Paradoxical regulation of ChAT and nNOS expression in animal models of Crohn’s colitis and ulcerative colitis

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Winston JH, Li Q, Sarna SK. Paradoxical regulation of ChAT and nNOS expression in animal models of Crohn’s colitis and ulcerative colitis. Am J Physiol Gastrointest Liver Physiol 305: G295–G302, 2013.—Paradoxical regulation of ChAT and nNOS expression in animal models of Crohn’s colitis and ulcerative colitis was studied.

Paradoxical regulation of ChAT and nNOS expression in animal models of Crohn’s colitis and ulcerative colitis was studied. Treatment with dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) induced colitis significantly decreased ChAT and nNOS expression in the colonic myenteric plexus neurons. In contrast, treatment with TNBS induced colitis significantly decreased ChAT expression and increased nNOS expression, whereas treatment with DSS induced colitis significantly decreased nNOS expression and increased ChAT expression. These findings suggest that inflammation-induced changes in the expression of ChAT and nNOS are dependent on the type and severity of the inflammatory response.

The data support the hypothesis that the expression of ChAT and nNOS is regulated by the inflammation type and severity, which could be of significance in the development of new therapeutic strategies for the treatment of inflammatory bowel disease.

Additional key words: enteric nervous system—neurotransmitter synthesis—mucosal immunity—animal models
METHODS

Animal models. Male Sprague Dawley rats, 6–10 wk old, were used in all experiments. The institutional animal care and use committee at the University of Texas Medical Branch at Galveston approved all procedures performed on animals. Rats received a single colonic infusion of either 50 or 80 mg/kg TNBS in 0.25 ml 40% ethanol through a catheter positioned 7 cm from the anus or they received 5% DSS in drinking water for 7 days. Experiments were performed on day 7 posttreatment.

Protein and mRNA measurements. Muscularis externae containing the circular and longitudinal muscle layers and myenteric plexus were dissected from distal colon by removing mucosal and submucosal layers. RNA was prepared using the Qiagen micro RNA kit (Qiagen, Valencia, CA), and reverse transcription was performed using the Superscript III kit from Invitrogen (Grand Island, NY). Real-time PCR was performed on an Applied Biosystems Step-One Plus using 18S as a normalizer (Applied Biosystems, Carlsbad, CA). Primers/probe sets for ChAT, nNOS, protein gene product (PGP) 9.5, and 18S were purchased from Applied Biosystems. Western blots were performed as described previously (45), and results were visualized and analyzed with the Li-cor Odyssey system (Li-Cor, Lincoln, Nebraska). Antibodies used were goat anti-ChAT 1:200 (Millipore, Bedford, MA), rabbit anti-nNOS 1:200 (Millipore), rabbit anti-PGP9.5 1:1,000 (AbD Serotec, Raleigh, NC), and mouse anti-β-actin 1:5,000 (Sigma, St. Louis, MO).

In vitro experiments. Distal colon muscularis externae strips isolated from naïve rats were incubated for various times in DMEM containing 10% fetal bovine serum, 2× antibiotic/antimycotic, gentamycin, and amphoterin (Invitrogen, Grand Island, NY). H2O2 was diluted from a 30% stock. IL-1β, TNF-α, and MG-132 were used at concentrations indicated in Figs. 1–7.

Immunofluorescence. Longitudinal muscle myenteric plexus preparations (LMMP) were fixed for 2 h in 4% paraformaldehyde in 1× PBS at room temperature. After extensive washing in PBS, LMMP were treated for 5 min with pronase and then blocked for 2 h in PBS containing 5% normal donkey serum and 0.3% Triton X-100. LMMP were incubated for 72 h at 4°C in blocking solution containing primary antibodies, ChAT 1:200 (Millipore) rabbit anti-nNOS 1:200 (Millipore), and mouse anti-HU 1:100 (Invitrogen). Appropriate Alexa Fluor 350, 488, and 594 antibodies from Invitrogen were used at 1:250 dilution. Fluorescent images were acquired on a Nikon epi800 microscope using Metaview software (Molecular Devices, Sunnyvale, CA).

