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Role of neuronal nitric oxide synthase in colonic distension-induced hyperalgesia in distal colon of neonatal maternal separated male rats

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Abstract

Background—Nitric oxide (NO) is implicated in the pathogenesis of irritable bowel syndrome (IBS) but the underlying mechanism is unclear. Thus, the aim of the present study is to examine the role of NO synthase (NOS) expression in the distal colon of neonatal maternal separation (NMS) model rats employed in IBS studies.

Methods—Male neonates of Sprague-Dawley rats were randomly assigned into NMS and normal control (N) groups. Rats of NMS group were subjected to 3-hr daily maternal separation on postnatal day 2–21. Rats were administrated non-selective NOS inhibitor L-NAME (100mg/kg), selective neuronal NOS (nNOS) inhibitor 7NINA (10mg/kg), selective inducible NOS (iNOS) inhibitor, endothelial NOS (eNOS) inhibitor (10mg/kg) or Vehicle (Veh; distilled water) intraperitoneally 1 hour prior to the experiment for the test and control groups, respectively.

Key results—The amount of NO was significantly higher in the NMS Veh rats compared with unseparated N rats. Western-blotting and real-time quantitative PCR studies showed that protein and mRNA expression of nNOS were higher in the NMS group than that in the N rats; whereas no significant change in iNOS and eNOS was found in either groups. NMS Veh rats showed low pain threshold and increased electromyogram (EMG) activity in response to colonic distension stimuli. L-NAME and 7NINA increased pain threshold pressure and attenuated EMG activity in the NMS rats. In addition, L-NAME and 7-NINA substantially reduced oxidative marker malondialdehyde level in NMS rats.

Conclusions & Inferences—NMS increased the NO generation by nNOS upregulation that interact with reactive oxygen species contributing to the visceral hypersensitivity in IBS.

Keywords

Irritable bowel syndrome; distal colon; neonatal maternal separation; colonic distension; visceral hyperalgesia; nitric oxide synthase

Introduction

Irritable bowel syndrome (IBS) is a prevalent chronic functional bowel disorder characterized by visceral hyperalgesia causing symptoms such as abdominal pain, flatulence, alteration of bowel habits, diarrhea and constipation without any structural cause^{1–4}. It affects approximately 10–20% of world's population⁵. Although IBS is not fatal, it is highly disruptive to a patient's daily life⁶. The etiology of IBS is multi-factorial including genetic and environmental effects^{7, 8}, diet⁹, psychological and autonomic nervous system disturbances^{10, 11}. Early-life psychological stress including maternal deprivation, and childhood emotional or physical abuse have been implicated in the pathogenesis of IBS^{12–14}. Yet the underlying mechanism is still not well understood.

Nitric oxide (NO) is a gaseous messenger which plays an essential role in the physiology and pathophysiology of the gastrointestinal tract^{15, 16}. NO is synthesized from *L*-arginine catalyzed by NO synthase (NOS). Three isoforms of NOSs have been identified: neuronal NOS (nNOS) and endothelial NOS (eNOS) are calmodulin-dependent constitutive enzymes which are involved in smooth muscle and vascular relaxation^{17, 18}; while inducible NOS (iNOS) is calcium-insensitive, which is induced in response to inflammation¹⁹. Clinical studies have reported elevated rectal and plasma NO in IBS patients^{20, 21}. Blockade of NOS increased threshold to rectal pain in IBS patients and rodent model^{22, 23}. These evidences suggest that NOS is likely to be involved in the development of IBS. However the role of NOSs in the psychological stress-induced IBS is not known. Therefore, the aim of the present study is to investigate changes in the expression of NOSs in the distal colon of the neonatal maternal separated rat model, a well developed powerful early-life stress model causing permanent alteration of the brain-gut axis leading to visceral hyperalgesia and gut dysfunctions^{12, 24}. The results of this study may elucidate the possible role of NOSs in early-life stress induced visceral hypersensitivity and dysmotility in IBS.

Methods

Chemicals

L-NAME (NG-Nitro-*L*-arginine methyl ester hydrochloride), 7-NINA (7-Nitroindazole monosodium salt), 1400W (*N*-[[3-(Aminomethyl) phenyl] methyl]-ethanimidamide dihydrochloride), and *L*-NIO (*N*⁵-(1-Iminoethyl)-*L*-ornithine dihydrochloride) were purchased from Tocris Bioscience (Ellisville, Missouri, USA).

