Involvement of the NO-cGMP-K\(_{\text{ATP}}\) channel pathway in the mesenteric lymphatic pump dysfunction observed in the guinea pig model of TNBS-induced ileitis

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Mathias R, von der Weid P-Y. Involvement of the NO-cGMP-K\(_{\text{ATP}}\) channel pathway in the mesenteric lymphatic pump dysfunction observed in the guinea pig model of TNBS-induced ileitis. Am J Physiol Gastrointest Liver Physiol 304: G623–G634, 2013. First published December 28, 2012; doi:10.1152/ajpgi.00392.2012.—Mesenteric lymphatic vessels actively transport lymph, immune cells, fat, and other macromolecules from the intestine via a rhythmical contraction-relaxation process called lymphatic pumping. We have previously demonstrated that mesenteric lymphatic pumping was compromised in the guinea pig model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ileitis, corroborating clinical and experimental observations of a dilated and/or obstructed phenotype of these vessels in inflammatory bowel disease. Many mediators released during the inflammatory process have been shown to alter lymphatic contractile activity. Among them, nitric oxide (NO), an inflammatory mediator abundantly released during intestinal inflammation, decreases the activity that occurred as a consequence of the intestinal inflammation caused by TNBS.

lymphatic vessel; lymphatic pumping; ileitis; Crohn’s disease; inflammation

THE LYMPHATIC SYSTEM IS A CRUCIAL COMPONENT OF both the immune and cardiovascular system. Composed of a dense network of initial and collecting lymphatic vessels as well as lymph nodes, the lymphatic system helps maintain tissue homeostasis while playing a role in immune cell trafficking and nutrient uptake and transport. In the small intestine, the lymphatic vasculature begins with lacteals, blind-ended tubes lined with endothelial cells in the center of the villi; it progresses to submucosal and muscular initial lymphatics of the same structure and finally to collecting lymphatics, beginning at the intestinal-mesenteric border (36). Thanks to layers of lymphatic muscle, mesenteric collectors exert a powerful rhythmical contractile activity able to propel their contents through draining lymph nodes and postnodal lymphatics back into the blood stream (54, 64). During inflammation, the lymphatic system is critical in transporting dendritic cells, macrophages, and lymphocytes from inflamed sites to lymph nodes, as well as resolving the associated edema (57). It has been noted that the increased interstitial fluid causes an increase in lymphatic contractility and lymph flow and that the various inflammatory mediators present during an inflammatory episode can cause alterations in lymphatic function (3, 45, 56).

ATP-sensitive potassium (K\(_{\text{ATP}}\)) channels have been shown to control membrane potential of vascular smooth muscle, but also lymphatic muscle (10, 52). Opening of the channel leads to membrane hyperpolarization, thereby decreasing voltage-dependent Ca\(^{2+}\) channel activity and inhibiting smooth muscle contraction (13, 27, 40, 43). Although several tissue-dependent isoforms exist, the Kir6.1/SUR2B isoform predominates in vascular smooth muscle, with a four and four subunit stoichiometry (7). The channel activity is heavily modulated by protein kinases (5, 6, 11, 24, 26, 42, 44, 46, 48, 52, 59, 61). Upregulation of the channel subunits has been shown to occur in disease states such as sepsis (7) and colitis (28), where inflammation is involved. These situations are often accompanied by an increase in nitric oxide (NO) production. Importantly, NO potently inhibits lymphatic pumping (38, 41, 52, 55) with a concomitant lymphatic muscle hyperpolarization (52, 55). These studies have shown that the hyperpolarizing and dilatory effects of NO can be reversed by administration of the K\(_{\text{ATP}}\) channel antagonist glibenclamide (52). Synthetic agonists that directly act on the K\(_{\text{ATP}}\) channel subunits such as cromakalin and pinacidil have been shown to cause a hyperpolarization of lymphatic muscle and an inhibition of lymphatic pumping (30, 39, 52).

Following an earlier study demonstrating inhibition of lymphatic pumping in mesenteric vessels from 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated guinea pigs, an animal model of intestinal inflammation (60), we assessed the causes of the dysfunction and the role played by NO and K\(_{\text{ATP}}\) channels.
METHODS

Ethical approval. The animal handling and experiments were approved by the University of Calgary Animal Care and Ethics Committee and conformed to the guidelines established by the Canadian Council on Animal Care.