Statistics. Data were expressed as means ± SE. Means were compared using either t-test or ANOVA where appropriate. Post hoc analysis was performed using the Tukey test.

RESULTS

Differential effects of TNBS and DSS inflammation on ChAT and nNOS expression. To determine whether TNBS- and DSS-induced inflammation differentially affects ChAT and nNOS expression, we measured ChAT and nNOS protein and mRNA in distal colon muscularis externae from TNBS-, DSS-, and saline-treated control rats 7 days after initiation of inflammation. PGP9.5 has been proposed as a valid marker that relates to the total number of neuronal cell bodies (16). Therefore, we also tested the expression levels of PGP9.5 in these tissues. We normalized expression of the above enteric neuronal proteins by using β-actin, a housekeeping protein.

DSS-induced colitis significantly increased ChAT (170 ± 30%, P < 0.05) and PGP9.5 (170 ± 6%, P < 0.05) protein expression (Fig. 1A); DSS inflammation also increased ChAT mRNA without affecting the mRNA of PGP9.5 (Fig. 1B). By contrast, this type of inflammation did not alter the protein expression of the neuronal marker protein gene product (PGP) 9.5.
expression of nNOS (Fig. 1A); however, it significantly decreased the nNOS dimer-to-monomer ratio (Fig. 1C).

Immunofluorescence staining of whole mount LMMP preparations from DSS-treated and control rats showed no difference in the percentage of ChAT (31 vs. 33%)- or nNOS (37 vs. 41%)- immunopositive neurons per ganglia or the mean number of neurons per ganglia (55 ± 5 vs. 59 ± 5) (Fig. 2). These findings supported a direct effect of DSS inflammation on protein expression rather than an increase in neuron numbers. These findings together with the quantitative RT-PCR data suggested that ChAT upregulation in DSS colitis was a transcriptional event. However, increase of PGP9.5 protein without an accompanied increase in its mRNA suggested a posttranscriptional effect.

By contrast, inflammation induced by 80 mg/kg TNBS suppressed ChAT (35 ± 5%, \( P < 0.05 \)), nNOS (42 ± 8%, \( P < 0.05 \)), and PGP9.5 (44 ± 6%, \( P < 0.05 \)) protein expression compared with naïve rats (Fig. 3A). Similar results were obtained in rats treated with 50 mg/kg TNBS (Fig. 3A). The nNOS dimer-to-monomer ratio did not change significantly in TNBS-treated rats (data not shown). In TNBS colitis, we

![Fig. 2. ChAT (blue), nNOS (red), and the neuronal marker Hu C/D (green) immunofluorescence on whole mount longitudinal muscle myenteric plexus preparations from control and DSS distal colon. No significant differences were observed in the mean number of ChAT- or nNOS-immunoreactive neurons per ganglia.](image1)

![Fig. 3. Downregulation of ChAT, nNOS, and PGP9.5 in trinitrobenzene sulfonic acid (TNBS)-induced colitis. A: Western blots showing that TNBS treatment at 50 (T50) or 80 (T80) mg/kg suppressed the expression of ChAT, nNOS, and the neuronal marker PGP9.5 in the distal colon muscularis externa of rats (\( *P < 0.05 \)); normalized to \( \beta \)-actin (\( *P < 0.05 \)) and compared with naïve rats (\( n = 4 \)). B: bar graph displaying the results of quantitative RT-PCR assays measuring relative mRNA levels of ChAT and PGP9.5, normalized to 18S RNA, in muscularis externa of rats treated with either 50 or 80 mg/kg TNBS and controls.](image2)
observed significant decline in ChAT (56 ± 7%, \( P < 0.05 \)) and PGP9.5 mRNA (55 ± 3% relative to 18S expression, \( P < 0.05 \); Fig. 3B). There are reports of declines in neuron numbers in TNBS colitis (18, 33, 34). Similar results were obtained with another neuronal marker, β-III-tubulin (data not shown). The decline in PGP9.5 protein expression observed in the present study may be indicative of this neuronal loss.

