PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Distribution of constitutive nitric oxide synthase in the jejunum of adult rat

Yan-Min Chen, Zhong-Ming Qian, Jian Zhang, Yan-Zhong Chang, Xiang-Lin Duan

Yan-Min Chen, Jian Zhang, Yan-Zhong Chang, Xiang-Lin Duan, Life Science College, Hebei Normal University, Shijiazhuang 050016, Hebei Province, China
Zhong-Ming Qian,Department of Applied Biology and Chemical Technology,The Hong Kong Polytechchnic University, Hung Hom, Kowloon, Hong Kong
Supported by Natural Science Foudation of Hebei Province; Education Department Foundation of Hebei Province. No. 2002136.
Correspondence to: Xiang-Lin Duan, Life Science College, Hebei Normal University, Shijiazhuang 050016, Hebei Province, China. dxlzhl@sj-user.he.cninfo.net
Telephone: +86-311-6049941 Ext.86480 Fax: +86-311-5828784
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Abstract

AIM: **To study the distribution of the constitutive nitric oxide synthase (NOS) in the jejunum of adult rat.**

METHODS: **The distribution of endothelial NOS (eNOS) was detected by immunohistochemistry. Immunofluorescence histochemical dual staining technique were used for studying the distribution of neuronal NOS (nNOS) and eNOS. The dual stained slides were observed under a confocal laser scanning microscope.**

RESULTS: **Positive neuronal NOS (nNOS) and endothelial NOS (eNOS) cells were found to be distributed in lamina propria of villi, and the epithelial cell was not stained. eNOS was mainly located in submucosal vascular endothelia, while nNOS was mainly situated in myenteric plexus. Some cells in the villi had both nNOS and eNOS. More than 80% of the cells were positive for both nNOS and eNOS, the rest cells were positive either for nNOS or for eNOS.**

CONCLUSION: **The two constitutive nitric oxide synthases are distributed differently in the jejunum of rat. nNOS distributed in myenteric plexus is a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves. eNOS distributed in endothelial and smooth muscle cells of blood vessels plays vasodilator role. eNOS and nNOS are coexpressed in some cells of lamina propria of villi. NO generated by those NOS is very important in the physiological and pathological process of small intestine.**

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INTRODUCTION

Nitric oxide (NO) is an intercellular and endocellular signal molecule, and has an important role in the physiological process of intestine. For example, NO can regulate muscular contraction and blood circulation of the intestine[1,2]. Nitric oxide synthase (NOS) is widely distributed in the intestine, and has several isoforms, such as constitutive nitric oxide synthase (nNOS and eNOS) and inducible NOS (iNOS)[3]. In previous studies, NOS was mostly located in small intestine and could be shown by enzyme cytochemistry, but different isoforms of NOS[4-8] coud not be distinguished. In order to study the characteristics and distribution of NOS, immunohistochemistry and immunofluorescence histochemical duel staining technique were used to investigate the distribution of the constitutive nitric oxide synthase (NOS) in the jejunum of adult rat, to provide morphological basis of digestive physiology.

MATERIALS AND METHODS

Specimens

Segments (1-2cm) of the jejunum were removed from decapitated male Sprague-Dawley rats (250-300g) and placed immediately in a fixative consisting of 4% paraformaldehyde and 0.1 M phosphate buffer (PB, pH7.4). The fixed jejunum segments were rinsed for at least 12h at 4℃ in 0.1 M PB (pH7.2) containing 30% sucrose, and then cryostat sections were made at 6μm thickness and mounted onto glass slides.

Reagents

Rabbit anti-rat eNOS antibody and SP kit were purchased from Beijing Zhongshan Biotechinical Company. Mouse anti-rat nNOS antibody, FITC-conjugated anti-mouse IgG and PE-conjugated anti-rabbit IgG were purchased from Wuhan Boster Biological Technology Company.

Immunohistochemistry

Immunohistochemical staining for eNOS was performed using SP technique with the following procedure.

(1)The slides were washed in 0.01 M phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by 0.3% H_2O in methanol for 25 minutes, followed by incubation in normal goat serum for 30 minutes at room temperature. (2)The slides were the incubated with a 1∶75 dilution of the primary rabbit anti-rat eNOS antibody for 12 hour at 4℃. A biotin-streptavidin detection system was employed with diaminobenzidine as the chromogen. (3)Then the slides were washed with PBS and incubated with a reagent (biotinylated anti-immunoglobulin) for 60 minutes at 37℃. After rinsing in PBS, the slides were incubated with the peroxidase-conjugated streptavidin label for 60 minutes at 37℃, and incubated with diaminobenzidine and H_2O_2 for 5 minutes. Finally the sections were counterstained with hematoxylin.