Surgical induction of inflammation. Male Hartley albino guinea pigs (Charles River, Montreal, Canada) between 7–15 days of age were housed at constant temperature (22°C) on a 12:12-h light-dark cycle, with food and water ad libitum. Animals were fasted 4 h and anesthetized with isoflurane (induction 4%, maintenance 2.5–3% in oxygen) before a midline laparotomy was performed. The small intestine was exteriorized and placed on a piece of damp gauze soaked in saline and TNBS (Sigma-Aldrich; 0.425 ml, 30 mg/ml in 30% ethanol in 0.9% NaCl) was injected into the lumen of the ileum, 8–10 cm proximal to the ileocecal junction. An equal volume of saline was injected into the ileum lumen of the sham group to control for volume effects and surgical procedure. The laparotomy was surgically closed and the animals were allowed to recover in the controlled environment of the animal care facility (60). Animals were euthanized by anesthetic overdose followed by exsanguination either 1 or 3 days after surgery to perform quantitative real-time PCR and in vitro analysis of the lymphatic vessel contractile activity, respectively.

Assessment of inflammation. Inflammation was macroscopically assessed in affected area of the ileum and surrounding tissue immediately after being excised from the euthanized animal according to previously established scoring criteria (60). Briefly, ileal segments were macroscopically assessed for the level of severity (0, none; 1, moderate; 2, severe) of erythema, hemorrhage, edema, strictures, ulceration, adhesion, and presence of mucus, with a maximum score of 14. Lymphatic vessels from TNBS-treated animals with a damage score below 6 displayed a contractile behavior similar to vessels from control and sham-treated animals (see also Ref. 60). Lymphatic vessels from animals with a damage score above 10 did not respond to any pharmacological treatments (see Contraction frequency measurements below) and were not considered further. Weight loss was assessed daily as a measure of animal health and used as a reliable indicator of inflammation.

Tissue preparation. Lymphatic vessels were prepared as previously described (9, 16, 58). Briefly, the small intestine was rapidly dissected and placed in 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$_2$PO$_4$; 11 glucose. The pH was maintained at 7.4 by constant bubbling with 95% O$_2$-5% CO$_2$. Small mesenteric collecting lymphatic vessels from animals with a damage score above 10 did not respond to any pharmacological treatments (see Contraction frequency measurements below) and were not considered further. Weight loss was assessed daily as a measure of animal health and used as a reliable indicator of inflammation.

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Contraction frequency measurements. The bath containing the lymphatic vessel preparations was mounted on the stage of an inverted microscope and continuously superfused with PSS heated to 36°C at a flow rate of 3 ml/min causing a changeover time of <7 s, as previously described (9, 16, 58). Impalements of lymphatic muscle cells were obtained from the adventitial side of a lymphatic vessel by using conventional glass intracellular microelectrodes filled with 0.5 M KCl (resistance 150–250 MΩ). Electrodes were connected to an amplifier (Intra 767, World Precision Instruments, Sarasota, FL) through an Ag-AgCl half-cell. Resting membrane potential was monitored on a digital oscilloscope (VC6525, Hitachi) and simultaneously recorded on a computer via an analog-to-digital converter (PowerLab/4SP, ADInstruments, Mountain View, CA).

Electrophysiology. Mesenteric preparations were mounted in a small organ bath (volume 100 μl), placed on the stage of an inverted microscope and continuously superfused with PSS heated to 36°C at a flow rate of 3 ml/min causing a changeover time of <7 s, as previously described (9, 16, 58). Impalements of lymphatic muscle cells were obtained from the adventitial side of a lymphatic vessel by using conventional glass intracellular microelectrodes filled with 0.5 M KCl (resistance 150–250 MΩ). Electrodes were connected to an amplifier (Intra 767, World Precision Instruments, Sarasota, FL) through an Ag-AgCl half-cell. Resting membrane potential was monitored on a digital oscilloscope (VC6525, Hitachi) and simultaneously recorded on a computer via an analog-to-digital converter (PowerLab/4SP, ADInstruments).

Lymphatic muscle impalements were characterized by a sharp drop in potential that settled after 10–15 s to a value typically more negative than −45 mV in vessels from control and sham-treated animals. Antagonists and inhibitors were typically applied through the superfusion, and their effect on membrane potential was monitored for 15–20 min. Quantitative real-time PCR. Mesenteric lymphatic vessels, arteries, and ileum from control, sham-treated, and TNBS-treated guinea pigs were quickly microdissected out, flushed with PBS to remove luminal contents, pinned down of surrounding tissue, and immediately immersed into RNase- and DNase-free collection tubes containing the RNA stabilization reagent RNAlater (Qiagen, Mississauga, ON, Canada). The RNA-stabilized, microdissected tissue was homogenized and disrupted by using sonication QIASHredder (Qiagen) and underwent RNA extraction with the Qiagen Micro RNeasy kit (Qiagen). Subsequent CDNA synthesis was undertaken using Superscript II Reverse Transcriptase enzyme (Invitrogen) with oligo(dT) primers. Real-time amplification of target genes was performed with QuantiTect SYBR
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Green (Qiagen) in an iCycler IQ Real-Time PCR Instrument (Bio-Rad). Guinea pig real-time primer sequences for SUR2B, Kir6.1, inducible NO synthase (iNOS), and endothelial NO synthase (eNOS) were designed by using the first-release guinea pig genome sequence published on ensembl (www.ensembl.org) by using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Expression of β-actin cDNA was used to normalize expression of the genes under study. Real-time primer sequences, as well as the associated ensembl accession numbers, are listed in Table 1. cDNA amplification for all genes was performed using an annealing temperature of 55°C and 50 cycles. Real-time PCR results were analyzed by the ∆ΔCt method. PCR products sequences were verified by the University of Calgary Core DNA service.

