Am J Physiol Gastrointest Liver Physiol 291: G566–G574, 2006.
First published June 29, 2006; doi:10.1152/ajpgi.00058.2006.

Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis

Theresa F. Wu,[1,2] Colin J. Carati,[3] Wallace K. MacNaughton,[1] and Pierre-Yves von der Weid[1,2]

[1]*Mucosal Inflammation Research Group and* [2]*Smooth Muscle Research Group, Department of Physiology and Biophysics, Faculty of Medicine, University of Calgary, Alberta, Canada; and* [3]*Microcirculation and Lymphology Laboratory, Department of Anatomy, School of Medicine, Flinders University, Adelaide, South Australia*

Submitted 6 February 2006; accepted in final form 27 April 2006

Wu, Theresa F., Colin J. Carati, Wallace K. MacNaughton, and Pierre-Yves von der Weid. Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis. *Am J Physiol Gastrointest Liver Physiol* 291: G566–G574, 2006. First published June 29, 2006; doi:10.1152/ajpgi.00058.2006.—The ability of the lymphatic system to actively remove fluid from the interstitium is critical to the resolution of edema. The response of the lymphatics to inflammatory situations is poorly studied, so we examined mesenteric lymphatic contractile activity in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) model of guinea pig ileitis, a well-accepted animal model of intestinal inflammation, by videomicroscopy in vivo and in vitro 1, 3, and 6 days after induction of ileitis. Lymphatic function (diameter, constriction frequency, amplitude of constrictions, and calculated stroke volume and lymph flow rate) of isolated vessels from TNBS-treated guinea pigs were impaired compared with sham-treated controls. The dysfunction was well correlated with the degree of inflammation, with differences reaching significance ($P < 0.05$) at the highest inflammation-induced damage observed at *day 3*. In vivo, significantly fewer lymphatics exhibited spontaneous constrictions in TNBS-treated than sham-treated animals. Cyclooxygenase (COX) metabolites were suggested to be involved in this lymphatic dysfunction, since application of nonselective COX inhibitor (10 μM indomethacin) or a combination of COX-1 and COX-2 inhibitors (1 μM SC-560 and 10 μM celecoxib) markedly increased constriction frequency or induced them in lymphatics from TNBS-treated animals in vivo and in vitro. The present results demonstrate that lymphatic contractile function is altered in TNBS-induced ileitis and suggest a role for prostanoids in the lymphatic dysfunction.

inflammatory bowel disease; inflammation; lymph drainage; indomethacin

THE LYMPHATIC SYSTEM is an essential component of the cardiovascular and the immune systems. Lymphatic vessels collect fluid, extracellular proteins, lipids, and antigenic substances from the interstitium and, through rhythmical phasic contractions of the smooth muscle in the vessel wall, propel this material along a network of collecting vessels through lymphoid tissue (e.g., lymph nodes) and back to the blood stream (41, 44). Both tissue fluid homeostasis and immune cell transport are critical in inflammatory reactions; inflammatory mediators cause an increase in vascular permeability, and the resultant elevation in interstitial fluid and protein concentration contributes to edema. Edema generally increases lymph flow (2, 3), and the inflammatory mediators and immune cells, which gain access to the lymph during these events, may well directly influence lymphatic function. Many inflammatory mediators have been shown to directly affect lymphatic contractile activity (see Ref. 42), but these effects remain complex and poorly defined in vivo.

Interstitial (submucosal) edema has been consistently reported in inflammatory bowel disease (IBD) (23, 25, 36), including chronic inflammatory conditions of still poorly known etiology, such as Crohn's disease and ulcerative colitis. The presence of dilated lacteals and submucosal lymphatic vessels often observed in IBD suggested that the edema could be consequent to lymphatic obstruction (16, 25). An alternative explanation for this observation is that the contractile function of the mesenteric collecting lymphatic vessels was impaired.

Whether consequent to lymphatic obstruction or vessel dysfunction, the presence of edema suggests that lymph flow is diminished in IBD, causing mucosal hypoxia and fibrosis, which are intimately associated with chronic inflammation in Crohn's disease (14, 40). The poor drainage of interstitial fluid could also lead to impaired transport of large molecules, particles, dead cells, and bacteria away from the intestine, which may promote infection and delay the immune response. Lymphatic circulation is thus likely to play a crucial role during IBD, but the involvement of lymphatic contractile function in inflammatory diseases and its role during IBD has never been addressed. Although the complexity of human IBD disorders cannot be reproduced in any animal model, the TNBS model of guinea pig ileitis is a well-described model of intestinal inflammation (29, 30, 32), which reproduces many features of these human diseases (10). The present study investigated whether the contractile function of mesenteric lymphatic vessels is altered in the TNBS model of guinea pig ileitis and the role of cyclooxygenase (COX) metabolites in this effect.

METHODS

Surgical Induction of Inflammation

Young male albino guinea pigs (10–20 days old; Charles River, Montreal, Canada) were housed at constant temperature (22°C) on a 12:12-h light-dark cycle, with food and water ad libitum. This study was approved by the University of Calgary Animal Care Council and carried out in accordance with the guidelines of the Canadian Council on Animal Care. Young guinea pigs were used to limit adipose tissue around the lymphatic vessels that occurs in older animals and impairs visualization of these vessels.

After fasting for 18–24 h, guinea pigs were anesthetized with halothane (induction 4%, maintenance 2.5–3% in oxygen). The distal

Address for reprint requests and other correspondence: P.-Y. von der Weid, Dept. of Physiology and Biophysics, Univ. of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1 (e-mail: vonderwe@ucalgary.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ileum was identified and exteriorized through a midline laparotomy. 2,4,6-Trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich) was injected intraluminally into the ileum (0.425 ml; 30 mg/ml in 30% ethanol) 10 cm proximal to the ileo-cecal junction. An equivalent volume of physiological saline (0.9% NaCl) was injected into the ileum lumen of the sham group to control for volume effects. The midline laparotomy was surgically closed, and the animals were maintained in a controlled environment for 1, 3, or 6 days.

Assessment of Inflammation

Macroscopic damage was assessed on ileal and duodenal tissues excised immediately after death by awarding a value of 0 (not evident), 1 (mild), or 2 (severe) to the following parameters: erythema, hemorrhage, edema, presence of strictures, presence of mucus, ulceration, and adhesions. Microscopic signs of inflammation were assessed from adjacent 5-μm ileal transverse sections fixed in buffered formalin and processed for routine histological hematoxilin-eosin staining. Scores of 0, 1, or 2 were assigned to three sections from three different slides for each animal by a blinded investigator to the following parameters: neutrophil infiltration, distance of neutrophil infiltration, submucosal thickness, hemorrhage, villi tip damage, villi surface damage, and dilation of the central lacteals. Scores for each parameter were added to give composite macroscopic and microscopic scores.

Assessment of Bowel Wall Edema

Ileum samples of 50–100 mg were harvested, thoroughly rinsed in sterile saline solution, blotted dry, and carefully weighed. Samples were allowed to dry to a constant weight for weeks and weighed again. The weight difference was calculated and taken as the weight of water in the sample: % water weight = [wet weight (mg) − dry weight (mg)]/[wet weight (mg)] × 100.