Immunofluorescence histochemical dual-staining technique

The slides were incubated with normal goat serum for 30 minutes, followed by incubation with rabbiu anti-rat eNOS antibody and mouse anti-rat nNOS antidody for 48 hour at 4℃. Then, These were washed with PBS and incubated with FITC-conjugated anti-mouse IgG and PE-conjugated anti-rabbit IgG for 24 hour at 4℃. After rinsing in PBS, the slides were observed under a confocal laser scanning microscope (MR/A_2, Nikon). Excitation of FITC and PE were 488 and 495 nm respectively, emission of FITC and PE were 525 and 578 nm. 0.01 M PBS was used as a substitute for primary antibody for negative control groups.

RESULTS

Immunohistochemistry showed that eNOS was lealid in the cytoplasm solely. eNOS was distributed mainly in the endothelia of submucosa vessels. Part of the smooth muscle of submucosa vessels was also positive

for eNOS (Figure 1). There were strongly positive substances in the cells of lamina propria of the villi, and in the cells near the striated border of villous epithelia. The epithelial cells were unstained (Figure 2).

Under the confocal laser scanning microscope, the positive substances of nNOS labeled by FITC were green, and those of eNOS labeled by PE were red. The positive substances of nNOS were distributed mainly in myenteric plexus, rarely in the submucosal plexus (Figure 3). In the lamina propria of the villi, more than 80% of the cells were positive for both nNOS and eNOS, the rest of them were positive either for nNOS or for eNOS (Figure 4 and 5).

