Roles of nitric oxide and prostaglandins in pathogenesis of delayed colonic transit after burn injury in rats

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Submitted 29 October 2004; accepted in final form 9 January 2005

Gan, Hua Tian, and J. D. Z. Chen. Roles of nitric oxide and prostaglandins in pathogenesis of delayed colonic transit after burn injury in rats. Am J Physiol Regul Integr Comp Physiol 288: R1316–R1324, 2005.—Burn injury has been shown to impair gut transit, but the exact mechanism remains unknown. The present study investigated whether nitric oxide synthase (NOS) and cyclooxygenase (COX) mediated changes in burn-induced colonic transit. After rats underwent 30% total body surface area burn injury, they were injected with S-methylisothiourea (SMT, selective inducible NOS inhibitor), 7-nitroindazole (7-NI, selective neuronal NOS inhibitor), and nimesulide (NIM, selective COX-2 inhibitor), respectively. The protein and mRNA of NOS and COX-2 were measured by Western blot analysis and real-time RT-PCR, and localization of NOS and COX-2 protein was determined by immunohistochemistry. Our results showed that colonic transit assessed by the geometric center was delayed from 3.47 ± 0.28 in controls to 2.21 ± 0.18 after burn (P < 0.009). SMT and NIM significantly improved colonic transit in burned rats but had no effect in sham-operated rats. 7-NI failed to modify delayed transit in burned rats but significantly delayed colonic transit in sham-operated rats. Both protein and mRNA of inducible NOS and COX-2 increased significantly but not neuronal NOS in burned rats. Inducible NOS protein expression was noted not only in epithelial cells but also in neurons of the myenteric ganglia in burned rats. These findings suggest that nitric oxide (NO) produced by neuronal NOS plays an important role in mediating colonic transit under the physiological condition. NO produced by inducible NOS and prostaglandins synthesized by COX-2 are both involved in the pathogenesis of delayed colonic transit after burn injury. Inducible NOS expression in neurons of the myenteric ganglia may contribute to dysmotility with burn injury.

Colonic dysmotility is a common complication of burn injury. Previous studies have demonstrated that burn injury impairs colonic motility (8), but the exact mechanisms remain unknown. A potential mediator of burn-induced colonic dysmotility is nitric oxide (NO), an important nonadrenergic, noncholinergic (NANC) neurotransmitter that induces gastrointestinal smooth muscle relaxation and regulates gastrointestinal motility (6, 48). NO is synthesized from L-arginine by the activation of NO synthase (NOS) (24). Three major isoforms of NOS have been identified (13): two types of constitutive NOS participate in normal physiological responses and are Ca$^{2+}$/calmodulin-dependent, including neuronal NOS (nNOS), which is mainly localized to neurons (5) and endothelial NOS (eNOS), which is mainly localized to endothelium (42). The third type of NOS is called inducible NOS (iNOS), which is Ca$^{2+}$/calmodulin-independent and is not present in tissues under normal physiological conditions but is expressed in a wide variety of cells in response to tissue injury and inflammatory stimulation, such as cytokines and LPS (10, 22). NO has been reported to play a key role in colonic transit under physiological conditions by mediating descending relaxation of colon, thereby facilitating colonic transit (28). However, the pathophysiological role of NO on colonic transit in the circumstance of burn injury remains unclear.

Multiple studies have suggested that prostaglandins synthesized from arachidonic acid acts as local regulatory agents that modulate gastrointestinal motility (21, 39). Synthesis of prostaglandins is carried out by cyclooxygenase (COX), which exists as two isoenzymes, COX-1 and COX-2. The constitutive form COX-1 is believed to be present in most tissues and cell types. COX-2, however, the inducible form within certain cells, is usually absent under normal conditions and is expressed in many cells in response to tissue injury and inflammatory stimulation (11, 43). Interestingly, it has been observed that the NO and the COX pathway share a number of similarities and play fundamental roles in similar physiopathological conditions (54). Recent studies have shown that there is a cross-talk interaction between the NO and the COX pathway; that is, NO regulates COX-2 activity and expression. In contrast, the prostaglandins synthesized by COX-2 may also influence NOS activity and expression (37).

