DIFFERENTIAL DENSITIES OF NITRIC OXIDE SYNTHESIZING NERVES IN THE SPHINCTERIC AND NON-SPHINCTERIC SMOOTH MUSCLES OF HUMAN GUT DURING FETAL DEVELOPMENT[1]

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SUMMARY: Nitric oxide (NO) is involved in the inhibitory neurotransmission in the sphincteric and non-sphincteric smooth muscles. However, the relative contribution of nitric oxide synthesizing innervation to these functionally diverse parts of the gut, particularly during development, is unknown. Gastrointestinal sphincters and adjoining non-sphincteric bowel segments were obtained from 14 human fetuses with gestational ages between 12 and 23 weeks. NO synthesizing nerves were examined by nicotinamide adenine deoxinucleotide phosphate (NADPH) diaphorase histochemistry. The densities of NADPH positive nerves in the smooth muscle were quantified using a computerized image analyzing system on random sections. The NO synthesizing nerve density in intestinal smooth muscles decreased during fetal development as a result of increased interspacing between myenteric ganglia and a disproportionately larger increase in smooth muscle area than neuronal area. Similarly, the nerve densities were lower in sphincteric regions than adjoining non-sphincteric regions at the same gestational ages. There is a relative reduction of the density of NO synthesizing nerves in intestinal smooth muscle particularly in sphincteric regions during development. These findings may have relevance to the occurrence of congenital dysmotility disorders of the sphincteric regions.

SUBJECT HEADINGS: Nitric oxide, non-adrenergic, non-cholinergic innervation. Sphincteric smooth muscle. Fetal development, human.

INTRODUCTION

Gastrointestinal sphincters are important components of the alimentary tract, regulating onward transit of gut contents and preventing its reflux. Unlike smooth muscle of non-sphincteric regions which exhibit phasic contractions smooth muscle are characterized by tonic contractions[12]. Both sets of smooth muscles are under enteric neuronal control which involves adrenergic, cholinergic as well as non-adrenergic non-cholinergic (NANC) nerves. The inhibitory NANC neurons are believed to be concerned with the relaxation of the gastrointestinal sphincters and the descending inhibition during intestinal peristalsis[9]. The exact identity of the NANC neurotransmitter remains unresolved: adenosine 5' triphosphate (ATP)[8] and the neuropeptide, vasoactive intestinal peptide (VIP)[13] have both been implicated as possible mediators. More recently, nitric oxide (NO) has been shown to be involved in the NANC inhibitory pathway[7,21,26,27].

Congenital dysmotility disorders, including achalasia, hypertrophic pyloric stenosis and Hirschsprung's disease are characterized by the failure of relaxation of the lower oesophageal sphincter, pylorus and internal anal sphincter respectively. The NANC inhibitory pathway is believed to play an important role in the pathophysiology of these conditions and abnormalities of nitric oxide synthesizing neurons have been identified[5,10,16,18,24,25].

Nitric oxide synthesizing innervation in the gut can be studied by nitric oxide synthase immunohistochemistry or nicotinamide adenine deoxinucleotide phosphate (NADPH) diaphorase histochemistry as previously shown in co-localization studies[11,15,18,26]. Using these methods, nitric oxide synthesizing nerves have been demonstrated to appear in the human gut as early as 18 weeks' gestation in one study[22] and at 12 weeks' gestation in another study[6]. Both are morphological studies and have not attempted either to quantify the changes of NO

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synthesizing innervation with increasing gestational ages, or more importantly to differentiate between the innervation densities of sphincteric regions and their adjoining non-sphincteric parts. The aim of the present study was to determine the relative contribution of nitric oxide synthesizing innervation to these functionally diverse parts of the alimentary tract during development.

METHOD

Bowel segments from distal esophagus, gastro-pyloric-duodenal region, ileocolonic region and rectosigmoid of 14 fetuses with gestation ages from 12-23 weeks were obtained from routine autopsy examination. All materials were acquired with maternal consent. The study has been approved by the Central Oxford Research Ethical Committee and performed in accordance with the guidelines on the use of fetal tissue in research. In the last two fetuses (16 and 23 weeks of gestation ages) the internal anal sphincters were also isolated for individual analysis.

