Endothelial Nitric Oxide Synthase Regulates Microlymphatic Flow via Collecting Lymphatics

Jeroen Hagendoorn, Timothy P. Padera, Satoshi Kashiwagi, Naohide Isaka, Fatima Noda, Michelle I. Lin, Paul L. Huang, William C. Sessa, Dai Fukumura, Rakesh K. Jain

Abstract—Functional interactions between the initial and collecting lymphatics, as well as the molecular players involved, remain elusive. In this study, we assessed the influence of nitric oxide (NO) on lymphatic fluid velocity and flow, using a mouse tail model that permits intravital microscopy and microlymphangiography. We found that NO synthase (NOS) inhibition decreased lymphatic fluid velocity in the initial lymphatics, without any effect on their morphology. Using the same model, we found a similar effect in eNOS−/− mice and in mice treated with a selective endothelial NOS (eNOS) inhibitor. Next, we uncoupled the superficial initial lymphatics from the deeper collecting lymphatics by ligating the latter and found that lymphatic fluid velocity in NOS-inhibited mice became equal to that in control animals. Surprisingly, lymphatic fluid velocity was significantly increased after ligating the collecting lymphatics, and there was a concomitant increase in injection rate and mean lymphatic vessel diameter. Our results provide the first in vivo evidence that eNOS affects function of the whole microlymphatic system and that it is regulated via the collecting lymphatics. (Circ Res. 2004;95:204-209.)

Key Words: microlymphatics ■ eNOS ■ lymphatic function ■ edema ■ lymphangiography

The lymphatic network plays a physiologically crucial, dual role in both immune function and the maintenance of tissue interstitial fluid balance through the transport of interstitial fluid back to the blood circulation. Despite its importance in pathologies such as edema and cancer, mechanistic insights into lymphatic function have been hampered by a lack of quantitative research models. In addition, molecular factors that may affect these mechanisms have remained elusive.

Structurally, the microlymphatic system has been well characterized. First, interstitial fluid is taken up by blind-ended, capillary structures (~60 μm in diameter) known as the initial lymphatics. These consist of adjacent lymphatic endothelial cells, which lack a continuous basement membrane and possess overlaps that act as primary valves. The initial lymphatics are dynamically coupled to collagen fibers of the interstitium via anchoring filaments, so that increased interstitial volume and resultant radial tension on the lymphatics leads to increased convective interstitial-lymphatic fluid transport. Then, fluid is transported to larger lymphatic structures (100 to 150 μm in diameter) that have a smooth muscle layer and intraluminal valves, which divide the lymph vessels into functional units called lymphangions. From these collecting lymphatics, lymph fluid is transported, via lymph nodes and lymphatic trunks, to the thoracic duct and right lymphatic duct and, eventually, drained into the jugular and subclavian veins.

Functionally, determinants of lymph flow are extrinsic propulsive forces such as the lymph formation rate, respiration, and skeletal muscle movement, and the intrinsic contractility of the smooth muscle layer of the collecting lymphatics. Although there is a positive pressure difference between the thoracic duct and dorsal foot lymphatics in humans in upright position, lymph flow is present during basal physiological conditions in caudocranial direction. It is speculated, therefore, that the contractile collecting lymphatics act as a primary driving force for lymph propulsion. A number of studies have confirmed systematic contractions of the collecting lymphatics in various ex vivo preparations. Moreover, oxygen tension is lower in mesenteric collecting lymphatics than in the surrounding interstitial fluid, implying in vivo energy consuming contractile processes of the lymphatic vessel wall. Thus, the transient contraction of each lymphangion forces fluid into the proximal lymphangion and, because one-way valves prevent backflow, this would result in net fluid flow toward the heart. The relative importance of collecting lymphatic vessel contractility on overall lymph flow and the interactions of initial and collecting lymphatics have, however, not been described.

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Nitric oxide (NO), a major regulator of (micro)vascular function, was found to be generated by lymphatic endothelial cells. Lymphatic endothelial cells express nitric oxide synthase (NOS). Exogenous NO inhibits the pacemaking activity of lymphatic smooth muscle cells by activating protein kinases via the cyclic GMP pathway. Applied NO was shown to resemble flow induced inhibition of contraction frequency of mesenteric lymphatics, whereas N-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase inhibitor, could partially attenuate this effect. Nevertheless, data on the differential role of the NOS isoforms on the components of the microlymphatic system in vivo are needed.