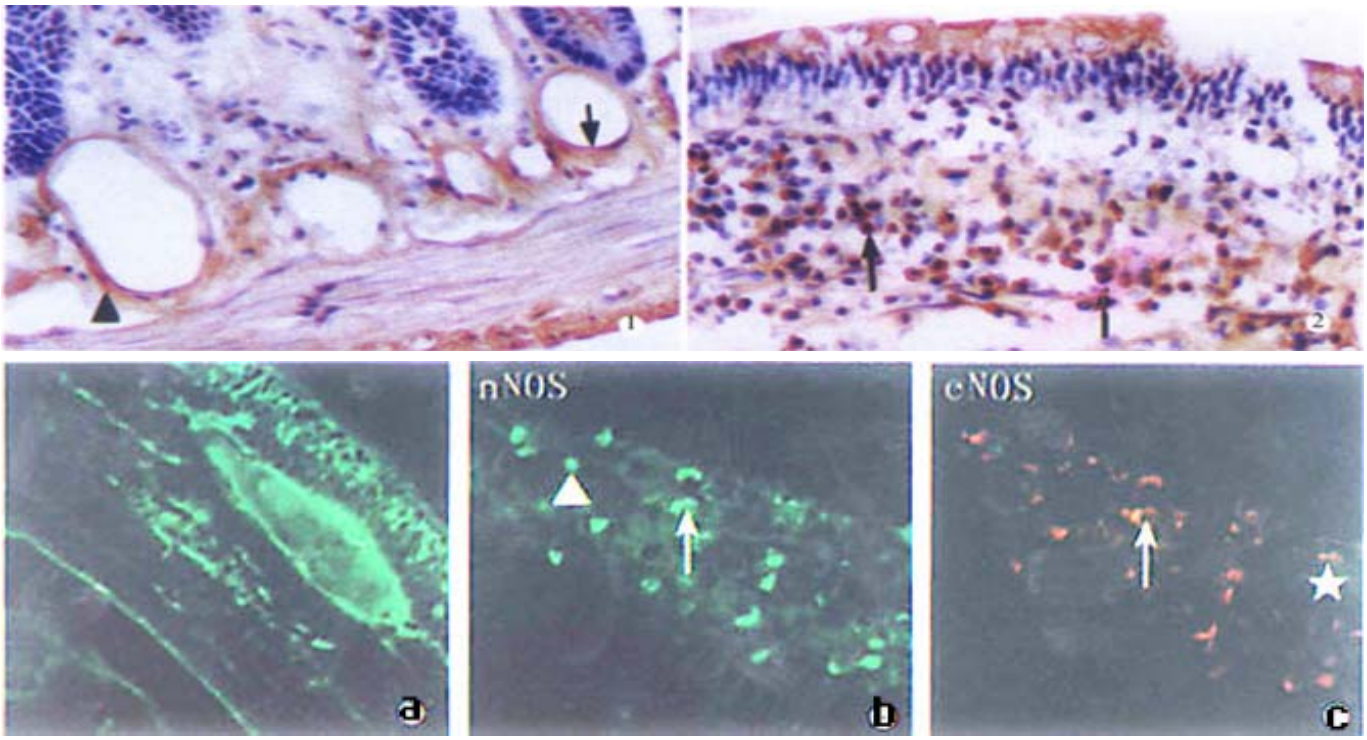

Figure 1 Immunohistochemical stain of eNOS in jejunal submucosa, showing the positive endothelium(↑) and microvascular smooth muscle(▲). ×170
Figure 2 Immunohistochemical stain of eNOS in jejunal villi,showing the positive cell in the proper layer(↑).×170
Figure 3-4-5 Immunofluorescence histochemical double-stain in jejunum observed under a confocal laser scanning microscope, fig3 showing the positive substances of nNOS were distributed mainly in myenteric plexus. ×350 fig4 and fig5 showing the nNOS-positive cell(▲), the eNOS-positive cell(★)and double-stained cell(↑)in the proper layer of villi. ×250

DISCUSSION

The two constitutively expressed, Ca^{2+}-dependent NOS isoforms previously identified in neurons (nNOS) and endothelial cells (eNOS) are now known to be distributed more widely[9-11]. eNOS is found in cardiac myocytes[12,13], epithelial cells[14-16], human platelets[17] and various neurons, particularly the pyramidal neurons of the hippocampus, where it is coexpressed with nNOS[18]. nNOS is found in the cytoskeleton of fast-contracting skeletal muscle fibers[19]. In our study, we found nNOS and eNOS were coexpressed in some cells of lamina propria of the villi. NO generated by those cells plays an important role in absorption and protection of microvasculature. Inhibition of endogenous NOS by N_G-nitro-L-auginine methyl ester (L-NAME) caused secretion of water and ions, and this secretion was reversed by administration of the NOS substrate L-arginine[20]. Previous studies indicated that norepinephrine[21, 22], somatostatin[23], and neuropeptide Y[24] increased ileal water and ion absorption at a similar magnitude to that observed with L-arginine. It is consistent with the hypothesis that endogenous NO has a proabsorptive influence in the intestine in the basal state. Furthermore, endogenous NO can reduce the vascular albumin leakage provoked by lipopolysaccharide (LPS)[25] and maintain microvascular integrity[26-29].

Our study showed that nNOS was distributed mainly in the myenteric plexus, rarely in submucosal plexus. Recent pharmacological and physiological studies demonstrated that NO is a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves of the gut[30-34]. During nerve stimulation, NO generated by nNOS in nerve terminals regulates the release of vasoactive intestinal polypeptide (VIP) when diffuses to muscle cells to participate in muscle relaxation[35-38]. Teng *et al*[39] found eNOS was selectively expressed in rabbit gastric and human intestinal smooth muscle cells. In turn, VIP acts on smooth muscle cells to generate NO. The NO formed in the muscle cells constitutes the predominant component (60-80%) of NO formed during nerve stimulation.

Using NOS histochemistry and endothelial cell immunohistochemisty, Nichols *et al*[40] provided the first anatomic evidence of NOS in both endothelial and smooth muscle cells of submucosal blood vessels in the intestines of rat and human, but he could not distinguish the isoform. We found eNOS was distributed in the endothelial and smooth muscle cells of submucosal blood vessels. This particular localization of eNOS was unexpected since only the inducible isoform of NOS had been reported in the vascular smooth muscle cells[41-44]. These anatomical data strongly supported the proposed vasodilator role of NO in the mammalian gastrointestinal tract. There is both basal and stimulated release of NO from the endothelium. Stimulated NO release is affected by certain antagonists (acetylcholine, ATP, or bradykinin) or by physical stimuli such as fluid shear stress[45-49] or low arterial PO_2[50]. Vascular smooth muscle-derived NO behaves as an autocrine factor that plays a role in modulation of vasodilator tone and represents a reserve pool of NOS, which may be required when the tissue is under a local stress. The

source of NO within the vascular wall, either intimal or medial, should be a consideration in future studies in terms of the relative contribution of these sources to vasodilator tone in the gut wall.