Chemicals and drugs. Glibenclamide and indomethacin were purchased from Sigma-Aldrich; ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) from Alexis (San Diego, CA); spermine NONOate and 1400W from Cayman Chemical; and cromakalim from Tocris Cookson (Ellisville, MO). Stock solutions of indomethacin were prepared in 100% ethanol; 1400W, glibenclamide, cromakalim, and ODQ in DMSO; and spermine NONOate in 0.01 NaOH. The compounds were then diluted in PSS to achieve the appropriate concentration. The final concentration of each vehicle was always ≤0.1% (vol/vol), a concentration that had no effect on lymphatic contractile and electrical activities.

Data and statistical analysis. Data are expressed as means ± SE. Concentration-response curves were built and the concentration of the agonist causing half-maximal pumping inhibition (IC50) was estimated by computerized nonlinear regression analysis (Prism, GraphPad software). Statistical significance was assessed by a two-tailed paired and unpaired Student’s t-test and ANOVA with appropriate post hoc tests (as specified in the text), with P < 0.05 being considered significant.

RESULTS

Nitric oxide inhibits lymphatic pumping and hyperpolarizes the lymphatic muscle in control guinea pigs. We used spermine NONOate to assess the effect of NO on rhythmically contracting lymphatic vessels isolated from control animals. One-minute applications of 100 and 300 μM of the NO donor caused a concentration-dependent decrease in contraction frequency (n = 10, Fig. 1, A and B). NO-induced pumping inhibition was significantly reduced in the presence of 10 μM ODQ, a soluble guanylyl cyclase inhibitor (n = 5, Fig. 1, C and D). Spermine NONOate also caused a hyperpolarization of the lymphatic muscle membrane potential, which was concentration dependent (n = 3; Fig. 1E) and inhibited by 10 μM ODQ (Fig. 1F).

Activating KATP channels inhibits lymphatic pumping and hyperpolarizes lymphatic muscle in control guinea pigs. A 1-min application of the synthetic KATP channel opener cromakalim resulted in a significant inhibition of pumping in lymphatic vessels from control animals. This effect was concentration dependent with an IC50 of 126 ± 1 nM (n = 5, Fig. 2, A and B). In the continuous presence of 1 μM cromakalim, vessels contracting at a frequency of 9 ± 1 contractions/min became totally quiescent. The contractile activity was restored to its initial frequency upon administration of the KATP channel blocker glibenclamide (10 μM, n = 4, Fig. 2, C and D). Consistently, cromakalim induced a reversible and concentration-dependent hyperpolarization of the lymphatic muscle (IC50 322 ± 1 nM, n = 4; Fig. 2, E and F) that was significantly inhibited by glibenclamide (1–10 μM, n = 4; Fig. 2, E and G).

NO-induced lymphatic pump inhibition and hyperpolarization requires KATP channel activation. Lymphatic pumping inhibited by 100 and 300 μM spermine NONOate was significantly restored in the presence of 10 μM glibenclamide (n = 6, Fig. 3, A and B). Similarly, hyperpolarization induced by spermine NONOate was inhibited by 1 μM glibenclamide (n = 4; Fig. 3, C and D).

Lymphatic vessels are dysfunctional in TNBS-treated guinea pigs. Surgical instillation of TNBS into the ileum of guinea pigs caused an acute intestinal inflammation as evidenced by increased macroscopic damage scores, compared with sham-treated animals (9 ± 0.4, n = 12 vs. 2 ± 0.4, n = 8 on day 1 and 7 ± 0.3, n = 50 vs. 1 ± 0.2, n = 14 on day 3 for TNBS- and sham-treated animals, respectively). The inflammation was associated with a marked decrease in body weight (5 ± 0.3% of the initial body weight on day 1 and 15 ± 1.6% on day 3; n = 6). Importantly, contractile activity of the mesenteric collecting lymphatics draining the inflamed ileal site in the TNBS-treated animals was noticeably compromised. Specifically, the vessels were quiescent and significantly dilated, characteristics they retained after isolation even when luminal perfused at high flow rate (Fig. 4A). A good correlation was observed between the severity of intestinal inflammation as assessed by macroscopic damage scoring and the increase in vessel diameter (Fig. 4B).

