\( \alpha_2 \)-Adrenergic regulation of NO production alters postoperative intestinal smooth muscle dysfunction in rodents

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Submitted 18 December 2003; accepted in final form 5 May 2004


\( \alpha_2 \)-Adrenergic receptor stimulation of the inflamed postoperative intestinal muscularis plays a significant role in aggravating postoperative ileus through an enhanced induction of iNOS mRNA and increased release of NO from manipulated intestinal muscularis.

Yohimbine

POSTOPERATIVE ILEUS IS A COMMON complication after intra-abdominal surgery leading to increased morbidity and prolonging hospital stay (18). Failure to tolerate oral feeding and dysfunctional bowel motility during postoperative ileus are the most common reasons for delayed hospital discharge after abdominal surgery. Inflammatory and neuronal mechanisms have been implicated in postoperative dysmotility, but the sequence of these events and their relative contribution to the pathogenesis remain poorly understood. Neural pathways, including spinal reflexes (19), neuropeptides (52), and the activation of adrenergic receptors, especially \( \alpha_2 \)-adrenergic receptors (37, 46), have been found to play an important role in the development of this disorder. \( \alpha_2 \)-Adrenergic receptor antagonists have been shown to ameliorate postoperative gastrointestinal motility abnormalities after abdominal surgery in rats (37, 46), horses (15), and humans (6). It has been suggested that this effect is probably due to the blockade of \( \alpha_2 \)-adrenergic receptors on vagal nerve terminals and postganglionic cholinergic nerves in the myenteric plexus (46), because activation of \( \alpha_2 \)-adrenergic receptors results in decreased acetylcholine release from enteric and autonomic nerves (1, 7, 41).

Our laboratory has previously focused on local inflammatory mechanisms within the intestine contributing to postoperative ileus, showing in both humans and rodents that surgical manipulation of the gut leads to a marked molecular and cellular inflammatory response within the intestinal muscularis (22, 23, 25, 26). The resulting inflammatory response has been shown to be proportional to the degree of gut ileus, as demonstrated by a decrease in gastrointestinal transit and a suppression of in vitro circular smooth muscle contractility (23, 48). It has also been shown that leukocyte adhesion molecule blocking antibodies prevent the recruitment of monocytes, neutrophils, and mast cells into the muscularis and also avert postoperative dysfunction (9, 23). This observation underlines the importance of inflammatory changes within the intestinal muscularis in the etiology of postoperative ileus. Additionally, it has been demonstrated that leukocyte-derived inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) play a significant role in the pathogenesis of rodent postoperative ileus (24, 39). We demonstrated that intestinal manipulation induces iNOS and COX-2 mRNA and protein within resident muscularis macrophages and recruited monocytes. Pharmacological inhibition or genetic manipulation of either iNOS or COX-2 resulted in improved postoperative in vitro jejunal circular muscle contractility (23, 48).

It has been reported that macrophages and monocytes possess both \( \alpha \)- and \( \beta \)-adrenergic receptors (30, 40). These receptors regulate various cell functions, such as energy metabolism (30), cytokine production (21, 43), and nitrite and superoxide secretion (40). When stimulating peritoneal murine macrophages with LPS, the \( \alpha_2 \)-receptor antagonist idazoxan reduced endotoxin-induced macrophage nitrite production (40). The \( \alpha \) - and \( \beta \)-adrenergic receptors appear to have different and sometimes antagonizing effects on regulating the immune responses of macrophages and monocytes. Interestingly, norepinephrine and epinephrine have been found in murine peritoneal macrophages by HPLC (42), and it was observed that \( \alpha_2 \)-adrenergic agonists increase and \( \alpha_2 \)-antagonists decreased TNF-\( \alpha \) production in an autocrine fashion (21, 42, 43).
Parasympathetic and sympathetic responses also typically have antagonistic effects. Recently, Tracey and colleagues (4, 29, 47, 50) have delineated in a series of articles the existence of a “cholinergic anti-inflammatory pathway” that consists of the vagal release of acetylcholine, which acts through the \(\alpha_2\)-nicotinic receptor subunit on macrophages to posttranscriptionally decrease the release of TNF-\(\alpha\) and other cytokines during sepsis.