On the basis of these observations, we hypothesized that NOS and COX-2 play a major role in mediating the impairment of colonic transit that occurs during burn injury. Accordingly, the aims of our study were to determine whether the expression of NOS and COX-2 were changed during burn injury and whether blocking the production of NO and prostaglandins ameliorated the disrupted colonic transit associated with burn injury.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan Laboratories, Houston, TX) weighing 250–300 g were obtained and housed in microisolator cages at room temperature (25°C) with alternating 12:12-h light-dark cycles. Standard rat chow and water were provided during a minimum stabilization period of 7 days. Before the experiments, all rats were starved overnight with free access to water. This study was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, Texas.
Experimental Procedure

Under general anesthesia with xylazine (7 mg/kg) and ketamine (60 mg/kg), an abdominal midline incision of rats was performed. A small catheter was inserted via the cecum (1 cm proximal to the cecocolic junction) into the proximal colon (1 cm distal to the cecocolic junction) and fixed with sutures. The catheter was tunneled through the anterior abdominal wall subcutaneously and placed outside the neck skin of rats. Three days after surgery, the rats underwent burn injury as previously described (9). Briefly, rats were anesthetized with xylazine (7 mg/kg) and ketamine (60 mg/kg), and the dorsum of the rats was shaved. Then the rats were placed on a Plexiglas plate, and the delimited area was burned by immersion in 95°C water for 10 s, which resulted in a full-thickness burn involving 30% of the total body surface area. The rats in the sham-operated group were treated identically, except that they were dipped in a 25°C water bath for 10 s. All rats received buprenorphine (0.5 mg/kg sc) for analgesia.

Each of the burned rats and control rats (sham-operated) were divided into four groups: saline, S-methylisothiourea (SMT), 7-nitroindazole (7-NI), and nimesulide (NIM). The saline group of sham-operated control rats and burned rats were injected with saline (5 ml/kg ip) immediately after and 12 h after the burn injury (or sham operation). In the other three groups of burned or control rats, saline was replaced with SMT (selective inducible NOS inhibitor, 5 mg/kg ip), 7-NI (selective neuronal NOS inhibitor, 20 mg/kg ip), and NIM (selective COX-2 inhibitor, 10 mg/kg ip), respectively. The drugs were purchased from Sigma (St. Louis, MO), and the doses were selected based on the previous in vivo studies, which confirmed that these selected doses resulted in effective inhibition of NO and prostaglandins biosynthesis (9, 31, 32, 46).

Analysis of Colon Transit

Thirty minutes after the second injection, the measurement of colonic transit was performed by using a technique as previously described (20). Briefly, 1.5 ml of saline containing 0.75 mg nonabsorbable phenol red was injected via the colonic catheter, and the catheter was flushed with saline (0.5 ml). Ninety minutes later, the rats were killed under anesthesia and the entire colon was immediately removed and divided into six segments of equal length. The contents released from the anus were collected and referred to as segment 7 for the measurement of possible phenol red. The content of each segment was placed in 100 ml of 0.1 N NaOH and homogenized. The homogenate was allowed to keep for 1 h at room temperature. Five milliliters of the supernatant was added to 0.5 ml of 20% trichloroacetic acid solution to precipitate the protein. After centrifugation at 10,000 g for 30 min, 4 ml of 0.5 N NaOH was added to the supernatant. Phenol red was determined by measuring the absorption at a 560 nm using a spectrophotometer (Beckman Instruments, Palo Alto, CA). Colonic transit was calculated as the geometric center (GC) of distribution of phenol red described as follows: Geometric center = Σ (counts of phenol red per segment) / A (segment number).