Lymphatic muscle in vessels from TNBS-treated animal are hyperpolarized. The inability of lymphatics from inflamed animals to contract might be due to a depressed lymphatic muscle membrane potential. To test this hypothesis, we measured lymphatic muscle membrane potential in vessels from TNBS- and sham-treated guinea pigs with intracellular microelectrodes. As illustrated in Fig. 4, C and D, the mean membrane potential value recorded in vessels from sham-treated animals (−56 ± 1 mV, n = 19) was not different from that measured in control animals (−52 ± 1 mV, n = 13). In contrast, membrane potential value recorded in vessels from TNBS-treated animals was strongly and significantly hyperpolarized (−68 ± 1 mV, n = 22; P < 0.05 vs. sham and control).

Inhibiting iNOS partially restores lymphatic pumping in TNBS-treated animals. To assess whether NO could be involved in the contractile dysfunction, we first examined the contractile behavior of the vessels in the presence of the

Table 1. Real Time PCR primer sequences (all 5′-3′)

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<th>Ensembl Accession No.</th>
<th>Forward</th>
<th>Reverse</th>
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selective iNOS inhibitor 1400W. A partial restoration of pumping in lymphatic vessels from TNBS-treated animals was observed after a 10-min superfusion with 10 µM 1400W (Fig. 5A). Restoration of pumping was significant in the five vessels examined, with contraction frequency increased from 0 to 8 ± 2 contractions/min during administration of 1400W (P < 0.05; Fig. 5B), a value not significantly different from the contraction frequency observed in vessels from sham-treated animals (n = 6; 10 ± 1 contractions/min). However, as illustrated in Fig. 5A, the pumping behavior was not as regular as in vessels from sham-treated animals, with occasional interruptions of the contraction-relaxation cycle. Membrane potential in vessels from inflamed animals was slightly but significantly depolarized by 1400W treatment (Fig. 5C). Neither contraction frequency nor membrane potential was affected by 1400W in vessels from sham-treated animals.

Inhibiting soluble guanylyl cyclase fully restores pumping in TNBS-treated animals. Addition of the soluble guanylyl cyclase (sGG) inhibitor ODQ (10 µM) to the superfusion caused a significant and sustained reactivation of pumping in vessels from TNBS-treated animals (9 ± 2 contraction/min) over the baseline period (0.4 ± 0.2 contractions/min, n = 6, P < 0.001; Fig. 6, A and B). ODQ did not cause a significant change in contraction frequency in vessels isolated from sham-treated animals.

Fig. 1. The NO donor spermine NONOate inhibited pumping and hyperpolarized lymphatic muscles. A: original trace of vessel diameter changes (downward deflections represent contractions) in an actively contracting lymphatic vessel from the guinea pig mesentery in response to 100 µM spermine NONOate, applied for 1 min (horizontal bar). B: concentration-dependent relationship of the spermine NONOate-induced inhibition of contraction frequency. C: original trace from the same preparation as depicted in A showing the inhibition of the spermine NONOate-induced decrease in contraction frequency in the presence of 10 µM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). D: concentration-dependent relationship of the spermine NONOate-induced decrease in contraction frequency in control conditions and in the presence of ODQ. Columns represent contractions per minute (mean ± SE, n = 10 and 5, respectively). *P < 0.05 and ***P < 0.001 vs. control (paired Student’s t-test). E: concentration-response relationship of the effect of spermine NONOate on lymphatic muscle membrane potential (n = 3). F: original intracellular microelectrode recordings displaying the hyperpolarization caused by a 1-min application of 100 µM spermine NONOate in control conditions (left trace) and in the presence of 10 µM ODQ (right trace).
animals. Membrane potential of vessels from TNBS-treated animals was depolarized in the presence of ODQ, but not more importantly than in vessels from sham-treated animals (Fig. 6C).

**Blocking K\textsubscript{ATP} channels restores pumping in dysfunctional lymphatic vessels.** Opening of K\textsubscript{ATP} channels hyperpolarizes lymphatic muscle and inhibits lymphatic pumping. We thus assessed the involvement of these channels in the inflammation-induced lymphatic dysfunction and incubated the vessels with glibenclamide. Lymphatic vessels from TNBS-treated animals, showing no activity resumed contractile activity in the presence of glibenclamide (10 \textmu M) (10 ± 2 contractions/min, n = 12, P < 0.0001; Fig. 7, A and B). This value was not significantly different from the contraction frequency recorded in vessels from sham-treated animals (11 ± 2 contractions/min, n = 8). In the latter vessels, glibenclamide had no significant effect on contraction frequency. Administration of glibenclamide (1–10 \textmu M) also caused membrane potential of lymphatic muscle from TNBS-treated animals to depolarize (Fig. 7C). Glibenclamide also depolarized membrane potential in vessels from sham-treated animals as already reported in control vessels (see Fig. 2F).

