A role of nitric oxide in the transitional segment of Hirschsprung's disease*

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Özet

Hirschsprung hastalığının transizyonel segmentinde nitrik oksitin rolü

Son yapılan fizyopatolojik çalışmalar nitrik oksit (NO)'in insan barsağındaki inhibitör innervasyonda bir nörotransmiter olabileceğini göstermektedir. NO'nun Hirschsprung hastalığındaki önemini açıklığa kavuşturmak amacı ile düzenlenen bu çalışmada, dört Hirschsprung'lu olgudan (yaşları 6 ay ile 2 yıl arasında değişen 3 erkek ve bir kız) elde edilen kolon örneklerinde enterik sinir cevapları incelenmiştir. Değişik otonom sinir blokerleri (L-NMMA ve L-arginin) uygulanması öncesi ve sonrasında, adrenerjik ve kolinerjik sinirlerin elektriksel alan uyarımına (EAU), kolonun verdiği in vitro cevaplar mekanograf kullanılarak değerlendirildi. Şu sonuçlar elde edildi: 1) Nonadrenerjik nonkolinerjik (NANK) inhibitör sinir lifleri ganglionik segmentte etkilidirler ve transizyonel segmentte bu etki azalmaktadır. Buna karşın aganglionik segmentteki enterik sinir lifleri üzerinde hiçbir etkileri yoktur. 2) NO, ganglionik segmentte NANK inhibitör sinir liflerinin relaksasyon cevabını ayarlamaktadır. NO'nun bu etkisi transizyonel segmentte daha az belirgin olmakta, aganglionik segmentte ise hiç gözlenmemektedir. Hirschsprung hastalığında gözlenen motilite bozukluğu, NO gibi NANK inhibitör sinir liflerinin azalmış veya ortadan kalkmış etkinliği ile yakından alakalı olabilir.

Anahtar kelimeler: *Hirschsprung hastalığı, nitrik oksit, gastrointestinal motilite, nöronlar, nöral inhibisyon*

Summary

Recent pathophysiological studies have shown that nitric oxide (NO) should be a neurotransmitter of inhibitory innervation in the human gut. To clarify the significance of NO in Hirschsprung's disease (HD), we have investigated enteric nerve responses in colonic tissue obtained from 4 patients (3 boys and 1 girl, from age of 6 months to 2 years) with this disease. A mechanography was used to evaluate in vitro colonic responses to electrical field stimulation (EFS) of adrenergic and cholinergic nerve before and after treatments with various autonomic nerve blockers, NG-monomethyl-L-arginine (L-NMMA), and L-arginine. The following results were obtained: 1) Non-adrenergic non-cholinergic (NANC) inhibitory nerves were found to act on the ganglionic segment and to a lesser extent in the transitional segment, but had no effect on the enteric nerves in aganglionic segment. 2) NO mediates the relaxation reaction of NANC inhibitory nerve in the ganglionic segment and to a lesser extent in the transitional segment, but no effect was observed in the aganglionic segment. Diminution and absence of action of NANC inhibitory nerves such as NO may be largely related to the impaired motility observed in HD.

Key words: *Hirschsprung disease, nitric oxide, gastrointestinal motility, neurons, neural inhibition*

Introduction

The clinical picture of Hirschsprung's disease (HD) arises from a disordered motor function of the distal alimentary tract, and congenital diminution and absence of ganglion cells in the affected bowel segment represents the fundamental pathology [4,6,11,15].

Hypoganglionosis is a histological feature of the intestine proximal to the aganglionic segment in HD, and this is so-called transitional zone [1,7]. At present, the definitive treatment for this disease consists of surgical removal of the transitional and aganglionic segment. However, the pathophysiology of HD remains controversial.