NO-secreting macrophages/monocytes are a known major component of the leukocytic inflammatory response within the muscularis after surgical manipulation of the intestine (25, 35) and significantly suppress intestinal contractility. iNOS expression is known to be modulated by adrenergic agonists (3, 40). Therefore, we hypothesized that an “adrenergic proinflammatory pathway,” which consists of \(\alpha_2\)-adrenergic upregulation of NO production in the postoperatively recruited monocytes and resident muscularis macrophages, plays a significant role in intensifying the postsurgical intestinal inflammatory response.

To investigate the adrenergic modulation of the postoperative inflammatory response hypothesis, we determined whether the specific \(\alpha_2\)-antagonist yohimbine would modulate the effect of intestinal surgery on in vivo gastrointestinal transit and in vitro jejunal smooth muscle contractility, muscularis externa iNOS mRNA expression, and NO release for the isolated inflamed muscularis. Furthermore, \(\alpha_2\)-receptors were immunolocalized to specific cell types within the inflamed postsurgical muscularis.

MATERIALS AND METHODS

Operative procedure and experimental groups. Sprague-Dawley male rats (220–300 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). The University of Pittsburgh Institutional Animal Care and Use Committee approved all experimental animal protocols. Animals were housed in a pathogen-free facility that is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with the requirements of humane animal care as stipulated by the United States Department of Agriculture and the Department of Health and Human Services. The rats were maintained on a 12:12-h light-dark cycle and provided with commercially available chow and tap water ad libitum.

The small bowel of the animals was subjected to surgical manipulation as described previously (25). Unoperated and vehicle-injected animals served as corresponding controls. Animals were anesthetized with methoxyflurane inhalation and a midline incision was made into the peritoneal cavity. The small bowel was everted onto moist gauze and the entire small bowel was lightly manipulated between two moist cotton applicators. After manipulation, the laparotomy was closed by using a double-layer running suture. The bowel manipulation procedure caused no mesenteric vascular bleeding or mortality. The animals recovered quickly from surgery and generally began to eat and drink within 2 h. For this study, animals were killed between 0 and 24 h after manipulation, and the intestine was used for in vitro organ bath contractile recordings, in vivo gastrointestinal transit studies, mRNA extraction, immunohistochemistry, and NO measurement. The specific \(\alpha_2\)-adrenergic antagonist yohimbine (Sigma, St. Louis, MO) was dissolved in DMSO (stock solution) and diluted in saline to the required concentration. Yohimbine was administered at 1 mg/kg sc at the start of surgery and at 21 h postoperatively. Control rats received the respective amount of vehicle.

Functional motility studies. Gastrointestinal transit was measured in controls and manipulated animals at 24 h postoperatively by evaluating the intestinal distribution of fluorescent FITC-labeled dextran (Molecular Probes, Eugene, OR). Animals were given FITC-labeled dextran (100 \(\mu\)l of 25 mg/ml stock solution) via gavage. Ninety minutes after administration, the entire GI tract (stomach to colon) was removed. The small bowel was divided into 10 equal parts, and the colon was divided into three equal parts (22). The intestinal contents were processed according to our previous description, and the fluorescent signal in each bowel segment was measured. The geometric center, \(\Sigma\) (percentage of total fluorescent signal in each segment times the segment number), was then calculated (32).

Mechanical activity was measured from circular muscle strips dissected from the midjejunum or midcolon as previously described (13). The spontaneous activity and the response of the circular muscle to increasing doses of the muscarinic agonist Bethanechol (0.3–300 \(\mu\)mol) were recorded by using an analog-to-digital computer hardware and software package (Biopac, Santa Barbara, CA). Contractions between different muscle strips were normalized by converting grams of contraction to grams per square millimeter of tissue. This was done by determining the cross-sectional area by the following equation (muscle density assumed to be 1.03 mg/mm\(^3\); mm\(^3\) = [wet muscle weight (mg)/muscle length (mm) \(\times\) muscle density (mg/mm\(^3\))].

