Tyrphostin AG126 Inhibits the Development of Postoperative Ileus Induced by Surgical Manipulation of the Murine Colon

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Running Title: Tyrosine Kinase Inhibition and Postoperative Ileus


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Abstract

Background: 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 pro-inflammatory 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. The phosphorylation of tyrosine residues on regulatory proteins by protein tyrosine kinases (PTK’s) occurs at multiple steps in the signaling cascades that regulate the expression of pro-inflammatory genes. Aim: To determine whether inhibition of PTK activity will attenuate the inflammatory response associated with colonic ileus, leading to improved function. Methods & Results: 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 s.c.) prior to surgery significantly attenuates the surgically induced impairment of colonic contractility both in vivo and in vitro. The improvement in function was associated with a reduction in the magnitude of the inflammatory cell infiltrate and with a decrease in the transcription of the genes encoding the pro-inflammatory mediators interleukin (IL)-1β, monocyte chemo attractant protein (MCP)-1, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. Furthermore, Tyrphostin AG-126 pretreatment significantly inhibited the activation of the multifactorial transcription factor NF-κB, which could form the basis for the reduction in pro-inflammatory mediator expression. Conclusions: These data demonstrate for the first time that inhibition of protein tyrosine kinase activity may represent a novel approach for the management of ileus in the clinical setting.
Introduction

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 post-operative ileus have greatly increased our understanding of the mechanisms that underlie surgically induced intestinal dysmotility. Studies in our laboratory and others 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 pro-inflammatory cytokines (IL-6, IL-1β) and chemokines (MCP-1), expression of adhesion molecules (ICAM-1) and the recruitment of circulating leukocytes (12; 17; 19; 39). Both resident and recruited leukocytes are important sources of prostaglandins (derived from the inducible isoform of cyclooxygenase, COX-2) and nitric oxide (derived from the inducible isoform of nitric oxide
synthase, iNOS), mediators that have direct inhibitory effects on intestinal smooth muscle contractility (8; 15; 18; 27; 35; 38). The 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 pro-inflammatory 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 (PTK’s). Tyrosine phosphorylation is linked to the activation of cytosolic transcription factors, which leads to enhanced gene transcription and the production of pro-inflammatory mediators. PTK’s play an integral role in this process, acting at multiple steps within signaling cascades. The enhanced activity of PTK’s has been implicated in the pathophysiology of many diseases that are associated with local (arthrosclerosis, 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 AG126, 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 current study were to determine whether inhibition of protein tyrosine kinase 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 protein tyrosine kinase inhibitor AG126 on postoperative colonic smooth muscle dysmotility and
on the pro-inflammatory pathways known to be involved in the development of post-operative ileus.

**Materials and Methods**

**Animals**

C57Bl6 male mice (20-25 g) were from Harlan (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 of 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-hour light/dark cycle and provided with commercially available rodent chow and tap water *ad libitum*.

**Experimental Groups and Operative Procedures**

Mice were anesthetized with inhaled isoflurane (IsoFlo - North Chicago, IL) and the abdomen opened by midline laparotomy. The colon was eventrated and then gently compressed along its entire length using sterile, moist, cotton applicators in a manner designed to simulate the manipulation of the colon that occurs clinically during abdominal surgery. Animals were divided into five experimental groups: (1) colonic manipulation without further intervention, (2) injection with 15 mg/kg sub-cutaneous tyrphostin AG126 (Sigma-Aldrich, St. Louis, MO) 1 hour prior to colonic manipulation, (3) identical to the second except that animals were injected with vehicle (60 µl of 50% DMSO in sterile saline), (4) age-matched un-operated control receiving no intervention, and (5) un-operated control injected with AG-126.
Dose selection for AG126 (5 to 15 mg/kg) resulted from preliminary experiments using gastrointestinal transit in vivo and circular muscle contractility in vitro as experimental endpoints (data not shown). Optimal effects on gastrointestinal function were achieved with a subcutaneous injection of 15 mg/kg AG126.