Approximately 1 cm of the selected levels were snap-frozen in liquid nitrogen and embedded in tissue-teck OCT compound (Miles Inc, Elkhart. IN). Cryostat sections of 10 micron thick were cut at right angle to the longitudinal axis of gut and mounted on gelatin coated slides. Histochemical staining for NADPH-diaphorase activity was performed by a published technique[18].

Images from several sections were selected for histomorphometry using a computerized image analyzing system (Roche Pathology Workstation). A software called VIDAS 25 was used for automatic measurements. The selected areas to be measured were defined by a binary cursor. These areas were individualized by increasing the contrast for the adjacent areas using a low pass filter and excluding all the adjacent image. The pictures were taken using an Ultra High Resolution Color Camera (Kontron, UK) and printed using Adobe Photoshop/Microsoft Powerpoint in a Polaroid CI 5000 (Digital Palette Film Recorder). Measurements of the area of the longitudinal and circular muscle layers, and the area of the NADPH diaphorase positive myenteric plexus within the adjacent muscle layers were made. Twenty measurements were performed for each of the selected areas. A ratio between the neuronal area the adjacent muscle area was calculated and expressed in percentages.

The unrelated "t" test for independent random samples was used for assessing the differences between two means of neuronal densities. $p < 0.05$ was used to reject the null hypothesis.

The means of the neuronal densities were grouped as follows; 12/13 weeks, 14/15 weeks, 16/17 weeks and 23 weeks. A degree of linear correlation between the means of all neuronal densities of the combined specimens and the increasing gestational age was established. The association between the two variables was described by a regression equation.

RESULTS

The arrangement of the enteric plexuses stained for NADPH-diaphorase of the developing human intestine is similar to that reviewed by Furness & Costa (1987)[5]. However, in the sphincter regions (pylorus and internal anal sphincter), ganglia can be seen inside the circular muscle layer and there is a predominance of Dogiel type II neurons as compared to the non-sphincter regions.

The means of the densities of enteric nitric oxide synthesizing neurons with their respective standard errors of the means in relationship to gestational ages are expressed in Table 1. At the most advanced gestational age studied (23 weeks), the three sphincteric regions i.e. lower oesophageal sphincter, pylorus and internal and sphincter showed significantly lower neuronal densities than the adjacent non-sphincteric regions ($p < 0.01$).

Table 1 - Ratios between the NADPH diaphorase positive myenteric ganglia and the adjacent innervated gut muscles from all the selected gut levels. Results expressed in % (Mean +/- SEM)

	12/13 weeks	14/15 weeks	16/17 weeks	23 weeks
Oesophagus	11.8+/-0.6	9.4+/-0.6	7.1+/-1.0	3.5+/-0.2
Stomach	12.2+/-0.9	8.6+/-0.7	8.2+/-0.6	5.6+/-0.5
Pylorus	8.4+/-0.8	5.8+/-0.8	4.7+/-0.5	3.0+/-0.4
Duodenum	19.9+/-1.0	18.5+/-0.6	17.5+/-0.6	13.5+/-0.5
Ileum	24.4+/-1.7	18.5+/-0.8	16.1+/-0.9	8.5+/-0.5
Rectum	24.8+/-0.7	18.2+/-0.6	14.7+/-0.7	8.4+/-0.5
I.A.S.*			9.6+/-0.6	4.6+/-0.3

*I.A.S. = Internal anal sphincter

In all selected levels, there is a progressive decrease of the densities of the nitric oxide synthesizing neurons. This is the result of the increasing interspacing between the myenteric ganglia and the disproportionately greater increase of the smooth muscle volume than the neuronal volume.

