Tyrphostin AG 126 inhibits development of postoperative ileus induced by surgical manipulation of murine colon

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Moore, Beverley A., Andreas Türler, Michael A. Pezzone, Kevin Dyer, Jennifer Grandis, and Anthony J. Bauer. Tyrphostin AG 126 inhibits development of postoperative ileus induced by surgical manipulation of murine colon. Am J Physiol Gastrointest Liver Physiol 286: G214–G224, 2004. First published September 25, 2003; 10.1152/ajpgi.00312.2003.—Manipulation of the bowel during abdominal surgery leads to a period of ileus, which is most severely manifested after procedures that directly involve the colon. Ileus is associated with the increased expression of proinflammatory cytokines and chemokines, a leukocytic infiltration into the muscularis, and the release of mediators from resident and infiltrating leukocytes that directly inhibit intestinal smooth muscle contractility. Phosphorylation of tyrosine residues on regulatory proteins by protein tyrosine kinases (PTKs) occurs at multiple steps in the signaling cascades that regulate the expression of proinflammatory genes. The purpose of this study was to determine whether inhibition of PTK activity will attenuate the inflammatory response associated with colonic ileus and lead to improved function. Using a rodent model of colonic postoperative ileus, we demonstrate that a single bolus injection of the PTK inhibitor tyrphostin AG 126 (15 mg/kg sc) before surgery significantly attenuates the surgically induced impairment of colonic contractility both in vivo and in vitro. Improvement in function was associated with a reduction in magnitude of inflammatory cell infiltrate and with a decrease in transcription of genes encoding proinflammatory mediators IL-1β and monocyte chemotactrant protein (MCP)-1, inducible nitric oxide synthase, and cyclooxygenase-2. Furthermore, tyrphostin AG 126 pretreatment significantly inhibited activation of multifactorial transcription factor NF-κB, which could form the basis for reduction in proinflammatory mediator expression. These data demonstrate for the first time that inhibition of PTK activity may represent a novel approach for management of ileus in the clinical setting.

protein tyrosine kinase; smooth muscle; macrophage; monocyte chemoattractant protein-1; interleukin-1β

POSTOPERATIVE GASTROINTESTINAL dysmotility, or ileus, continues to be a common consequence of abdominal surgery, causing significant patient discomfort (intestinal stasis, abdominal distension, nausea, emesis), and often leads to more serious problems (acute gastric dilatation, aspiration, respiratory compromise, cardiac arrhythmia, perforation). Both experimental and clinical observations suggest that the extent and duration of ileus is dependent on the degree to which the bowel is disturbed during abdominal surgery (13, 19), with the most severe ileus and protracted recovery times occurring after procedures that directly involve the colon (36, 42). Indeed, the colon appears to be particularly sensitive to disturbances within the abdominal cavity, exhibiting recovery times twice that of the stomach and three times that of the small bowel, even when not directly involved in the surgical procedure (13, 41). The effective duration of ileus is, therefore, mainly dependent on the return of colonic motility, and clinical interventions designed to prevent or improve postoperative ileus must take into account the effects of such interventions on the colon.

Animal models of postoperative ileus have greatly increased our understanding of the mechanisms that underlie surgically induced intestinal dysmotility. Studies in our laboratory and others (12, 17, 19, 39) have shown that mild manipulation of the small bowel or colon initiates an inflammatory cascade within the gastrointestinal muscularis. This results in the activation of macrophages normally resident within the muscularis with the subsequent release of proinflammatory cytokines (IL-6, IL-1β) and chemokines [monocyte chemotactrant protein-1 (MCP-1)], expression of adhesion molecules (ICAM-1), and the recruitment of circulating leukocytes. Both resident and recruited leukocytes are important sources of prostanoids [derived from the inducible isoform of cyclooxygenase (COX-2)] and nitric oxide (NO) [derived from the inducible isoform of NO synthase (iNOS)], mediators that have direct inhibitory effects on intestinal smooth muscle contractility (8, 15, 18, 27, 35, 38). Suppression of contractile activity correlates temporally with macrophage activation and the onset of leukocyte infiltration into the intestinal muscularis (16). Therefore, interventions that attenuate inflammatory responses early in the inflammatory cascade may provide a means to reduce the expression of proinflammatory mediators, and subsequently leukocyte recruitment, serving to prevent or attenuate the development of postoperative ileus.