**Protein Tyrosine Kinase Activity**

PTK activity was assayed in extracts of colonic muscularis externae in order to ensure that the dose of AG126 (15 mg/kg) found to provide the maximum protection against colonic dysmotility was also sufficient to block the surgically induced increase in protein tyrosine kinase (PTK) activity. The muscularis externa was harvested from control animals, and 4 hours post laparotomy from animals that underwent colonic manipulation. Tissues were immediately homogenized in ice cold 20 mM Tris.HCl buffer, pH 7.5, containing 10 mM EGTA, 2 mM EDTA, 2 mM activated sodium vanadate and protease inhibitors (1 mM PMSF, 50 µg/ml leupeptin, 25 µg/ml aprotinin, 10 µg/ml pepstatin A, and 2 mM dithiothreitol). Membrane and cytosolic fractions were separated by centrifugation. Cytosolic proteins were concentrated 50X by centrifugation through 3,000 MW cut-off filters (Amicon). PTK activity was determined in duplicate using a commercially available PTK assay kit (Sigma), according to the manufacturer’s instructions. Units of PTK activity were calculated from standard curves generated from known quantities of epidermal growth factor receptor (EGF-R). Results were normalized to tissue wet weight.
Myeloperoxidase (MPO) Histochemistry

Muscularis whole-mounts were prepared from the mid-colon collected 24 hr postoperatively as described previously (17). Paraformaldehyde-fixed tissues were treated with Hanker-Yates reagent (Polysciences, Inc., Warrington, PA) for detection of polymorphonuclear cells (PMN’s) exhibiting myeloperoxidase (MPO) activity and inspected using light microscopy. PMN infiltrate was determined as the mean number of MPO positive cells counted in five adjacent 200X optical fields centered between the mesenteric and anti-mesenteric borders.

Functional Studies

Intra-colonic pressure was recorded in vivo in awake, restrained mice (n=6/experimental group) utilizing intra-colonic balloon-tipped catheters fashioned from condom reservoir tips (1 cm length) and PE50 tubing which was attached to a Harvard mini-pump and a Statham pressure transducer to permit simultaneous balloon volume adjustment and pressure recording (30). Lubricated catheters were inserted anally 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. Following a 1 hr acclimatization period, intra-colonic 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 using the MacLab data acquisition package (AD Instruments Pty. Ltd., Castle Hill, Australia).

Intestinal transit was measured in controls and manipulated animals 24 hours postoperatively by evaluating the intestinal distribution of non-absorbable fluorescein-labeled dextran (molecular weight = 70,000) fed orally (100 μl of 25 mg/ml stock solution). Ninety
minutes after administration, animals were sacrificed, 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 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 (GC) 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 sacrificed 24 hours postoperatively when ileus is fully established (16). Muscle strips harvested from the mid colon were affixed to isometric force transducers (WPI, Sarasota, FL) and mounted in standard horizontal mechanical organ chambers that were continuously superfused with pre-oxygenated Krebs-Ringer bicarbonate buffer (KRB: in mM: 137.4 Na\(^+\), 5.9 K\(^+\), 2.5 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 134 Cl\(^-\), 15.5 HCO\(_3\)\(^-\), 1.2 H\(_2\)PO\(_4\)\(^-\), and 11.5 glucose), gassed with 97% O\(_2\)/3% CO\(_2\) to establish a pH of 7.4, at 37°C. Tissues were equilibrated for 1 hour and then incrementally stretched to an optimum length which 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-minute sampling period. Tonic contraction response curves were generated by exposing the tissues to increasing concentrations of the muscarinic agonist bethanechol (0.3 to 300 \(\mu M\)) for 10 minutes with intervening 10-minute 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/mm\(^2\)/sec).
Pro-Inflammatory Gene Expression