Western Blot Analysis

To investigate the potential changes that might occur in the protein level of the NOS and COX-2, Western blot analysis was performed using a method previously described (38). In short, the proximal and the distal colon tissues were cut into small pieces and homogenized in lysis buffer [50 mM Tris HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaVO₄, 1 mM NaF, and 1 µg aprotinin/leupeptin/pepsatin]. Equal amounts of protein (50 µg) were separated by SDS-PAGE and transferred to PVDF membrane (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline solution containing Tween 20 (TBST) [20 mM Tris-HCl, 120 mM NaCl (pH 7.6), 0.05% Tween 20], and then incubated at 4°C overnight with the anti-iNOS antibody (1:2,500, BD Transduction Lab., San Jose, CA), anti-nNOS antibody (1:2,500, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-COX-2 antibody (1:500, Santa Cruz Biotechnology). After being washed three times with TBST solution, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at room temperature. After incubation, the membrane was washed extensively with TBST solution. The immune complexes were visualized using the ECL system (Amersham Pharmacia Biotech, Arlington, IL), and the bands were measured by a Fluorchem imaging system (Alpha Innotech, San Leando, CA) for quantitative densitometric analysis.

Immunohistochemistry Staining

To determine where NOS and COX-2 protein was expressed in situ, immunohistochemistry staining was used. Briefly, the tissues collected from the proximal and distal colon were fixed in 10% formalin and embedded in paraffin. Sections (4 µm thick) were deparaffinized and rehydrated in xylene and degraded concentration of ethanol series. After blocking of endogenous peroxidase activity by incubating the sections in 0.3% hydrogen peroxide in distilled H₂O for 30 min at room temperature, sections were treated for antigen retrieval by placing in 10% target retrieval solution (Dakocytomation, Carpin teria, CA) at 95°C for 30 min. Nonspecific binding was blocked with 5% normal goat serum in PBS for 20 min at room temperature. Sections were then incubated with the primary antibody (anti-iNOS antibody, 1:200; anti-nNOS antibody, 1:300; and anti-COX-2 antibody, 1:200) for 1 h at room temperature. A biotin-conjugated antibody (Vector Laboratories, Burlingame, CA) was used as a secondary antibody. After being incubated with avidin-biotin complex (Vector kit, Vector Laboratories) for 30 min, the sections were stained in diaminobenzidine tetrahydrochloride with 0.03% hydrogen peroxide. As a negative control, sections of the same specimens were processed by the same method but excluding the primary antibody. The number of NOS-immunopositive cells in 20 ganglia was counted in each section, and the average number of nNOS-immunopositive cells per ganglion was determined in each section as described by Mizuta et al. (27). Special care was taken to ensure that each neuron was counted only once.

Real-Time Quantitative RT-PCR

To further investigate whether the changes in NOS mRNA and COX-2 mRNA were responsible for the changes in NOS and COX-2 protein, real-time quantitative RT-PCR was used. Briefly, total RNA was isolated from the proximal and distal colonic tissues using RNAqueous (Ambion, Austin, TX). The amount of RNA was estimated by measuring the absorbance at 260 nm. The Applied Biosystems (Foster City, CA) assays-by-design or assays-on-demand 20× assay mix of primers and TaqMan MGB probes (FAM dye-labeled) were used for all of the target genes and predeveloped 18S rRNA (VIC-dye-labeled probe). TaqMan assay reagent (P/N 4319413E) were used for endogenous control. These assays are designed to span exon-exon junctions so as not to detect genomic DNA, and all these primers and probe sequences were searched against the Celera database to confirm specificity. The primer and probe sequences used were as follows: COX-2: probe: 5'-CCAGCAACACCCCG; primer: sense 5'-GAGTCACTACACAGACAGATTGCT, antisense 5'-TACAAG GTTGGAACATTCCTT, nNOS: probe: 5'-AGGAGAATCTCG ACTGTCGGCCATCACG, GenBank accession number: NM-052799, iNOS: probe: 5'-TATTTTCCACGGCCAACACACAGGAT, GenBank accession number: NM-012611. (The sequence information of nNOS and iNOS primers are considered proprietary by Applied Biosystems and are not available.) Separate tubes (singleplex) one-step RT-PCR was performed with 80 ng RNA for both target genes and endogenous control. The reagent we used was TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR was reverse transcription 48°C for 30 min, AmplifiTag activation 95°C for 10 min, denaturation 95°C for 15 s, and annealing/
extension 60°C for 1 min (repeated 40 times) on ABI7000. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T (ΔΔC_T) method as described by the manufacturer (Applied Biosystems). The amount of target (2^(-ΔΔC_T)) was obtained by normalizing it to an endogenous reference (18S rRNA) and relative to a calibrator.