The means of the combined neuronal densities of all the selected levels in each age group were correlated with the gestational ages. The results are expressed in Fig. 1. There is a significant reverse linear correlation between the two parameters, which can be expressed by the following regression equation:

$$y = 30.158 - 1.0313x$$

y - density of nitric oxide synthesizing neurons

x - gestational age expressed in weeks

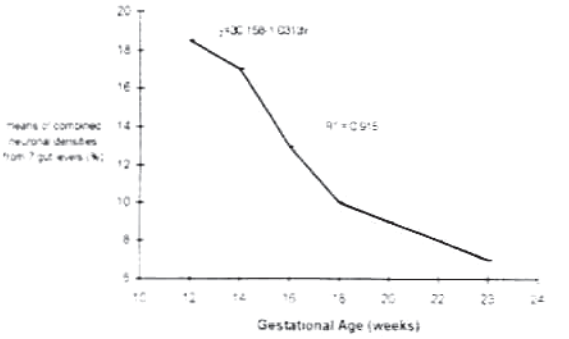

Fig. 1 - Correlation of the means of the combined neuronal densities of all the gut levels with gestational age.

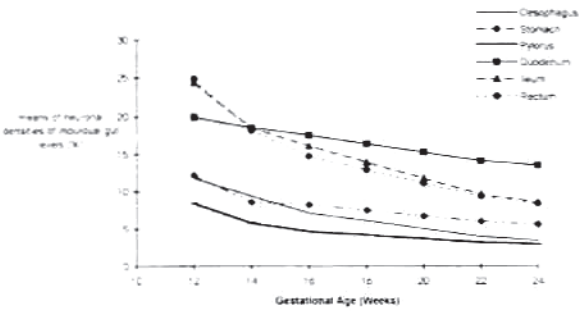

Fig. 2 - Correlation of the means of the neuronal densities of individual gut levels with gestational age.

The individual curves of the means of the neuronal densities of all selected gut levels are expressed in Fig. 2.

DISCUSSION

Sphincteric and non-sphincteric regions are functionally diverse parts of the gut. Knowledge of the differential densities of neurons in these regions could contribute towards better understanding of possible differences in their underlying regulatory mechanisms. Nevertheless, systematic studies to compare the densities of neurons in sphincteric versus non-sphincteric regions of the gut have rarely been carried out owing to technical problems, especially when the subject of interest is NANC inhibitory innervation. In particular immunohistochemical studies of neuropeptides such as VIP which has been implicated in inhibitory NANC nerves are difficult to perform in sphincteric regions because of their thickness[3].

Nitric oxide has been shown to be involved in the inhibitory NANC neurotransmission in both the sphincteric and non-sphincteric regions of the gut[4,19,21,23]. In a previous study[6,18], we have demonstrated co-localization of nitric oxide synthase and NADPH diaphorase in human gut. In the present study, we have quantified the nitric oxide synthesizing neurons in both the sphincteric regions and the adjoining non-sphincteric regions of human fetal gut using computerized image analysis to measure the areas of NADPH diaphorase positive myenteric plexus and its adjoining muscle layers in histochemical sections.

It has previously been shown that nitric oxide synthesizing neurons appears in human gut as early as 12 weeks' gestation[6] proliferating and increasing in sophistication with development[6,22]. This study shows that in parallel with such development, the density of nitric oxide synthesizing neurons in all parts of the gastrointestinal tract paradoxically decreases with advancing gestational age. The explanation is two-fold. First, the myenteric plexus develops initially as a complete ring. With maturation, ganglion cells aggregate into ganglia with progressive increase in spaces between the ganglia. Secondly, there is a greater increase in muscle volume, compared to neuronal volume during development.