Animal and neonatal maternal separation

The animal experimental procedures as detailed below were approved by the Animal Ethics Committee of the Chinese University of Hong Kong and the Institutional Animal care and Use of Committee of the University of Maryland-Baltimore. All male Sprague-Dawley pups were grouped to 6 pups per dam on postnatal day 2 (P2; date of the birth is designated as P0). Pups were randomly assigned to neonatal maternal separation (NMS) or unseparated control (N) groups according to well established protocol^{25, 26}. In brief, pups in the NMS group were separated from their mothers and placed into individual cages in an adjacent room maintained at 22°C for 3 hours (09:00–12:00) on P2–P21. The pups were then returned to the maternal cages after the separation on each period day. While N group of rats were allowed to remain in standard cages with their dams. All pups were weaned on day 22 and housed (5 rats per cage) on 12:12-hr light-dark cycle (Lights on at 06:00) with free access to food and water ad libitum.

Implantation of electromyogram electrode

The visceral motor response to colonic distension (CRD) was measured by recording electromyogram (EMG). Rats were anesthetized by inhalation of 2% isoflurane (in oxygen, 0.5L/min). A pair of EMG electrodes was surgically implanted at the lower left abdominal area to expose the external oblique abdominal musculature and the electrode were tunneled subcutaneously, exteriorized and secured at the back of the neck for subsequent EMG recording.

Administration of NOS inhibitors

The NMS rats were randomly divided into 5 groups (8 rats per group), with four groups being dosed intraperitoneally with L-NAME (100mg/kg), 7NINA (10mg/kg), 1400W (10mg/kg) or L-NIO (10mg/kg) in volume of 1ml/kg in distilled water 1 hr prior to the CRD experiment. The 5th group, designed as “Veh” and the N rats were administered intraperitoneally with distilled water.

Colonic distension-induced visceral hyperalgesia

The CRD study commenced 7 days after EMG electrodes implantation. The rats were anesthetized with 2% isoflurane (in oxygen, 0.5L/min) to facilitate placement of the inflatable balloon, constructed from a latex glove finger attached to a Rigidflex balloon dilator via a Y connector to a syringe pump and a sphygmomanometer into the descending colon²⁷. The balloon catheter was inserted into the rectum. The rats were allowed to recover for 30 min prior to the experiment. EMG signals in response to CRD were recorded with a PowerLab 16/30 instrument, and analyzed using Chart software (AD instruments, Bella Vista, Australia). The pain threshold pressure was defined as minimum pressure that evokes an observatory signal. EMG signal responses to CRD were measured at 10, 20, 40, 60 and 80mmHg. The changes of the EMG signal response to CRD were determined by calculating the change of area-under-curve (AUC) of raw EMG amplitude responses to CRD, based on the formula $\Delta AUC \% \text{ baseline} = (\text{AUC during CRD} / \text{AUC before CRD})$.

Tissue preparation

Rats were anesthetized immediately after the completion of the CRD study, the distal colon dissected, immediately frozen in liquid nitrogen, and stored at -70°C .

Measurement of NO concentration

Frozen samples of the distal colon were homogenized in phosphate-buffered saline and centrifuged at 10,000g for 20min at 4°C . The concentration of NO in the supernatant was determined using a NO colorimetric assay kit (Abam HK Ltd, 168 Connaught Road Central, Hong Kong) according to the manufacturer's instructions. The handled sample was measured at 540nm using a microplate reader (Fluostar Optima spectrometer, BMG Labtech, Offenbury, Germany). The concentration of NO was expressed as μM , was determined by using a standard calibration curve.

Real-time quantitative PCR

Frozen distal colons were homogenized with Trizol reagent (Invitrogen, USA), and the total RNA was extracted following the manufacturer's instructions. Real-time PCR reactions were carried out in a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers and taqman fluorogenic probes were purchased from Applied Biosystems, and their sequences are shown in table 1. DNA was employed to quantify mRNA encoding β -actin, which is used as a non-regulated reference gene. The data were analyzed with the Step One software and relative gene expression was determined using the $2^{-\Delta\Delta}$ cycle threshold (C_T) Method²⁸. In brief, the C_T values were normalized by subtracting

from the β -actin control ($\Delta C_T = C_T \text{ NOS mRNA} - C_T \beta\text{-actin control}$). The expression of the mRNA of NOS in the NMS group relative to the N group was calculated by subtracting the normalized C_T values in the N group from those in the NMS group ($\Delta\Delta C_T = C_T \text{ NMS} - \Delta C_T \text{ N}$), and the relative expression ($2^{-\Delta\Delta C_T}$) was thus determined.

