Homeostatic and therapeutic roles of VIP in smooth muscle function: myo-neuroimmune interactions

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Shi X-Z, Sarna SK. Homeostatic and therapeutic roles of VIP in smooth muscle function: myo-neuroimmune interactions. Am J Physiol Gastrointest Liver Physiol 297: G716–G725, 2009. First published August 6, 2009; doi:10.1152/ajpgi.00194.2009.—We tested the hypothesis that spontaneous release of vasoactive intestinal peptide (VIP) from enteric neurons maintains homeostasis in smooth muscle function in mild inflammatory insults and that infusion of exogenous VIP has therapeutic effects on colonic smooth muscle dysfunction in inflammation. In vitro experiments were performed on human colonic circular smooth muscle tissues and in vivo on rats. The incubation of human colonic circular smooth muscle strips with TNF-α suppressed their contractile response to ACh and the expression of the pore-forming α1C subunit of Cα1.2 channels. VIP reversed both effects by blocking the translocation of NF-κB to the nucleus and its binding to the κB recognition sites on hox1C1b promoter. The translocation of NF-κB was inhibited by blocking the degradation of IkBβ. Induction of inflammation by a subthreshold dose of 17 mg/kg trinitrobenzene sulfonic acid (TNBS) in rats moderately decreased muscularis externa concentration of VIP, and it had little effect on the contractile response of circular smooth muscle strips to ACh. The blockade of VIP and putitary adenylate cyclase-activating peptide receptors 1/2 during mild inflammatory insult significantly worsened the suppression of contractility and the inflammatory response. The induction of more severe inflammation by 68 mg/kg TNBS induced marked suppression of colonic circular muscle contractility and decrease in serum VIP. Exogenous infusion of VIP by an osmotic pump reversed these effects. We conclude that the spontaneous release of VIP from the enteric motor neurons maintains homeostasis in smooth muscle function in mild inflammation. The experiments were performed on circular smooth muscle strips obtained from human and rat colons as well as on primary cultures of human and rat colonic circular smooth muscle cells. We established the translational relevance of our cellular findings by using the trinitrobenzene sulfonic acid (TNBS) model of experimental inflammation in adult Sprague-Dawley rats.

MATERIALS AND METHODS

Primary cultures of human colonic circular smooth muscle cells. Human tissues were obtained from the descending and sigmoid colons of patients undergoing surgery for colon cancer with approval of the University of Texas Medical Branch Institutional Review Board, as described previously (31, 33). Full-thickness tissues were dissected from disease-free margins of resected colon segments. The microdissected circular muscle layer was collected in ice-cold HEPES buffer (in mmol/L: 120 NaCl, 2.6 KH2SO4, 4 KCl, 2 CaCl2, 0.6 MgCl2, 25 HEPES, 14 glucose, and 2.1% essential amino acid mixture, pH 7.4). Two successive digestions with papain and collagenase dispersed smooth muscle cells. The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in the presence of 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 0.25 μg/ml of amphotericin B. The cells in passages 2–5 were used in experiments. All cells were cultured in low serum (1%) for 24 h before experiments to minimize the potential effects of growth factors.

Induction of colonic inflammation by TNBS. Sprague-Dawley rats weighing 180–280 g (Harlan Laboratories, Indianapolis, IN) were used. Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, Texas approved all proce-
dures. Colonic inflammation was induced by intracolonic administration of 17-mg/kg or 68-mg/kg (0.25 ml in 40% ethanol) doses of TNBS (Sigma Chemical, St. Louis, MO) under 2% isoflurane anesthesia, as described elsewhere (6). Twenty-four hours before infusion of TNBS, drinking water was replaced with Colyte, and rats were fasted overnight to cleanse the colon. This step minimized variable absorption of TNBS by different quantities of fecal pellets among rats and allowed consistent exposure of a given dose of TNBS to mucosal surface. The control rats received 0.25 ml of saline. The animals were euthanized 7 days later to obtain tissues from the distal colon.

