NOS-expressing neurons showed a great variability both in size and morphology. This may be a reflecting of different subclasses of neurons with different functional properties. According to the results, the area of the perikarya and nuclei of NOS-expressing neurons increased sharply around the early postnatal period, and diminished by degrees until 12 months of age. The nuclear:cytoplasmic size ratio of NOS-positive neurons was highest at 2 weeks when compared with that at 2, 4, 6 and 12 months (P<0.05). Hence, we can deduce that neurons expressing NOS are almost mature at 2 months. The nuclear:cytoplasmic ratio of NOS neurons in the adult cavia cobaya recorded by Cracco and Filogamo (1994) was lower than that obtained here; 0.21 vs. 0.28 at 12 months, possibly because of species differences. As far as developmental stage and location are concerned, the nuclear:cytoplasmic ratios of NADPH-d neurons in the duodenum and ileum, respectively, were higher than those in the jejunum from 2 weeks to 2 months, and this relationship was then reversed from 4 months on. These results seem to indicate that development of the myenteric nerve plexus in the duodenum and ileum was ahead of that in the jejunum.

Conclusions

In summary, the present study showed the differences in pattern of development and distribution of NADPH-d neurons in myenteric plexus of developing goat small intestines. Large numbers of NADPH-d neurons could be found in the goat small intestine, and were mainly located in the myenteric nerve plexus. The NADPH-d neurons were essentially mature by as early as 2 months. The development and maturation of NADPH-d neurons in the developing goat small intestine may affect the functional maturation of small intestine such as nutrient digestion and absorption. These findings may help to develop new feeding strategies for improving the health and growth performance in goat especially during weaning. And further studies are needed to investigate the detailed function and in the functional maturation of the small intestine in goats.

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