In Vitro Experiments

One, three, or six days after surgery, guinea pigs, fasted for 24 h and were euthanized by decapitation under halothane-induced anesthesia, and lymphatic vessels were prepared as previously described (12, 43). Briefly, the small intestine and associated mesentery were dissected out and placed in a physiological saline solution (PSS) of the following composition (in mM): 2.5 $CaCl_2$, 5 KCl, 2 $MgCl_2$, 120 NaCl, 25 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose, with pH maintained at 7.4 by constant bubbling with a 95% O_2-5% CO_2 gas mixture. A section of mesentery, together with the associated arteries, veins, and collecting lymphatic vessels, was dissected and pinned onto the Sylgard-coated base of a 2-ml organ bath, which was placed on the stage of an inverted microscope (CK40, Olympus) and continuously superfused (3 ml/min) with warm (36–37°C) PSS. Selected collecting lymphatic vessels were cut open, and a fine glass cannula was inserted into their lumen and passed through at least one lymphatic valve to minimize back flow. Vessels were perfused with a low (0.3 mM) calcium solution to prevent precipitate obstructing the cannula (43). Lymphatic activity was observed on a television monitor connected to a video camera; the change in vessel diameter was monitored using a video-dimension analyzer (model V94; Living Systems Instrumentation, Burlington, VT) and recorded on a computer via an analog-to-digital converter and the "Chart" software (PowerLab/4SP; ADInstruments, Mountain View, CA).

The rate of luminal perfusion was initially set at 2.5 μl/min, which induces a consistent rhythmical constriction in control lymphatic vessels (12). Following a 30-min equilibration period, the average number of beats/min over a 5-min period served as the baseline control activity. The vessel was considered inactive if it did not exhibit constrictions within 30 min of perfusion at 2.5 μl/min. Changes in lymphatic activity were assessed while intraluminal flow was increased from 0.5 to 10 μl/min in 2.5-μl/min increments for 10 min at each rate. After the perfusion rate reached 10 μl/min, indomethacin (nonselective COX inhibitor; Sigma-Aldrich), celecoxib (COX-2 selective inhibitor; NicOx, Sophia Antipolis, France), or SC-560 (COX-1 selective inhibitor; Boehringer-Ingelheim, Ingelheim, Germany) were added to the superfusion for 20 min, followed either by addition of a second antagonist for 20 min or by a wash-out period of 10 min in normal PSS. Alternatively, vessels were superfused with indomethacin, celecoxib, or SC-560 in PSS while increasing intraluminal flow. Following 10 min at 10 μl/min, the tissue was treated with a second antagonist for 20 min or washed with regular PSS.

A minimum of two tissue preparations were taken from each animal from an area as close to (proximal sample) the site of injection as permitted by the severity of the inflammation, use of the mesentery directly at the injection site being sometimes precluded by bowel wall adhesions. To assess lymphatic contractile function in an area potentially less affected by the inflammation, a section of duodenal mesentery was also collected at least 8 cm away (distal sample) from the injection site.

In Vivo Experiments

Intravital microscopy was used to examine lymphatic contractile function in vivo. Guinea pigs, fasted for 18–24 h prior to experimentation at 1, 3, and 6 days postsurgery, were anesthetized with intramuscular injection of ketamine (0.27 ml/kg of 100 mg/ml), xylazine (0.375 ml/kg of 20 mg/kg), and diazepam (0.375 ml/kg of 5 mg/ml). Half-dose supplements were administered as required. Following anesthesia, the carotid artery and trachea were cannulated with polyethylene tubing (0.8 and 1.8 mm inner diameter, respectively) to monitor arterial pressure (Harvard Apparatus) and ensure spontaneous respiration. Saline was continuously infused (0.03 ml/min) through the carotid artery to maintain adequate hydration of the animal.

A 2-cm incision was made in the lower abdomen, and a section of the small intestine and associated mesentery was externalized. The animal was transferred onto a holding plate, the externalized loop of ileum was placed onto a clear pedestal placed under a stereomicroscope (MZ 12.5, Leica) or an upright microscope (Axioscope, Zeiss), and lymphatics were visualized through a camera (Hyper HAD, Sony) and television monitor. Animal body temperature was monitored and maintained using a homeothermic blanket control unit (Harvard Apparatus). The exposed tissue was continuously superfused with PBS of the following composition (in mM): 140 NaCl, 7.5 Na_2HPO_4, and 2.5 NaH_2PO_4 (pH 7.4), supplemented with 1% BSA (Sigma-Aldrich) and maintained at 37°C. The protein-enriched PBS was used to prevent osmotic imbalance between the observation bath and tissue (1). Exposed bowel was covered with moist gauze to minimize dehydration of the tissue.

The preparation was allowed to stabilize for 30 min, after which a 10-min period was used to determine the baseline activity of lymphatic vessel constriction rate and diameter. A vessel that showed no constrictions within 1 min of visualization was defined as "inactive." Responsiveness of lymphatic vessels to indomethacin was assessed after a 30-min superfusion with 10 μM indomethacin, followed by a 10-min wash-out period using regular PBS. Constriction frequency and changes in diameters were monitored by video microscopy.

Data Analysis and Statistics

Constriction frequency, systolic and diastolic diameters, and amplitude changes of lymphatic vessels were assessed in vitro from the Chart software traces and in vivo by directly measuring the parameters from video images on the monitor screen. Indices for stroke volume and volume flow of lymph through a section of a mesenteric lymphatic vessel were calculated according to Benoit et al. (3). These calculations give the unit stroke volume as the difference between systolic and diastolic unit volumes and multiplying this by constriction frequency to give volume flow (cf. cardiac output = stroke

volume of the heart × heart rate). In vivo measurements were obtained from at least 3 vessels in 3 or more separate fields of view in the mesentery of each animal.

Statistical significance was assessed using either the Student's *t*-test or one- and two-way repeated measures ANOVA, followed by appropriate post hoc tests. *P* values <0.05 were considered statistically significant.

RESULTS

Assessment of Inflammation

Administration of TNBS caused significant macroscopic evidences of inflammation at *days 1*, *3*, and *6* compared with sham-treated animals. Microscopic damage score was significantly higher 1 and 3 days after TNBS administration, returning to sham-treated values at *day 6* (Fig. 1).

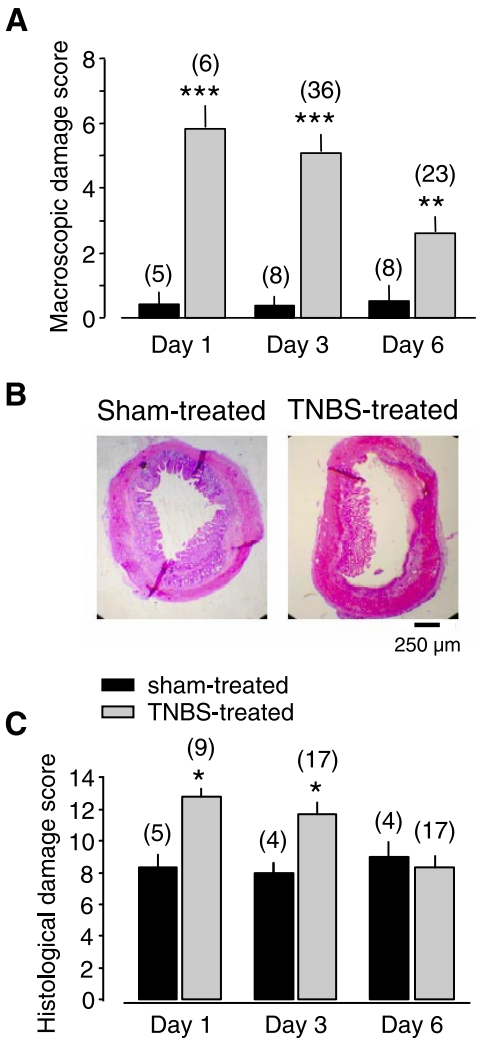

Fig. 1. Assessment of inflammation in the ileum of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated guinea pigs. Macroscopic (*A*) and microscopic assessment from histology sections (*B* and *C*) of ileum in sham- and TNBS-treated animals 1, 3, and 6 days after surgical instillation of TNBS. *A*: damage scores assessed by the macroscopic parameters. *B*: representative hematoxylin and eosin staining sections at *day 3*. *C*: damage scores assessed by the microscopic parameters. Legends in *C* apply to both graphs. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. respective sham-treated groups (nonparametric ANOVA, with Dunn's post hoc test).