We used a previously described and modified macroscopic scoring system to assess mucosal damage (3, 22, 34) as follows: 0 = normal mucosa; 1 = localized hyperemia but no erosions, ulcers, or scars; 2 = linear ulcer or scar with inflammation at one site >2 mm but <5 mm; 3 = two or more sites of ulceration and/or inflammation, each up to 5 mm; and 4 = two or more major sites of inflammation and ulcerations >5 mm each or one major site of inflammation extending >1 cm along the length of the mucosa.

**Systemic administration of VIP with osmotic pump.** A 7-day osmotic pump filled with 0.1 μl of saline or human-, rat-, porcine-, and bovine-specific VIP or VIP and pituitary adenylate cyclase-activating peptide receptor (VPAC1,2) antagonist (p-chloro-d-Phe6, Leu17)-VIP, Bachem, King of Prussia, PA) was implanted subcutaneously in the thigh, as previously described (35), 4 h before the induction of inflammation. The infusion rate was 0.5 μl/h.

**Human and rat tissue preparation for muscle bath studies.** Freshly obtained full-thickness human colon tissues were stored on ice for no longer than 1 h in carbenogated Krebs solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaC2, 1 NaH2PO4, 1.2 MgCl2, 1 NaHCO3, 11 d-glucose, and 25 NaHCO3). The mucosal layer, submucosal layer, and taenia coli were removed under magnifying glass. Circular muscle strips (2 mm x 10 mm) were cut along the circumferential axis and placed in DMEM solution at 37°C (6, 28, 31, 34, 35). A similar procedure was used to obtain circular muscle strips from the rat colon (35). The contractile activity of colonic circular muscle strips was recorded with Grass isometric force transducers and amplifiers connected to Biopac data acquisition system (Goleta, CA). The contractile response to ACh was obtained at concentrations of 10−6 M to 10−2 M, after equilibrating the muscle strips under 1 g of tension for 1 h. The bathing solution was replaced every 15 min during equilibration and about 5 min after obtaining data with each new concentration of ACh. After wash, the tension was readjusted to 1 g and the muscle strips left to equilibrate for at least 15 min before adding the next concentration of ACh. At this time, the baseline tension was similar to that before administration of the previous concentration of ACh. The contractile response of circular muscle was quantified as increase in area under contractions for at least 15 min after addition of ACh to the bath over the baseline area under contractions during 4 min before addition of ACh. Western blotting. Whole cell extracts and cytoplasmic and nuclear proteins were processed as described previously (32). The proteins in the samples were resolved by standard immunoblotted method using equal quantities (10 μg or 20 μg) in each lane. β-Actin was used as internal control. The antibody (1:200 to 1:400) for αC subunit of Ca,1,2 channels was purchased from Alomone Laboratories, Jerusa-

**RESULTS**

**VIP reverses TNF-α-induced suppression of αC gene expression in primary cultures of HCCSMCs.** The incubation of human colonic circular smooth muscle strips with 20 ng/ml TNF-α for 24 h significantly suppressed expression of the pore-forming αC subunit of Ca,1,2 channels (Fig. 1A) and the contractile response to ACh (Fig. 1B). The incubation of these strips with 10−7 M VIP and TNF-α together for 24 h almost completely reversed the suppression of αC subunit and suppression of contractile response to ACh (Fig. 1, A and B). Note that incubation with VIP alone enhanced the expression of αC subunit and contractility of smooth muscle strips as reported previously (31). Circular smooth muscle strips incubated with vehicle (sterile PBS) served as controls. The above effects of TNF-α and VIP were mediated at the level of αC expression and transcription. The incubation of primary cultures of HCCSMCs transfected with the hαC promoter luciferase gene construct (33) for 24 h with TNF-α expression (33) for 24 h with TNF-α significantly suppressed the expression of promoter activity by TNF-α. VIP alone enhanced hαC promoter activity. Cell cultures incubated with vehicle served as controls.
The role of transcription factor NF-κB in maintaining homeostasis in smooth muscle function by VIP in response to TNF-α. The human α1C1b promoter (hα1C1b) has two κB recognition sites, GGGGACCCCG at −1,499/−1,490 and GGGGATGC (−1,481/−1,472), that mediate the suppression of α1C gene by TNF-α (33). We found that TNF-α treatment of HCCSMCs for 60 min significantly increases the translocation of p50 and p65 subunits of NF-κB to the nucleus (Fig. 2A) as well as their bindings to both NF-κB recognition sites (RE1 and RE2) (Fig. 2B). However, the incubation of the HCCSMCs with TNF-α in the presence of VIP significantly reduces the nuclear translocation of both NF-κB subunits as well as their binding to the κB recognition sequences.