In this study, we adapted a previously described model of the microlymphatic circulation for the combined and separate assessment of initial and collecting lymphatic function in the mouse tail. This model uses constant pressure infusion of a fluorescent dye in the interstitium, which is taken up only by the lymphatics (Figure 1). Intravital microscopy is then performed to visualize the lymphatic vessels. RTD indicates region of intravital microscopy of lymphatic transport and residence time distribution analysis. Arrow indicates location of ligation of collecting lymphatic vessels.

Materials and Methods

Animals
Studies were performed in 7- to 10-week-old female C57BL/6 and nude mice. In addition, male and female eNOS−/− mice backcrossed for 10 generations to C57BL/6 background were compared with age- and gender-matched wild-type animals from the same heterozygote-heterozygote breeding pair. All animals were bred and maintained in animal facilities at Massachusetts General Hospital. Fifty-six mice for ligation experiments, and 17 additional controls. All procedures were performed following the guidelines of the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

Experimental Design
Mice received a subcutaneous osmotic pump (ALZET Model 1003D, Durect Corp) 3 days before the lymphatic function measurements for continuous infusion of L-NMMA or N-monomethyl-L-arginine (D-NMMA) (controls) at 350 mg/kg daily, as described. For selective eNOS inhibition, mice received a daily intraperitoneal injection of cavtratin, a cell-permeable peptide derived from caveolin-1, at 2.5 mg/kg or the control peptide AP at 1.2 mg/kg, during 3 days. The following groups were studied: group 1 (n = 4), L-NMMA administration; group 2 (n = 4), D-NMMA administration; group 3 (n = 4), eNOS−/− mice; group 4 (n = 4), wild-type mice; group 5 (n = 4), cavitran administration; group 6 (n = 4), control peptide (AP) administration; group 7 (n = 8), L-NMMA administration plus bilateral collecting lymph vessel ligation; group 8 (n = 7), D-NMMA administration plus bilateral collecting lymph vessel ligation. Additional control groups consisted of: nude mice (n = 3) to confirm that lymphatic fluid velocities were consistent with our previous data and C57BL/6 mice (n = 4) without pump implantation.

Surgical Procedure
Mice in the experimental groups 7 and 8 underwent ligation of the deep collecting lymphatic vessels of the tail immediately before the microlymphangiography, so that development of edema was avoided (Figure 1). Mice were anesthetized intramuscularly (90 mg/kg ketamine and 9 mg/kg xylazine) and placed on a heated surgical microscopy table. The translucent collecting lymphatic vessels were separated from the tail veins with microsurgical forceps through small, bilateral incisions in the axial direction, and ligated with a 10-0 nonabsorbable suture (Prolene, Ethicon). The incision site was closed with surgical glue, taking care to avoid circumferential tension on the tail that could interfere with superficial lymphatic function.

Quantitative Lymph Flow Measurements Using Residence Time Distribution Analysis
Fluorescence intensity measurements were performed using residence time distribution (RTD) analysis as described previously. Briefly, mice were anesthetized and placed on a small plate. FITC-dextran (2.5%) (MW = 2 million; Sigma) in PBS was infused into the interstitial tissue of the tail tip, with a constant pressure of 40 cm H₂O via a 30-gauge needle. Thus, changes in blood vessel permeability would not affect RTD measurements of initial lymphatic fluid velocity. The mouse was transferred to an epifluorescence microscopy setup as described previously. Eight adjacent fluorescent images of the tail, with a field dimension of 3.5 × 2.5 mm, were obtained from distal to proximal, every 10 minutes until saturation was reached in the most proximal region. The temporally consecutive fluorescent images were analyzed offline using NIH Image Analysis software. The average fluorescence intensity was determined for each image and used to calculate the mean residence time for each region, the lymphatic fluid velocity in the tail lymphatic network, and the mean LV diameter.

Immunohistochemistry
Lymphatic vessels of the tail were histologically identified using ferritin lymphangiography (type I ferritin, M, 480 000; Sigma Chemical Co) as described before. Distribution of the NOS isoforms on lymphatic vessel walls was examined immunohistochemically using monoclonal antibodies against eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS) (BD Transduction Laboratory, Inc) as described before.