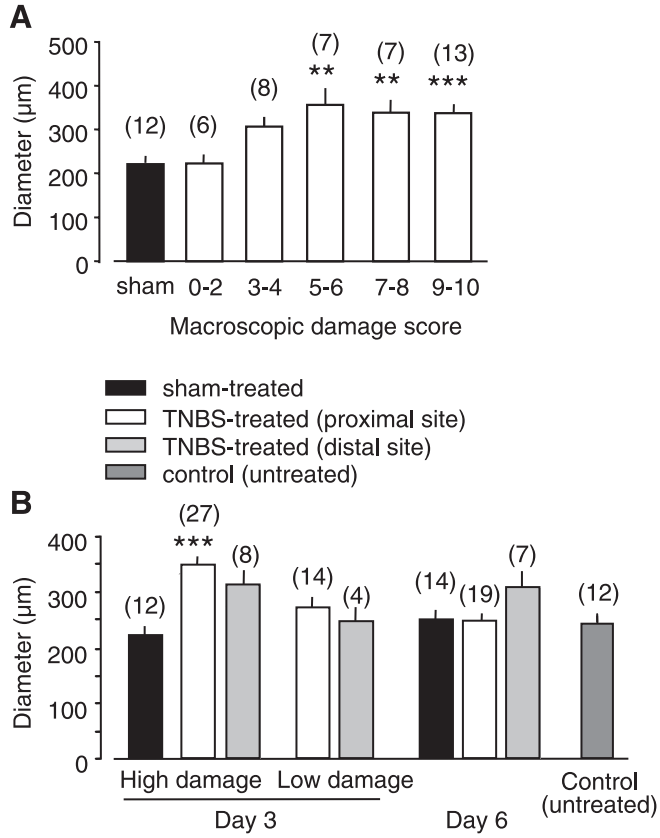

Fig. 2. Effect of TNBS treatment on diastolic diameter of lymphatic vessels in vitro. *A*: relationship between vessel diameter and macroscopic damage score at *day 3*. Diameters are significantly increased at high damage scores. *B*: diameters from TNBS- and sham-treated vessels at *days 3* and *6*. Diameters of vessels from highly inflamed ileum, close from the TNBS injection site, are significantly higher than their sham counterpart. TNBS-treated animals at *day 3* are divided into "high-" and "low-damage" score subgroups (see *Vessel diameter* for details), and vessels were analyzed proximal and distal (≥8 cm away) to the injection site. Numbers of vessels analyzed are indicated in parentheses. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. respective sham-treated groups (ANOVA, with Tukey-Kramer's post hoc test).

Assessment of Bowel Wall Edema

To evaluate edema, the submucosal thickness, one of the parameters included in the microscopic evaluation of inflammation, was considered. In the TNBS-treated animals, submucosal thickness was increased to 162 ± 16% ($P < 0.05$) and to 135 ± 16% of sham values at *days 3* and *6*, respectively. The percent water weight was also measured in ileum samples. In the sham-treated animals, it was 78.5 ± 0.7% ($n = 8$) 1 day after surgery and did not change significantly at *days 3* and *6* ($n = 10$). In contrast, the percent water weight in the TNBS-treated animals was significantly increased at *day 3* (80.5 ± 0.4%, $n = 10$, $P < 0.01$) and *day 6* postsurgery (81.7 ± 0.7%, $n = 10$, $P < 0.01$).

Lymphatic Vessel Contractile Function in TNBS-Treated Guinea pigs: In Vitro Data

Vessel diameter. In vitro lymphatic diastolic diameter increased with the severity of inflammation in the TNBS-treated group, reaching a maximum at damage scores between 5 and 8 (Fig. 2*A*) on *day 3*. There was variation in the degree of

inflammation in this group, and consequently, it was subdivided into a "low-damage" subgroup (macroscopic damage score of 0–4) and a "high-damage" subgroup (damage score over 5). In the high-damage subgroup, the mean diastolic vessel diameter was significantly greater than in sham-treated vessels for tissue taken from an area of the mesentery proximal to the TNBS injection site but not different in vessels taken more distally (Fig. 2*B*). Six days after induction of the ileitis, vessel diameters of the TNBS-treated group were not significantly different from their sham-treated counterparts, which were not significantly different from control (untreated) vessels (Fig. 2*B*).

Constriction frequency. In control and sham-treated animals, the frequency of lymphatic vessel constrictions increased as perfusion rate increased from 0.5 μl/min, reaching a maximum at 5.0–7.5 μl/min. Data from the *day 3* group was subdivided according to macroscopic damage scores (see *Vessel diameter* above). Constriction frequency of vessels in the high-damage subgroup remained significantly lower than in sham-treated animals over the whole range of perfusion rates (Fig. 3). Vessels of the high-damage subgroup taken distal (>8 cm) to the TNBS injection site showed weak contractile activity that remained significantly lower than sham-treated and control animals at all perfusion rates. In lymphatic vessels from the low-damage subgroup, constrictions could be stimulated at all perfusion rates, even in tissues that were taken directly from the injection site, albeit at lower frequencies than their respective sham controls. Lymphatic contractile activity in TNBS-treated animals 6 days following the induction of inflammation

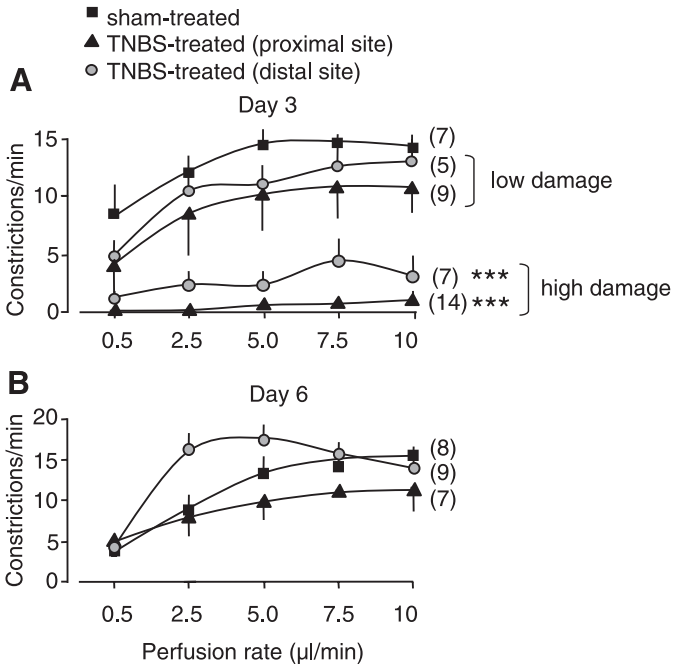

Fig. 3. Effect of TNBS treatment on lymphatic vessel constriction frequency in vitro. Comparison of constriction frequencies between lymphatics from sham- and TNBS-treated guinea pigs at *day 3* (*A*) and *day 6* (*B*) during increase in luminal perfusion rate. TNBS-treated animals at *day 3* (*A*) are divided into high- and low-damage score subgroups (see *Vessel diameter* for details), and vessels were analyzed proximal and distal to the injection site. Values are means of 1 vessel from *n* animals (in parentheses) ± SE. ***$P < 0.001$ vs. sham-treated group at each respective perfusion rate (ANOVA, with Tukey-Kramer's post hoc test).