**H₂O₂ increases the expression of ChAT and nNOS.** To explain these observations, we tested the hypothesis that H₂O₂, a component of oxidative stress, and Th1-type proinflammatory mediators differentially alter the expression of ChAT and nNOS in vitro. First, we examined the effects of treatment of distal colon muscularis externae strips from naïve rats in vitro for 24 h with increasing concentrations of H₂O₂. ChAT and nNOS mRNA were incubated in vitro for 1, 3, or 6 h with 50 μM H₂O₂. Both ChAT and nNOS mRNA levels were significantly increased, \( n = 4, * P < 0.05 \).

ChAT and nNOS proteins decreased concentration-dependently on incubation of distal colon muscularis externae strips from naïve rats at 1, 3, and 6 h (Fig. 4B). These findings supported a direct transcriptional effect for H₂O₂.

**Role of NF-κB.** Because the transcription factor NF-κB is activated by both cytokines and H₂O₂, we examined its role in the regulation of ChAT and nNOS expression. Treatment of 24 h (data not shown). H₂O₂ treatment significantly increased both ChAT and nNOS mRNA at 1, 3, and 6 h (Fig. 4B). These findings supported a direct transcriptional effect for H₂O₂.

ChAT and nNOS proteins decreased concentration-dependently on incubation of distal colon muscularis externae strips from naïve rats for 24 h with IL-1β, but no significant effects were observed on PGP9.5 expression (Fig. 5B) (\( P < 0.05 \)). Incubation with IL-1β for 3 and 6 h had no effect on any of the above proteins. Incubation with 20 ng/kg TNF-α had similar effects (data not shown). Incubation of muscularis externae strips with H₂O₂ (50 μM) and IL-1β (20 ng/ml) together for 24 h significantly decreased ChAT and nNOS protein, without a significant effect on PGP9.5 (Fig. 5B). Together, these findings suggested that in vitro these inflammatory cytokines produced a delayed decrease in ChAT and nNOS expression without altering PGP9.5.
no significant effect on ChAT or nNOS expression (Fig. 6).

Distal colon muscle strips from naïve rats were incubated for 24 h with either IL-1β (20 ng/ml) or H2O2 (50 μM) in the presence of the NF-κB inhibitor MG-132. MG-132 failed to prevent changes in ChAT or nNOS expression in response to incubation with IL-1β or H2O2 (*P < 0.05 vs. control).

muscle strips with the NF-κB inhibitor MG-132 was without effect on IL-1β-induced decrease or the H2O2-induced increase in ChAT and nNOS expression (Fig. 6). MG-132, by itself, had no significant effect on ChAT or nNOS expression (Fig. 6).

**DISCUSSION**

In the hierarchy of the enteric nervous system, the intrinsic sensory neurons with nerve endings in mucosa code the mechanical/chemical stimuli from the digesta into impulses that are transmitted directly or via interneurons to the excitatory and inhibitory motor neurons in the myenteric ganglia (Fig. 7). Excitation of the excitatory and inhibitory motor neurons, respectively, releases ACh and NO in the microenvironment of smooth muscle cells. The coded signal arriving at the excitatory and inhibitory motor neuron stimulates action potentials that, respectively, release ACh and NO to activate excitation-contraction and excitation-inhibition couplings, respectively, in smooth muscle cells (35, 36, 38, 41). The amplitude, duration, and type of resulting contraction (RPCs, tone, or GMCs) in smooth muscle depend on the relative contributions of the two types of competing couplings.