Western-blotting study

Frozen distal colons were homogenized in the lysis buffer containing 10ml of protease inhibitor cocktail (Calbiochem, USA), and centrifuged at 15,000g for 20 minutes at 4°C, and the supernatants were mixed with the Laemmi buffer, incubated at 70°C for 3min. The resulting protein samples were separated individually in 7.5% SDS-polyacrylamide mini-gels by electrophoresis. The gels were transferred to polyvinylidene-difluoride membranes using a transblotting apparatus (Bio-Rad laboratories, USA) and blocked with 5% skimmed milk in TBS buffer at room temperature for 2 hours. The membranes were then incubated with primary antibody nNOS, iNOS, eNOS or β -actin (Santa Cruz Biotechnology Inc, USA) for overnight at 4°C. Immunoreactivity was detected with the secondary antibodies, anti-mouse IgG conjugated to HRP for nNOS and β -actin, or anti-rabbit IgG conjugated to HRP for iNOS and eNOS (Santa Cruz Biotechnology Inc, USA). The blots were developed with a chemiluminescence's reagent, and the films exposed and analyzed with the aid of Image J (National Institutes of Health, USA).

Measurement of malondialdehyde

The distal colon tissue was homogenized with phosphate-buffered saline and the homogenate (100 μ l) was mixed with the thiobarbituric acid reagent containing thiobarbituric acid (1500 μ l of 0.8% solution), acetic acid (1500 μ l of 20% solution), SDS (200 μ l of 8.1% solution) and distilled water (700 μ l). The sample was incubated at 95 °C for 1 hr, centrifuged at 3,000g for 10min and then measured spectrophotometrically at 586nm using a microplate reader (Fluostar Optima spectrometer, BMG Labtech, Offenbury, Germany). Standard curves were constructed with 1,1,3,3-tetraethoxypropane as a standard. The malondialdehyde (MDA) concentration in the distal colon was normalized to wet tissue weight (mg) and expressed as μ mol/mg.

Statistical analysis

Data were expressed as mean $\pm$ SE. Statistical analyses were performed using Prism 4.0 software (GraphPad software Inc, La Jolla, CA, USA). Statistical significances were determined by the unpaired *t*-test and differences were considered significant at $^*p < 0.05$.

Results

7NINA and L-NAME attenuate CRD-induced visceral hyperalgesia in NMS rats

The threshold to exhibit EMG signals in response to CRD was markedly reduced by 45% in the NMS (Veh) rats as compared to the N rats (N=8 per group; $p < 0.01$) (Figure 1). On average, the threshold pressure in the NMS and N groups were 14.4 ± 0.6 and 25.0 ± 1.3 mmHg, respectively. Pre-treatment with the non-selective inhibitor L-NAME (100mg/kg) and the selective nNOS inhibitor 7-NINA (10mg/kg) significantly elevated threshold to pain by 48% (N=8; $p < 0.05$) and 43% (N=8; $p < 0.05$), respectively, when compared with Veh animals in the NMS rats (Figure 1). By contrast, selective iNOS inhibitor 1400W (10mg/kg) and eNOS inhibitor L-NIO (10mg/kg) had no statistically significant effect on the threshold to pain in the Veh group (Figure 1). In the EMG recording, EMG activities were found to be pressure-dependent among the test groups during ascending CRD with the magnitude of EMG signals in response to CRD substantially higher in the NMS (Veh) group than that in the N group at each distension pressure ($p < 0.01$,

Figure 2A–D). L-NAME (100mg/kg) and 7-NINA (10mg/kg) substantially reduced the elevated EMG signals of the Veh group at each distension pressure (Figure 2A–D), but the 1400W (10mg/kg) and L-NIO (10mg/kg) treatments showed no apparent effect on the EMG magnitude in the NMS rats (Figure 2A–D).

Elevated NO production from the distal colon of NMS rats

NO production from the distal colon in N and NMS rats were measured using colorimetric assay. There were no significant difference in colonic NO concentration before and after CRD in N rats (Figure 3A). The amount of NO from the distal colon was significantly increased by about 2-fold in NMS rats when compared with N control rats before CRD (N=8; $p<0.01$) and after CRD (N=8; $p<0.01$) (Figure 3A). L-NAME and 7NINA substantially attenuated NO level by 53% and 50% in NMS rats; while neither 1400W nor L-NIO had any effect on NO level in NMS rats (Figure 3B).