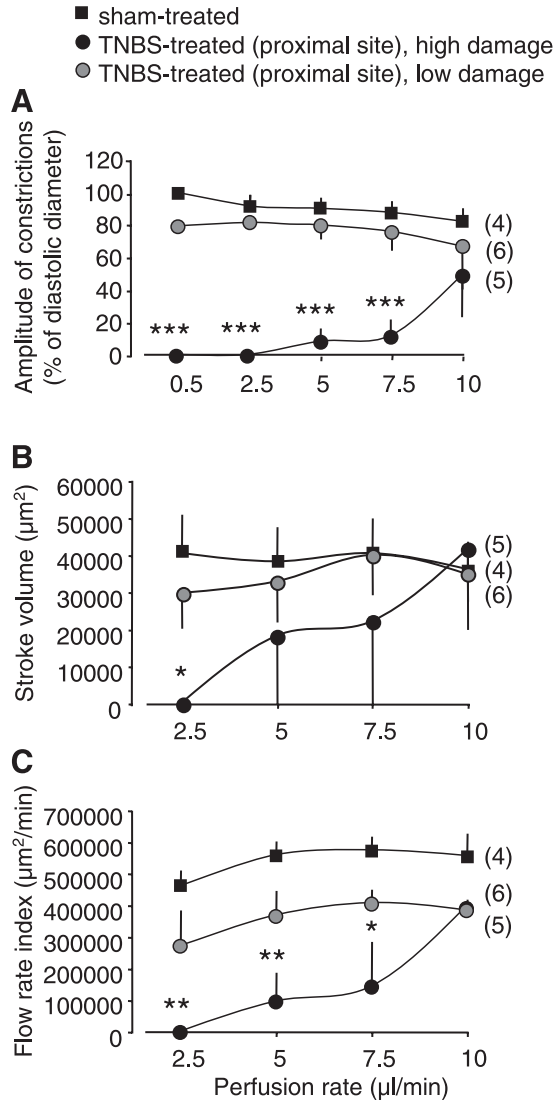

Fig. 4. Effect of TNBS-treatment on lymphatic vessel constriction amplitude (*A*), stroke volume (*B*), and index of active lymph flow (*C*) in vitro. Parameters are compared between lymphatics taken from close to the injection site (proximal site) of sham- and TNBS-treated guinea pigs at *day 3* during increase in luminal perfusion rate. TNBS-treated animals are divided into high- and low-damage score subgroups. Values are means of 1 vessel from *n* animals (in parentheses) ± SE. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. sham-treated group at each respective perfusion rate (ANOVA, with Tukey-Kramer's post hoc test).

was not significantly different from control or sham values at all perfusion rates. Lymphatic vessels from a distal origin exhibited higher constriction frequencies at lower perfusion rates than that of vessels taken proximal to the injection site at all perfusion rates (Fig. 3*B*).

Amplitude of constrictions. Many lymphatic vessels were quiescent in the *day 3* high-damage subgroup; when constrictions occurred, their amplitude was significantly lower than that of sham-treated vessels. In these vessels, constrictions only increased at high flow rates (Fig. 4*A*), in contrast to the sham group, the *day 3* low-damage subgroup (Fig. 4*A*), and the TNBS-treated vessels at *day 6* ($n = 8$, data not shown), where the amplitude of constrictions were similar and slightly decreased with increasing intraluminal flow.

Calculated indices for stroke volume and lymph flow. Stroke volumes in the *day 3* high-damage subgroup were low at low perfusion rates, increasing steadily with increase in intraluminal flow and reaching the same values observed in vessels from sham-treated animals (which remained almost constant across the flow range; Fig. 4*B*). The same trends were observed in lymph flow rate (Fig. 4*C*). Stroke volumes and lymph flow rate values in the *day 3* low-damage subgroup and in TNBS-treated vessels at *day 6* ($n = 8$, data not shown) were not significantly different from the sham-treated vessels.

Lymphatic Vessel Contractile Function in TNBS-Treated Guinea pigs: In Vivo Data

Vessel diameter. Lymphatic vessels proximal to the TNBS injection site were significantly larger than vessels in sham-treated animals at 1, 3, and 6 days postsurgery (Fig. 5*A*). Diameters of vessels distal to the site of injection were not significantly different from those in sham-treated animals.

Contractile activity and constriction frequency. Spontaneous constrictions were observed in 55 ± 4% of proximal vessels from sham-treated animals but only 27 ± 5% and 23 ± 4% of proximal vessels on *days 1* and *3* in TNBS-treated animals, respectively ($P < 0.05$). The proportion of constricting vessels in areas distal to the TNBS injection site was not significantly different from that in sham-treated animals or in animals 6 days after induction of inflammation (Fig. 5*B*). In vessels that were constricting, the frequency distribution followed a normal gaussian curve with the same mean (8.8 ± 0.5 and 8.2 ± 0.4 constrictions/min) and median (8.0) value in all observed TNBS- and sham-treated lymphatic vessels. There was no significant difference in the frequency of constrictions between treatment group, day, and distance from the injection site.

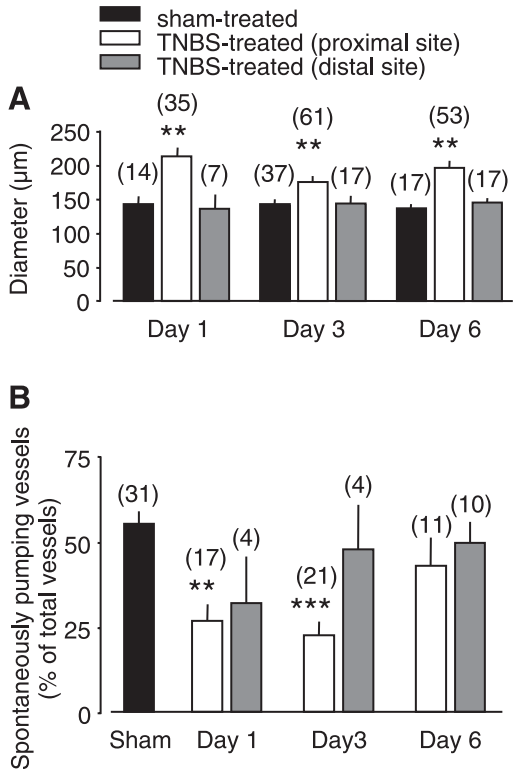

Fig. 5. Effect of TNBS-treatment on lymphatic vessel diastolic diameter (*A*) and spontaneously constricting vessels (*B*) in vivo. Diameters and spontaneous constrictions were measured during intravital observation in lymphatic vessels proximal and distal (>8 cm away) to the injection site in sham- and TNBS-treated animals at *days 1*, *3*, and *6*. Numbers in parentheses refer to the number of vessels (6–10 animals, *A*) and to the number of mesenteric field examined (1–5 per animal, *B*). Values are means ± SE. Data from sham-treated groups were not significantly different and were pooled together for assessment against each other group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. respective sham-treated groups (ANOVA with Tukey-Kramer's post hoc test).