There is no generally accepted explanation as to why the diminution and lack of ganglions cells in the intestinal wall should result in contraction of that segment of the intestine [6,15]. There has been re-

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newed interest in the intrinsic intestinal innervation. Besides the classical adrenergic and cholinergic nervous systems, the non-adrenergic and non-cholinergic (NANC) nervous systems have been suggested to play important roles in the regulation of gastrointestinal motility [5,16]. Recently, one possible explanation for relaxation failure in HD is a deficiency of NANC inhibitory nerves that normally mediate the descending inhibition of the peristaltic reflex.

It has also been proposed that in NANC nervous systems, peptide-like vasoactive intestinal polypeptide (VIP), substance P, or neurotensin, which was initially thought to be a gut hormone, may act as a neurotransmitter or neuromodulator [14,15]. Diminution of neuropeptides such as VIP and substance P in the NANC nerves as well as other abnormalities of the enteric nervous system have been described [9,15]. However, the functional significance of their abnormalities has no been fully clarified. Recent pathophysiological studies have shown that nitric oxide (NO) should be as a messanger, or a neurotransmitter of inhibitory innervation in the human gut [12,18].

Therefore, NO synthase was localized by histochemical staining in cells in the enteric plexus and in neuronal processes in the human gut [8]. Vanderwinden et al [19] reported that NO synthase was selectively absent in the plexus area and musculature of aganglionic segments, whereas moderate staining was observed in the hypertrophied nerve bundles in the submucosa. But, there are no reports on NO in the transitional segment. The present study investigated the effects of NO on transitional, aganglionic, and ganglionic bowel segments derived from patients with HD. Mechanographical techniques were used to study the specimens in vitro.

Material and Methods

Sixteen preparations were taken from the aganglionic, hypoganglionic (transitional zone) and ganglionic colon of patients (3 boys and 1 girl, from age of 6 months to 2 years) with HD, respectively. Damaged tissues were carefully removed from the preparations and histological examinations were performed to check not only their integrity, but also the diminution and absence of ganglion cells in the specimens taken from patiens with HD. The mucosa was removed from each colon specimen, and muscle strips approximately 15 mm in length and 4 mm in width were prepared in the direction of the circular muscle.

The muscle strips were placed in a 10 ml organ bath containing Krebs solution heated at 37^0 C and gassed with 95 % O_2 /5 % CO_2. Changes in motility were recorded with an isotonic transducer (ME Commercial, model ME-4012, Japan) and isotonic movements in the direction of the circular muscle were recorded using a pen recorder (Rikadenki, R-10, Japan) once the muscle strips had become stabilized after 1 hour. Electrical field stimulation (EFS) using repetitive 0.5 miliseconds rectangular pulses at 5 Hz (such low frequency stimulations are considered to stimulate only nerve fibers) and 50 V from a constant current stimulator (ME Commercial, model ME-6052, Japan) was performed for periods of 30 seconds. The stimulation were sent through the silver clip (ME Commercial, selfine clip, Japan) itself. Mechanographical techniques were performed for obtaining responses to EFS in vitro.

The following drug preparations were used: atropine sulfate ($1x10^{-7}$ g/ml; Sigma USA), phenoxibenzamine ($5x10^{-6}$ g/ml; Sigma, USA), propranolol ($5x10^{-6}$ g/ml; Sigma, USA), tetrodotoxin ($5x10^{-7}$ g/ml; Sankyo, Japan), N^G-monomethyl-L-arginine ($1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$ g/ml; Peninsula Labor, USA), L-arginine ($1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$ g/ml, Nakarai, Japan). The X^2 test (two-tailed) was used for statistical analysis and a "p value" of less than 0.05 was regarded as significant.

Results

Experiment 1: To determine whether the NANC inhibitory nerves were present in the colon specimens, the response to EFS before and after blockage of the adrenergic and cholinergic nerves, and after the addition of tetrodotoxin was studied.