Statistical Analysis

Data were expressed as means ± SE. Statistical analysis was performed using the Student t-test or ANOVA. A value of P < 0.05 was considered statistically significant.

RESULTS

Effects of Burn Injury on Colonic Transit

Colonic transit was significantly delayed with burn injury (Figs. 1–3). The GC was 3.47 ± 0.28 in the sham-operated control rats with saline (n = 11) and reduced to 2.21 ± 0.18 in the burned rats with saline (n = 12) (P < 0.009), a reduction of ~36%.

Involvement of NO with burn-induced delayed colonic transit. Selective blockade of iNOS with SMT significantly improved delayed colonic transit in the burned rats (Fig. 1). The GC was 2.21 ± 0.18 in burned rats with saline and increased to 3.19 ± 0.24 in burned rats with SMT (n = 10, P < 0.002). However, SMT had no effect in the sham-operated control rats with a GC of 3.36 ± 0.15 (n = 10) compared with sham-operated control rats with saline. Administration of 7-NI failed to modify delayed transit in the burned rats (Fig. 2). The GC was 2.4 ± 0.19 (n = 11) in the burned rats with 7-NI, which was similar to that in the burned rats with saline (2.21 ± 0.18). In the sham-operated control rats, however, the administration of 7-NI significantly delayed colonic transit: the GC was reduced to 2.68 ± 0.21 (n = 10) (P < 0.04) compared with the sham-operated control rats with saline.

Involvement of COX-2 with the burn-induced delayed colonic transit. As shown in Fig. 3, selective blockade of COX-2 with NIM significantly increased colonic transit in the burned rats. The GC was 2.21 ± 0.18 in the burned rats with saline and increased to 3.08 ± 0.16 in the burned rats with NIM (n = 11) (P < 0.001). However, the colonic transit in the sham-operated control rats was not altered with NIM (3.47 ± 0.28 with saline vs. 3.39 ± 0.14 with NIM, n = 10).

Effects of Burn Injury on NOS and COX-2 Protein Expression in the Colon

Western blot analysis. The densitometry evaluation showed that the burn injury significantly increased the expression of iNOS and COX-2 protein in both proximal and distal colon (Figs. 4 and 5). However, it had no effect on the expression of the nNOS protein, in either the proximal or the distal colon, although we observed a tendency of decreased nNOS expression in the proximal colon from burned rats (P = 0.09 vs. sham-operated control rats, Fig. 6). There was also no difference in the expression of iNOS, nNOS, or COX-2 between the proximal colon and the distal colon in either burned or sham-operated control rats (Figs. 4, 5, 6).

Immunohistochemistry staining. In the burned rats, iNOS staining was noted and localized in the surface and in the upper half of colonic epithelial cells in both proximal and distal colon (Fig. 7A). The epithelial iNOS staining, in most instances, seemed more intense on the upper half of the cells than the surface of cells and abruptly diminished and disappeared in the...
bottom of epithelia. The expression of iNOS was cytoplasmic. In the sham-operated control rats, however, no staining of iNOS was observed in either the proximal or the distal colon (Fig. 9A). Interestingly and importantly, it was also noted that neurons in myenteric ganglia in both proximal and distal colon of the burned rats were also noticeably and consistently stained for iNOS (Fig. 7A). On the other hand, the intense staining of COX-2 was also observed in the burned rats. The staining of COX-2 was localized to epithelial cells on the surface and the upper half of crypts (Fig. 8B). There was also expression of COX-2 in smooth muscle cells of muscle layers and in scattered lamina propria mononuclear cells in burned rats, whereas, in the sham-operated rats, there was no staining for COX-2 in either the proximal or the distal colon (Fig. 8A). The immunolocalization of COX-2 was also cytoplasmic. In addition, nNOS-immunoreactive neuronal cells and nerve fibers were found throughout the myenteric plexus of the colon in both burned (Fig. 7B) and the sham-operated control rats (Fig. 9B). There was no difference in the average number of nNOS-immunoreactive cells between the proximal and the distal colon or between the sham-operated control rats and burned rats (data not shown). Nonneuronal tissues (e.g., muscle cells, endothelial cells, epithelial cells, and mononuclear cells) were unstained.