The above findings suggest that VIP counters the adverse effects of TNF-α on smooth muscle cells by suppressing the translocation of NF-κB to the nucleus. NF-κB is retained in the nucleus by inhibitory proteins of the IκB family (2). The cell-signaling pathways for the activation of NF-κB stimulated by most extracellular stimuli converge on the family of IκB kinases, IKKα, and IKKβ. The activation of these enzymes...
phosphorylates IκBs (predominantly Iκα and Iκβ), which dissociates them from the cytoplasmic NF-κB, resulting in their ubiquitination and proteasomal degradation. This un-masks the nuclear localization sequence of NF-κB, and it translocates to the nucleus. We found that TNF-α induces degradation of both IκBs within a few minutes of its exposure to HCCSMCs (Fig. 2C). Concurrent incubation with VIP has no effect on the degradation of Iκα, but it blocks the degradation of Iκβ (Fig. 2C). TNF-α, by itself, phosphorylates both IKKα and IKKβ (Fig. 2D). However, in the presence of VIP, it is unable to phosphorylate IKKβ (Fig. 2D). These data suggest that VIP inhibits NF-κB activation by TNF-α by blocking the cell signaling pathways leading to the phosphorylation of IKKβ.

Homeostatic role of endogenous VIP in preserving smooth muscle function during mild inflammatory insult. Our experiments indicated that 17 mg/kg TNBS is a subthreshold dose that does not significantly increase MPO in the muscularis externa (Fig. 3A), and it has no significant effect on weight gain over a 7-day postinfammation period, when compared with control rats (Fig. 3B). This dose of TNBS also does not significantly increase the concentrations of cytokines, such as IL-1β, and chemokines, such as RANTES, in the muscularis externa tissues of the distal colon (Fig. 3C and D). In addition, there is little damage to the mucosal surface visualized by hematoxylin and eosin staining (Fig. 3E, left and middle). This dose of TNBS has marginal effect on the reactivity of colonic circular smooth muscle strips to ACh (Figs. 4A and 5). Finally, a 17-mg/kg dose of TNBS has no significant effect on the expression of α1C subunit (Fig. 4B) or the nuclear binding of p65 (Fig. 4C) and p50 (Fig. 4D) subunits of NF-κB.

We tested the hypothesis that spontaneous release of endogenous VIP maintains homeostasis in smooth muscle function and restrains the induction of inflammation by the subthreshold inflammatory insult. We applied the subthreshold insult during blockade of VPAC1/2 receptors by a 7-day systemic infusion of 20 nmol/day (p-chloro-D-Phe6, Leu17)-VIP with a surgically implanted osmotic pump. The infusion started 4 h before the application of subthreshold inflammatory insult. During the blockade of VPAC1/2 receptors, the subthreshold insult significantly increases MPO (Fig.
A), the concentrations of IL-1β (Fig. 3C), and RANTES (Fig. 3D) in the muscularis externa, and it significantly bluntsthe weight gain of rats (Fig. 3B) during the 7-day postinsult period. The visual macroscopic damage score by subthreshold inflammatory insult during the blockade of VPAC1/2 receptors, 2.7 ± 0.2, is significantly greater than that in control rats infused with vehicle and subjected to the same insult, 1.8 ± 0.2. This score is 0 ± 0 in naive control rats. Hematoxylin and eosin staining supports these findings (Fig. 3E).

The blockade of VPAC1/2 receptors during mild inflammatory insult also significantly worsens the suppression of contractility (Figs. 4A and 5) and the suppression of α1C subunit (Fig. 4B), and it increases the nuclear binding of p65 and p50 subunits of NF-κB (Fig. 4, C and D), respectively.