The profiles of the inflammatory mediators in the muscularis externae differ between TNBS- and DSS-induced inflammation (45). TNBS treatment increases the Th1-type inflammatory mediators as well as H2O2 in the colon muscularis externae, whereas DSS treatment increases primarily H2O2 (45). Our findings show that DSS colitis upregulates the expression of ChAT, indicating an increase in ability to synthesize ACh. The increase of ChAT mRNA indicated that the upregulation of ChAT was a transcriptional event. The total number of ChAT-reactive neurons per ganglia did not increase in DSS colitis. These in vivo effects were selective, since the expression of nNOS remained unaffected. In vitro experiments confirmed that graded concentrations of H2O2, a component of oxidative stress, upregulate ChAT expression. It

![Fig. 6. Effects of NF-κB inhibitor on inflammatory IL-1β- and H2O2-induced alterations in ChAT and nNOS expression. Distal colon muscle strips from naïve rats were incubated for 24 h with either IL-1β (20 ng/ml) or H2O2 (50 μM) in the presence of the NF-κB inhibitor MG-132. MG-132 failed to prevent changes in ChAT or nNOS expression in response to incubation with IL-1β or H2O2 (*P < 0.05 vs. control).](image)

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![Fig. 7. Summary of alterations in enteric neural and smooth muscle regulation of motility function induced by TNBS and DSS inflammation. Mucosal stimulation by digesta stimulates intrinsic sensory neurons that transmit the signal to excitatory and inhibitory motor neurons. The release of ACh and NO induces excitation-contraction and excitation-inhibition couplings, respectively, in smooth muscle cells that compete to determine the amplitude, duration, and type of resulting contraction (RPCs, tone, or GMCs). ISN, intrinsic sensory neuron.](image)
is noteworthy that in vitro a significant upregulation of ChAT occurred in a narrow range of H2O2 concentration. Immunohistochemical staining also found no change in the proportion of ChAT-reactive neurons in the affected regions of the myenteric plexus in ulcerative colitis patients (31). By contrast, DSS colitis upregulated PGP9.5 protein expression in the absence of a concurrent increase in PGP9.5 mRNA, suggesting posttranscriptional regulation of PGP9.5 in DSS colitis. The regulation of PGP9.5 protein in the absence of changes in the number of neurons observed in DSS colitis raises questions regarding previous assertions of this marker to accurately reflect neuron number (16).

The expression of nNOS did not change in DSS inflammation. However, there was a significant decrease in the dimer-to-monomer ratio, consistent with a decline in NO production/function (2, 19, 51). A major difference between the in vivo and in vitro data was that, in vitro, nNOS expression was increased by H2O2, whereas it was not altered in the in vivo DSS colitis. This difference cannot be explained by a differential sensitivity to H2O2 concentration, since the in vitro concentration-response curves are similar for both ChAT and nNOS. A possible explanation could be that the longer duration of exposure in vivo, 7 days in the DSS model, as opposed to 6 to 24 h in vitro may lead to loss of increased nNOS expression. In addition, we cannot rule out the contribution of other inflammatory products of DSS colitis that target the expression of nNOS; we tested only H2O2 in vitro.

By contrast, TNBS inflammation upregulates both H2O2 and recruitment of Th1-type immune cells, resulting in the upregulation of proinflammatory cytokines such as IL-1β and TNF-α (45). In vitro treatment with IL-1β or TNF-α concentration-dependently decreased the expression of ChAT and nNOS. Even though H2O2 by itself increased the expression of these proteins, the net effect of concurrent application of H2O2 and IL-1β was the suppression of both ChAT and nNOS. NF-κB is a prominent regulator of inflammatory response (20). However, we found this transcription factor does not mediate the suppression of ChAT or nNOS, even though it regulates suppression of the pore-forming α1C-subunit of Ca1.2b channels in smooth muscle cells during colonic inflammation (44).