SYBRgreen real-time RT-PCR. iNOS mRNA expression was determined by SYBRgreen two-step, real-time RT-PCR. The small intestinal muscularis externa was collected at three time points (0, 3, and 6 h after surgery). The muscularis was isolated, as described previously (39), and was immediately snap frozen in liquid nitrogen and stored at \(-80^\circ\)C. Total mRNA extraction was performed as previously described by using the guanidinium-thiocyanate phenol-chloroform extraction method (13). The DNA pellets were resuspended in RNA-secure resuspension solution (Ambion, Austin, TX). Equal aliquots (200 ng) of total RNA from each sample, quantified by spectrophotometry, were then processed for complementary DNA (cDNA) synthesis.

Primers were designed by using Primer Express software (PE Applied Biosystems, Foster City, CA) and purchased from Life Technologies (Rockville, MD). GAPDH was used as an endogenous control. The sequences of the real-time PCR primers are listed in Table 1. The efficiency and equality of the real-time PCR primer pairs were determined by amplifying serial dilutions of colonic muscularis cDNA. For each target gene, different MgCl\(_2\) (3–5 mM) concentrations were tested to optimize the PCR amplification. Agarose gel electrophoretic analysis was used to verify the presence of a single product and to ensure that the amplified product corresponded to the size predicted for the amplicon. The PCR reaction mixture was prepared by using the SYBRgreen PCR core reagents (PE Applied Biosystems). PCR conditions on an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) were as recommended by

<table>
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<th>Target</th>
<th>Primer Sequence</th>
<th>MgCl(_2), mM</th>
<th>Slope</th>
<th>Correlation Coefficient (R^2)</th>
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<td></td>
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<td>Antisense: 5'-GGGATGAATTGGAGCTGGA-3'</td>
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Values are means ± SE. iNOS, inducible nitric oxide synthase.
the manufacturer. Relative quantification was performed by using the comparative cycle threshold method as described previously (38).

Tissue culture preparation and NO measurement in the supernatant. The tissue source for NO release was determined by using the isolated intestinal muscularis. The small intestines of control and manipulated rats after 24 h were removed under sterile conditions. The small intestine was transferred to a sterile beaker containing HBSS (Sigma) with 200 U/ml penicillin G and 200 μg/ml streptomycin. The muscularis was isolated, and aliquots of 100–150 g were incubated in culture dishes containing supplemented DMEM-F-12 culture medium in a CO2-controlled incubator (NuAire, Plymouth, MN). The muscularis from manipulated animals was incubated in the following: 1) DMEM-F-12 culture medium alone, 2) yohimbine (1 mg/l) in vitro, or 3) 1-N[1-iminoethyl]lysinine (1-NIL; 50 μM) (Sigma), a selective iNOS inhibitor. After an incubation period of 24 h, the cultured tissue was inspected for contamination, and supernatant was frozen in liquid nitrogen and stored at −80°C. The muscle tissue was blotted dry and weighed.

Culture supernatants were assayed for the measurement of NO release by the Griess reaction (16). Supernatant from the tissue culture was incubated with granulated cadmium (Oxford Biomedical Research, Oxford, MI) to convert nitrate to nitrite. The total amount of nitrite per sample was then measured by using Griess reagent. The absorption at 540 nm was measured with a microplate reader and compared with standard dilutions of sodium nitrite. Total nitrite produced was normalized to 100 mg of muscle tissue.