Increased nNOS expressions from the distal colon of the NMS rats

The protein expressions of the NOS isoforms, namely, nNOS, iNOS and eNOS in the distal colon of the NMS and N rats were examined by a western-blotting study. Figure 4A shows the nNOS, iNOS and eNOS expressions for the distal colon of NMS and N rats. Pre and Post-CRD nNOS expression from the distal colon was significantly higher in the NMS group as compared to the N group (N=8 per group; $p<0.01$) (Figure 4B). No apparent differences were observed in the iNOS and eNOS expressions in both groups (Figure 4B). The relative mRNA expressions of nNOS, iNOS and eNOS were examined by real-time quantitative PCR. The nNOS mRNA expression was significantly higher in the distal colon of the NMS group than that in the N group before CRD (N=8 per group; $p<0.05$) (Figure 5). There was an increase in nNOS mRNA expression in the distal colon of the NMS group, but not in the N group after CRD. There were no significant differences in the iNOS and eNOS expressions between the N and NMS rats either pre- or post CRD (Figure 5).

7-NINA and L-NAME attenuated oxidative stress in the distal colon of NMS rats response to CRD

The level of oxidative marker MDA in the distal colon was significantly increased by 70% in the NMS Veh group when compared with N control rats ($92.4\pm4.7\mu\text{mol/mg}$ versus $54.3\pm4.5\mu\text{mol/mg}$; $p<0.05$) (Figure 6). The MDA level was significantly reduced by 21% and 19% in NMS rats treated with L-NAME and 7NINA, respectively; while neither 1400W nor L-NIO had any effect on the MDA level in NMS rats (Figure 6).

Discussion

This study is the first to show elevated nNOS expression and NO production from the distal colon of NMS rats. Recent studies have reported that early life stress contributes to the development of IBS^{29–31}. The results of our study showed a reduced threshold to pain and elevated EMG activity in response to CRD in NMS rats. This finding is consistent with our previous study and those of others that NMS alters hypothalamus-pituitary-axis and neurochemical changes, resulting in permanent visceromotor alterations that mimicking the main symptoms of IBS in human^{25, 26, 32}. Apart from alteration in colonic sensitivity, changes in visceral perception accessed via colonic balloon distension could be through changes in colonic compliance. However, vivo study has reported an increased visceral hypersensitivity response to CRD without changes in compliance³³. Clinical studies have also shown that no differences in rectal compliance between IBS patients and healthy subjects³⁴ were observed, and the administration of opioid kappa receptor agonist asimadoline reduced pain perception response to CRD without affecting colonic compliance³⁵.

It is well known that NO is synthesized and released from the myenteric plexus of the distal colon. NO exerts its action via cyclic guanosine 3',5'-monophosphate dependent pathway by activating soluble guanylyl cyclase (sGC)³⁶. sGC in turn activates phosphodiesterases and protein kinase G that modulates motility and peristalsis of rat distal colon³⁷⁻³⁹. Yet excessive NO production is associated with motility and motor disorders in gastrointestinal tract¹⁵. Clinical studies have reported that plasma and rectal NO level is significantly higher in IBS patients than normal healthy subjects^{20, 21}. Our data showed an increase in the amount of NO in the distal colon of NMS rats and the level was sustained after CRD stimulus. Xu and colleagues also reported an enhanced neurotransmitter NO in the myenteric plexus of the colon in rodent model of IBS⁴⁰. These observations indicate that early-life stress mediates endogenous NO production. Our in vivo study demonstrated that the non-selective NOS inhibitor L-NAME increased threshold to pain and reduced EMG activity in NMS rats in response to CRD. Mizuta and colleagues also showed that L-NAME significantly delayed colonic transit³⁷. These findings confirmed our conclusion that NO is involved in visceral hypersensitivity in IBS. We observed in the present study that NMS up-regulated the expression of nNOS in the distal colon of rats. In addition, it is shown that the nNOS inhibitor 7NINA, but not the iNOS inhibitor 1400W, nor the eNOS inhibitor L-NIO, reduced NO level, elevated pain threshold and depressed EMG activity evoked by CRD in NMS rats. These findings suggest that NMS enhances endogenous NO production mediated by nNOS upregulation, which may have a direct effect on intestinal nerves resulting in visceral hyperalgesia. On the other hand, iNOS was found to be linked to inflammatory colonic dysfunction in IBS patients^{23, 41}, and that it is involved in impaired colonic transit and visceral pain under proinflammatory conditions such as intestinal injury⁴², inflammatory bowel diseases and colitis^{43, 44}. Therefore, it is likely that iNOS and nNOS play different roles in the pathogenesis of gastrointestinal tract disorders.