Neuronal cell loss in TNBS colitis (18, 33, 34), which may be reflected by a reduction of PGP9.5 protein in the present study, appears to contribute to the downregulation of ChAT and nNOS; the decrease in ChAT and nNOS mRNA was not different from that of PGP9.5 (all relative to β-actin) in TNBS-treated rats. Other reports found that the loss of neurons was due to apoptosis, it occurred within a few hours of the application of TNBS, and it persisted after recovery from inflammation (5, 21, 33, 34). The loss of neurons is preventable by suppressing inflammation with steroids or anti-neutrophil serum treatment (5, 34). However, the identity of the inflammatory mediator that causes apoptosis early on after induction of inflammation remains unknown. Our findings suggest that H2O2 and proinflammatory cytokines IL-1β and TNF-α may not cause apoptosis at the concentrations used. Loss of neurons did not occur by the elevation of H2O2 in DSS-treated rats, and in vitro incubation of muscularis with IL-1β or TNF-α at concentrations that induced transcriptional downregulation of ChAT and nNOS did not suppress PGP9.5.

Some studies noted architectural abnormalities in the myenteric neurons of patients with inflammatory bowel disease (12, 55). However, the findings of these studies are diffuse, and the studies did not control for the duration and severity of disease or ongoing treatment. None of these studies evaluated the expression of enzymes that regulate the generation of ACh or NO.

The motor abnormalities in inflammatory bowel disease patients are the suppression of RPCs and tone and increase in the frequency of GMCs (1, 3, 6, 7, 17, 24, 46, 56). The suppression of RPCs and tone disrupts the ordered slow propulsion and extensive mixing of the digesta; both changes undermine uniform and extensive exposure of the digesta to mucosa for optimal absorption. On the other hand, the stimulation of GMC frequency increases the frequency of mass movements, resulting in urgency and frequency of defecation (42, 43). Our present and previous findings along with those of others help in putting together a composite picture to show how inflammation might concurrently produce two opposing motor functions, suppression of RPCs and tone on one hand and stimulation of GMCs on the other. ACh is the physiological mediator of RPCs and tone (30, 38, 40, 50), whereas substance P stimulates GMCs in the colon (15, 52). Note that the generation of RPCs and GMCs utilizes different cell-signaling pathways of excitation-contraction coupling (37, 39).

It appears that inflammation of the muscularis externae affects all components of neuromuscular regulation of gut motor function (Fig. 7). However, some of these effects are specific to the types of inflammatory mediators generated by inflammation. Crohn’s colitis-like inflammation induced by TNBS sensitizes the intrinsic sensory neurons and strengthens neurotransmission (22, 26) that implies an amplified input to the motor neurons (Fig. 7A). However, this type of inflammation suppresses the expression of ChAT and nNOS as well as the release of ACh and NO that would reduce the excitatory and inhibitory inputs to smooth muscle cells. At the next level, TNBS inflammation impairs excitation-contraction coupling in smooth muscle cells that reduces smooth muscle reactivity to Ach, resulting in suppression of RPCs and tone (45). While the cholinergic excitatory output is suppressed in the colon of Crohn’s disease patients and its animal models, the substance P (SP) and neurokinin 1 receptors are upregulated, and SP-immunoreactive fibers increase in Crohn’s disease patients (14, 28, 48). As discussed above, SP stimulates GMCs in the colon.

The effects of ulcerative colitis-like inflammation induced by DSS treatment on the intrinsic sensory neuron excitability or neurotransmission remain unknown (Fig. 7B). As discussed above, this type of inflammation upregulates the expression of ChAT that has the potential to increase RPCs. However, at the smooth muscle level, DSS inflammation suppresses the smooth muscle reactivity to Ach; the overall effect of the two opposing alterations is to suppress RPCs in DSS inflammation (Fig. 7B) (13, 45). On the other hand, the proportion of SP-immunoreactive neurons more than doubles in the myenteric plexus of ulcerative colitis patients (31). In addition, H2O2 generated by oxidative stress has been shown to increase the frequency of GMCs (13).

We conclude that different compositions of the inflammatory milieu in ulcerative colitis-like and Crohn’s colitis-like inflammation induced by DSS and TNBS exert differential effects on the gene expression of proteins regulating the synthesis of two prominent neurotransmitters, ACh and NO. In intact animals, inflammation induced by DSS upregulates the expression of ChAT without affecting that of nNOS. On the other hand, TNBS inflammation downregulates the expression of ChAT and nNOS.
REFERENCES