Histochemistry and immunohistochemistry. Whole mounts of the intestinal muscularis were investigated for the presence of resident and recruited leukocytes, as well as the cellular localization of α2-adrenergic receptors and tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines. Midjejunal segments were cut from the bowel and immersed into Krebs Ringer buffer in a chilled cold Krebs Ringer buffer. Muscularis whole mounts were also used for immunohistochemical analysis. The muscularis whole mounts were incubated for 24 h at 4°C in the primary antibody. We performed immunohistochemical stainings for α2A-adrenergic receptors (rabbit anti-rat antibody, 1:50; Affinity BioReagents, Golden, CO), ED1 (monocyte-specific antibody, mouse anti-rat 1:75; Serotec, Indianapolis, IN) and tyrosine hydroxylase (mouse monoclonal antibody, 1:20; Novocastra Laboratories). The specimens were then incubated in the appropriate secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit antibody, 1:1,000; Molecular Probes, or Cy3-conjugated donkey anti-mouse antibody, 1:1,000; Immunoresearch Laboratories, West Grove, PA) at 4°C overnight. Whole mounts were placed on coverslips and inspected by light or fluorescent microscopy after staining (Nikon FXA; Fryer, Huntley, IL). Nonspecific isotype-matched antibodies and primary antibody incubation without secondary antibody were used as negative controls. Controls, as well as rats, 12 and 24 h after intestinal manipulation were studied.

In a separate experiment, we investigated the presence of α2A receptors and tyrosine hydroxylase on peritoneal macrophages. Rats were killed 24 h postoperatively. Unoperated animals served as controls. At 24 h, 1 ml of sterile saline was injected intraperitoneally and reaparated after 2–3 min. This peritoneal lavage fluid was incubated in culture dishes containing small glass slides in DMEM culture medium for 24 h. The slides were subsequently stained for the monocyte-specific marker ED1 (1:150) and costained for α2A-adrenergic receptors (1:100), as well as for tyrosine hydroxylase (1:40).

Calculations and statistics. Data are presented as means ± SE. Data were assessed for normal distribution by a standardized normal probability plot using software from STATA (College Station, TX). When two groups were compared, the unpaired or paired Student’s t-test was used, as appropriate. Multiple groups were evaluated statistically by ANOVA followed by a Scheffé’s multiple-comparison test. Data that lacked normal distribution were assessed by the Wilcoxon’s rank sum test (for paired data) or the Mann-Whitney U-test (for unpaired data). Data were considered statistically significant at P < 0.05 (2-tailed).

RESULTS

α2-Adrenergic blockade improves postoperative gastrointestinal transit. Atipamezole, a selective α2-adrenergic antagonist, has previously been shown to improve gastrointestinal transit a few hours after laparotomy in rats (46). We sought to determine whether α2-adrenergic blockade would ameliorate postoperative ileus that develops over a period of 24 h after surgical manipulation of the rat small intestine (22). In this experiment, gastrointestinal transit was measured in controls and surgically manipulated animals 24 h after surgery with or without yohimbine (1 mg/kg), which was administered subcutaneously at the start of surgery and 21 h postoperatively. Figure 1 shows the gastrointestinal distribution of orally fed fluorescein-labeled dextran in the four experimental groups of animals. Yohimbine did not alter transits in control animals as determined by a similarly calculated geometric center (controls: 9.21 ± 0.4; controls with yohimbine: 9.59 ± 0.18). Surgical manipulation caused a significant delay in gastrointestinal transit, decreasing the average geometric center to 6.71 ± 0.88. Treatment with yohimbine significantly improved the manipulation-induced delay, increasing the mean geometric center to 8.69 ± 0.27 (P < 0.03).