**Expression of iNOS mRNA increases and eNOS mRNA decreases in lymphatic vessels from TNBS-treated animals.** Relative expression of iNOS and eNOS mRNA was measured in sham- and TNBS-treated animals by real-time quantitative PCR. Analysis showed a significant increase in relative iNOS expression in the lymphatic vessels from TNBS-treated (0.4 ± 0.1, n = 5) vs. sham-treated animals (0.01 ± 0.003, n = 5, P < 0.001). Comparison of eNOS mRNA expression showed a downregulation in lymphatic vessels from TNBS-treated animals (0.3 ± 0.2, n = 8) compared with sham-treated animals (3.7 ± 0.9, n = 7, P < 0.0001; Fig. 8A).

**Expression of K\textsubscript{ATP} channel subunit mRNA increases in lymphatic vessels from TNBS-treated animals.** Real-time quantitative PCR data showed that K\textsubscript{ATP} channel subunits are

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**Fig. 2.** The K\textsubscript{ATP} channel opener cromakalim inhibited pumping and hyperpolarized lymphatic muscles. A: original traces of vessel diameter changes (downward deflections represent contractions) in an actively contracting lymphatic vessel from the guinea pig mesentery in response to 0.1 and 0.3 \textmu M cromakalim, applied for 1 min (horizontal bar). B: concentration-dependent relationship of the cromakalim-induced inhibition of contraction frequency (n = 5). C: original trace of the cromakalim-induced pumping inhibition and its reversal by glibenclamide. D: summary bar graph of the inhibitory action of glibenclamide on the cromakalim-induced pumping inhibition. Columns represent contractions per minute (mean ± SE, n = 4). **P < 0.01 vs. control (paired Student’s t-test). E: original intracellular microelectrode recordings displaying the hyperpolarization caused by a 1-min application of 0.1 \textmu M cromakalim (horizontal bars) in control conditions (left trace) and in the presence of 1 \textmu M glibenclamide (right trace). F: concentration-response relationship of the cromakalim-induced hyperpolarization (n = 4). G: summary bar graph of the inhibitory action of glibenclamide on the cromakalim-induced hyperpolarization (n = 3). **P < 0.01 vs. control (paired Student’s t-test).
expressed in lymphatic vessels of sham-treated animals in roughly equal concentrations (Fig. 8B). Our analysis revealed that expression of both channel subunits was upregulated in vessels from TNBS-treated animals, with a three- to fourfold increase in relative expression of Kir6.1 \( (n = 5–6; \ P < 0.01) \) and SUR2B \( (n = 5–8; \ P < 0.001; \ \text{Fig. 8B}) \). Expression levels of Kir6.1 and SUR2B mRNA were comparable in TNBS-treated animals.

Sensitivity to $K_{\text{ATP}}$ channel agonists is increased in lymphatic vessels from TNBS-treated animals. We investigated next whether the increase in $K_{\text{ATP}}$ subunit mRNA expression was translated into increased sensitivity to cromakalim in lymphatics isolated from TNBS-treated animals. To cause these normally quiescent vessels to rhythmically contract and according to findings that inhibition of iNOS (current study) and COX (60) reactivate pumping, we incubated the vessels in a combination of ODQ (10 µM) and indomethacin (10 µM). In these conditions, vessels from TNBS-treated animals contracted at 10 ± 1 contractions/min, a frequency not different from sham-treated and control vessels (Fig. 9, inset), and were significantly more sensitive to cromakalim than vessels from sham and control animals, as illustrated by the rightward shift of the concentration-response curve (IC$_{50}$ 93 nM ± 1 nM, \( n = 4 \), \( P < 0.01 \) vs. sham; \text{Fig. 9A}). Response to cromakalim in

Fig. 3. Glibenclamide inhibited spermine NONOate-induced lymphatic pumping inhibition and hyperpolarization. A: original traces of vessel diameter changes (downward deflections represent contractions) in the same actively contracting lymphatic vessel used in Fig. 1A in response to 100 µM spermine NONOate, applied for 1 min (horizontal bar) in the presence of glibenclamide. B: concentration-dependent relationship of the spermine NONOate-induced inhibition in control conditions and in the presence of glibenclamide. Columns represent contractions per minute (mean ± se, \( n = 6 \)). **$P < 0.05$ and ***$P < 0.01$ vs. control (paired Student’s $t$-test). C: original intracellular micro-electrode recordings displaying the hyperpolarization caused by a 1-min application of 100 µM spermine NONOate in control conditions (left trace) and in the presence of 1 µM glibenclamide (right trace). D: summary bar graph of the inhibitory action of glibenclamide on the spermine NONOate-induced hyperpolarization \( (n = 4) \). **$P < 0.01$ vs. control (paired Student’s $t$-test).