One early event in the induction of inflammatory pathways is the phosphorylation of proteins on tyrosine residues by protein tyrosine kinases (PTKs). Tyrosine phosphorylation is linked to the activation of cytosolic transcription factors, which leads to enhanced gene transcription and the production of proinflammatory mediators. PTKs play an integral role in this process, acting at multiple steps within signaling cascades. The enhanced activity of PTKs has been implicated in the pathophysiology of many diseases associated with local (arthritis, psoriasis, pleurisy, inflammatory bowel disease) or systemic (sepsis and septic shock) inflammation (3, 5, 6, 28, 31). Members of the tyrphostin family of PTK inhibitors have shown considerable promise for the treatment of inflammatory...
conditions, and tyrphostin AG 126, in particular, has demonstrated efficacy with low toxicity in the treatment of inflammatory diseases in other organ systems (3, 5, 6, 28, 31). Therefore, the aims of the present study were to determine whether inhibition of PTK activity would prevent or modulate the development of colonic postoperative ileus and to identify the mechanism by which this might occur. Functional and molecular techniques were used to assess the effects of the PTK inhibitor tyrphostin AG 126 on postoperative colonic smooth muscle dysmotility and on the proinflammatory pathways known to be involved in the development of postoperative ileus.

MATERIALS AND METHODS

Animals. C57BL/6 male mice (20–25 g) were from Harlan Sprague Dawley (Indianapolis, IN). The protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mice 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 U.S. Department of Agriculture and the Department of Health and Human Services. Mice were maintained on a 12:12-h light-dark cycle and were provided with commercially available rodent chow and tap water ad libitum.

Experimental groups and operative procedures. Mice were anesthetized with inhaled iso-flurane and then gently compressed along its entire length by rodent chow and tap water ad libitum.

Intracolonic pressure was recorded in vivo in awake, restrained mice (n = 6/experimental group) utilizing intracolonic balloon-tipped catheters fashioned from condom reservoir tips (3-cm length) and polyethylene PE-50 tubing attached to a Harvard minipump and a Statham pressure transducer to permit simultaneous balloon volume adjustment and pressure recording (30). Lubricated catheters were inserted analy and positioned with the balloon 2.5–3.0 cm from the anus. The animals were placed in a rodent restraining device (Harvard Apparatus, Holliston, MA) covered with a drape to minimize environmental stimulation. After a 1-h acclimatization period, intracolonic pressure was recorded for a period of 30 min. Contraction magnitude (area under the trace) and peak amplitude were analyzed for the entire 30-min recording period by using the MacLab data acquisition package (AD Instruments, Castle Hill, Australia).

Intestinal transit was measured in controls and manipulated animals 24 h postoperatively by evaluating the intestinal distribution of non-absorbable fluorescein-labeled dextran (70,000 molecular weight) fed orally (50 mg/kg) and the time required for the dye to reach the anus. Mice were killed, and the contents of the stomach, small bowel (divided into 10 equal segments), cecum, and colon (3 segments) were collected. The fluorescent signal in each sample was determined in duplicate by using a fluorescence plate reader. Data were expressed as the percentage of total fluorescence signal in each segment and plotted in a distribution histogram. For statistical analyses, gastrointestinal transit was calculated as the geometric center of the distribution of labeled dextran along the gastrointestinal tract (26).

In vitro circular muscle mechanical activity was measured as previously described (11). Briefly, mice were killed 24 h postoperatively when ileus is fully established (16). Muscle strips harvested from the midcolon were affixed to isometric force transducers (WPI, Sarasota, FL) and mounted in standard horizontal mechanical organ chambers that were continuously superfused with preoxygenated Krebs-Ringer bicarbonate buffer (KRB; in mM: 137.4 Na⁺, 5.9 K⁺, 2.5 Ca²⁺, 1.2 Mg²⁺, 134 Cl⁻, 15.5 HCO₃⁻, 1.2 H₂PO₄⁻, and 11.5 glucose), gassed with 97% O₂–3% CO₂ to establish a pH of 7.4, at 37°C. Tissues were equilibrated for 1 h and then incrementally stretched to an optimum length that produced the maximum spontaneous contractile amplitude, as described previously (19). Frequency of spontaneous contractions was determined by calculating the number of contractions over a 10-min sampling period. Tonic contraction response curves were generated by exposing the tissues to increasing concentrations of the muscarinic agonistbethanechol (0.3–300 μM) for 10 min with intervening 10-min washes with KRB. Contractile activity in response to bethanechol was calculated by integrating the area under the trace and normalizing the response to the tissue area (g·mm²·s⁻¹).