EFS responses before blockade of the adrenergic and cholinergic nerves: As shown in Table Ia, the ganglionic muscle strips demonstrated relaxations at a frequency of 75.0 %. These reactions were 31.6 %

Table I. Experiment 1

(a)	No reaction	Contraction	Relaxation	
Ganglionic segment	0 % (0/16)	25.0 % (4/16)	75.0 % (12/16)	*
Transitional segment	0 % (0/16)	68.4 % (11/16)	31.6 % (5/16)	*
Aganglionic segment	0 % (0/16)	100 % (16/16)	0 % (0/16)	*
(b)				
Ganglionic segment	0 % (0/16)	6.3 % (1/16)	93.7 % (15/16)	*
Transitional segment	6.3 % (1/16)	43.8 % (7/16)	50.0 % (8/16)	
Aganglionic segment	93.7 % (15/16)	6.3 % (1/16)	0 % (0/16)	
(c)				
Ganglionic segment	93.7 % (15/16)	6.3 % (1/16)	0 % (0/16)	
Transitional segment	87.5 % (14/16)	12.5 % (2/16)	0 % (0/16)	
Aganglionic segment	93.7 % (15/16)	6.3 % (1/16)	0 % (0/16)	

*EFS: electrical field stimulation, *p<0.05.*
(a) Response to EFS before blockade of the adrenergic and cholinergic nerves.
(b) Response to EFS after blockade of the adrenergic and cholinergic nerves.
(c) Response to EFS following administration of tetrodotoxin.

of the transitional muscle strips. However, the aganglionic muscle strips demonstrated contractions at a frequency of 100 %. Significant differences were noted between the frequency of relaxation reaction in ganglionic and transitional and aganglionic colon ($p<0.05$, $p<0.0001$, respectively). Figure 1a illustrates the results of a typical experiment.

EFS responses after blockade of the adrenergic and cholinergic nerves: As shown in the Table Ib, EFS of ganglionic muscle strips produced relaxation reaction than before blockade. The relaxation frequency observed in the ganglionic muscle strips was 93.3 %. These reactions were 43.8 % of the transitional muscle strips. However, the aganglionic muscle strips exhibited contraction at a frequency of 7.3 %. The remainder of the muscle strips demonstrated no responses. Significant differences were noted between the frequency of relaxation reaction in ganglionic and transitional colon ($p<0.05$). Figure 1b illustrates the results of a typical experiment.

Response to EFS following administration of tetrodotoxin after blockade of the adrenergic and cholinergic nerves: It was thus uncertain whether EFS responses reacted via the nerves or by direct effect on the smooth muscle. To clarify this point, a study was made of the effects of EFS after blocking the total enteric nervous systems with tetrodotoxin. Tetrodotoxin almost abolished the EFS responses in

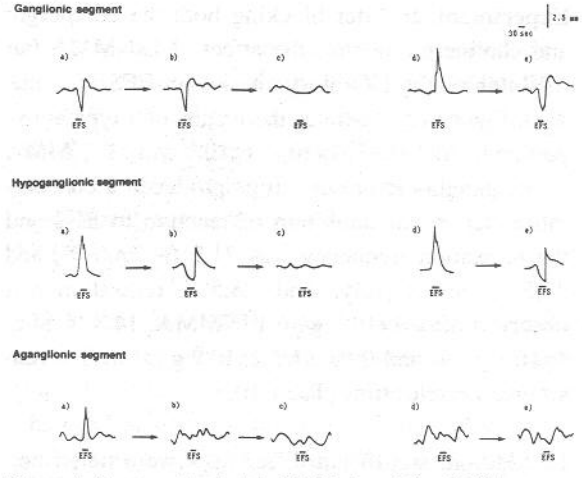

Figure 1. Response to electrical field stimulation (EFS).
(a) Response to EFS before blockade of the adrenergic and cholinergic nerves.
(b) Response to EFS after blockade of the adrenergic and cholinergic nerves.
(c) Response to EFS following administration of tetrodotoxin after blockade of the adrenergic and cholinergic nerves.
(d) Effect of L-NMMA (1x10-6 g/ml) to EFS after blockade of the adrenergic and cholinergic nerves.
(e) Effect of L-arginine (1x10-6 g/ml) to EFS following administration of L-NMMA (1x10-6 g/ml) after blockade of the adrenergic and cholinergic nerves.

ganglionic, transitional and aganglionic muscle strips (Table Ic). Figure 1c illustrates the results of a typical experiment. These results indicated that the responses mediated by NANC inhibitory nerves occurred in the ganglionic colon and to a lesser extent in transitional colon, but not in the aganglionic colon.