Fig. 4. 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced ileitis lead to lymphatic vessel pumping inhibition and membrane potential hyperpolarization. A: original traces of lymphatic vessel pumping in an actively contracting vessel from sham-treated (left trace) and a quiescent, dilated vessel from TNBS-treated animals (right trace). B: summary graph of the correlation between macroscopic scoring of the ileal inflammation and diameter of lymphatic vessels draining the inflamed area ***$P < 0.001$ vs. sham (ANOVA, with Dunnett’s post hoc test). C: original traces illustrating the difference in lymphatic muscle membrane potential between sham and TNBS-treated animals. D: summary bar graph of the resting membrane potential recorded in lymphatic vessels from control, sham, and TNBS-treated animals. Number of experiments are indicated in parentheses (B and D); ***$P < 0.001$ vs. sham (unpaired Student’s $t$-test).

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sham-treated vessels was not significantly different from that of control vessels (IC$_{50}$ 239 ± 1 nM, n = 4 and 315 ± 1 nM, n = 4, respectively, P > 0.05). We then assessed the hyperpolarizing response of inflamed vessels to cromakalim. As illustrated in Fig. 9B, the increase in sensitivity was not observed and the concentration-dependent relationship was not different from that obtained in sham or control vessels (n = 3–4).

**DISCUSSION**

Following our previous study demonstrating that collecting mesenteric lymphatic vessels draining the guinea pig ileum were dilated and quiescent during an inflammatory episode caused by the instillation of TNBS, we reveal here the critical role played by NO in the lymphatic contractile dysfunction. Pumping inhibition was markedly reversed after treatments with the iNOS-selective inhibitor 1400W and the sGC inhibitor ODQ. Our data also revealed the pivotal involvement of K$_{ATP}$ channels in the lymphatic contractile dysfunction. Specifically, we showed that mRNA expression of the subunits forming K$_{ATP}$ channels was upregulated in collecting mesenteric lymphatic vessels draining the ileum affected by the TNBS-induced inflammation leading to an increased sensitivity to the K$_{ATP}$ channel activator cromakalim. Our findings that lymphatic pumping and membrane potential rates were restored by 1400W and ODQ as shown in Figs. 5 and 6.

**Fig. 5.** The inducible NO synthase (iNOS) inhibitor 1400W partially restored pumping and resting membrane potential in lymphatic vessels from TNBS-treated animals. A: original traces of lymphatic vessel pumping in an actively contracting vessel from sham-treated (top traces) and TNBS-treated animals (bottom traces) in control condition (left) and during treatment with 10 µM 1400W (right). Summary bar graphs of the effect of 1400W on lymphatic pumping (B) and membrane potential (C) on vessels from sham and TNBS-treated animals. *P < 0.05 vs. control (paired Student’s t-test).

**Fig. 6.** The guanylate cyclase inhibitor ODQ restored pumping and resting membrane potential in lymphatic vessels from TNBS-treated animals. A: original traces of lymphatic vessel pumping in an actively contracting vessel from sham-treated (top traces) and TNBS-treated animals (bottom traces) in control condition (left) and during treatment with 10 µM ODQ (right). Summary bar graphs of the effect of ODQ on lymphatic pumping (B) and membrane potential (C) on vessels from sham and TNBS-treated animals. *P < 0.05 and ***P < 0.001 vs. control (paired Student’s t-test).
phatic vessels from inflamed animals were hyperpolarized and that both membrane potential and pumping were restored following administration of the selective K<sub>ATP</sub> channel blocker, glibenclamide, also strongly support a role for K<sub>ATP</sub> channels in the inhibition of lymphatic pumping seen in TNBS-treated animals. Importantly, we demonstrated that activation of the K<sub>ATP</sub> channels is strongly linked to the action of NO since NO-induced inhibition of pumping was associated with a hyperpolarization of the lymphatic muscle, both fully reversed by glibenclamide. A schematic illustration of the signaling mechanisms proposed to be involved in the inflammation-induced lymphatic contractile dysfunction is outlined in Fig. 10.

Role of NO in inflammation-induced pumping inhibition.

Under physiological conditions, NO is constitutively produced by the lymphatic endothelium under the catalytic action of NO synthase (NOS) isoform 3 or eNOS. This enzyme has been shown by immunohistology to be substantially expressed and localized in the endothelium of collecting lymphatics (22, 32, 34) and to be particularly abundant in the valve region (4). Pharmacological studies have consistently demonstrated that relaxation of lymphatic vessels and pumping inhibition strongly depends on NO. These studies investigated contractile responses to NO release by the lymphatic endothelium either via pharmacological stimulation (15, 32, 53, 62) or via shear forces caused by lymph flow increase during phasic contraction (20, 21, 38, 51). The chronotropic inhibition of lymphatic contraction frequency by NO has been associated with a lymphatic muscle membrane potential hyperpolarization and a decrease in size and frequency of spontaneous transient depolarizations, electrical events shown to initiate action potentials and contractions in guinea pig mesenteric lymphatics (53, 55). Gasheva et al. (21) demonstrated that the NO pathway played a key role in the regulation of the contraction-generated reduction of lymphatic tone in the rat thoracic duct, suggesting that it was responsible for the self-regulatory adjustment of lymphatic pumping to the changes in lymph flow pattern. More recently, the role of NO was confirmed by use of genetically modified mice. Intravital microscopy was used to study contractile activity in mouse popliteal collecting lymphatic vessels and to demonstrate that pharmacological and genetic inhibition