Proinflammatory gene expression. Colonic muscularis externa specimens were harvested 3 h postoperatively, a time point when inflammatory mediator expression associated with colonic ileus is well established (39). Total RNA extraction was performed by using the guanidium-thiocyanate phenol-chloroform extraction method as described previously (11). After treatment to remove potentially contaminating DNA (DNA-Free kit; Ambion, Austin, TX), aliquots of extracted RNA were quantified by spectrophotometry and diluted to generate RNA stock solutions containing 40 ng/μl total RNA. Primers were designed according to published sequences (β-actin; 29) and GenBank accession numbers by using Primer Express software (PE Applied Biosystems) and were summarized in Table 1. Gel electrophoresis was performed for each primer to confirm the absence of nonspecific bands and that the amplicons were of the correct size. Efficiency of PCR amplification of target cDNA was determined by
measuring colinearity of dilution. Serial threefold dilutions of target cDNA were performed in triplicate, and standard curves were generated by plotting cycle threshold (Ct) values vs. relative input copy number. Slopes of $-3.22 \pm 0.2$ ($r^2 = 0.99$) with corresponding efficiencies of 97–110% were considered to be acceptable.

Target mRNA levels were quantified in duplicate by SYBRgreen two-step, real-time RT-PCR by using SYBRgreen PCR Core Reagents (PE Applied Biosystems). Samples were estimated in duplicate by using the conditions recommended by the manufacturer, and data were plotted as the change in emission intensity of the fluorescence signal ($\Delta$Ct) vs. the cycle number. Quantification of mRNA expression was normalized to the $\beta$-actin endogenous reference gene and calculated relative to control by using the comparative Ct method as described by Schmittgen et al. (33) (see also User Bulletin #2, PE Applied Biosystems, Foster City, CA). To exclude PCR amplification of contaminating genomic DNA, RT-negative controls (samples containing RNA that were not reverse transcribed) were included in each PCR reaction. Melting curve analysis was performed for each PCR reaction to ensure amplification of a single product.

EMSA. Colonic muscularis was harvested 1.5 h postoperatively to detect the activation of the transcription factors, STAT protein and NF-κB. Binding reactions were carried out by using 20 μg of extracted protein. STAT proteins were detected by using the radiolabeled DNA-binding element high-affinity serum-inducible element (hSIE) duplex oligonucleotide. hSIE preferentially binds STAT1 and STAT3 to form protein-DNA complexes that consist of serum-inducible factor (SIF)-A (STAT3 homodimer), SIF-B (STAT1-STAT3 heterodimer), and SIF-C (Stat1 homodimer). NF-κB activation was assayed by using radiolabeled α-lysophosphatidylcholine (New England Nuclear) and NF-κB consensus oligonucleotides (Santa Cruz Laboratories, Santa Cruz, CA). EMSAs were performed on 4% nondenaturing polyacrylamide gels. Band intensities of the SIF-A and NF-κB oligonucleotide complexes were quantified by Phospholmage analysis or by densitometry.

Data analysis. Data were compiled as means ± SE. Statistical analysis was performed by using the unpaired Student’s t-test for single comparisons or ANOVA for multiple comparisons by using the Bonferroni post hoc test. Statistical significance was assumed at $P \leq 0.05$.

RESULTS

PTK activity. PTK activity within the colonic muscularis externa was measured 3 h postoperatively to determine if whether an increase in PTK activity occurred in response to surgical manipulation of the colon, and 2) whether tyrphostin AG 126 (15 mg/kg sc) administered in vivo was adequate to inhibit this activity. After surgical manipulation, PTK activity was significantly increased in the cytosolic fraction of muscularis tissue homogenates (Fig. 1). Pretreatment with tyrphostin AG 126 resulted in complete blockade of the surgically induced increase in cytosolic PTK activity, whereas vehicle had no effect. PTK activity in isolates of the membrane fraction proved to be below the detection limits of the assay and was not examined further.

Inflammatory cell infiltrate. Surgical manipulation of the small bowel (17) or colon (39) typically results in a massive cellular inflammatory response within the muscularis. The effect of tyrphostin AG 126 pretreatment on recruitment of MPO-positive leukocytes into the colonic muscularis of control and manipulated animals is shown in Fig. 2. In control mice, MPO-positive cells were rare (Fig. 2A). Colonic manipulation resulted in a massive increase in the number of MPO-positive cells infiltrating the muscularis 24 h postoperatively (Fig. 2B). This infiltrate was markedly reduced in animals pretreated with tyrphostin AG 126 (Fig. 2C) but not in those treated with vehicle (Fig. 2D). Modulation of the neutrophilic inflammatory response by tyrphostin AG 126 is summarized in Fig. 2E for statistical comparison. Infiltrate was reduced by 65% after treatment with tyrphostin AG 126. Tyrphostin AG 126 by itself had no effect on inflammatory cell infiltrate in unoperated mice compared with naïve controls.