α2-Adrenergic blockade attenuates the postoperative suppression in muscle contractility. We then sought to determine whether this improvement in postoperative gastrointestinal contractility after yohimbine was restricted to a systemic in vivo response or whether it could also be observed in vitro. We (24, 39) have previously shown that intestinal manipulation causes a decrease in jejunal circular smooth muscle strip contractility and that this decrease is, in part, caused by inflammatory events. Again, we studied the four groups of rats and assessed spontaneous in vitro circular smooth muscle contractility and mechanical responses to increasing doses of the muscarinic agonist bethanechol. Figure 2 shows intestinal smooth muscle contractility in control and in surgically manipulated rats with and without yohimbine in vivo treatment. α2-Adrenergic blockade with yohimbine did not alter spontaneous or bethanechol-stimulated smooth muscle contractility in control rats (contractility at 100 μM bethanechol: controls: 4.12 ± 0.79 4.14 ± 0.69 g·mm−2·s−1; controls with yohimbine, 4.14 ± 0.69 g·mm−2·s−1). Intestinal manipulation caused a distinct decrease in contractility (P < 0.01). This decrease was attenuated by yohimbine (contractility at 100 μM bethanechol: intestinal manipulation, 2.26 ± 0.41 g·mm−2·

s−1; intestinal manipulation with yohimbine, 3.63 ± 0.68 g·mm−2·s−1; P < 0.03).
Yohimbine does not alter postoperative leukocytic infiltration. Previously, we (23, 24) have shown that postoperative jejunal smooth muscle dysfunction is caused by recruitment of leukocytes into the intestinal muscularis that develops postoperatively over a period of 24 h. Therefore, we sought to determine whether yohimbine would alter the cellular inflammatory infiltrate by quantifying myeloperoxidase-positive cells within intestinal muscularis whole mounts. Intestinal manipulation caused a significant increase in myeloperoxidase-positive cells per \( \times 10^2 \) field (control: 1.2 ± 0.2 vs. intestinal manipulation with yohimbine: 61.7 ± 14.8; \( P < 0.001 \) (Fig. 3). Yohimbine did not significantly alter the extent of this leukocytic infiltrate in controls or in surgically manipulated rats (controls with yohimbine: 1.1 ± 0.2 vs. intestinal manipulation with yohimbine: 69.0 ± 0.1).

Yohimbine decreases iNOS mRNA and NO release from the postoperative intestinal muscularis. Because leukocytic recruitment did not appear to be the basis for improved intestinal function, we explored the alternative hypothesis that resident and infiltrating leukocyte function may be altered by adrenergic blockade. Previously, we (24) have shown that iNOS and its product NO contribute significantly to the development of postoperative intestinal smooth muscle dysfunction in rodents. It was found that inducible NO, mainly produced by infiltrating monocytes and macrophages within the intestinal muscularis, plays an essential role in inhibiting gastrointestinal motility after intestinal surgery. Interestingly, \( \alpha_2 \)-adrenergic receptors have also been reported to regulate NO release in macrophages (40). We, therefore, studied iNOS mRNA upregulation within the intestinal muscularis with its extensive network of resident...
Fig. 3. Histogram of infiltrating leukocytes in muscularis whole mounts in control rats and rats after IM. IM caused a marked increase in the muscularis leukocytic infiltrate as measured by a myeloperoxidase stain (P < 0.001). Yohimbine did not affect this infiltrate (n = 6).

Fig. 4. Histogram showing the relative increase in inducible nitric oxide (NO) synthase iNOS messenger RNA (mRNA) after IM, as measured by real-time RT-PCR. Yohimbine did alter iNOS mRNA at baseline. IM caused an 8-fold increase 3 h and a 14-fold increase 6 h after IM. Yohimbine prevented this increase in iNOS mRNA postoperatively (*P < 0.05, n = 5–9).

macrophages and subsequent monocytic infiltrate at 3 and 6 h postoperatively with and without yohimbine (Fig. 4). Yohimbine did not significantly affect baseline iNOS mRNA expression in control animals. Intestinal surgery caused a marked upregulation of iNOS mRNA at 3 h (8.9 ± 1.9-fold) and at 6 h (13.6 ± 4.3-fold) postoperatively, compared with control levels. Yohimbine significantly blunted this postsurgical increase (P < 0.05), because iNOS mRNA expression only increased 4.9 ± 1.1-fold at 3 h and 2.7 ± 0.7-fold at 6 h after surgery.