Our study also showed that an increase in MDA level from the distal colon of NMS rats after CRD, which is consistent with a report that oxidative stress is implicated in the development of visceral pain⁴⁵. Oxidative stress underpins overproduction of reactive oxygen species (ROS). At physiological condition, ROS tends to be catalytically converted to hydrogen peroxide (H₂O₂) catalyzed by superoxide dismutase (SOD) enzyme and subsequently H₂O₂ is subsequently detoxified by catalase or glutathione peroxidase. Our data showed that the administration of 7-NINA to NMS rats significantly reduced the MDA level from the distal colon after CRD. This suggested that ROS can react with NO to form the reactive molecule preoxynitrite that inhibits the catalytic function of SOD by nitrating the enzyme⁴⁶, which can subsequently prevent the breakdown of ROS leading to the development of persistent hyperalgesia. However the mechanistic role of preoxynitrite in pain generation still needs to be clarified.

In conclusion, the present study showed elevated NO production and upregulation of nNOS expression in the distal colon of NMS rats. These results suggest that elevated NO generation mediated by nNOS is involved in sustaining visceral hyperalgesia in NMS rats, and provide a new information that NO is involved in the mechanism of early-life stress in the pathogenesis of IBS.

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Figure 1.

Effects of NOS inhibitors on pain threshold pressure among groups. The pain threshold was significantly lowered in the NMS Veh group as compared with the N group (N=8 per group). The non-selective NOS inhibitor, L-NAME (N=8; 100mg/kg), and the selective nNOS inhibitor 7NINA (N=8; 10mg/kg), treated NMS group substantially increased pain threshold pressure relative to NMS Veh group. Selective iNOS inhibitor 1400W (N=8; 10mg/kg) and eNOS inhibitor L-NIO (N=8; 10mg/kg) had no effect on pain threshold pressure in NMS rats. Statistical significance is indicated by ** $p < 0.01$ unpaired t -test compared with N group and # $p < 0.05$ versus NMS Veh group, one way ANOVA.

Figure 2.

Effects of NOS inhibitors on electromyographic activity response to ascending intensities of CRD in the NMS and N rats (A–D). Data are presented as mean±SEM. Statistical significance is indicated by ** $p < 0.01$ unpaired t -test compared with N group (N=8) and ## $p < 0.01$ versus NMS Veh group (N=8), one way ANOVA.

Figure 3.

(A) NO concentration from distal colon of NMS and N rats. The amount of NO in distal colon before and after CRD were significantly increased in NMS rats (N=8) compared with N rats (N=8). (B) Effects of NOS inhibitors (N=8 per group) on NO levels in the NMS rats and N rats. Statistical significance is indicated by ** $p < 0.01$ unpaired t -test compared with N group and ## $p < 0.01$ versus NMS Veh group, one way ANOVA.

Figure 4.

(A) Protein levels in NOSs from distal colon in NMS and N rats. (B) Summary of protein expressions of NOSs in NMS and N groups. nNOS expression in NMS rats was higher than that of N rats (N=8 per group). Statistical significance is indicated by ** $p < 0.01$ unpaired t -test compared with N group.

Figure 5.

Summary of relative mRNA NOSs expression from distal colon in NMS and N rats. Relative nNOS expression was significantly increased in NMS rats as compared with N rats (N=8 per group). Statistical significance is indicated by ** $p < 0.01$ unpaired *t*-test compared with N group.

Figure 6.

Effect of NOS inhibitors on the MDA level of NMS rats. The MDA level was substantially increased in NMS group compared with N group. L-NAME and 7-NINA, but not 1400W and L-NIO, significantly reduced MDA level NMS rats (N=8 per group). Statistical significance is indicated by ** $p < 0.01$ or unpaired t -test compared with N group and ## $p < 0.01$ versus NMS Veh group, one way ANOVA.

Table 1

Primer sequences for PCR amplification of target (nNOS, iNOS and eNOS) and endogenous (β -actin) genes.

Primer	Primer Sequence 5'-3'
nNOS (forward)	5'-TCA TTA GCA ATG ACC GAA GC-3'
nNOS (reverse)	5'-AAC ATT GGA AAG ACC TTG GG-3'
Probe (sense)	5'-TCC GCC ACA TAC GTG AGG CG-3'
iNOS (forward)	5'-GAG ACA GGA AG TCG GAA GC-3'
iNOS (reverse)	5'-GTG TTG AAG GCG TAG CTG AA-3'
Probe (sense)	5- TAG CCA GGG ACC TGG CTG CC-3'
eNOS (forward)	5'-TGTAGC TGT GCT GGC ATA CA-3'
eNOS (reverse)	5'-TTG AGT TGG CTC ATC CAT GT-3'
Probe (sense)	5'-TGT GCT GGG CCC TCT GCA CT-3'
β -actin (forward)	5'-AGC AGA TGT GGA TCA CCA AG -3'
β -actin (reverse)	5'-AAC AGT CCG CCT AGA AGC AT-3'
Probe (sense)	5'-CCT CCA TCG TGC ACC GCA A-3'