Effects of COX Inhibition on the TNBS-Induced Decrease in Lymphatic Contractility

The role of prostanoids in the TNBS-induced inhibition of lymphatic contractile function was assessed first in vitro using COX inhibitors. In the presence of the nonselective COX inhibitor indomethacin (10 μM) applied at the end of the in vitro increased perfusion rate protocol, lymphatic vessels from the high-damage subgroup of *day 3* TNBS-treated animals had a significantly higher constriction frequency than when superfused with PSS alone, although the mean constriction frequency was still significantly less than in sham-treated animals (Fig. 6*A*). Indomethacin had no effect on constriction frequencies of lymphatic vessels from sham-treated and *day 6* TNBS-treated animals, which were already close to control values.

The effect of increasing perfusion rate on constriction frequency of lymphatic vessels of the *day 3* high-damage subgroup was enhanced by indomethacin, but constriction frequency was still significantly lower than that measured in vessels from the sham-treated (Fig. 6*B*) and control groups (data not shown). Constriction frequency of vessels from the low-damage subgroup was not significantly affected by indomethacin ($n = 3$, data not shown). Addition of indomethacin to the superfusate of vessels from *day 6* animals slightly increased (without reaching significance) constriction frequency at all perfusion rates ($n = 7$, data not shown); constriction frequency in these vessels were not different from sham control vessels.

Lymphatic vessels were defined as inactive if they did not show any contractile activity at any of the perfusion rates tested during the in vitro experiments. In 73% of these inactive vessels from TNBS animals, a 10–15 min (mean ± SE; 11.9 ± 0.6 min) of superfusion with 10 μM indomethacin was able to initiate constrictions (Fig. 7*A*). The indomethacin-stimulated constriction frequency was irregular, alternating periods of activity (20.7 ± 4.7 beats/min for 2.0 ± 4.7 min), with periods of inactivity of 2.5 ± 0.5 min.

As described in the previous section, lymphatic vessels that were still rhythmically constricting in vivo in animals with highly (macroscopic score of 5 or above) inflamed ileums had a range of constriction frequencies not significantly different from that of sham-treated animals. To evaluate the effect of indomethacin on the contractile activity of lymphatic vessels in vivo, we performed experiments on vessels from the *day 3* high-damage subgroup that display a low contractile activity (range of 1–8 constrictions/min) similar to that recorded in vitro at a high perfusion rate (as illustrated in Fig. 6). After being superfused with indomethacin (10 μM) for 5–7 min, the constriction frequency of these vessels increased to values that were not significantly different from that of sham-treated vessels (Fig. 7*B*).

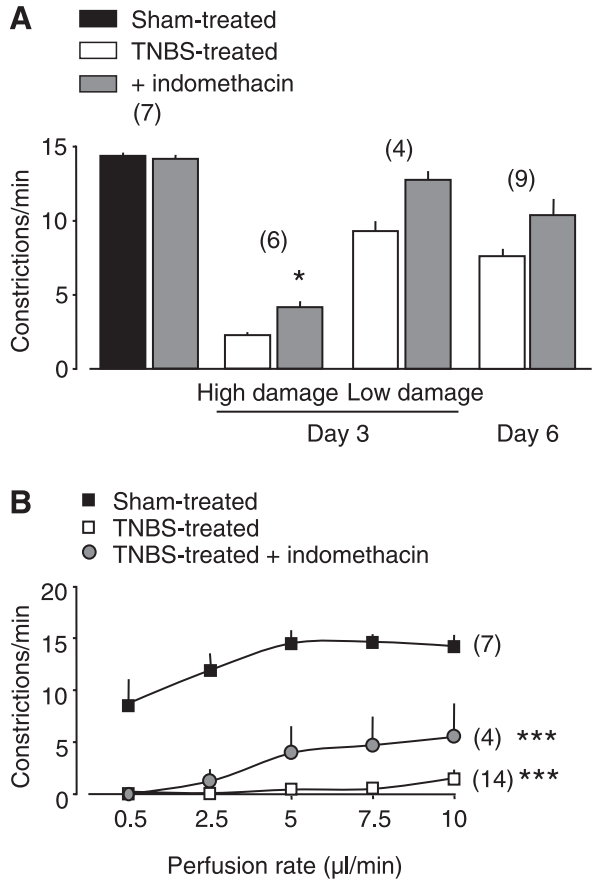

Fig. 6. Effect of indomethacin on TNBS-induced decrease in constriction frequency in vitro. *A*: indomethacin, applied at the higher perfusion rate (10 μl/min), significantly enhanced constriction frequency in vessels obtained from the *day 3* high-damage subgroup and did not effect those from sham-treated or the less inflamed TNBS-treated animals. *B*: constriction frequencies of vessels from the *day 3* high-damage subgroup were increased across the intraluminal perfusion range in the presence of indomethacin, however not significantly. Values are means of 1 vessel from *n* animals (in parentheses) ± SE. *A*: indomethacin groups are compared with their respective control groups. $*P < 0.05$ (paired Student's *t*-test). *B*: TNBS-treated groups with and without indomethacin compared with sham-treated groups at each respective perfusion rate. $***P < 0.001$ (ANOVA, with Tukey-Kramer's post hoc test).

To determine whether inhibition of COX-1 or COX-2 was specifically responsible for the indomethacin-induced increase in constriction frequency, response to increase in perfusion rate was investigated in vitro in vessels belonging to the *day 3* high-damage subgroup in the presence of the selective COX-1 and COX-2 inhibitors SC-560 or celecoxib, respectively. The application of celecoxib (10 μM) did not change the constriction frequency response to increases in perfusion rate in these vessels compared with PSS superfusion (data not shown and Fig. 8*Aa*). However, when SC-560 (1 μM) was added to the celecoxib-containing PSS at the highest (10 μl/min) perfusion rate, constriction frequency was significantly increased (Fig. 8*Aa*). Similarly, constriction frequency was not increased by SC-560 alone at any perfusion rate (data not shown and Fig. 8*Ab*) but was significantly augmented after additional application of celecoxib at a perfusion rate of 10 μl/min (Fig. 8*Ab*). No difference was observed with any combination of the COX inhibitors in the *day 3* low-damage subgroup (Fig. 8*B*) or in the *day 6* group (data not shown).