At this point, we were interested to see whether this decrease in iNOS mRNA expression after in vivo yohimbine treatment would translate into a decrease in NO release by the inflamed intestinal muscularis. Figure 5 shows a histogram of NO production as measured by the Griess reaction. In vivo treatment with yohimbine did not alter baseline NO release levels from the isolated cultured unoperated muscularis (control: 5.4 ± 0.6 μM/100 mg tissue; control + yohimbine: 6.1 ± 1.0 μM/100 mg tissue). Intestinal surgery caused a significant 5.8-fold increase in NO release from the muscularis harvested 24 h postoperatively compared with control (28.8 ± 0.9 μM/100 mg tissue; P < 0.001). Yohimbine treatment in vivo or after tissue harvest in culture significantly decreased the stimulated increase in NO levels in the cultured inflamed muscularis supernatant by about one-third (18.8 ± 3.2 and 19.6 ± 2.2 μM/100 mg tissue, respectively, P < 0.05). Incubating the inflamed intestinal muscularis with the specific iNOS inhibitor L-NAME (50 μM) decreased NO levels to baseline levels (8.9 ± 0.5 μM/100 mg tissue), suggesting that most of the NO produced by the postsurgical intestinal muscularis was produced by iNOS.

Localization of α2-adrenergic receptors to infiltrating monocytes. In Yohimbine decreases iNOS mRNA and NO release from the postoperative intestinal muscularis: we showed that α2-adrenergic blockade with yohimbine improved postsurgical gastrointestinal motility in vivo (gastrointestinal transit) and in vitro (circular smooth muscle contractility) and suppressed the postsurgical increase in iNOS mRNA expression and NO production. We then sought to localize α2-adrenergic receptors within the inflamed muscularis by staining intestinal muscularis whole mounts using immunohistochemistry. α2-Adrenergic receptors were identified on a large population of round infiltrating cells after intestinal surgery (Fig. 6A). Costaining the inflamed whole mounts with the macrophage/microcyte-specific antibody ED1 (Fig. 6B) demonstrated colocalization with the α2-adrenergic fluorescent signal (Fig. 6C). The infiltrating monocytes in the intestinal muscularis were also found to visually express tyrosine hydroxylase (not shown). The infiltrating monocytes appeared to be rather homogenously spread within the intestinal muscularis, and no specific proximity was seen to sympathetic nerve fibers.

In a separate series of experiments, we sought to study whether the localization of α2-adrenergic receptors and tyrosine hydroxylase in infiltrating monocytes within the muscularis also pertained to peritoneal monocytes obtained via lavage of the peritoneal cavity. Peritoneal lavage revealed only a few ED1-positive peritoneal monocytes in control rats. Visually all of these control peritoneal macrophages were also positive for α2-adrenergic receptor antibody. After intestinal manipulation, a noticeably larger number of ED1+ peritoneal macrophages was retrieved via peritoneal lavage (Fig. 7A). Again, all of these were visually assessed to be positive for

Fig. 5. Histogram demonstrating a significant increase in nitrite accumulation in intestinal muscularis tissue culture supernatants of the intestinal muscularis compared with control (P < 0.001). This response was prevented by the presence of the specific iNOS inhibitor L-N6-(1-iminoethyl)lysine (L-NIL). Yohimbine, both in vitro (adding Yohimbine to the tissue culture supernatant) and in vivo (incubating tissue from Yohimbine-treated rats) decreased postoperative NO production by the intestinal muscularis by about 1/3 (P < 0.03; n = 5–7).
α2-adrenergic receptors (Fig. 7B). Peritoneal macrophages also stained positive for tyrosine hydroxylase (Fig. 7C).