This trend is accentuated when the sphincteric regions and non-sphincteric regions are compared. The disproportionately greater increase in muscle bulk in the sphincteric regions result in the lower esophageal sphincter, pylorus and internal anal sphincter having significantly lower densities of nitric oxide synthesizing neurons than the adjoining non-sphincteric regions. These findings may appear difficult to reconcile with the known occurrence of higher VIP concentrations demonstrated by radioimmunoassay[1] and higher VIP gene expression demonstrated by molecular biological studies[2] in the sphincteric regions compared to the non-sphincteric regions, particularly as there have been suggestions that both VIP and NO may be transmitters involved in inhibitory NANC innervation[14,17,19,20]. The different methods used in these studies and our study made comparison of results difficult. It is not known whether VIP or NO is the primary NANC transmitter. It is possible that not all VIP neurons produce NO and vice versa. In addition, the VIP concentrations and gene expression were measured on adult sphincteric tissue whereas our study on NO synthesizing nerves were made on developing tissues. The neuronal density measurement used in our study has the advantage that the growth of the end-organ, namely the muscle which the neurons innervate is properly recognized in the data presented.

The nature of the present study, namely a histomorphometric examination, precludes firm conclusions on functional or developmental significance. Nevertheless, it is tempting to hypothesize that the progressive decrease in the density of nitric oxide synthesizing neurons in the sphincteric regions of human gut during fetal development may account for the vulnerability of the sphincters to developmental anomalies. A precipitous reduction, from whatever unknown cause, in the inhibitory NANC input to the sphincteric regions which are already delicately balanced could result in the onset of sphincter spasm. This can in turn initiate a vicious circle resulting in the development of anomalies such as esophageal achalasia, hypertrophic pyloric stenosis or internal sphincter achalasia respectively.

REFERENCES

1. ALUMETS. J., SCHAAFFALITZKY DE MUCKADELL O., FEHRENKRUG, J., SUNDLER, F., HAKANEON, R., UDDMAN, R. - A rich VIP nerve supply is characteristic of sphincters. *Nature, 280:* 155-156, 1979.

2. BANDYOPADHYAY, A., CHAKDER, S., LYNN, R.B., RATTAN, S. - Vasoactive intestinal polypeptide gene expression is characteristically higher in opossum gastrointestinal sphincters. *Gastroenterology, 106:* 1467-1476, 1994.

3. BANDYOPADHYAY, A., CHAKDER, S., LYNN, R.B., RATTAN, S. - Reply to letter. VIP messenger RNA in the sphincters in smooth muscle. *Gastroenterology, 107:* 1912, 1994.

4. BAYGUINOV, O. & SANDERS, K.M. - Role of nitric oxide as an inhibitory neurotransmitter in the canine pyloric sphincter. *Am. J. Physiol, 264:* G775-G783, 1993.

5. BEALER, J.F., NATUZZI, E.S., BUSCHER, C., URSELL, P.C., FLAKE, A.W., ADZIICK, N.C., HARRISON, M.R. - Nitric oxide synthase is deficient in the aganglionic colon of patients with Hirschsprung's disease. *Pediatrics, 93:* 647-651, 1994.

6. BRANDT C.T., TAM P.K.H., GOULD S.J. - Nitrergic innervation of the human gut during early fetal development. *J. Pediatr. Surg. 31:* 661-664, 1996.

7. BULT H., BOECKXSTAENS G.E., PELCKMANS P.A., JORDAENS F.H., VAN MAERCK Y.M., HERMAN A.G. - Nitric oxide as an inhibitory nonadrenergic noncholinergic neurotransmitter. *Nature, 345:* 346-347, 1990.

8. BURNSTOCK, G. - Purinergic Nerves. *Pharmacol Rev, 24:* 509-551, 1972.

9. BURNSTOCK, G. - Review Lecture. Neurotransmitters and trophic factors in the autonomous nervous system. *J. Physiol (Lond), 313:* 1-35, 1981.

10. CUFFARI C., RUBIN C., KRANTIS C. - Routine use of nitric oxide stain in the differential diagnosis of Hischsprung's disease. *J. Pediatr. Sur. 28:* 1202-1204, 1993.

11. DAWSON T.M., BREDT D.S., FOTUHI M., HWANG P.M., SYNDER S.H. - Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Nat. Aca. Sci. (USA), 88:* 7797-7801, 1991.