DISCUSSION

Morphological and histological changes occur in intestinal lymphatic vessels during IBD (16, 20, 22). Pathologists have noted for decades that marked submucosal edema and engorgement of capillaries and lymphatics are one of the first and most consistent morphological changes that occur in intestinal inflammation (25, 36). In the present study, we showed significant macroscopic damage, microscopically evident edema, and increased tissue fluid in TNBS-treated animals at *day 3*, the latter continuing into *day 6* after TNBS treatment. Typically, edema would result in increased lymphatic contractility and

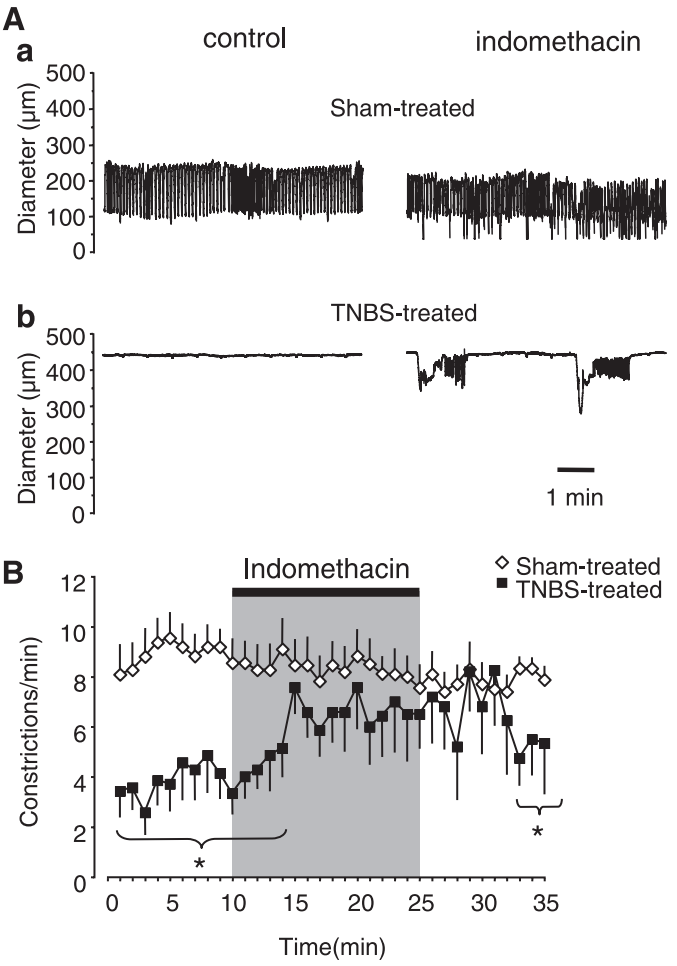

Fig. 7. Effect of indomethacin on the contractile activity of mesenteric lymphatic vessel. *A*: original traces of vessel diameter changes (downward deflections indicate vessel constrictions) in an in vitro-perfused lymphatic preparation from sham-treated (*a*) and TNBS-treated animal (high-damage subgroup, *b*) before (control, *left traces*) and after 15 min of superfusion in indomethacin (10 μM, *right traces*). The constrictions depicted in *Ab* (indomethacin) are the first exhibited by the vessel, which was quiescent before and during the first 15 min of indomethacin administration. Also, note the larger diastolic diameter in the TNBS-treated vessel (*Ab*). *B*: comparison of the effect of indomethacin (10 μM) on the constriction frequency of sham-treated and TNBS-treated animals during intravital (in vivo) recordings. A group of vessels ($n = 7$) from TNBS-treated animals displaying a low constriction frequency comparable to that observed in vitro was selected for these experiments. In these vessels, the constriction frequency increased to values not significantly different ($*P < 0.05$, ANOVA, with Tukey-Kramer's post hoc test at each respective time) from those of sham-treated animals after about 5 min in the presence of indomethacin and decreased back to control values during indomethacin washout.

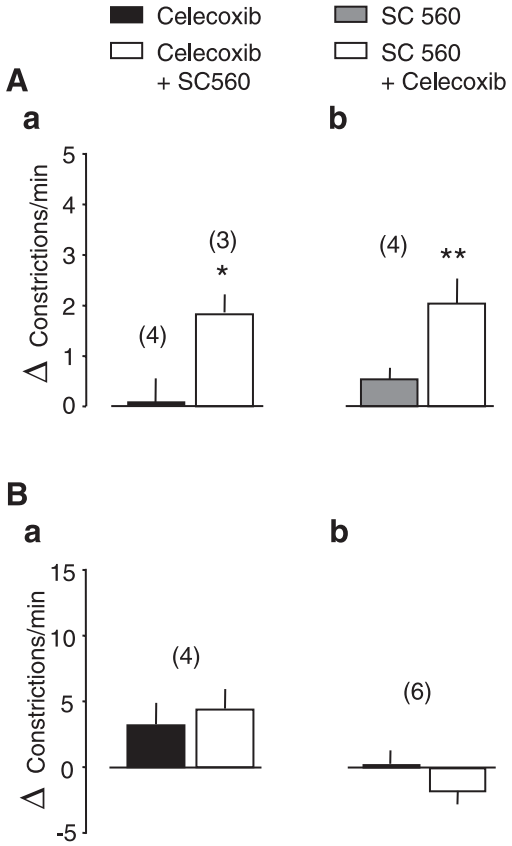

Fig. 8. Effect of cyclooxygenase selective inhibitors (SC-560 and celecoxib) on the TNBS-induced decrease in constriction frequency in vitro. Difference in constriction frequency (Δ constrictions/min) measured at a perfusion rate of 10 μl/min in *day 3* high-damage (*A*) and low-damage (*B*) subgroups of TNBS-treated guinea pigs between physiological saline solution (PSS) superfusion and administration of celecoxib (10 μM) or celecoxib plus SC-560 (1 μM, *a*) and SC-560 or SC-560 plus celecoxib (*b*). Legend applies to both *A* and *B*. $*P < 0.05$ and $**P < 0.01$ vs. their respective control (PSS) groups (ANOVA, with Tukey-Kramer's post hoc test).

diameter, such as has been reported (3) in hemodilution-induced edema of the small bowel. Thus we would expect increased spontaneous lymphatic constriction in vivo and increased constriction frequency and diameter at both *day 3* and *day 6*, because there was significant edema at this time. While lymphatic diameters were increased, spontaneous constriction of lymphatics was reduced (at least at *day 3*), and constriction frequency of those vessels that were constricting was not different from control values, despite an increased interstitial driving pressure sufficient to increase lymphatic diameter. The highest edema was recorded on *day 6*, yet the proportion of spontaneously pumping lymphatic vessels and their constriction frequencies were not different from control values derived from nonedematous control tissue. Clearly, this indicates that lymphatic contractility was compromised in TNBS-treated bowel. The TNBS-induced edema is likely to be responsible for the significant dilation in the diastolic diameter of collecting lymphatic vessel both in vitro and in vivo in the mesentery of TNBS-treated guinea pigs, especially because there is a good correlation between dilation of these lymphatic vessels and the severity of inflammation at *day 3*. A possible cause for the dilated lymphatic vessels could be downstream lymphatic obstruction due to the inflammatory conditions. Lymphatic obstruction was observed in human patients who were undergoing surgery for Crohn's disease (16), and lesions similar to those in IBD have been reported in animals with experimentally obstructed mesenteric lymphatic vessels (21, 22, 34), although not always (8, 16). Lymphatic vessel obstruction was not directly assessed in the present study. However, we observed no major lymph nodes or obvious sites of obstruction between vessels that were proximal or distal to the TNBS-affected ileum (unpublished observations). Since the distal vessels were largely unaffected by the TNBS treatment (i.e., their behavior did not seem to be affected by any downstream obstruction), the impaired lymph movement in vessels proximal to the TNBS-affected ileum must be due to impaired lymphatic contractile function. Furthermore, vessels from inflamed animals (*day 3*, high-damage subgroup) had larger diameters that were maintained in vitro, where vessels were removed from the influence of any possible obstruction. This impairment was accompanied by constrictions of reduced frequencies and amplitudes, leading to stroke-volume values and flow-rate indices that reached control values only at the highest in vitro perfusion rate. The contractile function was partially restored in vivo and in vitro by the topical application of indomethacin, which is unlikely to have "unblocked" downstream lymphatics. Taken together, these findings suggest that TNBS-induced ileitis resulted in impairment of lymphatic contractile function, which was likely to decrease drainage of lymph from the inflamed ileum. It is possible that edema consequent to severe inflammation leads to an increase in fluid load and lymphatic intraluminal pressure sufficient to overpower lymphatic contractile ability and convert the lymphatics to passive conduits. However, we often observed that small particles and cells that would usually be flowing down normal lymphatic vessels remained stagnant in vessels proximal to the TNBS-affected ileum (unpublished observation), suggestive of lymph stasis. Moreover, the active pump of mesenteric lymphatics has been described as able to sustain constrictions even at high imposed flow rates (13, 24). This robust contractile activity was confirmed in our in vitro experiments, where vessels from sham-treated animals were able to increase their constriction frequency with increasing perfusion rates before reaching a plateau. By contrast, frequency and amplitude of these constrictions were significantly lower in vessels isolated from animals with severe inflammation (*day 3*, high-damage subgroup), and they were less responsive to increasing perfusion rates. In summary, the inhibition of lymphatic contractility in TNBS-inflamed ileum must include direct effects of inflammation on the lymphatic vessel itself and is likely to contribute to reduced lymphatic drainage from the inflamed bowel.