Colonic muscularis externa specimens were harvested 3 hours post-operatively, a time point when inflammatory mediator expression associated with colonic ileus is well established (39). Total RNA extraction was performed using the guanidium-thiocyanate phenol-chloroform extraction method as described previously (11). After treatment to remove potentially contaminating DNA (DNA-Free Kit, Ambion Inc., Austin), 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 using Primer Express software (PE Applied Biosystems), and are summarized in Table 1. Gel electrophoresis was performed for each primer to confirm the absence of non-specific bands and that the amplicons were of the correct size. Efficiency of PCR-amplification of target cDNA was determined by measuring co-linearity of dilution: Serial 3-fold dilutions of target cDNA were performed in triplicate, and standard curves were generated by plotting C_T values versus 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 SYBR Green two-step, real-time RT-PCR using SYBR Green PCR Core Reagents (PE Applied Biosystems). Samples were estimated in duplicate using the conditions recommended by the manufacturer, and data were plotted as the ΔR_0 fluorescence signal versus the cycle number. Quantification of mRNA expression was normalized to the β-actin endogenous reference gene and calculated relative to control using the comparative C_T 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.

**Electrophoretic Mobility Shift Assay (EMSA)**

Colonic muscularis was harvested 1.5 hr postoperatively to detect the activation of the transcription factors, signal transducer and activator of transcription (STAT) protein and nuclear factor-κB (NFκB). Binding reactions were carried out using 20 μg of extracted protein. STAT proteins were detected using the radio-labeled DNA-binding element hSIE (high affinity serum inducible element) duplex oligonucleotide. hSIE preferentially binds STAT1 and STAT3 to form a protein-DNA complexes that consist of serum-inducible factor (SIF)-A (STAT3 homodimer), SIF-B (STAT1-STAT3 heterodimer) and SIF-C (Stat1 homodimer). Nuclear factor-κB activation was assayed using radio-labeled α-dCTP (New England Nuclear, now Perkin-Elmer) and NFκB concensus oligonucleoties (Santa Cruz Laboratories, CA). EMSA were performed on 4% non-denaturing polyacrylamide gels. The band intensities of the SIF-A and NF-κB oligonucleotide complexes were quantified by Phospho-Imager analysis or by densitometry.

**Data Analysis**

Data were compiled as mean ± standard error of the mean. Statistical analysis was performed using the unpaired Student’s t test for single comparisons or analysis of variance (ANOVA) for multiple comparisons using the Bonferonni post-hoc test. Statistical significance was assumed at $P \leq 0.05$. 
RESULTS

Protein Tyrosine Kinase Activity

Protein tyrosine kinase (PTK) activity within the colonic muscularis externa was measured 3 hr postoperatively to determine; first, whether an increase in PTK activity occurred in response to surgical manipulation of the colon, and second, whether tyrphostin AG126 (15 mg/kg s.c.) administered in vivo was adequate to inhibit this activity. Following surgical manipulation, PTK activity was significantly increased in the cytosolic fraction of muscularis tissue homogenates (Figure 1). Pretreatment with tyrphostin AG126 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 AG126 pretreatment on recruitment of myeloperoxidase (MPO)-positive leukocytes into the colonic muscularis of control and manipulated animals is shown in Figure 2. In control mice, MPO+ cells were rare (Fig. 2A). Colonic manipulation resulted in a massive increase in the number of MPO+ cells infiltrating the muscularis 24 hr postoperatively (Fig. 2B). This infiltrate was markedly reduced in animals pretreated with AG126 (Fig. 2C) but not in those treated with vehicle (Fig. 2D). Modulation of the neutrophilic inflammatory response by AG126 is summarized in Fig. 2E for statistical comparison. Infiltrate was reduced by 60% after treatment with AG126. AG126 by
itself had no effect on inflammatory cell infiltrate in unoperated mice compared to naïve controls.