**DISCUSSION**

In the present study, we demonstrated that yohimbine, an α2-adrenergic antagonist, downregulates the surgical induction of leukocytic iNOS mRNA and that the subsequently decreased release of NO results in a functional improvement in postoperative motility. The α2-adrenergic receptors were localized within the postoperative inflamed muscularis to macrophage/monocytes by immunohistochemistry. However, because macrophages/monocytes are prime NO producers via iNOS and yohimbine did not alter the nonmanipulated intestine, these data indicate that endogenous catecholamine stimulation of the α2-adrenergic receptor by neuronal, hormonal, and/or autocrine sources plays a significant role in intensifying the postoperative inflammatory generation of NO from macrophages/monocytes.

The observation that the infiltrating monocytes and peritoneal macrophages stained positive for the rate limiting enzyme in catecholamine synthesis tyrosine hydroxylase suggests that these cells are able to produce catecholamines themselves in an autocrine fashion. The ability of macrophages to synthesize catecholamines has previously been well documented (5, 8). It has been shown (43) that macrophage-derived norepinephrine can alter LPS-associated cytokine production. Autocrine production of catecholamines by macrophage/monocytes may thus be contributing to postoperative adrenergic regulation of NO production within the intestinal muscularis. However, we have not ruled out the significance of neuronal sources of catecholamines in this study.

Previous studies (2, 9, 27) have identified the importance of both neurogenic and local inflammatory mechanisms for causing prolonged postoperative ileus. Our current demonstration of α2-adrenergic modulation of postoperative production of the inhibitory mediator NO by infiltrating monocytes creates a mechanistic link between the postoperative inflammatory events that we previously delineated within the intestinal muscularis (22, 24, 25, 39) and the involvement of neuronal pathways, such as α2-adrenergic receptor activation (15, 37, 46) in the pathogenesis of postoperative ileus.

NO is a major inhibitory neurotransmitter of gastrointestinal motility. It is well established that NO causes hyperpolarization of gastrointestinal circular muscle and inhibits spontaneous contractions (44, 45, 53). Additionally, we have shown that NO produced by leukocyte-derived iNOS inhibits gastrointestinal motility after intestinal manipulation in rodents (24). Application of the specific iNOS inhibitor L-NIL significantly improved postsurgical smooth muscle contractility and iNOS knockout mice displayed significant resistance to the development of postoperative ileus (24). Immunohistochemistry localized iNOS predominantly to infiltrating monocytes after intestinal manipulation. Thus considering the present data, it seems

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**Fig. 6.** Immunohistochemical staining of jejunal muscularis whole mounts from rats 24 h after IM for α2-adrenergic receptors showed a large population of round infiltrating cells after intestinal surgery (A; original magnification, ×400). Costaining the inflamed whole mounts with the macrophage/monocyte-specific antibody ED1 (B) demonstrated colocalization with the α2-adrenergic fluorescent signal (C). Original magnification, ×400.
likely that α2-adrenergic receptors on infiltrating monocytes in the intestinal muscularis are involved in altering postoperative NO release from these cells.

Macrophages (35) and monocytes (12) have been shown to produce NO by various NOS isoforms. The regulation and function of the inducible isoform iNOS is mainly associated with inflammatory and immune responses (35). We have previously shown that a surgically induced leukocytic infiltrate, consisting of neutrophils and monocytes within the intestinal muscularis mediates the development of postoperative ileus (23). On the other hand, adrenergic receptors have been found to regulate macrophage and monocyte functions, including the release of nitrite (40). Norepinephrine can alter the energy metabolism (30) and the oxygen consumption (31) of human peripheral blood monocytes via adrenergic receptors in the setting of inflammation. α-Adrenergic receptors on monocytes were found to modulate complement component synthesis (28). Adrenergic receptors can also regulate free radicals, such as hydrogen and superoxide, as well as NO release by peritoneal macrophages in mice (40). In this particular study, both the α-antagonist idazoxan and the β-antagonist propranolol inhibited free radical release by peritoneal macrophages. However, only idazoxan, but not propranolol, inhibited NO release. In our study, α2-adrenergic receptors were heavily expressed on infiltrating and peritoneal monocytes within the intestinal muscularis. The mononuclear phagocyte system is defined as a population of cells derived from the bone marrow that differentiate to form blood monocytes initially circulating in the blood to then enter tissues to become resident tissue macrophages (20). Because α2-receptors were primarily seen on infiltrating and peritoneal monocytes, it appears that the expression of α2-adrenergic receptors is an early developmental and inflammatory characteristic that diminishes as monocytes become resident tissue macrophages. Similarly, functional α1-receptors have been described on peripheral blood mononuclear cells in patients with juvenile rheumatoid arthritis leading to an increased production of the proinflammatory cytokine IL-6 (17). In contrast, functional α1-adrenergic receptors were absent on peripheral blood mononuclear cells of healthy individuals. Although speculative, the differential expression of functional adrenergic receptors on monocytes may represent an acute modulation of the immune response in the setting of inflammation.