Colonic contractility. To determine whether inhibition of PTKs would alter the functional manifestations of postsurgical ileus, effects of surgical manipulation and treatment with tyrphostin AG 126 on colonic contractility were assessed. Representative intracolonic pressure recordings from the distal colon obtained from control and surgically manipulated mice are shown in Fig. 3, A–C. A motility index generated by calculating integrated pressure per unit time (area under trace) for the five groups of mice is shown in Fig. 3F. The colon of control mice generated spontaneous low-amplitude pressure waves with superimposed larger phasic pressure waves of relatively short duration (amplitude = 84 ± 7 cmH2O, Fig. 3A; n = 6). Twenty-four hours after manipulation, this motility pattern was markedly changed with a significant reduction in

![Fig. 1. Pretreatment with tyrphostin AG 126 suppresses the surgically induced increase in muscularis protein tyrosine kinase (PTK) activity. PTK activity in cytosolic fractions of colonic muscularis extracts was significantly increased after colonic manipulation (CM) compared with control. Activity was completely inhibited by tyrphostin AG 126 treatment but not by vehicle. Data are means ± SE and are normalized per milligram of tissue wet weight. ∗Compares CM with controls; †compares CM + AG 126 with CM; ‡compares CM + vehicle with CM + AG 126; P < 0.001. n = 5 animals.](http://ajpgi.physiology.org/doi/10.22033/ajpgi.102301)
the amplitudes of the phasic waves (32 ± 8 cmH2O; Fig. 3B) and a decrease in the motility index (Fig. 3F). In tyrphostin AG 126-pretreated animals (Fig. 3C), the colon generated significantly more contractile activity compared with the untreated animals. These pressure patterns were relatively low in amplitude (46 ± 3 cmH2O) but of a longer duration compared with naive animals; however, the motility index was similar to control levels (Fig. 3F). Treatment with vehicle had no effect on the manipulation-induced changes in motility pattern (Fig. 3D) or motility index. Tyrphostin AG 126 treatment by itself did not significantly alter the amplitude of colonic pressure records of unoperated mice (82 ± 12 cmH2O/min; Fig. 3E) or the integrated motility index compared with naive controls (Fig. 3B).

Colonic ileus is characterized by delayed transit throughout the gastrointestinal tract (36, 42). Figure 4 summarizes the effects of tyrphostin AG 126 treatment on upper gastrointestinal transit measured 24 h postoperatively. Distribution histograms of the fluorescein-labeled dextran present in each bowel segment of naive control and surgically manipulated mice are plotted in Fig. 4A. In naive animals, labeled dextran was distributed primarily within the terminal small bowel, cecum, and proximal colon 90 min after oral ingestion. Colonic manipulation resulted in a decrease in upper gastrointestinal transit with accumulation of the label occurring within the distal half of the small intestine. Transit times were restored to normal in manipulated animals pretreated with tyrphostin AG 126 but not in those treated with vehicle (Fig. 4B). Tyrphostin AG 126 had no effect on transit in unoperated mice. Calculated geometric center results are summarized in Fig. 4C, in which higher values of geometric center indicate a more distal distribution of the fluorescent signal; i.e., more rapid transit.

Inflammatory events associated with surgical manipulation of the colon have been shown to inhibit spontaneous contractility of intestinal circular smooth muscle, as well as the capacity of the muscle to contract in response to muscarinic agonists (39). Effects of PTK inhibition on colonic circular muscle contractility in vitro are summarized in Fig. 5. Representative traces of spontaneous contractility of colonic circular muscle are shown in Figs. 5, A–E. Control midcolonic circular muscle strips generated high amplitude and rhythmic contractions with a mean contractile force of 1.1 ± 0.3 g at a frequency of 0.8 ± 0.2 min (Fig. 5A; n = 8). After colonic manipulation, the amplitude of spontaneous contractions was reduced by 82% (0.3 ± 0.1 g). Contractile frequency appeared normal at times but often degenerated into periods of irregular contractile activity or periods of no activity that persisted for several minutes (compare Fig. 5, B and D). Overall contraction frequency declined to 0.3 ± 0.1 contractions/min. Treatment with tyrphostin AG 126 attenuated the postoperative reduction in contractile force (0.8 ± 0.2 g) and maintained spontaneous rhythmicity (0.7 ± 0.2 min, Fig. 5C), whereas treatment with vehicle had no effect (Fig. 5D; contractile force 0.2 ± 0.1 g; frequency 0.2 ± 0.1 min). Treatment of unoperated mice with tyrphostin AG 126 (Fig. 5E) had no effect on contractile force (1.2 ± 0.4 g) or frequency (0.7 ± 0.2 min).