**Colonic Contractility**

In order to determine whether inhibition of PTK’s would alter the functional manifestations of postsurgical ileus, the effects of surgical manipulation and treatment with tyrphostin AG126 on colonic contractility were assessed. Representative intracolonic pressure recordings from the distal colon obtained from control and surgically manipulated mice are shown in Figure 3A-C. A motility index generated by calculating integrated pressure per unit time (area under trace) for the five groups of mice is shown in Figure 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 cm H₂O, Fig. 3A; n=6). Twenty-four hours after manipulation, this motility pattern was markedly changed with a significant reduction in the amplitudes of the phasic waves (32 ± 8 cm H₂O; Fig. 3B) and a decrease in the motility index (Fig. 3F). In AG126 pretreated animals (Fig. 3C) the colon generated significantly more contractile activity compared to the untreated animals. These pressure patterns were relatively low in amplitude (46 ± 3 cm H₂O) but of a longer duration compared to naïve 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 AG126 treatment by itself did not significantly alter the amplitude of colonic pressure records of un-operated mice (82 ± 12 cm H₂O/min; Fig. 3E) or the integrated motility index compared to naïve controls (Fig. 3B).
Colonic ileus is characterized by delayed transit throughout the gastrointestinal tract (36; 42). Figure 4 summarizes the effects of tyrphostin AG126 treatment on upper gastrointestinal transit measured 24 hours 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 minutes after oral ingestion. Colonic manipulation resulted in a decrease in upper gastrointestinal transit, with accumulation of the label occurring within the distal 1/2 of the small intestine. Transit times were restored to normal in manipulated animals pretreated with AG126 but not in those treated with vehicle (Fig. 4B). AG-126 had no effect on transit in unoperated mice. Calculated geometric center (GC) results are summarized in Fig. 4C, whereby higher values of GC 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). The effects of PTK inhibition on colonic circular muscle contractility \textit{in vitro} are summarized in Figure 5. Representative traces of spontaneous contractility of colonic circular muscle are shown in Figures 5A-E. Control circular mid-colonic muscle strips generated high amplitude, rhythmic contractions with a mean contractile force of 1.1±0.3 g at a frequency of 0.8±0.2 min\(^{-1}\) (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. 5B and 5D). Overall contraction frequency declined to 0.3±0.1 contractions min\(^{-1}\).
Treatment with AG-126 attenuated the postoperative reduction in contractile force (0.8±0.2 g) and maintained spontaneous rhythmicity (0.7±0.2 min⁻¹, Fig. 5C); whereas treatment with vehicle had no effect (Fig. 5D; contractile force 0.2±0.1 g; frequency 0.2±0.1 min⁻¹). Treatment of unoperated mice with Tyrphostin (Fig. 5E) had no effect on contractile force (1.2±0.4 g) or frequency (0.7±0.2 min⁻¹).

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

Pro-Inflammatory 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 using SYBR green real-time RT-PCR. Figure 6 shows that IL-6 and IL-1β gene expression was increased 1260-fold and 100-fold, respectively, in manipulated mice relative to unoperated controls. IL-1β mRNA expression was reduced by 30% to 70-fold in mice pretreated with tyrphostin AG-126. 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 immuno-competent 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 AG126. ICAM-1 expression, an adhesion molecule that promotes immune cell adhesion to the vascular endothelium prior to targeted transmigration, was increased 23-fold and this response was not altered by treatment with AG126. 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 AG126 inhibited the induction of these mediators by 45% and 85%, respectively. In all cases, tyrphostin pretreatment had no effect on basal 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 NFκB, and the JAK/STAT signaling pathway with enhanced induction of STAT3. The effects of colonic manipulation and tyrphostin pretreatment on transcription factor activation are shown in Figure 7. Muscularis extracts from mice that underwent colonic manipulation demonstrated a significant increase in NF-κB band intensity compared to controls (Fig. 7A). Phospho-Imager analysis (Fig. 7B) showed that tyrphostin treatment decreased postsurgical activation of NF-κB by approximately 50% (n=4). Activation of STAT3 homodimers (SIF-A) also was significantly elevated in the manipulated colon.
compared to control (Fig. 7C). Densitometer analysis showed that pretreatment with tyrphostin, did not result in a significant reduction in STAT3 band intensity relative to the untreated manipulated group (n=4).
DISCUSSION