Effects of Burn Injury on NOS and COX-2 Gene Expression in the Colon

A significant increase in the amount of iNOS and COX-2 mRNA was observed in both the proximal and distal colon in

Fig. 4. Western blot analysis of iNOS protein expression. A: Western blot results display protein of iNOS in the tissues obtained from the proximal colon and distal colon of both burned rats and sham-operated rats. The density of the iNOS-immunoreactive band at 130 KDa was significantly increased in both proximal and distal colon obtained from burned rats compared with those from sham-operated rats. B: densitometric analysis of iNOS expression. The data were expressed as means ± SE (each group, n = 10). *P < 0.002 vs. sham-operated rats.

Fig. 5. Western blot analysis of cyclooxygenase-2 (COX-2) protein expression. A: Western blot results display protein of COX-2 in the tissues obtained from the proximal colon and distal colon of both burned rats and sham-operated rats. The density of the COX-2-immunoreactive band at 72 KDa was significantly increased in both proximal and distal colon obtained from burned rats compared with those from sham-operated rats. B: densitometric analysis of COX-2 expression. The data were expressed as means ± SE (each group, n = 10). *P < 0.009 vs. sham-operated rats.

Fig. 6. Western blot analysis of nNOS protein expression. A: Western blot results display protein of nNOS in the tissues obtained from the proximal colon and distal colon of both burned rats and sham-operated rats. There was no difference in the density of the nNOS-immunoreactive band at 155 KDa between burned rats and sham-operated rats. B: densitometric analysis of nNOS expression. The data were expressed as means ± SE (each group, n = 10).
the burned rats compared with the sham-operated rats (Table 1). The amount of nNOS mRNA was, however, not altered with the burn injury in either the proximal colon or distal colon but was significantly more abundant in the proximal colon than the distal colon, which was not noted with the iNOS mRNA or COX-2 mRNA (Table 1).

DISCUSSION

The present study showed that 1) NO produced by nNOS played an important role in mediating colonic transit under physiological conditions; NO produced by iNOS and prostaglandins synthesized by COX-2 were both involved in the pathogenesis of delayed colonic transit induced by burn injury, suggesting that nNOS and iNOS played different roles in mediating colonic transit under different physiological and pathophysiological states. To the best of our knowledge, this is the first report demonstrating that upregulation of iNOS and COX-2 mediate burn injury-induced delay in colonic transit in rats. 2) Previous reports had showed that iNOS was expressed in epithelial cells and macrophage; our results demonstrated that iNOS was also expressed in neurons in myenteric ganglia of colon in burned rats, which may contribute to the dysmotility seen in the burned condition.