Fig. 2. Tyrphostin AG 126 pretreatment reduces the surgically induced increase in the number of leukocytes infiltrating the colonic muscularis. A–D: photomicrographs of muscularis whole mounts stained for polymorphonuclear leukocytes exhibiting MPO activity. A: only a few weakly positive cells were found in whole mounts from unoperated control mice (arrowheads). B: whole mount from a mouse having undergone CM showing a massive increase in the numbers of infiltrating MPO-positive cells 24 h postoperatively. C: treatment with tyrphostin AG 126 led to a reduction in the number of infiltrating cells, whereas treatment with vehicle had no effect (D). E: histogram summarizing the number of MPO-positive cells counted in muscularis whole mounts for the 4 experimental groups. The number of infiltrating cells was increased 90-fold after CM compared with control. Tyrphostin AG 126 treatment but not vehicle resulted in a 65% decrease in the number of infiltrating cells. Differences between CM and CM + vehicle were not significant. Data are means ± SE, n = 6 animals. *Compares CM with controls; †compares CM + AG 126 with CM; ‡compares CM + vehicle with CM + AG 126; P < 0.01.
Fig. 3. Pretreatment with tyrphostin AG 126 attenuates the surgically induced impairment of colonic contractility in vivo. A–E: representative traces from intracolonic pressure recordings. A: pressure recordings from an unoperated control mouse showing spontaneous low-amplitude pressure waves with superimposed larger phasic pressure waves of relatively short duration. B: pressure magnitude is markedly reduced after CM compared with control. C: in manipulated mice treated with tyrphostin AG 126, contraction amplitude and duration of the contractions are increased, but this is not the case in animals treated with vehicle (D). E: tyrphostin AG 126 treatment alone had no effect on contractility in control animals. F: motility index generated by integration of contractile area under the pressure curves. Motility index was significantly reduced after CM but restored to control levels in animals treated with tyrphostin AG 126. Differences between CM and CM + vehicle were not significant. Data are means ± SE, n = 6 animals. *compares effects of CM relative to control; †compares CM + AG 126 with CM; ‡compares CM + vehicle with CM + AG 126; P < 0.01.

The addition of bethanechol (0.3 to 300 μM) to the superfuse elicited tonic contractions with magnitudes that were concentration dependent. Complete bethanechol-stimulated dose-response curves for colonic circular muscle are shown in Fig. 5F. Surgical manipulation led to a ~50% reduction in the capacity of the muscle to contract in response to bethanechol. Pretreatment with tyrphostin AG 126, but not vehicle, completely prevented the postsurgical impairment of contractility. Responses from unoperated animals treated with tyrphostin AG 126 were not different from naive controls (not shown). Figure 5G summarizes peak contractile responses to 100 μM bethanechol for the five experimental groups for statistical comparison.

Proinflammatory gene expression. We demonstrated previously that proinflammatory mediators are upregulated early during colonic postoperative ileus (39), some of which play a role in leukocyte recruitment. Therefore, the effect of tyrphostin AG 126 on proinflammatory mediator mRNA expression was evaluated by using SYBRgreen real-time RT-PCR. Figure 6 shows that IL-6 and IL-1β gene expressions were increased 1,260-fold and 100-fold, respectively, in manipulated mice relative to unoperated controls. IL-1β mRNA expression was reduced by 30% to 70% in mice pretreated with tyrphostin AG 126. Tyrphostin AG 126 tended to decrease the expression of IL-6 message, but this did not reach statistical significance. The expression of MCP-1, a chemokine that plays an important role in the targeted transmigration of immunocompetent cells into the small intestinal and colonic muscularis (16, 40), was increased 256-fold after colonic manipulation and reduced by 43% to 145-fold in animals treated with tyrphostin AG 126. Expression of ICAM-1, an adhesion molecule that promotes immune cell adhesion to the vascular endothelium before targeted transmigration, was increased 23-fold, and this response was not altered by treatment with tyrphostin AG 126. Leukocytes, both resident within the intestinal muscularis and those recruited during inflammatory events, synthesize and secrete mediators derived from the induction of COX-2 and iNOS that have potent inhibitory effects on intestinal smooth muscle contractility. COX-2 and iNOS expression after colonic manipulation were increased 4.5-fold and 10-fold, respectively. Pretreatment with tyrphostin AG 126 inhibited the induction of these mediators by 45 and 85%, respectively. In all cases, tyrphostin AG 126 pretreatment had no effect on basolateral mediator expression in unoperated animals, nor did treatment of manipulated animals with vehicle alter surgically induced increases in mediator expression.