Fig. 7. Glibenclamide restored pumping and resting membrane potential in lymphatic vessels from TNBS-treated animals. A: original traces of lymphatic vessel pumping in an actively contracting vessel from sham-treated (top traces) and TNBS-treated animals (bottom traces) in control condition (left) and during treatment with 10 μM glibenclamide (right). Summary bar graphs of the effect of glibenclamide on lymphatic pumping (B) and membrane potential (C) on vessels from sham and TNBS-treated animals. *P < 0.05 and ***P < 0.001 vs. control (paired Student’s t-test).

Fig. 8. Expression of iNOS and K<sub>ATP</sub> subunits mRNA is upregulated in mesenteric lymphatic vessels from TNBS-treated animals. Quantitative real-time PCR analysis of iNOS and NO synthase (eNOS) mRNA expression (A) and SUR2B and Kir6.1 (B) in lymphatic vessels from sham and TNBS-treated animals. Data are means ± se of vessels from 5–8 animals, expressed relative to the number of copies of β-actin. **P < 0.01 and ***P < 0.001 vs. sham (unpaired Student’s t-test).
In the present study, we observed that NOS expressions in guinea pig mesenteric lymphatic vessels were altered in the inflammatory setting caused by TNBS intestinal instillation, with eNOS being downregulated and iNOS upregulated. The predominant role of iNOS-produced NO in causing lymphatic contractile dysfunction was further suggested by the substantial restoration of pumping observed during inhibition of iNOS with 1400W. Although this restoration was significant in that it did cause response in otherwise completely quiescent vessels, it was usually not sustained and not recapitulating the contractile behavior observed in vessels from sham-treated animals. The more robust restoration observed during ODQ administration suggests that other sources of NO may contribute to pumping inhibition by activating guanylyl cyclase. Indeed, during intravital experiments, administration of the eNOS inhibitor Δ^5-nitro-L-arginine (100 μM) to minimally active mesenteric lymphatics from TNBS-treated guinea pigs slightly improved pumping (TF Wu, unpublished data) suggesting a small involvement of eNOS-derived NO in the contractile dysfunction. It is however, also possible that the inflammation present in the guinea pig ileitis model was so severe and iNOS overexpression so important that it surmounted the inhibitory ability of 1400W.

The persistence of lymphatic pumping inhibition in the in vitro experiments and the iNOS overexpression in the lymphatic isolated preparation may suggest that NO was produced in part by the vessel itself. However, possible contribution of other nonlymphatic cells present in the surrounding mesentery in producing and releasing NO or overexpressing iNOS cannot be totally excluded and the exact cellular origin of the mediator has yet to be investigated. It is even more plausible that in vivo, immune cells circulating in the lymph or around the lymphatic vessels may also participate to the release of NO and lymphatic dysfunction. Liao et al. (33) reported that the strong inhibition of lymphatic pumping observed during inflammation was mediated by NO produced by bone marrow-derived cells colonizing the vicinity of the lymphatics. Furthermore, elevated production of NO by peritoneal macrophages reported in dextran sodium sulfate (DSS) mice, another animal model of inflammatory bowel disease (1), could also contribute to impaired lymphatic function.

Interaction between NO and prostaglandin pathways. In addition to NO, other proinflammatory vasodilators are likely to be involved in the lymphatic contractile dysfunction. Indeed,
improvement of pumping with indomethacin reported in the same TNBS model (60) strongly suggest a role for products of arachidonic acid metabolism. Prostacyclin and prostaglandin E2 (PGI2 and PGE2) have been directly associated with lymphatic pumping inhibition in the same mesenteric preparation (8, 45). Action of these mediators could further explain the incomplete effect of 1400W in restoring contractile activity in vessels from TNBS-treated animals. PGE2 could also indirectly influence NO action, since it has been shown to downregulate iNOS at both the mRNA and protein levels (37, 50).