Factors affecting lymphatic contractile function include soluble factors released by the microvascular endothelia and hormonal, humoral, or neural factors. Significant among these are the prostaglandins, the production of some of which has been shown to increase during IBD (26, 39) as a consequence of inducible COX-2 upregulation (17, 37). Most studies to date show that acute administration of COX metabolites exert a cytoprotective action on the mucosa, whereas other investigations (4, 11, 38, 45) demonstrate an exacerbation of inflammation in animals with experimental colitis and humans upon long-term administration of NSAIDs. To date, there has been no investigation of the effect of prostaglandins on lymphatics during intestinal inflammation, but we know that prostaglan-

dins, among the most important modulators of lymphatic function, can either enhance or inhibit lymphatic contractility (18, 42). Our present results clearly indicate that COXs might play a role in the dysfunction observed in lymphatic vessels of TNBS-treated guinea pigs, as inhibition of COX-1 and COX-2 with indomethacin was able to partly restore in vitro, and augment in vivo, rhythmical contractile activity. With respect to the metabolites that could be responsible for these effects, PGE_2 and prostacyclin are plausible candidates, as they have been shown to potently dilate rat iliac lymphatics (31) and inhibit contractility in bovine as well as guinea pig mesenteric vessels (6, 15, 19). Studies involving deletion of prostaglandin receptors and neutralizing antibodies to PGE_2 have reported (33) PGE_2 and PGI_2 as the primary prostanoids involved in inflammation. A study by Brock et al. (5) demonstrated that induction of COX-2 with LPS resulted in the preferential production of PGE_2 and prostacyclin (accounting for ~86% of the total prostaglandin produced) rather than simply increasing total prostaglandin production. TNBS-induced intestinal inflammation triggered production of COX-2 (35). It is possible that, as a consequence of our experimental conditions, the profile of prostaglandin synthesis was shifted towards the inhibitory metabolites, leading to the inhibition of lymphatic contractile activity and its partial restoration under COX inhibition with indomethacin. If indeed the production of prostacyclin and PGE_2 from COX-2 resulted in the suppression of contractile function, one would expect that the selective inhibition of COX-2 would yield results similar to general COX inhibition by indomethacin. However, results from this current study showed that inhibition of COX-2 alone did not reverse significantly the decrease in constriction frequency; the suppression of the activity of both isoforms of COX was required to produce an effect. One possible explanation for this could be that a small amount of inhibitory prostaglandins can be produced from COX-1. Brock et al. (5) showed that prostacyclin and PGE_2 accounted for ~26% of total COX-1 derived metabolites. Another explanation for the inefficacy of COX-2 inhibition alone to produce a significant effect on constriction frequency could be that the production of the inhibitory prostaglandins is due to upregulation of the selective prostaglandin E synthase (7), an enzyme downstream from COX in the metabolism of arachidonic acid, rather than the preferential production inhibitory prostaglandins by COX-2. Clearly, the role of COX metabolites in lymphatic function during inflammation needs further investigations.

We have demonstrated dilatation and inhibition of contractile function in mesenteric lymphatic vessels of guinea pigs with TNBS-induced ileum inflammation. The evidence presented strongly suggests a decrease in drainage during experimental ileitis, as lymphatic pumping is known to be the main driver of lymph flow (9, 27, 28). Our study gives new information on the effect of intestinal inflammation on the lymphatic system and its capability to drain interstitial fluid and resolve edema. We believe the lymphatic drainage system is a significant contributor to the development of intestinal inflammation.

ACKNOWLEDGMENTS

We thank Simon Roizes and Martin Bratschi for excellent technical assistance.

GRANTS

This study was supported by grants from the Crohn's and Colitis Foundation of Canada and the Alberta Heritage Foundation for Medical Research (AHFMR). P.-Y. von der Weid is an AHFMR Scholar and W. K. MacNaughton is an AHFMR Senior Scholar.