Transcription factor activation. Initiation of the proinflammatory events associated with postoperative ileus has been shown to involve the activation of both NF-κB and the Janus kinase (JAK)/STAT signaling pathway with enhanced induction of STAT3. The effects of colonic manipulation and tyrphostin AG 126 pretreatment on transcription factor activation are shown in Fig. 7. Muscularis extracts from mice that underwent colonic manipulation demonstrated a significant increase in NF-κB band intensity compared with controls (Fig. 7A). PhospholImager analysis (Fig. 7B) showed that tyrphostin AG 126 treatment decreased postsurgical activation of NF-κB by ~50% (n = 4 animals). Activation of STAT3 homodimers (SIF-A) also was significantly elevated in the manipulated colon compared with control (Fig. 7C). Densitometer analysis showed that pretreatment with tyrphostin AG 126 did not result
that consists of the induction of proinflammatory cytokines, the massive recruitment and extravasation of inflammatory leukocytes into the muscularis, and the release of mediators that directly inhibit smooth muscle contractility (17, 35, 38). In the present study, we hypothesized that the inhibition of protein tyrosine phosphorylation, an important process occurring at key points in the initiation of inflammatory pathways, would attenuate the development of ileus. Findings reported here demonstrate that pretreatment with the PTK inhibitor tyrphostin AG 126 1 h before surgery significantly attenuated the molecular and cellular inflammatory responses associated with postoperative ileus and that PTK inhibition subsequently prevented the surgically induced impairment of gastrointestinal motility.

Infiltrating leukocytes and the mediators they release play a central role in the development of intestinal smooth muscle dysmotility that accompanies intestinal inflammation (17, 39) whereby the time course and magnitude of the cellular infiltrate correlates with the onset and degree to which intestinal contractility is impaired (16). In the present study, as predicted, surgical manipulation of the colon resulted in a marked increase in the number of infiltrating leukocytes measured 24 h postoperatively, with slowing of gastrointestinal transit and impairment of colonic contractility. Treatment of mice with a single dose (15 mg/kg) of tyrphostin AG 126, sufficient to block the surgically induced increases in PTK activity, led to a 65% reduction in the number of leukocytes infiltrating the colonic muscularis. This reduction in infiltrate was associated with a marked improvement in the contractile function of the colonic muscularis; indeed, both gastrointestinal transit in vivo and colonic contractility in vitro were not different from those of naive controls. However, qualitative differences in the colonic contractile pattern in vivo remained in manipulated animals treated with tyrphostin AG 126. Intracolonic pressure recordings demonstrated that, although the overall capacity of the manipulated colon to generate contractile pressure per unit time was similar to controls, differences in the magnitude and duration of the contractions persisted (see Fig. 3). It has been suggested from studies of dissociated smooth muscle cells in vitro that PTK inhibitors, including tyrphostins, can directly inhibit smooth muscle protein phosphorylation and thus alter contractile activity (9, 43). Such a process could potentially account for the altered contractile pattern observed in vivo. However, our results would argue against this notion, because the contractile pattern, peak amplitude, and contractile pressure (motility index) in unoperated mice treated with tyrphostin AG 126 were not different from naive controls. Furthermore, tyrphostin AG 126 pretreatment had no effect on spontaneous and bethanechol-induced contractile activity in vitro in circular smooth muscle strips harvested from unoperated mice. Therefore, it is unlikely that the single bolus of tyrphostin AG 126 given before laparotomy had significant effects on the smooth muscle cell contractile apparatus 24 h later. A more likely scenario is that inflammatory events after abdominal surgery were not completely restored to control levels by PTK inhibition.

We have proposed that molecular inflammatory events leading to the expression of cytokines, chemokines, and adhesion molecules form the basis for the targeted transmigration of circulating leukocytes into the intestinal muscularis (1, 11, 14, 14a, 40). Real-time RT-PCR analyses demonstrated that, as

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**DISCUSSION**

Postoperative ileus is, at least in part, caused by the initiation of an inflammatory cascade within the intestinal muscularis

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![Graph A](image1.png)

**A**

![Graph B](image2.png)

**B**

![Graph C](image3.png)

**C**

**Fig. 4.** Tyrphostin AG 126 prevents the surgically induced delay in intestinal transit. **A**: median histogram comparing the distribution of nonabsorbable fluorescein-labeled dextran along the gastrointestinal tract 1.5 h after oral administration in control mice and in mice subjected to CM. In control mice, marker accumulated in the distal small bowel, cecum, and proximal colon. CM caused a significant delay in intestinal transit with marker accumulating in the mid small bowel. **B**: histogram comparing distribution of dextran in manipulated mice treated with tyrphostin AG 126 or vehicle. Note that distribution of label in manipulated animals treated with tyrphostin AG 126 is comparable to that observed in control mice in A (solid bars). **C**: summary of the calculated geometric center for the 4 experimental groups. Geometric center was significantly reduced after CM and restored to control values after treatment with tyrphostin AG 126 but not by vehicle. Differences between CM and CM + vehicle were not significant. Data are means ± SE, n = 6 animals. *Compares CM vs. control; †compares CM + AG 126 with CM; ‡compares CM + vehicle with CM + AG 126; P < 0.01. Stom, stomach; sb, small bowel; col, colon.