Table II. Experiment 2

	No reaction	Contraction	Relaxation
Ganglionic segment			
L-NMMA			
$1x10^{-8}$ g/ml	0% (0/16)	18.7% (3/16)	81.3% (13/16)
$1x10^{-7}$ g/ml	6.3% (1/16)	18.7% (3/16)	75.0% (12/16) * *
$1x10^{-6}$ g/ml	12.5% (2/16)	50.0% (8/16)	37.5% (6/16) *
Transitional segment			
L-NMMA			
$1x10^{-8}$ g/ml	7.1% (1/14)	64.3% (9/14)	28.5% (4/14)
$1x10^{-7}$ g/ml	7.1% (1/14)	78.6% (11/14)	14.3% (2/14)
$1x10^{-6}$ g/ml	14.3% (2/14)	85.7% (12/14)	0% (0/14)
Aganglionic segment			
L-NMMA			
$1x10^{-8}$ g/ml	100% (16/16)	0% (0/16)	0% (0/16)
$1x10^{-7}$ g/ml	100% (16/16)	0% (0/16)	0% (0/16)
$1x10^{-6}$ g/ml	100% (16/16)	0% (0/16)	0% (0/16)

*EFS: electrical field stimulation, *p<0.05.*
Effects of NG-monomethyl-L-arginine (L-NMMA; 1x10-8, 1x10-7, 1x10-6 g/ml) to EFS after blockade of the adrenergic and cholinergic nerves.

Experiment 2: After blocking both the adrenergic and cholinergic nerves, the effect of L-NMMA (an inhibitor of the NO-biosynthesis) on EFS was studied. Figure 1d illustrates the results of a typical experiment. At $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$ g/ml, L-NMMA in the ganglionic muscle strips produced a concentration-dependent inhibition of reaction to EFS and the relaxation frequency was 81.3 %, 75.0 %, and 37.5 %, respectively. And, 28.5 % relaxation was observed after $1x10^{-8}$ g/ml L-NMMA, 14.3 % after $1x10^{-7}$ g/ml, and 0 % after $1x10^{-6}$ g/ml in the transitional muscle strips (Table II).

In addition, significant differences were noted between the relaxation frequency in $1x10^{-8}$ and $1x10^{-6}$ g/ml and between $1x10^{-7}$ and $1x10^{-6}$ g/ml in the ganglionic muscle strips ($p<0.05$). The effects of L-NMMA on aganglionic muscle strips was also investigated. None of the aganglionic muscle strips exhibited a response.

Experiment 3: Following experiment 2, the effect of L-arginine (a precursor of the NO-biosynthesis) on the L-NMMA induced inhibition of evoked relaxation to EFS was also investigated. As shown in Figure 1e, the inhibition of relaxation reaction to EFS in the ganglionic and transitional colon was reversed. Thus, 42.3 % and 7.1 % relaxation was observed after $1x10^{-8}$ g/ml L-arginine, 64.2 % and 21.4 % after $1x10^{-7}$ g/ml, and 85.7 % and 35.7 % after $1x10^{-6}$ g/ml in the ganglionic and transitional muscle strips, respectively (Table III). Significant differences were noted between the frequency of relaxation reaction to $1x10^{-8}$ and $1x10^{-6}$ g/ml L-arginine ($p<0.05$). Reactions of aganglionic muscle strips to EFS were unaffected by L-arginine.