Postoperative ileus is at least in part caused by the initiation of an inflammatory cascade within the intestinal muscularis that consists of the induction of pro-inflammatory 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 current 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. The findings reported here demonstrate that pretreatment with the protein tyrosine kinase inhibitor, tyrphostin AG126, one-hour prior to surgery significantly attenuated the molecular and cellular inflammatory responses that are 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 current study, as predicted, surgical manipulation of the colon resulted in a marked increase in the number of infiltrating leukocytes measured 24 hr postoperatively, with slowing of gastrointestinal transit and impairment of colonic contractility. Treatment of mice with a single dose (15 mg/kg) of tyrphostin AG126, sufficient to block the surgically induced increases in PTK activity, led to a 60% 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 naïve controls. However, qualitative differences in the colonic contractile pattern in vivo remained in manipulated animals treated with AG126. Intracolonic pressure recordings demonstrated that, whereas 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). Pressure recordings from control mice demonstrated high amplitude, short duration contractions that were virtually absent after surgical manipulation. After AG126 treatment, contractions generated from the manipulated colon remained relatively low in amplitude compared to controls, but longer in duration, indicating that a complete return to normal function was not achieved. 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 as the contractile pattern, peak amplitude and contractile pressure (motility index) in un-operated mice treated with AG126 were not different from naïve controls. Furthermore, tyrphostin pre-treatment had no effect on spontaneous and bethanechol-induced contractile activity in vitro in circular smooth muscle strips harvested from un-operated mice. Therefore, it is unlikely that the single bolus of tyrphostin given prior to laparotomy had significant effects on the smooth muscle cell contractile apparatus 24 hours later. A more likely scenario is that inflammatory events following abdominal surgery were not completely blocked and 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; 20; 40). Real time RT-PCR
analyses demonstrated that, as predicted, the levels of IL-6, IL-1β, ICAM–1 and MCP-1, were elevated 3 hours postoperatively in muscularis extracts harvested from mice that had undergone colonic manipulation. Pretreatment with AG126 significantly reduced IL-1β and MCP-1 mRNA expression, but did not have a significant effect on IL-6 or ICAM-1 message 3 hr 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 AG126 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 have demonstrated that the release of prostaglandins (derived from COX-2) and nitric oxide (derived from iNOS) from both activated resident macrophages and recruited leukocytes have potent inhibitory effects on intestinal smooth muscle contractility (8; 15; 18; 27; 35; 38). RT-PCR analyses in the current study demonstrated a significant increase in iNOS and COX-2 message 3 hours postoperatively. Pretreatment with AG126 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 from the small bowel (18; 34). While 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 synthesis, and that inhibition of NO production also reduces prostaglandin release (32). In the rodent colon, pharmacologic 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 AG126 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 the 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 hours (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 hour time point was primarily from resident leukocytes. However, we assume that nitric oxide and prostaglandin production were also reduced secondarily as a result of the decreased ability of circulating leukocytes to target to the colonic muscularis in AG126 treated mice, and that this would contribute to the improved contractile function observed 24 hr post-laparotomy.

The target specificity of AG126 and thus the mechanism by which it might exert its protective effects against inflammation has not been completely identified. The induction of inflammatory mediator expression occurs through the activation of numerous transcription factors (for example, NF-IL6, STAT proteins, and NF-κB) via multiple signaling pathways. The relative contribution of each pathway to pro-inflammatory mediator production is dependent on species, cell type, and the pro-inflammatory 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 AG126. NF-κB activation requires tyrosine phosphorylation during phosphorylation-dependent ubiquitination of
the IκB-NFκB 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 pro-inflammatory 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, AG126 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 AG126 reduced the surgically induced inflammatory responses observed in the current 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 AG126. By reducing NF-κB activation, AG126 could act on pro-inflammatory mediator expression directly by inhibiting the upregulation of MCP-1, COX-2, and iNOS mRNA, and/or indirectly by inhibiting IL-1β expression and subsequently IL-1β mediated induction of MCP-1, COX-2, and iNOS expression.