12. FURNESS J.B., COSTA M. - The gastrointestinal sphincters. In Furness J.B., Costa M. *The Enteric Nervous System,* 1st Ed. Edinburgh Churchill Livingstone, 1987.

13. GOYA R.K., RATTAN S., SAID S.I. - VIP as a possible neurotransmitter of non-adrenergic non-cholinergic inhibitory neurons. *Nature, 288:* 378-380, 1980.

14. GRINDLER J.R., MARTHY K.S., JIN J.G., MAKHLOUF C.M. - Stimulation of nitric oxide from muscle cells by VIP prejunctional enhancement of VIP release. *Am. J. Physiol, 262:* 6774-6778, 1992.

15. HOPE B.T., MICHAEL G.H., HNIGGE K.M., VINCENT S.R. - Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. (USA), 88:* 2811-2814, 1991.

16. KOBAYASHI H., O'BRIAN D.S., PURI P. - Lack of expression of NADPH diaphorase and neural cell adhesion molecule (NCAM) in colonic muscle of patient with Hirschsprung's disease. *J. Pediatr. Sur, 29:* 301-304, 1994.

17. LI C.G., RAND M.J. - Nitric oxide and vasoreactive intestinal polypeptide mediate non adrenergic non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur J Pharmacol, 191:* 303-309, 1992.

18. O'KELLY T.J., DAVIES J.R., TAM P.K.H., BRADING A.F., MORTENSEN N.J.M.C. - Abnormalities of nitric producing neurons in Hirschsprung's disease morphology and implications. *J. Pediatr. Surg., 20:* 294-300, 1994.

19. RATTAN S & CHAKDER S. - Role of nitric oxide as a mediator of internal anal sphincter relaxation. *Am. J. Physiol., 262:* G107-G112, 1992.

20. SAID S.I. - Nitric oxide and vasoactive intestinal peptide: cotransmitters of smooth muscle relaxation. *News Physiol Sci., 7:* 181-183, 1992.

21. SHUTTLEWORTH C.W.R., MURPHY R., FURNESS J.B. - Evidence that nitric oxide participates in non-adrenergic inhibitory transmission to intestinal muscle in the guinea-pig. *Neurosci. Lett. 103:* 77-80, 1991.

22. TIMMERMANS J.P., BARBIERS M., SCHEUERMANN D.W., BORGERS J.J., ADRIANENSEN D., FEKETE E., MAYER B., VAN MAERCKE E.A., DE GROODT, LASSEAL M.H. - Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. *Cell. Tissue. Res., 275:* 235-245, 1994.

23. TOTTRUP A., SUANE D., FOREMAN A. - Nitric oxide mediating NANC inhibition in opossum lower esophageal sphincter. *Am. J. Physiol., 206:* G385-G389, 1991.

24. VANDERWINDEN J., DE LAET M., SCHIFFMAN S., MAILLEUX P., LOWENSTEIN C.J., SYNDER S.H., VANDERHAEGHEN J.J. - Nitric oxide synthase distribution in the enteric nervous system of Hirschsprung's disease. *Gastroenterology, 105:* 969-973, 1993.

25. VANDERWINDEN J., MAILLEUX P., SCHIFFMAN S.N., VANDERHAEGHEN J.J., DE LAET M.H. - Nitric Oxide synthase activity in infantile hypertrophic pyloric stenosis. *N. Eng. J. Med., 327:* 511-515, 1992.

26. YOUNG S.M., XUE C., SHUTTLEWORTH C.W.R., BREDT D.S., SYNDER S.H. - Co-localisation of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine. *Histochemistry, 97:* 375-378, 1992.

27. WARD S.M., XUE C., SHUTTLEWORTH C.W., BREDT D.S., SYNDER S.H., SANDERS K.M. - NADPH diaphorase and nitric oxide synthase co-localisation in nitric neurons of canine proximal colon. *Am. J. Physiol., 263:* G277-G284, 1992.

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