in a significant reduction in STAT3 band intensity relative to the untreated manipulated group (n = 4 animals).
predicted, the levels of IL-6, IL-1β, ICAM-1, and MCP-1 were elevated 3 h postoperatively in muscularis extracts harvested from mice that had undergone colonic manipulation. Pretreatment with tyrphostin AG 126 significantly reduced IL-1β and MCP-1 mRNA expression but did not have a significant effect on IL-6 or ICAM-1 message 3 h postoperatively, a time point when message expression for these mediators is well established (39). Leukocyte adhesion through expression of ICAM-1 and targeted transmigration in response to MCP-1 play important roles in intestinal leukocyte recruitment, and blockade of either mechanism significantly attenuates cellular inflammation and the associated intestinal smooth muscle dysfunction (17, 40). These results suggest that tyrphostin AG 126 reduces the cellular inflammatory response by inhibiting the targeted migration of leukocytes into the intestinal muscularis through reduced expression of MCP-1.

We and others (8, 15, 18, 27, 35, 38) have demonstrated that release of prostaglandins (derived from COX-2) and NO (derived from iNOS) from both activated resident macrophages and recruited leukocytes have potent inhibitory effects on intestinal smooth muscle contractility. RT-PCR analyses in the present study demonstrated a significant increase in iNOS and COX-2 message 3 h postoperatively. Pretreatment with tyrphostin AG 126 resulted in a 50% reduction in COX-2 message and the complete prevention of iNOS induction. We have demonstrated that blockade of either COX-2 or iNOS activity, whether pharmacologically or through gene deletion, significantly ameliorates postoperative ileus arising from surgeries involving the small bowel (18, 34). Although the mechanism is not completely understood, it has been proposed from studies using LPS-stimulated murine macrophages that NO derived from iNOS can stimulate COX-2-dependent prostaglandin syn-

Fig. 5. Tyrphostin AG 126 prevented the surgically induced impairment of colonic circular smooth muscle contractility in vitro. A–E: representative traces showing effects of tyrphostin AG 126 on spontaneous contractility. A: contractility pattern typical of unoperated controls. B: after CM, the amplitude of spontaneous contractions was markedly reduced and often degenerated into periods of irregular activity lasting for several minutes (area between arrowheads). Spontaneous contractility was improved in animals pretreated with tyrphostin AG 126 (C) but not in animals treated with vehicle (D). E: tyrphostin AG 126 had no effect on contractility in unoperated control mice. F: bethanechol dose-response curves demonstrating effects of tyrphostin AG 126 pretreatment on the surgically induced impairment in the capacity of colonic smooth muscle to contract in response to muscarinic agonists. After CM, contractions in response to increasing concentrations of bethanechol (0.3–300 μM) were markedly impaired. Magnitude of the bethanechol-stimulated contractile response in animals subjected to CM was reduced by ~50% throughout the concentration-response curve (○). Contractile responses were restored to control levels in mice treated with tyrphostin AG 126 but not with vehicle. G: summary of contractility data showing peak contractile response at 100 μM bethanechol. Peak response was reduced by 50% after CM but was restored to control levels by treatment with tyrphostin AG 126. Differences between CM and CM + vehicle were not significant. Data are means ± SE, n = 8 animals per group. *Compares CM vs. control; †compares CM + AG 126 with CM; ‡compares CM + vehicle with CM + AG 126; P < 0.01.
thesis and that inhibition of NO production also reduces prostaglandin release (32). In the rodent colon, pharmacological blockade in vitro of COX-2- and iNOS-dependent inhibition of smooth muscle contractility demonstrated that inhibition of COX-2 resulted in little improvement and that iNOS activity played the predominant role (39). Inhibition by tyrphostin AG 126 of COX-2 and the complete blockade of iNOS would attenuate both of these smooth muscle inhibitory pathways. The resulting decline in NO release could potentially lead to a further reduction in COX-2-dependent prostaglandin synthesis. It is also important to note that reduced expression of COX-2 and iNOS message was not merely a reflection of the decreased cellular inflammatory infiltrate, because leukocyte extravasation into the muscularis develops postoperatively after 4–6 h (16). Thus it can be deduced that the reduction in the smooth muscle kinetically active mediators generated by iNOS and COX-2 at the 3-h time point was primarily from resident leukocytes. However, we assume that NO and prostaglandin production were also reduced secondarily as a result of the decreased ability of circulating leukocytes to target to the colonic muscularis in tyrphostin AG 126-treated mice and that this would contribute to the improved contractile function observed 24 h postlaparotomy.