IL-6 mediated pro-inflammatory processes are less dependent on NF-κB activation, and also rely on Janus kinase (JAK)/STAT signaling (2). The Janus 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 AG490 are effective inhibitors of JAK signaling (7). In the current study, surgically-induced STAT3 activation was unaffected by AG126 pretreatment. Limitations associated with the PTK assay did not permit direct analysis of membrane associated tyrosine kinases, and a possible effect of tyrophostin AG126 on Janus
kinases in this system cannot be ruled out completely. However, others have shown that AG126 exhibits little efficacy at receptor tyrosine kinases (28). Thus, the continued activity of STAT3 transcription factors would result in a persistent level of pro-inflammatory signaling. This may account for the failure of AG126 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 \textit{in vivo} after treatment with tyrphostin AG126 can be explained in terms of ongoing residual inflammatory processes. Nevertheless, functional experiments demonstrate that colonic contractility was significantly improved in AG126 treated mice, and that despite incomplete recovery of the normal colonic contractile pattern \textit{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 AG126 does not result in a corresponding blockade of all pro-inflammatory signaling, as determined at the level of gene transcription. Signaling pathways dependent upon 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 manuscript, 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 protein tyrosine kinase inhibitor, tyrphostin AG126. We propose that the protective effects of AG126 against the development of colonic ileus are largely due to the inhibition of NF-κB activation, resulting in
the reduced expression of the pro-inflammatory mediators IL-1β and MCP-1 and subsequent amelioration of cellular inflammation. In addition, the 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 protein tyrosine kinases, such as tyrphostin AG126 can provide new insights into the mechanisms of pro-inflammatory 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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REFERENCE LIST

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41. **Waldhausen JH, Shaffrey ME, Skenderis BS, Jones RS and Schirmer BD.**


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FIGURE LEGENDS

**Figure 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 to control. Activity was completely inhibited by tyrphostin AG-126 treatment, but not by vehicle. Data are mean ± SEM, and are normalized per mg of tissue wet weight. * Compares CM with controls; † Compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; \( p < 0.001 \). n=5.

**Figure 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 polymorpho-leukocytes exhibiting myeloperoxidase (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 colonic manipulation showing a massive increase in the numbers of infiltrating MPO-positive cells 24 hr postoperatively. C. Treatment with tyrphostin AG-126 lead 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 four experimental groups. The number of infiltrating cells was increased 90-fold after colonic manipulation (CM) compared to control. Tyrphostin 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 mean ± SEM, n=6. * Compares CM with controls; † Compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; \( p < 0.01 \).
Figure 3. Pretreatment with tyrphostin AG-126 attenuates the surgically induced impairment of colonic contractility in vivo. A-E. Representative traces from intra-colonic pressure recordings. (A) Pressure recordings from an un-operated 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 colonic manipulation compared to control. (C) In manipulated mice treated with tyrphostin AG-126, contraction amplitude and duration of the contractions are increased, but not in animals treated with vehicle (D). (E) Tyrphostin 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 colonic manipulation, but restored to control levels in animals treated with tyrphostin. Differences between CM and CM+Vehicle were not significant. Data are mean ± SEM, n=6. * Compares effects of colonic manipulation (CM) relative to control; † Compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; $P < 0.01$.

Figure 4. Tyrphostin AG-126 prevents the surgically induced delay in intestinal transit. A. Median histogram comparing the distribution of non-absorbable fluorescein labeled dextran along the gastrointestinal tract 1.5 hours after oral administration in control mice and in mice subjected to colonic manipulation (CM). In control mice, marker accumulated in the distal small bowel, cecum, and proximal colon. Colonic manipulation 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 distribution of label in manipulated animals treated with tyrphostin AG-126 is comparable to that observed in
control mice in panel A (solid bars). C. Summary of the calculated geometric center for the four experimental groups. Geometric center was significantly reduced after colonic manipulation, and restored to control values after treatment with tyrphostin but not by vehicle. Differences between CM and CM+Vehicle were not significant. Data are mean ± SEM, n=6. * Compares CM versus control; † compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; P < 0.01. stom = stomach, sb = small bowel, col = colon