Effect of neuroimmune interactions on VIP concentration in muscularis externa. The nerves and immune cells interact to regulate the functions of each other. We found that subthreshold insult in naive rats, but significantly, reduces the tissue concentration of VIP in the muscularis externa with this dose of TNBS was significantly less than that with the subthreshold dose of TNBS (Fig. 6A). Further examination showed that the suppression of VIP concentration in the muscularis externa occurs within 24 h after the induction of inflammation, and it remains suppressed for at least 7 days (Fig. 6B). The 68-mg/kg dose of TNBS also significantly suppressed the serum concentration of VIP (Fig. 6C).

Therapeutic potential of VIP in maintaining homeostasis of smooth muscle function during severe inflammatory response. We reported recently that the systemic infusion of VIP by an osmotic pump elevates the serum concentration of VIP that is sustained throughout the period of infusion (35). Therefore, we investigated whether reestablishing serum concentration of VIP by an osmotic pump would compensate for the decrease in muscularis externa VIP by the inflammatory response and prevent smooth muscle dysfunction.

We found that inflammation induced with 68 mg/kg TNBS significantly increases the tissue concentrations of MPO in muscularis externa and mucosal/submucosal tissue (Fig. 7, A and B). It also significantly suppresses the contractile response to ACh (Figs. 5 and 7C) and the expression of α1C subunit (Fig. 7D), and it increases the nuclear abundance of p65 and p50 NF-κB subunits in the muscularis externa (Fig. 7, E and F). Systemic infusion of 20 nmol/day VIP for 7 days
by an osmotic pump, starting 4 h before the induction of inflammation with 68 mg/kg TNBS, significantly reverses all the adverse effects of the inflammatory response, including the nuclear translocation of p65 and p50 (Fig. 7). It reduced the visual macroscopic score from 3.4 ± 0.4 to 1.5 ± 0.2. Hematoxylin and eosin staining confirmed the reduction in inflammatory damage (Fig. 3F). The plasma VIP concentration on day 7 of infusion was about twofold greater than that before the start of infusion (Fig. 6C). The gain in body weight of rats receiving 68 mg/kg TNBS was significantly stunted over the 7-day postinflammation period, when compared with age-matched vehicle-treated animals (Fig. 8A). The rats ate significantly less food per day starting from day 1 of inflammation (Fig. 8B). The food intake improved gradually, but it was still significantly less on day 7, when compared with control food intake before the induction of inflammation (Fig. 8B). There was no significant decrease in food intake in rats subjected to severe inflammatory insult.

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while receiving 20 nmol/day VIP with an osmotic pump (Fig. 8).

DISCUSSION

Our findings show a three-way interaction between the enteric VIPergic neurons, circular smooth muscle cells, and immunocytes in the muscularis externa (Fig. 9, A and B). Under low-grade inflammatory conditions, the proinflammatory cytokines, such as TNF-α and IL-1β, attempt to suppress circular smooth muscle contractility, but the spontaneous release of endogenous VIP from the enteric VIPergic neurons opposes this effect and maintains homeostasis (Fig. 9A). At the same time, bilateral interaction between VIPergic neurons and immune cells results in small, but significant, suppression of VIP expression and the release of inflammatory mediators from activated immune cells in the muscularis externa. The residual VIP under these conditions is sufficient to prevent a significant increase of inflammatory mediators and MPO in the muscularis externa. As a result, the colonic circular smooth muscle contractility maintains nearly normal function. However, if the protective effects of endogenous VIP on immunocytes and smooth muscle cells are precluded by blocking VPAC1/2 receptors, the inflammatory response in the muscularis externa increases and smooth muscle function is impaired.