Colonic transit is a complex function regulated by colonic peristaltic reflex consisting of ascending contraction and descending relaxation (40). Ascending contraction causes the bolus to propagate through the long segment of the colon. Descending relaxation allows rapid propulsion of a large bolus by ascending giant motor contraction and prevents the development of tone in the distal segment so that it can accommodate the colonic contents (27). Hence, descending relaxation plays a key role in the coordination of propulsive motility in the colon (16, 27, 40). NANC inhibitory neurons are known to mediate descending relaxation (15). Recently, Mizuta et al. (28) found that colonic transit was significantly delayed by administration of nitro-L-arginine methyl ester (L-NAME) in conscious rats, thereby suggesting that NO played an important role in promoting colonic transit and confirmed that this promoting effect was modulated by NO through mediating descending relaxation. In our study, selective blockade of nNOS isoform by 7-NI significantly delayed the colonic transit in the sham-operated control rats, whereas selective blockade of iNOS isoform with SMT failed to exert the same effect, suggesting that it was nNOS that modulated colonic transit. This finding is new and extends the observation made by Mizuta et al. (28).
For a long period of time, studying the effects of burn injury on colonic transit has been neglected. Although extensive research has focused on the effects of burn injury on gastric and small-bowel motility (2, 32), no studies have been done on colonic transit after burn injury, except for one report published by Chen et al. (8) in 1982. However, although Chen et al. (8) demonstrated that intestinal and colonic motility in the rats were decreased after burn injury; however, the precise mechanisms responsible for the delay in colonic transit that occur during burn injury is not known. The present study shows that colonic transit is delayed significantly by burn injury, as reported by Chen et al. (8). What is more, we also find that this delay in transit is significantly reversed by the blockade of the iNOS isoform with SMT but not the blockage of nNOS with 7-IN. These results suggest that NO produced by iNOS plays a key role in mediating colonic transit after burn injury. In fact, nNOS is a well-known potent inhibitory neurotransmitter in the gastrointestinal tract, but only recently has iNOS been suggested to play a role in gastrointestinal dysmotility during endotoxemia and ischemia/reperfusion (17, 55). Nonetheless, the specific pathophysiological effect of iNOS on colonic transit after burn injury has not yet been elucidated. Here, our findings clearly demonstrated the role of iNOS in the pathogenesis of burn-induced delay in colonic transit. These results, together with those discussed in the previous paragraph, suggest that the role of NO in colonic transit may depend upon the condition, whether physiological or pathophysiological. Under the physiological conditions, NO produced by nNOS seems to play a major role in mediating the physiological colonic transit. However, under pathophysiological states, NO synthesized by iNOS may play a pivotal role in modulating delayed colonic transit.

In further studies, we assessed the cellular and molecular mechanisms of action involving NO. Using Western blot analysis, we found a significant increase in iNOS but not nNOS protein in the burned rats. These findings were consistent with the immunohistochemical staining in which iNOS, but not nNOS, was expressed intensely in the burned rats. The upregulated iNOS protein observed by Western blot analysis and immunohistochemical staining in the burned rats might result from the increased gene expression of iNOS, which was supported by the data obtained from the real-time RT-PCR. The iNOS mRNA expression was found to be significantly increased in the burned rats, indicating that the upregulated iNOS observed in burned rats was secondary to the increased gene transcription of iNOS. However, the nNOS mRNA expression was not altered with the burn injury.

Surprisingly, the iNOS protein expression was noted in neurons in myenteric ganglia in both proximal and distal colon in the burned rats. This was an important finding, indicating that NO from iNOS was locally synthesized in neurons of the myenteric plex us and might contribute to neuronal damage, which led to impaired colonic motility and to delayed colonic transit. This result provided us with histomorphological evidence that in addition to nNOS, iNOS also acts as inhibitory neurotransmitter and is involved in delayed colonic transit in rats. More importantly, this finding suggests that there may be an important and complex interaction between iNOS isoform and nNOS isoform. Actually, recent studies have suggested that there may be an inhibitory feedback mechanism modulating NOS isoforms’ own synthesis (3). Accumulating evidence has shown that iNOS expression can downregulate nNOS activity and expression (1, 3). Whether the upregulated iNOS observed in present study resulted in an inhibition of nNOS needs further investigation. However, there was indeed a tendency of a decrease for nNOS protein in proximal colon from the increased gene expression of iNOS, which was supported by the data obtained from the real-time RT-PCR. The iNOS mRNA expression was found to be significantly increased in the burned rats, indicating that the upregulated iNOS observed in burned rats was secondary to the increased gene transcription of iNOS. However, the nNOS mRNA expression was not altered with the burn injury.