REFERENCES

1. **Barber BJ, Babbitt RA, Dutta S, and Parameswaran S.** Changes in rat mesentery interstitial matrix due to superfusate. *Am J Physiol Heart Circ Physiol* 265: H852–H856, 1993.
2. **Benoit JN and Zawieja DC.** Effects of f-Met-Leu-Phe-induced inflammation on intestinal lymph flow and lymphatic pump behavior. *Am J Physiol Gastrointest Liver Physiol* 262: G199–G202, 1992.
3. **Benoit JN, Zawieja DC, Goodman AH, and Granger HJ.** Characterization of intact mesenteric lymphatic pump and its responsiveness to acute edemagenic stress. *Am J Physiol Heart Circ Physiol* 257: H2059–H2069, 1989.
4. **Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, and Lynch RG.** Rapid development of colitis in NSAID-treated IL-10-deficient mice. *Gastroenterology* 123: 1527–1542, 2002.
5. **Brock TG, McNish RW, and Peters-Golden M.** Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. *J Biol Chem* 274: 11660–11666, 1999.
6. **Chan AK, Vergnolle N, Hollenberg MD, and von der Weid P-Y.** Proteinase-activated receptor 2 modulates guinea-pig mesenteric lymphatic vessel pacemaker potential and contractile activity. *J Physiol* 560: 563–576, 2004.
7. **Claveau D, Sirinyan M, Guay J, Gordon R, Chan CC, Bureau Y, Riendeau D, and Mancini JA.** Microsomal prostaglandin E synthase-1 is a major terminal synthase that is selectively up-regulated during cyclooxygenase-2-dependent prostaglandin E2 production in the rat adjuvant-induced arthritis model. *J Immunol* 170: 4738–4744, 2003.
8. **Danese CA, Georgalas-Penesis M, Kark AE, and Dreiling DA.** Studies of the effects of blockage of intestinal lymphatics. I. Experimental procedure and structural alterations. *Am J Gastroenterol* 57: 54154–54156, 1972.
9. **Eisenhoffer J, Elias RM, and Johnston MG.** Effect of outflow pressure on lymphatic pumping in vitro. *Am J Physiol Regul Integr Comp Physiol* 265: R97–R102, 1993.
10. **Elson CO, Sartor RB, Tennyson GS, and Riddell RH.** Experimental models of inflammatory bowel disease. *Gastroenterology* 109: 1344–1367, 1995.
11. **Felder JB, Korelitz BI, Rajapakse R, Schwarz S, Horatagis AP, and Gleim G.** Effects of nonsteroidal antiinflammatory drugs on inflammatory bowel disease: a case-control study. *Am J Gastroenterol* 95: 1949–1954, 2000.
12. **Fox JL and von der Weid P-Y.** Effects of histamine on the contractile and electrical activity in isolated lymphatic vessels of the guinea-pig mesentery. *Br J Pharmacol* 136: 1210–1218, 2002.
13. **Gashev AA and Zawieja DC.** Physiology of human lymphatic contractility: a historical perspective. *Lymphology* 34: 124–134, 2001.
14. **Giatromanolaki A, Sivridis E, Maltezos E, Papazoglou D, Simopoulos C, Gatter KC, Harris AL, and Koukourakis MI.** Hypoxia inducible factor 1α and 2α overexpression in inflammatory bowel disease. *J Clin Pathol* 56: 209–213, 2003.
15. **Hanley CA, Elias RM, Movat HZ, and Johnston MG.** Suppression of fluid pumping in isolated bovine mesenteric lymphatics by interleukin-1: interaction with prostaglandin E2. *Microvasc Res* 37: 218–229, 1989.
16. **Heatley RV, Bolton PM, Hughes LE, and Owen EW.** Mesenteric lymphatic obstruction in Crohn's disease. *Digestion* 20: 307–313, 1980.
17. **Hendel J and Nielsen OH.** Expression of cyclooxygenase-2 mRNA in active inflammatory bowel disease. *Am J Gastroenterol* 92: 1170–1173, 1997.
18. **Johnston MG and Feuer C.** Suppression of lymphatic vessel contractility with inhibitors of arachidonic acid metabolism. *J Pharmacol Exp Ther* 226: 603–607, 1983.
19. **Johnston MG, Kanalec A, and Gordon JL.** Effects of arachidonic acid and its cyclo-oxygenase and lipoxygenase products on lymphatic vessel contractility in vitro. *Prostaglandins* 25: 85–98, 1983.
20. **Kaiserling E, Krober S, and Geleff S.** Lymphatic vessels in the colonic mucosa in ulcerative colitis. *Lymphology* 36: 52–61, 2003.

21. **Kalima TV.** Experimental lymphatic obstruction in the ileum. *Ann Chir Gynaecol Fenn* 59: 187–201, 1970.
22. **Kalima TV, Saloniemi H, and Rahko T.** Experimental regional enteritis in pigs. *Scand J Gastroenterol* 11: 353–362, 1976.
23. **Kirsner JB and Shorter RG.** *Inflammatory Bowel Disease*. Philadelphia, PA: Lea and Febiger, 1975.
24. **Koller A and Kaley G.** Shear stress-induced dilation of arterioles. *Am J Physiol Heart Circ Physiol* 274: H382–H383, 1998.
25. **Kovi J, Duong HD, and Hoang CT.** Ultrastructure of intestinal lymphatics in Crohn's disease. *Am J Clin Pathol* 76: 385–394, 1981.
26. **Ligumsky M, Karmeli F, Sharon P, Zor U, Cohen F, and Rachmilewitz D.** Enhanced thromboxane A2 and prostacyclin production by cultured rectal mucosa in ulcerative colitis and its inhibition by steroids and sulfasalazine. *Gastroenterology* 81: 444–449, 1981.
27. **McGeown JG, McHale NG, Roddie IC, and Thornbury K.** Peripheral lymphatic responses to outflow pressure in anaesthetized sheep. *J Physiol* 383: 527–536, 1987.
28. **McGeown JG, McHale NG, and Thornbury KD.** The role of external compression and movement in lymph propulsion in the sheep hind limb. *J Physiol* 387: 83–93, 1987.
29. **Miceli P, Morris GP, MacNaughton WK, and Vanner S.** Alteration in capsaicin-evoked electrolyte transport during the evolution of guinea-pig TNBS ileitis. *Am J Physiol Gastrointest Liver Physiol* 282: G972–G980, 2002.
30. **Miller MJ, Sadowska-Krowicka H, Jeng AY, Chotinaruemol S, Wong M, Clark DA, Ho W, and Sharkey KA.** Substance P levels in experimental ileitis in guinea pigs: effects of misoprostol. *Am J Physiol Gastrointest Liver Physiol* 265: G321–G330, 1993.
31. **Mizuno R, Koller A, and Kaley G.** Regulation of the vasomotor activity of lymph microvessels by nitric oxide and prostaglandins. *Am J Physiol Regul Integr Comp Physiol* 274: R790–R796, 1998.
32. **Moore BA, Stewart TM, Hill C, and Vanner SJ.** TNBS ileitis evokes hyperexcitability and changes in ionic membrane properties of nociceptive DRG neurons. *Am J Physiol Gastrointest Liver Physiol* 282: G1045–G1051, 2002.
33. **Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K, Gregory SA, and Isakson PC.** Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J Exp Med* 184: 883–891, 1996.
34. **Reichert FL and Mathes ME.** Experimental lymphedema of the intestinal tract and its relation to regional cicatrizing enteritis. *Annals Surg* 104: 610–616, 1936.
35. **Reuter BK, Asfaha S, Buret A, Sharkey KA, and Wallace JL.** Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. *J Clin Invest* 98: 2076–2085, 1996.
36. **Robb-Smith AH.** A bird's-eye view of Crohn's disease. *Proc R Soc Med* 64: 157–161, 1971.
37. **Singer I, Kawka DW, Schloemann S, Tessner T, Riehl T, and Stenson WF.** Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology* 115: 297–306, 1998.
38. **Singh VP, Patil CS, Jain NK, and Kulkarni SK.** Aggravation of inflammatory bowel disease by cyclooxygenase-2 inhibitors in rats. *Pharmacology* 72: 77–84, 2004.
39. **Taniguchi T, Tsukada H, Nakamura H, Kodama M, Fukuda K, Tominaga M, and Seino Y.** Effects of a thromboxane A2 receptor antagonist in an animal model of inflammatory bowel disease. *Digestion* 58: 476–478, 1997.
40. **Taylor CT.** Regulation of intestinal epithelial gene expression in hypoxia. *Kidney Int* 66: 528–531, 2004.
41. **Van Helden DF and Zhao J.** Lymphatic vasomotion. *Clin Exp Pharmacol Physiol* 27: 1014–1018, 2000.
42. **Von der Weid P-Y.** Lymphatic vessel pumping, and inflammation-the role of spontaneous constrictions and underlying electrical pacemaker potentials. *Aliment Pharmacol Ther* 15: 1115–1129, 2001.
43. **Von der Weid P-Y, Crowe MJ, and van Helden DF.** Endothelium-dependent modulation of pacemaking in lymphatic vessels of the guinea-pig mesentery. *J Physiol* 493: 563–575, 1996.
44. **Von der Weid P-Y, and Zawieja DC.** Lymphatic smooth muscle: the motor unit of lymph drainage. *Int J Biochem Cell Biol* 36: 1147–1153, 2004.
45. **Wallace JL, Keenan CM, Gale D, and Shoupe TS.** Exacerbation of experimental colitis by nonsteroidal anti-inflammatory drugs is not related to elevated leukotriene B4 synthesis. *Gastroenterology* 102: 18–27, 1992.