The suppression of neural VIP by immune cells increases with the intensity of inflammation. At higher intensities of inflammation, the inflammatory mediators reduce the concentrations of VIP in muscularis externa to about one-third of their basal levels. Under these conditions, the concentrations of the inflammatory mediators in the muscularis externa and MPO increase significantly, and they suppress smooth muscle contractility in the absence of basal protection by endogenous VIP. The suppression of VIP expression in the muscularis externa by inflammatory response seems to be a critical step in smooth muscle dysfunction. If the depletion of endogenous VIP is compensated by its exogenous infusion, the inflammatory response is suppressed and smooth muscle function is restored to near normal conditions. Concurrently, the exogenous VIP partially suppresses the inflammatory response in the mucosal/submucosal tissue. Therefore, the restoration of smooth muscle function by exogenous VIP is partially attributable to suppression of the inflammatory response and partially attributable to its direct effect on smooth muscle cells to block the deleterious effect of the residual inflammatory response.

Inflammatory insult rapidly suppresses the total VIP content in the muscularis externa (Fig. 6). Our findings and those of others show that the amplitude of VIP suppression correlates with the degree of inflammation (5, 18, 29). Immunohistological analysis of the muscularis externa revealed that the suppression of VIP expression by inflammatory mediators is a critical step in smooth muscle dysfunction. If the depletion of endogenous VIP is compensated by its exogenous infusion, the inflammatory response is suppressed and smooth muscle function is restored to near normal conditions. Concurrently, the exogenous VIP partially suppresses the inflammatory response in the mucosal/submucosal tissue. Therefore, the restoration of smooth muscle function by exogenous VIP is partially attributable to suppression of the inflammatory response and partially attributable to its direct effect on smooth muscle cells to block the deleterious effect of the residual inflammatory response.
chemical findings on the effects of inflammation on VIPergic neurons are divergent (5, 17–20, 25, 29, 30, 36). However, the consensus is that inflammation damages morphology of the enteric nervous system, including a reduction in the number of cell bodies and VIP release in Crohn’s disease, ulcerative colitis, and in experimental models of inflammation (4, 18, 25, 36). Taken together, these data show that, in severe inflammatory response, very little endogenous VIP may be available to counter inflammation and to protect the function of neighboring nonimmune cells in the muscularis externa and in mucosal/submucosal layers. Although the inflammatory response and VIP counter each other successfully in low-grade inflammation, the inflammatory response gains upper hand in severe inflammation by suppressing the expression of VIP and damaging axons (Fig. 9).

Severe inflammation stunts weight gain, and it reduces food intake. The exogenous administration of VIP reverses both effects. These data suggest that basal plasma circulation of VIP may play a critical role in maintaining appetite. This regulation may be a protective mechanism to prevent loading of the gut with food under conditions where its digestive functions are impaired by inflammation.

NF-κB is a ubiquitous transcription factor that induces transcription of genes encoding inflammatory mediators in immune as well as in nonimmune cells (15, 27, 28). In nonimmune cells, such as CCSMCs, NF-κB activation suppresses the transcription of genes encoding the cell-signaling proteins of the excitation-contraction coupling (33). The enteric VIPergic neurons directly innervate the circular smooth muscle cells as well as the immune cells of the gut lymphoid...
tissue (12, 39). Previous in vitro studies found that VIP suppresses inflammatory response in immune cells of the lamina propria by inhibiting translocation of NF-κB to the nucleus and its subsequent binding to κB recognition sites on the promoters of genes encoding the inflammatory mediators (8–10).

Our findings show that VIP also inhibits the translocation of NF-κB in circular smooth muscle cells to prevent suppression of contractility by proinflammatory cytokines. VIP inhibits the phosphorylation of IκKB in response to TNF-α, which prevents the degradation of IκBα, but not that of IκBα. The degradation of IκKB is associated with longer-term activation of NF-κB, whereas the degradation of IκBα results in transient activation of NF-κB (16, 40). By contrast, stimulation of immune cells by LPS degrades IκBα to induce the expression of inflammatory mediators (7). Therefore, it seems that VIP inhibits activation of the canonical pathway of activation of NF-κB regardless of the cell type and the involvement of species of IκB proteins and their kinases.