Previous studies (6, 15, 37, 46) have described an improvement in early postsurgical gastrointestinal motility abnormalities in different species when using various α2-adrenergic antagonists. Inhibition of gastrointestinal motility via α2-receptor stimulation has mainly been ascribed to the presence of α2-receptors on vagal nerve terminals and postganglionic cholinergic nerves in the myenteric plexus (10, 11, 51). α2-Adrenergic receptors in the brain have also been suggested to play a role (10, 14). Our study suggests for the first time, that α2-adrenergic receptors on monocytes may increase the postoperative release of NO, thus inhibiting gastrointestinal motility. α2-Adrenergic regulation of NO production has also been described as playing a direct role in chloride absorption by the thick ascending limb of the loop of Henle (36). These authors (36) showed that the α2-adrenergic receptor agonist clonidine decreased sodium chloride absorption in the thick ascending limb of the loop of Henle by activating α2-adrenergic recep-

Fig. 7. A: shows ED1-positive peritoneal macrophages obtained via peritoneal lavage after IM. Original magnification, ×200. B: all of these control peritoneal macrophages were also positive for α2-adrenergic receptor antibody. C: peritoneal macrophages also stained positive for tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines. Original magnification, ×200.
tors, which stimulated NOS and increasing production of endogenous NO via activation of phosphatidylinositol 3-kinase.

Yohimbine attenuated but did not completely reverse the effects of intestinal manipulation on gastrointestinal motility. As previously determined, the development of postsurgical intestinal smooth muscle dysfunction is multifactorial and several mechanisms may be involved. We (39) have recently shown that prostaglandin production via the induction of COX-2 plays an important role as a causative mechanism of postoperative ileus. The effect of yohimbine on postoperative prostaglandin production is presently unknown. Other possible factors that may compromise postsurgical intestinal smooth muscle function include free oxygen radicals, which are released during inflammation and have been shown (49) to cause a damaged contractile response in the GI tract. Cytokines, such as IL-6 and IL-1β, which are released in large quantities within the intestinal mucosal during postoperative ileus (33), have also been shown (34) to alter intestinal motility by affecting the intestinal muscularis during postoperative ileus (33).

In conclusion, our study demonstrates for the first time that α2-adrenergic receptors on infiltrating monocytes within the intestinal muscularis play a key role in regulating induction of iNOS and release of NO within the muscularis after intestinal manipulation. Thus α2-adrenergic receptors provide a crucial link between postsoperative intestinal inflammatory mechanisms and the activation of neuronal pathways in the pathogenesis of postoperative ileus. These observations also suggest that α2-adrenergic receptor activation on macrophages and monocytes may also represent an important neuroimmunomodulatory mechanism possibly involved in other gastrointestinal inflammatory disorders.

GRANTS
This work was supported by the National Institutes of Health Grants R01-GM58241, P50-GM-53789, and R01-DK-54824. C. Kreiss was supported by the Foundation for Digestive Health and Nutrition/American Gastroenterological Association/AstraZeneca Fellowship/Faculty Transition Award.

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