Target specificity of tyrphostin AG 126 and thus the mechanism by which it might exert its protective effects against inflammation have not been completely identified. Induction of inflammatory mediator expression occurs through the activation of numerous transcription factors (for example, NF-IL6, STAT proteins, and NF-kB) via multiple signaling pathways. The relative contribution of each pathway to proinflammatory mediator production is dependent on species, cell type, and the proinflammatory stimulus. All are dependent on protein phosphorylation at specific points within their activation cascades, and those pathways requiring tyrosine phosphorylation are potential targets for inhibition by tyrphostin AG 126. NF-kB activation requires tyrosine phosphorylation during phosphorylation-dependent ubiquitination of the IkB-NF-kB suppressor complex within the cytosol (4). It is clear from a large body of literature concerning other systems and cell types that IL-1β, either alone or in combination with other proinflammatory cytokines, can initiate the transcription of MCP-1, COX-2, and iNOS (4) and that NF-κB activation plays a significant role in these processes (21, 22, 37), including the transcriptional regulation of the IL-1β gene itself (44). Indeed, tyrphostin AG 126 has been shown to inhibit the production of IL-1β, COX-2, and iNOS in vivo in other models of local and systemic inflammation (5, 6, 10, 23). Thus inhibition of the NF-κB signaling pathway is a potential mechanism by which tyrphostin AG 126 reduced the surgically induced inflammatory responses observed in the present study during the development of colonic ileus. This hypothesis was supported by EMSA gel analysis showing that NF-κB activation was reduced by 50% in
manipulated mice pretreated with tyrphostin AG 126. By reducing NF-xB activation, tyrphostin AG 126 could act on proinflammatory mediator expression directly by inhibiting the upregulation of MCP-1, COX-2, and iNOS mRNA and/or indirectly by inhibiting IL-1B expression and subsequently IL-1B-mediated induction of MCP-1, COX-2, and iNOS expression.

IL-6-mediated proinflammatory processes are less dependent on NF-xB activation and also rely on JAK/STAT signaling (2). The JAKs kinases constitute a family of receptor-associated tyrosine kinases that, when tyrosine phosphorylated, provide docking sites for a variety of STAT proteins that are in turn tyrosine phosphorylated. STAT3 activation, in particular, has been linked to ICAM-1, MCP-1, COX-2, and iNOS induction (24, 25). Tyrophostins such as the specific JAK-2 inhibitor AG 490 are effective inhibitors of JAK signaling (7). In the present study, surgically induced STAT3 activation was unaffected by tyrphostin AG 126 pretreatment. Limitations associated with the PTK assay did not permit direct analysis of membrane-associated tyrosine kinases, and a possible effect of tyrphostin AG 126 on JAKs in this system cannot be ruled out completely. However, others (28) have shown that tyrphostin AG 126 exhibits little efficacy at receptor tyrosine kinases. Thus the continued activity of STAT3 transcription factors would result in a persistent level of proinflammatory signaling. This may account for the failure of tyrphostin AG 126 pretreatment to significantly alter the surgically induced increases in IL-6 and ICAM-1 gene expression and most likely accounts for the incomplete inhibition of MCP-1 and COX-2 expression. It then becomes clear that persistent abnormalities in colonic smooth muscle contractility patterns in vivo after treatment with tyrphostin AG 126 can be explained in terms of ongoing residual inflammatory processes. Nevertheless, functional experiments demonstrate that colonic contractility was significantly improved in tyrphostin AG 126-treated mice and that despite incomplete recovery of the normal colonic contractile pattern in vivo, gastrointestinal transit was restored to control levels.

It is evident from the results reported here that complete blockade of cytosolic PTK activity by tyrphostin AG 126 does not result in a corresponding blockade of all proinflammatory signaling, as determined at the level of gene transcription. Signaling pathways dependent on receptor tyrosine phosphorylation and those dependent on phosphorylation of proteins other than tyrosine are likely to continue. Furthermore, complex interactions between signaling pathways can occur at the level of protein synthesis and enzyme activity. An in-depth analysis of these interactions was beyond the scope of this article, and further study is warranted to understand the full extent of the effects of PTK inhibition on inflammatory signaling in postoperative ileus.

In conclusion, this study demonstrates that colonic dysmotility caused by surgical manipulation was significantly improved by treatment of mice with the PTK inhibitor tyrphostin AG 126. We propose that the protective effects of tyrphostin AG 126 against the development of colonic ileus are largely due to the inhibition of NF-xB activation, resulting in the reduced expression of the proinflammatory mediators IL-1B and MCP-1 and subsequent amelioration of cellular inflammation. In addition, inhibition of COX-2 induction and the complete blockade of iNOS induction, enzymes that synthesize mediators that directly inhibit smooth muscle contractility, contribute significantly to the improvement in colonic dysmotility. Inhibitors of PTK such as tyrphostin AG 126 can provide new insights into the mechanisms of proinflammatory signaling in postoperative ileus, leading to the identification of new targets for the development of drugs useful for the management of ileus in the clinical setting.

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