According to the results of experiment 1 and 2, relaxation reaction of NO was observed in the ganglionic colon and a lesser extent in the transitional colon, but was absent in the aganglionic colon. The decrease and loss of the effects of NO that mediate non-adrenergic inhibitory nerves may be largely related to the peristaltic abnormalities seen in transitional and aganglionic colon.

Discussion

It has been widely reported that NO plays a major role in the relaxation of the enteric smooth muscle of many different animal species including humans [8,12,18]. In 1990, Bult et al [2] provided evidence that NO is released by stimulation of the NANC inhibitory nerves in the gastrointestinal tract. Recent studies have shown that NO meets most criteria for neurotransmitters, supporting the role for NO as a neurotransmitter in the gastrointestinal tract [12,18]. However, histochemical studies have shown that NO is identical to nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-diaphorase) [8].

Table III. Experiment 3

	No reaction	Contraction	Relaxation	
Ganglionic segment				
L-arginine				
1x10-8 g/ml	7.1% (1/14)	50.0% (7/14)	42.8% (6/14)	*
1x10-7 g/ml	14.3% (2/14)	21.4% (3/14)	64.2% (9/14)	*
1x10-6 g/ml	14.3% (2/14)	0% (0/14)	85.7% (12/14)	
Transitional segment				
L-arginine				
1x10-8 g/ml	7.1% (1/14)	85.7% (12/14)	7.1% (1/14)	
1x10-7 g/ml	7.1% (1/14)	71.4% (10/14)	21.4% (3/14)	
1x10-6 g/ml	7.1% (1/14)	57.1% (8/14)	35.7% (5/14)	
Aganglionic segment				
L-arginine				
1x10-8 g/ml	100% (14/14)	0% (0/14)	0% (0/14)	
1x10-7 g/ml	100% (14/14)	0% (0/14)	0% (0/14)	
1x10-6 g/ml	100% (14/14)	0% (0/14)	0% (0/14)	

*EFS: electrical field stimulation, *p<0.05.*
Effects of L-arginine (1x10-8, 1x10-7, 1x10-6 g/ml) to EFS following administration of NG-monomethyl-L-arginine (L-NMMA; 1x10-6 g/ml) after blockade of the adrenergic and cholinergic nerves.

Recent studies reported that NO synthase was selectively absent in the plexus area and the muscle of aganglionic segments. Moderate staining was observed in the hypertrophied muscle bundles in the submucosa possibly originating from the pelvic plexuses [8]. In contrast, NO synthase was abundant in the ganglionic segment, and the pattern was similar to that in normal colon as demonstrated by NADPH-diaphorase histochemistry [3,10,12,19]. There are no reports on NO in the transitional segment [17].

Previously, we reported that diminution of action of NANC inhibitory nerves mediated by such factor as NO might be related to the impaired motility observed in patients with hypoganglionosis. These findings therefore indicate that the diminution and absence of NO, resulting in the diminution and absence of an inhibitory signal at the level of the smooth musculature, may be responsible for increased tone in the HD colon and a major feature in the pathophysiology of HD.

In this study, NANC inhibitory nerves were found to act on normal human colon and to a lesser extent on the transitional segment, but there was no effect on the enteric nerves in aganglionic segment. When the muscle strips of ganglionic colon were exposed to L-NMMA, an inhibitor of the NO-biosynthesis, the relaxation response to EFS after blocking both the adrenergic and cholinergic nerves was attenuated or replaced by a contractile response. The effect of L-NMMA was reversed in a concentration-dependent manner by L-arginine, a precursor of NO-biosynthesis. In contrast to ganglionic segment, none of the aganglionic segment showed any response to L-NMMA or L-arginine.

The effects of L-NMMA and L-arginine decreased in the transitional segment compared with the ganglionic segment. The relaxation reaction of NO was observed in ganglionic colon and to a lesser extent in the transitional colon but was absent in aganglionic colon.