Table 1. nNOS, iNOS, and COX-2 gene expression

<table>
<thead>
<tr>
<th>Group</th>
<th>nNOS mRNA</th>
<th>nNOS mRNA</th>
<th>iNOS mRNA</th>
<th>iNOS mRNA</th>
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<tbody>
<tr>
<td>Proximal col</td>
<td>10 1.03±0.22†</td>
<td>9 15.72±5.40</td>
<td>9 1.71±0.26</td>
<td></td>
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<tr>
<td>Distal colon</td>
<td>9 0.63±0.14†</td>
<td>10 100.75±37.45*</td>
<td>10 3.33±0.46*</td>
<td></td>
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<tr>
<td>Distal colon</td>
<td>10 0.45±0.12</td>
<td>9 7.15±2.08</td>
<td>9 1.63±0.36</td>
<td></td>
</tr>
<tr>
<td>Distal colon</td>
<td>10 0.23±0.07</td>
<td>10 111.28±43.07*</td>
<td>10 3.65±0.76*</td>
<td></td>
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</table>

Results are expressed as means ± SE; n, number of rats. Relative amounts of nNOS, iNOS, or COX-2 mRNA were obtained from internal standard (2^-ΔΔCT) and normalized to 18S rRNA. *P < 0.04 vs. sham groups †P < 0.04 vs. distal colon groups.
used as a marker of neurons in the gut. Therefore, nNOS might be stained by nNOS antibody; nonneuronal tissues (e.g., muscle cells, endothelial cells, epithelial cells and mononuclear cells) could not be stained (49). Consequently, we could infer that iNOS immunopositive staining reflects neurons in light of staining of nNOS from these sequence sections.

Numerous studies have shown that iNOS could be expressed in neurons from both central nervous system and peripheral nervous system (18, 19, 26, 47, 52, 53). However, the mechanism of iNOS expression in neurons remains unknown. Early studies have shown that the expression of iNOS was modulated by inflammatory cytokines (53) and relied on an NF-kB dependent mechanism (47). Valentine et al. (52) reported that IL-1β and TNF-α could stimulate the expression of iNOS on cultured myenteric neurons in vitro. Miampamba and Sharkey (26) also observed iNOS expression in myenteric neurons in rats with colitis. In an animal model, Heneka et al. (19) found that neuronal iNOS expression could be induced by using direct injection of proinflammatory cytokines. These results suggest that cytokines are involved in the expression of iNOS in neurons. Although the expression of cytokines was not assessed in our study, previous studies have shown that the levels of various cytokines were raised in rats and humans after burn injury (41). The expression of iNOS in neurons therefore might be caused by a cytokine-mediated process. Another likely explanation is that the hypoxia stress produced in burn injury might itself trigger the expression of iNOS by activating a hypoxia-responsive enhancer gene in promoter region of iNOS (25).

Results from our study differ from the previous study by Mizuta et al. (28), in which the blockage of NO was found to delay colonic transit; our results showed an increase of iNOS, but a simultaneous delayed colonic transit, in the burned rats. We postulate that this discrepancy is attributed to the amount or level of NO, that is, NO is excitatory on colonic transit at a level below a certain threshold but inhibitory at a level above the threshold. Under normal physiological conditions, there is a low level of NO synthesized by nNOS, which enhances colonic transit by enhancing descending relaxation (28, 41). At an excessive level, NO is expected to delay colonic transit. This is because the high level of NO not only enhances descending relaxation but also inhibits ascending contractions, resulting in impaired coordination of the peristaltic reflex. Although no comparison could be made between the level of NO induced by iNOS in this study and the hypothetical threshold, a growing evidence has indicated that once iNOS is functionally active, it synthesizes a large amount of NO, particularly during endotoxemia or burn injury (33, 35, 41).

Interestingly, although the level of nNOS mRNA expression was higher in the proximal colon than the distal colon in our present study, no significant differences in nNOS protein expression and the number of nNOS-immunoreactive cells between proximal and distal colon obtained from both burned rats and sham-operated rats were observed. The results were in disagreement with those of Takahashi and Owyang (49) who observed the average number of nNOS-immunoreactive cells, and the expression of nNOS protein was significantly higher in the tissue from the proximal colon than that from the distal colon. This difference between our data and studies by Takahashi and Owyang (49) cannot be completely explained, but it could be caused by a time lag between nNOS gene expression and synthesis of nNOS protein, which might have been present in the proximal colon during our study. In fact, this time delay between gene expression and protein synthesis has been widely reported (7, 34).