REFERENCES

1 Eskandari MK, Kalff JC, Billiar TR, Lee KK, Bauer AJ. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. *Am J Physiol* 1999;277:G478-486
2 Konomi H, Meedeniya AC, Simula ME, Toouli J, Saccone GT. Characterization of circular muscle motor neurons of the duodenum and distal colon in the Australian brush-tailed possum. *J Comp Neurol* 2002;443:15-26
3 Peng X, Wang SL. Nitric oxide and gastrointestinal movement. *Shijie Huaren Xiaohua Zazhi* 1998; 6:445-446
4 Peng X, Feng JB, Wang SL. Distribution of nitric oxide synthase in stomach wall in rats. *World J Gastroenterol* 1999;5:92
5 Nichols K, Staineds W, Krantis A. Nitric oxide synthase distribution in the rat intestine: a histochemical analysis. *Gastroenterology* 1993; 105:1651-1661
6 Bagyanszki M, Roman V, Fekete E. Quantitative distribution of NADPH-diaphorase-positive myenteric neurons in different segments of the developing chicken small intestine and colon. *Histochem J* 2000;32:679-684
7 Peng X, Feng JB, Wang SL. Nitric oxide synthase distribution in myenteric plexus of rat digestive tract. *Shijie Huaren Xiaohua Zazhi* 1998;6:250-252
8 Wilhelm M, Batori Z, Pasztor I, Gabriel R. NADPH-diaphorase positive myenteric neurons in the ileum of guinea-pig, rat,rabbit and cat: a comparative study. *Eur J Morphol* 1998;36:143-152
9 Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 1991;351:714-718
10 Busconi L, Michel T. Endothelial nitric oxide synthase: N-termianl myristoylation determines subcellular localization. *J Biol Chem* 1993; 25:8410-8413
11 Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T. Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett* 1992;307:287-293
12 Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T. Endothelial nitric oxide synthase targeting to caveolae: specific interactions with caveolin isforms in cardiac myocytes and endothelial cells. *J Biol Chem* 1996;271:22810-22814
13 Balligand JL, Kobzik L, Xan XQ, Kaye DM, Belhassen L, O'hera DS, Kelly RA, Smith TW, Michel T. Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial nitric oxide synthase in cardiac mytocytes. *J Biol Chem* 1995;270:14582-14586
14 Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 1993;9:371-377
15 Lamas S, Marsden PA, Li GK, Tempst P, Michel T. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc Natl Acad Sci* 1992;89:6348-6352
16 Shaul PW, North AJ, Wu LC, Wells LB, Brannon TS, Lau KS, Michel T, Margraf LR, Star RA. Endothelial nitric oxide synthase is expressed in cultured human bronchiolar epithelium. *J Clin Invest* 1994;94:2231-2236
17 Sase K, Michel T. Expression of constitutive endothelial nitric oxide synthase in human blood platelets. *Life Sci* 1995;57:2049-2055
18 Dinerman JL, Dawson TM, Schell J, Snowman A, Snyder SH. Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc Natl Acad Sci USA* 1994;91:4214-4218
19 Nakane M, Schmidt HW, Pollock JS, Forsermann U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 1993;316:175-180
20 Barry MK, Aloisi JD, Pickering SP, Yeo CJ. Nitric oxide modulates water and electrolyte transport in the ileum. *Ann Surg* 1994;219:382-388
21 Yano CJ, Couse NF, Antiohos C, Zinner MJ. The effect of norepinephrine on intestinal transport and perfusion pressure in the isolated perfused rabbit ileum. *J Surg Res* 1988;44:617-624
22 Yao CJ, Couse NF, Zinner MJ. Discrimination between alpha1 and alpha2 adrenergic receptors in the isolated perfused ileum. *Surgery* 1988;104:130-136
23 Anthone GJ, Bastidas JA, Orandle MS, Yao CJ. Direct proabsorptive effect of octreotide on ionic transport in the small intestine. *Surgery* 1990;108:1136-1142
24 Anthone GJ, Orandle MS, Wang BH, Yao CJ. Neuropeptide Y induced intestinal absorption: mediation by á2-adrenergic receptors. *Surgery* 1991;110:1132-1138
25 Crouser ED, Julian MW, Weinstein DM, Fahy RJ, Bauer JA. Endotoxin-induced ileal mucosal injury and nitric oxide dysregulation are temporally dissociated. *Am J Respir Crit Care Med* 2000;161:1705-1712
26 László F, Morschl E, Pávó I, Whittle BJR. Nitric oxide modulates the gastrointestinal plasma extravasation following intraabdominal surgical manipulation in rats. *Eur J Pharmacol* 1999;375:211-215