Present data confirm that the diminution of reaction of NANC inhibitory nerves in the transitional and lack of that in the aganglionic bowel segment of HD patients suggests abnormal innervation of the muscle.

The diminution and absence of NO as a neurotransmitter in the muscle is most likely to be responsible for the spasticity of the lesional, transitional and aganglionic segment of HD. Recently, NO and various other neuropeptides have been shown to coexist in single neurons [12]. Thus, further assessment of the relationship between NO and neuropeptides is required.

References

1. Bentley JRR: Some new observations on megacolon in infancy and childhood with special reference to the management of megasigmoid and megarectum. Dis Colon Rectum 7:462, 1964
2. Bult H, Boeckxstaens GE, Pelckmans PA, et al: Nitric oxide as a inhibition non-adrenergic non-cholinergic neurotransmitter. Nature 345:346, 1990
3. Cuffari C, Rubin SZ, Krantis A: Routine use of nitric oxide stain in differential diagnosis of Hirschsprung's diseases. J Pediatr Surg 28:1202, 1994
4. Doig CM: Hirschsprung's disease and mimicking conditions. Dig Dis 12:106, 1994
5. Furness JB, Costa M: Types of nerves in the enteric nervous system. Neuroscience 5:1, 1980
6. Holschneider AM, Meier-Ruge W, Ure BM: Hirschsprung's disease and allied disorders - A review. Eur J Pediatr Surg 4:260, 1994
7. Munakata K, Okabe I, Morita K: Hypoganglionosis. Pediatr Surg Int 7:8, 1992
8. Munakata K, Tomita R, Kurosu Y, et al: Histochemical studies on pathophysiology of Hirschsprung's disease with special reference to NADPH-diaphorase. Biomedical Res 15:421, 1994
9. Munakata K, Tomita R, Kurosu Y: Preliminary immunohistochemical new findings in the myenteric plexus of patients with intestinal neuronal dysplasia type B. Eur J Pediatr Surg 7:21, 1997
10. O'Kelly T, Davies JR, Brading AF, et al: Abnormalities of nitric oxide producing neurons in Hirschsprung's disease: Morphology and implications. J Pediatr Surg 29:294, 1994
11. Puri P: Hirschsprung's disease: Clinical and experimental observations. World J Surg 17:374, 1993
12. Stark ME, Bauer AJ, Sarr MG, et al: Nitric oxide mediates inhibitory nerve input in human and canine jejunum. Gastroenterology 104:398, 1993
13. Tomita R, Kurosu Y, Aoki N, et al: Study on non-adrenergic non-cholinergic inhibitory nerves in the colon of patients with diverticular disease. Nihon Univ J Med 35:15, 1992
14. Tomita R, Kurosu Y, Munakata K, et al: Physiological studies on gastrointestinal hormones in the colon of patients with diverticular disease. Nihon Univ J Med 35:253, 1993
15. Tomita R, Munakata K, Kurosu Y: Peptidergic nerves in Hirschsprung's disease and its allied disorders. Eur J Pediatr Surg 4:346, 1994
16. Tomita R, Munakata K, Tanjoh K, et al: Regulation of non-adrenergic non-cholinergic inhibitory nerves in the colonic segment of elongated sigmoid colon. Nihon Üniv J Med 37:1, 1995
17. Tomita R, Munakata K, Kurosu Y: Nitric oxide in Hypoganglionosis. Condon JR (ed). Collegium Internationale Chirurgiae Digestivae (XV). Monduzzi Editore, Bologna (Italy), 1996, p.747-51
18. Tomita R, Kurosu Y, Munakata K: Regulation between nitric oxide and non-adrenergic non-cholinergic inhibitory nerves in human lower esophageal sphincter. J Gastroenterol 32:1, 1997
19. Vanderwinden JM, Delant MH, Schiffmann SN, et al: Nitric oxide distribution in enteric nervous system in Hirschsprung's disease. Gastroenterology 105:969, 1993