The signaling pathways for the activation of NF-κB and the affinities of NF-κB dimmers to bind to their variant κB recognition sites on target promoters are cell, stimulus, and promoter specific (15). We found that both endogenous and exogenous VIP inhibit the translocation of NF-κB subunits p50 and p65 to the nucleus and their subsequent binding to promoter of the gene encoding the ε1C-subunit of Ca2+ channels in smooth muscle cells of the muscularis externa in vivo and in vitro. On the other hand, in immune cells, LPS translocates homodimers of p65 to induce the transcription of inflammatory mediators (7). These differences may contribute to the differential effects of NF-κB activation; in immune cells, the activation of this transcription factor enhances the transcription of genes, whereas, in smooth muscle cells, it suppresses the transcription of genes encoding proteins of the excitation-contraction coupling.

Two other groups previously investigated the therapeutic potential of exogenous administration of VIP in a murine model of TNBS inflammation and reported conflicting outcomes (1, 23). Both studies investigated only the therapeutic potential of VIP on inflammatory response and physical symptoms. Abad et al. (1) reported that one or more intraperitoneal bolus injections of VIP block the inflammatory response to TNBS and development of physical symptoms, such as mucosal damage and weight loss. Newman et al. (23) found that similar bolus injections of VIP or its constant infusion by an osmotic pump block chemokine-induced chemotaxis of mono-nuclear cells, but it fails to modify histological damages, cytokine markers of disease, or clinical symptoms, including weight loss. By contrast, we found that constant infusion of exogenous VIP by an osmotic pump in rats diminishes the inflammatory response, improves clinical symptoms, and growth in weight, and it restores suppression of smooth muscle contractility. Abad et al. (1) and Newman et al. (23) used much higher doses of TNBS, 142 mg/kg and 118 mg/kg, respectively, compared with 68 mg/kg used by our laboratory. Their doses produced massive inflammation, resulting in dramatic weight loss of about 20%, mortality of about 30%, bloody diarrhea, and rectal prolapse. Our dose of 68 mg/kg TNBS suppressed smooth muscle contractility, resulting in loose stools. This dose of TNBS stunted growth, but it did not cause weight loss or mortality. The inflammatory and clinical symptoms in our study are closer to those seen in acute and chronic inflammation in humans. The exogenous administration of VIP may not be able to overcome massive wasting disease.

VIP, injected as a bolus, is eliminated from the plasma within a few minutes (11, 13, 14, 21). It is not clear how such a short duration of VIP pulse could alter longer-term transcription of genes encoding inflammatory mediators (1). It is noteworthy that the severity of inflammatory response and its duration varied dramatically in the study of Abad et al. (1). This may be due to the variable amount of fecal pellets present in the colon at the time of administration of TNBS. Different quantities of fecal pellets may absorb different amounts of TNBS. In our study, we cleansed the colon by replacing drinking water with Colyte 24 h before TNBS administration followed by overnight fasting. Abad et al. (1) did not demonstrate the inhibition of NF-κB activation by bolus doses of VIP in their model of TNBS inflammation.

In conclusion, the gut wall is persistently exposed to hostile pathogenic environment, and the VIPergic neurons emanating from the myenteric and submucosal plexi densely innervate most immune and nonimmune cells in muscularis externa and mucosal/submucosal tissues. Our findings show that spontaneous release of VIP from these neurons may be one of the first lines of defense against mild/moderate inflammatory insults. The second line of defense, of course, is the generation of anti-inflammatory cytokines, but that happens after the inflammatory response has matured. The spontaneous release of VIP in the gut wall maintains homeostasis in both immune and nonimmune cells. Homeostasis in smooth muscle function is maintained by inhibiting the translocation of NF-κB to the nucleus attributable to stabilization of IκBα. A severe inflammatory insult suppresses the total content of VIP as well as its release from nerve varicosities, which allows the inflammatory response to mature and adversely affect the function of non-immune cells, such as smooth muscle cells. The suppression of VIP in gut tissues is critical for the inflammatory response to mature. The compensation for loss of neuronal VIP by its exogenous administration protects smooth muscle function directly by inhibiting the activation of NF-κB and indirectly by partially suppressing the inflammatory response.

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