The results of this study also showed that the selective inhibition of COX-2 ameliorated the delay in colonic transit after burn injury, suggesting that in addition to NOS, prostaglandins are also involved. Increasing evidence has demonstrated that endogenous prostaglandins are involved in the gastrointestinal dysmotility (21, 39). A number of studies have shown that nonselective COX-2 inhibitors increase small-bowel transit in a rodent model of postoperative ileus and improve gastric emptying in rats (21, 39). A similar role of endogenous prostaglandins in the inhibitory effects of burn injury on colonic transit was documented in our present study. It was also interesting to note that the epithelial cells expressed both iNOS and COX-2 and that the expression of iNOS and COX-2 localized to the epithelial cells had a similar pattern of distribution. These data suggest an important link between the NOS and COX pathways. In fact, cross-talk interactions between the NOS and COX system have been reported widely (37, 54). The COX and NOS systems have been shown to be present together and share some important features (37, 54). COX-2 can be activated by NO produced from iNOS. In contrast, the prostanooids from COX-2 may also enhance the expression of iNOS (37). Selective inhibition of iNOS by L-NIL inhibits not only NO but also prostanoid production (36). Previous studies have suggested that when iNOS and COX-2 are produced concurrently, a positive feedback loop exists by which they potentiate each other’s production (37, 54). The concurrent increases in iNOS and COX-2 in this study appeared to support the concept. However, further studies are required to better define such an interaction.

Although the method used in our study for colonic transit might have some limitations for the measurement of solid-content transit, we thought it was workable and could reflect well the propulsive colonic motor function. Actually, numerous studies have demonstrated that it is possible to use nonabsorbable dyes or radioactive materials, which are dissolved or mixed in liquid or semiliquid, as markers for testing colonic transit (23, 28, 29, 50, 51). A preponderance of evidence from several groups of investigators has shown that colonic transit could be measured by using Evans’ blue suspended in carboxymethylcellulose (50), technetium-99m mixed in methylcellulose (51), or Na51CrO4 (radiochromium) dissolved in saline (28). In addition, a semiliquid nonabsorbable marker for measuring colonic transit was reported by Monnikes et al. (29), and Lin et al. (23) injected 3 ml of technetium-99m directly through the cecal catheter. Moreover, the representation of transit using the GC has been commonly used, which provides an accurate quantitative measure for transit (28, 29).
The isoform selectivity of 7-NI or of SMT has been debated. However, we chose 7-NI and SMT on the basis of previous studies in which these compounds had been clearly demonstrated to be selective nNOS or iNOS inhibitors (4, 31, 44, 46). Szabo et al. (46) have reported that SMT is a more potent inhibitor of iNOS than any other known inhibitor of NOS. Similarly, Moore and colleagues (30, 31) have demonstrated that 7-NI is a useful experimental tool with which to study the roles of nNOS in vivo. Actually, none of the NOS inhibitors reported to date is adequately specific to achieve the pharmacological ideal of specific isoform inhibition; that is, none of these compounds can be considered absolutely selective for a given isoform. They were found only to have a relative selectivity or preference for one NOS isoform over another (12). In addition, for NOS inhibitors, there is a big difference in terms of potency and isoform selectivity both in vitro and in vivo. Early studies have demonstrated that although 7-NI inhibits both nNOS and eNOS in vitro, it does not inhibit endothelium-dependent relaxation of blood vessels, nor does it influence the blood pressure in vivo; thus 7-NI is not considered effective on eNOS in vivo (4, 31). Another study from Szabo and colleagues (46) has also showed SMT only slightly affects eNOS in vivo. Therefore, like many investigators, we used 7-NI as a nNOS inhibitor and SMT as an iNOS inhibitor.

In conclusion, NO produced by neuronal NOS plays an important role in mediating colonic transit under physiological conditions. Burn injury delays colonic transit in rats. NO produced by inducible NOS and prostaglandins synthesized by COX-2 are both involved in the pathogenesis of delayed colonic transit induced with burn injury.

ACKNOWLEDGMENTS

The authors thank Huiping Guo of the real-time PCR core facility of the University of Texas Medical Branch at Galveston for providing outstanding technical support.

REFERENCES