**Figure 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 AG126 on spontaneous contractility. (A) Contractility pattern typical of unoperated controls. (B) After colonic manipulation, the amplitude of spontaneous contractions was markedly reduced and often degenerated into periods of irregular activity lasting for several minutes (region between arrowheads). (C) Spontaneous contractility was improved in animals pretreated with tyrphostin AG126, but not in animals treated with vehicle (D). (E) Tyrphostin AG126 had no effect on contractility in un-operated control mice. F. Bethanechol dose-response curves demonstrating the effects of tyrphostin AG126 pretreatment on the surgically induced impairment in the capacity of colonic smooth muscle to contract in response to muscarinic agonists. After colonic manipulation (CM), contractions in response to increasing concentrations of bethanechol (0.3 to 300 μM) were markedly impaired. The magnitude of the bethanechol stimulated contractile response in animals subjected to colonic manipulation (♦) was reduced by ~50% throughout the concentration-response curve (○). Contractile responses were restored to control levels in mice treated with tyrphostin AG126 (▲), but not by vehicle (■). G. Summary of contractility data showing peak contractile response at 100 μM bethanechol. Peak response
was reduced by 50% after colonic manipulation, but was restored to control levels by treatment with AG126. Differences between CM and CM+Vehicle were not significant. Data are mean ± SEM, n=8 per group. * Compares CM versus control; † compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; P<0.01.

Figure 6. SYBR green real-time RT-PCR analysis of colonic muscularis extracts showing the effects of tyrphostin AG-126 on surgically induced increases in pro-inflammatory mediator expression. IL-6, IL-1β, ICAM-1, MCP-1, and COX-2 mRNA expression were increased 1200-fold, 100-fold, 23-fold, 250-fold, 5-fold and 2.5-fold, respectively, 3 hours after colonic manipulation (CM). Treatment with tyrphostin AG126 significantly attenuated IL-1β, MCP-1 and COX-2 mRNA expression and completely prevented the surgically induced increase in iNOS mRNA expression, whereas treatment with vehicle had no effect. In all cases, treatment of unoperated control mice with AG126 had no effect on mediator expression. Differences between CM and CM+Vehicle were not significant. Data are mean ± SEM and are presented as fold increase relative to naive control, n=5. * Compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; P < 0.05.

Figure 7. Tyrphostin AG126 inhibits nuclear factor-kappa B (NF-κB) activation in response to colonic manipulation (CM). (A) Electrophoretic mobility shift assay (EMSA) was performed using 20 μg of protein extracted from muscularis externae using radiolabeled α-dCTP to characterize activation of NF-κB in muscularis externae harvested from unoperated control mice and from mice having undergone CM. Figure compares representative examples from naive controls, AG-126 treated controls, and from manipulated animals receiving AG-126, vehicle, or
no treatment. NFκB band intensity is clearly reduced in manipulated mice treated with tyrphostin AG-126, but not by vehicle. (B) PhosphoImager analysis of NF-κB band intensity. Band intensity was significantly increased after colonic manipulation (CM). Treatment of manipulated animals with tyrphostin AG126, but not vehicle, significantly inhibited the CM-induced increase in band intensity. Differences between CM and CM+Vehicle were not significant. (C) Scanning densitometer analysis of signal transducer and activator of transcription (STAT)-3 homodimer (SIF-A) band intensity. Band intensity was significantly increased after CM. Treatment with AG126 or vehicle had no effect on band intensity. Data are mean ± SEM; n=4. * Compares CM with naïve control; † Compares CM+AG-126 with CM; ‡ Compares CM+Vehicle with CM+AG126; P < 0.05.
Figure 1
Figure 2

A. Mag: 200X

C.

D.

E. MPO-positive cells/field (200X mag.)

- Control
- Control + AG126
- CM
- CM + AG126
- CM + Vehicle

* p < 0.05
† p < 0.01
‡ p < 0.001
Figure 3

A

Control

B

CM

C

CM + AG126

D

CM + Vehicle

E

Control + AG126

F

Motility Index (cm H$_2$O.s)

*  
†  
‡
Figure 4
Figure 5

A. Control

B. CM

C. CM + AG126

D. CM + Vehicle

E. Control + AG126

F. Contractility (g/mm²/s) vs. Bethanechol [µM]

G. Bar graph showing contractility (g/mm²/s) with different treatments:
- Control
- Control + AG126
- CM
- CM + AG126
- CM + Vehicle

Significance levels: * p < 0.05, † p < 0.01, ‡ p < 0.001
Figure 7

A

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B

Counts/min x 10^6

Control | Control | CM | CM + AG126 | CM + Vehicle |

C

10^6 Arbitrary Units/mm^2

Control | Control | CM | CM + AG126 | CM + Vehicle |