27 Iwashita E, Miyahara T, Hino K, Tokunaga T, Wakisaka H, Sawazaki Y. High nitric oxide synthase activity in endothelial cells in ulcerative colitis. *J Gastroenterol* 1995;30:551-554
28 Tan B, He SY, Deng HW, Li YJ. Effect of quercetin on adhesion of platelets to microvascular endothelial cells *in vitro*. *Acta Pharmacol Sin* 2001;22:851-856
29 Laszlo F, Whittle BJ, Moncada S. Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. *Br J Pharmacol* 1994;111:1309-1315
30 Takeuchi T, Niioka S, Yamaji M, Okishio Y, Ishii T, Nishio H, Takatsuji K, Hata F. Decrease in participation of nitric oxide in nonadrenergic, noncholinergic relaxation of rat intestine with age. *Jpn J Pharmacol* 1998;78:293-302
31 Nakao K, Takahashi T, Utsunomiya J, Owyang C. Extrinsic neural control of nitric oxide synthase expression in the myenteric plexus of rat jejunum. *J Physiol* 1998;507:549-560
32 Shah S, Nathan L, Singh R, Fu YS, Chaudhuri G. E2 and not P4 increases NO release from NANC nerves of the gastrointestinal tract: implications in pregnancy. *Am J Physiol Regul Integr Comp Physiol* 2001;280:R1546-1554
33 Shah S, Hobbs A, Singh R, Cuevas J, Ignarro LJ, Chaudhuri G. Gastrointestinal motility during pregnancy: role of nitrergic component of NANC nerves. *Am J Physiol Regul Integr Comp Physiol* 2000; 279:R1478-1485
34 Correia NA, Oliveira RB, Ballejo G. Pharmacological profile of nitrergic nerve-, nitric oxide-, nitrosoglutathione-and hydroxylamine-induced relaxations of the rat duodenum. *Life Sci* 2000;68:709-717
35 Vittoria A, Costagliola A, Carrese E, Mayer B, Cecio A. Nitric oxide-containing neurons in the bovine gut, with special reference to their relationship with VIP and galanin. *Arch Histol Cytol* 2000;63:357-368
36 Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT. Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell Tissue Res* 2001;304:31-41
37 Ekelund M, Ekblad E. Intestinal adaptation in atrophic rat ileum is accompanied by supersensitivity to vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide and nitric oxide. *Scand J Gastroenterol* 2001;36:251-257
38 Konturek SK, Konturek PC. Role of nitric oxide in the digestive system. *Digestion* 1995;56:1-13
39 Teng B,Murthy KS,Kuemmerle JF, Grider JR, Sase K, Michel T, Makhlouf GM. Expression of endothelial nitric oxide synthase in human and rabbit gastrointestinal smooth muscle cells. *Am J Physiol* 1998; 275:G342-351
40 Nichols K, Staines W, Rubin S, Krantis A. Distribution of nitric oxide synthase activity in arterioles and venules of rat and human intestine. *Am J Physiol* 1994; 267:G270-275
41 Charpie JR, Webb RC. Vascular myocyte-derived nitric oxide is an autocrine that limits vasoconstriction. *Biochem Biophys Res Commun* 1993;194:763-768
42 Hirafuji M, Tsunoda M, Machida T, Hamaue N, Endo T, Miyamoto A, Minami M. Reduced expressions of inducible nitric oxide synthase and cyclooxygenase-2 in vascular smooth muscle cells of stroke-prone spontaneously hypertensive rats. *Life Sci* 2002;70:917-926
43 Teng X, Li D, Catravas JD, Johns RA. C/EBP-beta mediates iNOS induction by hypoxia in rat pulmonary microvascular smooth muscle cells. *Circ Res* 2002;90:125-127
44 Teng X, Zhang H, Snead C, Catravas JD. Molecular mechanisms of iNOS induction by IL-1 beta and IFN-gamma in rat aortic smooth muscle cells. *Am J Physiol Cell Physiol* 2002;282:C144-152
45 Busse R, Mulsch A. Endothelium-derived relaxing factor: nitric oxide. *Mol Aspects Inflam* 1991;42:189-205
46 Busse R, Mulsch A, Fleming I, Hecker M. Mechanisms of nitric oxide release from the vascular endothelium. *Circulation* 1993;87(Suppl.V):18-26
47 Ignarro LJ, Wood KS, Fukuto JM. Continuous basal formation of endothelium-derived relaxing factor and muscle-derived relaxing factor, both of which are nitric oxide. *J Cardiovasc Pharmacol* 1991;17(suppl.3):S229-S233
48 Lamontage D, Pohl U, Busse R. Mechanical deformation of vessel wall and shear stress determine the basal EDRF release in the intact coronary vascular bed. *Circ Res* 1992;70:123-130
49 Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow *in vivo*. *Hypertension* 1986;8:37-44
50 Pohl U, Busse R. Hypoxia stimulates the release of endothelium-derived relaxant factor. *Am J Physiol* 1989;256(Heart Circ Physiol 25):H1595-H1600

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