As in smooth muscle, KATP channels can be activated via the cyclic AMP (cAMP)-protein kinase A (PKA) pathway in lymphatic muscle (52). Cyclic nucleotides are regulated within the cell by phosphodiesterases (PDE) (17). In the cell, cAMP levels far exceed those of cGMP; however, cGMP is able to compete for the catalytic sites of the cGMP-inhibited PDE3, therefore decreasing hydrolysis of cAMP and increasing cellular signaling via the cAMP-PKA pathway (35, 63). This potential for cross talk between cyclic nucleotide pathways provides a possible explanation for why ODQ is so effective at restoring pumping. When soluble guanylyl cyclase is blocked by ODQ, the inhibitory effect of cGMP on PDE3 is removed, cAMP level decreases, as well as phosphorylation of KATP channels by PKA. It is possible that the increased production of NO by iNOS contributes to a permissive effect of cGMP on the cAMP-PKA pathway. Direct phosphorylation of the KATP channel by cGMP-dependent protein kinase G (PKG) has been reported (23) and could also account for the NO-dependent activation of KATP channel.

Role of KATP channels in inflammation-induced pumping inhibition. Our data confirm the pivotal role played by KATP channels in regulating lymphatic pumping (25, 30, 39, 45, 52, 58), as evidenced by the potent action of cromakalim to hyperpolarize lymphatic muscle and abolish the phasic contractions that mesenteric lymphatics characteristically exhibit (Fig. 2). More importantly, our findings strongly implicate KATP channels in inhibiting contractility of the lymphatic muscle during inflammation. First, membrane potential and pumping were restored in quiescent lymphatic vessels from TNBS-treated animals following administration of the selective KATP channel blocker glibenclamide. Second, expression of KATP channel subunits was increased in lymphatics isolated from inflamed animals. Third, and consistently with the KATP channel overexpression, vessels from inflamed animals were more sensitive to cromakalim than vessels from sham-treated animals. However, contractile activity in these vessels was measured under conditions in which perfusion rate was increased to 10 μl/min, a value that allowed us to cause restoration of pumping in the presence of the inhibitors used. At this flow rate, sensitivity to cromakalim in control (and sham) vessels was in fact decreased compared with that in control vessels at moderate flow rate (2.5 μl/min, see Fig. 2). Indeed, when hyperpolarizations in response to cromakalim were compared between vessels from sham- and TNBS-treated animals, a protocol in which perfusion rate was not a factor, enhanced sensitivity to cromakalim in vessels from TNBS-treated animals was not observed. It is possible that KATP channel overexpression in vessels from TNBS-treated animals occurred to counteract the increase in transmural pressure/flow rate the vessels were exposed during intestinal inflammation and that the increased sensitivity to cromakalim could only be revealed under conditions of high flow rate mimicking the inflamed situation. Our findings bear similarities with observations by Elias et al. (14), who described a decrease in pumping in mesenteric lymphatics of the sheep during endotoxemia and suggested that the administration of endotoxin could cause a rightward shift of the transmural pressure-fluid pumping curve, meaning that lymphatics vessels were still able to pump but that greater intraluminal pressures were required to elicit a similar level of pumping. The authors further hypothesized that a shift to the right of the transmural pressure-pumping curve would contribute to edema, because the lymphatic vessels would require accumulation of more fluid than normal to provide an increase in the transmural stimulation sufficient to activate the pump. However, such a shift in the curve to the right might be beneficial in edematous/inflammatory conditions because it could allow the lymphatic pump to be more efficient at transmural pressures that would have originally been over the peak of the pumping curve.

KATP channels have been implicated in dysregulation of the contractile activity in several other smooth muscles during inflammatory processes, such as colonic smooth muscle activity (2, 28) during DSS-induced colitis or hypotension (19, 31) and arterial vasodilation (47) during LPS-induced inflammation and endotoxemia.

Interestingly in the context of septic shock, increase in aortic NO could be blunted by selective inhibition of iNOS (12). This action was accompanied by a decrease in NF-κB activation and a downregulation of vascular KATP channel expression, suggesting that activation of this transcription factor by NO leads to overexpression of vascular KATP.

Implication for resolution of edema and inflammation. During inflammation, lymphatic vessels play a dual role. While they help with edema resolution at the inflamed site, they also aid in immune cell trafficking (49). Assuming that lymphatic pumping is critical to lymph flow regulation, high contraction frequency may increase lymph drainage and immune cells trafficking from the inflamed site to the draining lymph node, allowing an appropriate immune response to be mounted. Slowing or inhibiting pumping would then decrease immune cell trafficking and alter the immune response. Alternatively it may help to reduce a potentially exaggerated immune response. It is also possible, assuming that the increased interstitial fluid accumulated in the inflamed area produces a pressure gradient from the interstitial space to the lymphatic vessel lumen capable of maintaining a high lymph flow to the lymph nodes, that nonpumping lymphatic vessels act more efficiently as a conduit for the drainage of excess fluid and immune cells. Again, the consequence could be either an appropriate resolution of edema and immune response or promotion of an overwhelming one. To discriminate between these possibilities, efficiency of lymph flow in these inflammatory situations needs to be further examined.

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REFERENCES


