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

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

METHODS: The distribution of endothelial NOS (eNOS) was detected by immunohistochemistry. Immunofluorescence histochemical dual staining technique was 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 endothelial 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 intestine3,4. 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)5-7. In previous studies, NOS was mostly located in small intestine and could be shown by enzyme cytochemistry, but different isoforms of NOS could not be distinguished. In order to study the characteristics and distribution of NOS, immunohistochemistry and immunofluorescence histochemical dual 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 (pH 7.4). The fixed jejunum segments were rinsed for at least 12h at 4°C in 0.1 M PB (pH 7.2) 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 Biotechnical 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% H2O2 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°C. A biotin-streptavidin detection system was employed with diaminobenzidine as the chromogen. (3)Then the sections were washed with PBS and incubated with a reagent (biotinylated anti-immunoglobulin) for 60 minutes at 37°C. After rinsing in PBS, the slides were incubated with the peroxidase-conjugated streptavidin label for 60 minutes at 37°C, and incubated with diaminobenzidine and H2O2 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 rabbit anti-rat eNOS antibody and mouse anti-rat nNOS antibody for 48 hour at 4°C. 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°C. After rinsing in PBS, the slides were observed under a confocal laser scanning microscope (MR/A2, 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 endothelium 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).

**DISCUSSION**

The two constitutively expressed, Ca²⁺-dependent NOS isoforms
previously identified in neurons (nNOS) and endothelial cells (eNOS) are
now known to be distributed more widely⁹⁻¹¹. eNOS is found in cardiac
myocytes¹²⁻¹³, epithelial cells¹⁴⁻¹⁶, human platelets¹⁷ and various neurons,
particularly the pyramidal neurons of the hippocampus, where it is
coenzymed with nNOS¹⁸. nNOS is found in the cytoskeleton of fast-
contracting skeletal muscle fibers¹⁹. 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 NG-
nitro-L-arginine methyl ester (L-NAME) caused secretion of water and
ions, and this secretion was reversed by administration of the NOS
substrate L-arginine²⁰. Previous studies indicated that norepinephrine²¹,
²², somatostatin²³, and neuropeptide Y²⁴ 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 integrity²⁶⁻²⁹.

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 gut³⁰⁻³⁴. 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 relaxation³⁵⁻³⁷. Teng et al³⁹ 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
immunohistochemistry, Nichols et al⁴⁰ 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 cells⁴¹⁻⁴⁴. 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 stress⁴⁵⁻⁴⁷ or low arterial PO₂⁴⁸. 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.