Mean Arterial Blood Pressure
Eight-week-old female C57BL/6 mice were weighed and anesthetized. Mean arterial pressure (MAP) was measured by cannulation of the exposed left carotid artery with a PE-10 intravascular polyethy...
Results

NOS Inhibition Decreases Initial Lymphatic Fluid Flow

We performed immunohistochemistry for eNOS, iNOS, and nNOS on tail sections, after ferritin lymphangiography to identify the lymphatic vessels. eNOS protein was localized to the walls of the collecting lymphatic vessels of the mouse tail (Figure 3). There was no discernible staining of iNOS or nNOS in the lymphatics (data not shown). Next, we repeated the lymphatic function measurements in eNOS−/− mice and wild-type controls and found, consistent with the L-NMMA treated animals, that lymphatic fluid velocity was decreased (5.9 ± 0.6 versus 8.5 ± 0.7 μm/s, respectively; P < 0.05), without a significant difference in injection rate (23.5 ± 4.4 versus 20.7 ± 3.2 nL/min, respectively; NS), or in mean lymphatic vessel diameter (66.4 ± 2.6 versus 66.7 ± 1.6 μm, respectively; NS). On microlymphangiography, there were no evident morphological abnormalities in the initial lymphatics of eNOS−/− mice compared with wild-type mice. We then performed lymphatic function measurements in mice that had received 3 days of cavtratin, an eNOS inhibitor that does not have any effect on iNOS.24 The used dose of cavtratin caused a decrease in lymphatic fluid velocity (6.6 ± 0.3 versus 8.8 ± 0.2 μm/s, respectively; P < 0.05) (Figure 2A), without a significant difference in injection rate (14.9 ± 0.7 versus 17.2 ± 1.5 nL/min, respectively; NS) (Figure 2B), or in mean lymphatic vessel diameter (60.7 ± 2.3 versus 62.4 ± 1.9 μm, respectively; NS) (Figure 2C). These data delineate eNOS-derived NO in the regulation of lymphatic function. With the given dose of cavtratin, previously shown to have no effect on blood pressure,18 lymphatic fluid velocity appeared less decreased compared with L-NMMA–treated mice. Possibly, the relatively large molecular size of Cavtratin prevented an optimally effective concentration from reaching the lymphatic system. Taken together, these data show that eNOS inhibition decreases overall lymph flow.

eNOS Inhibition Does Not Affect Structure or Function of Uncoupled Initial Lymphatics

We hypothesized that eNOS inhibition affected lymphatic function via the collecting lymphatics. Therefore, we uncou-
Ligation of the Collecting Lymphatics

Initial Lymphatic Resistance Is Decreased After Ligation of the Collecting Lymphatics

Discussion

What is the role of eNOS expression and NO in these mechanistic processes? Our data show that lymph flow was a passive process, i.e., governed by Starling forces and driven purely by lymph formation rate, the microlymphatic system could be described in terms of an electrical circuit, where the relative resistances of the initial and collecting lymphatics determine the actual flow in both compartments. This circumstance may be true in states of high lymph formation rate, such as in our model, where the injection rate equals 10 to 20 times the baseline physiological lymph formation rate. The collecting lymphatics can constrict, resulting in increased outflow resistance for the entire network. Removing the collecting lymphatics functionally by ligation, removes the control of lymph flow. This leads to lower resistance in the initial lymphatics, an increased interstitial-lymphatic pressure gradient, and thus to increased lymph flow. In addition to the increased infusion rate, the amount of fluorescent solution near the interstitial injection site appeared less in the ligated animals. This suggests that, in conjunction with higher lymph fluid velocity and wider lymphatic vessel diameter, overall lymph formation must be augmented. Although establishing the exact mechanistic relationship between collecting and initial lymphatics warrants further investigation and may be revealed by mathematical modeling, these data represent strong evidence that collecting lymphatics act as regulators of microlymphatic lymph flow.

How do the flexible initial lymphatic vessels and the contractile collecting lymphatics act in concert to induce and sustain overall lymph fluid flow? If lymph flow were a passive process, governed by Starling forces and driven purely by lymph formation rate, the microlymphatic system could be described in terms of an electrical circuit, where the relative resistances of the initial and collecting lymphatics determine the actual flow in both compartments. This circumstance may be true in states of high lymph formation rate, such as in our model, where the injection rate equals 10 to 20 times the baseline physiological lymph formation rate. The collecting lymphatics can constrict, resulting in increased outflow resistance for the entire network. Removing the collecting lymphatics functionally by ligation, removes the control of lymph flow. This leads to lower resistance in the initial lymphatics, an increased interstitial-lymphatic pressure gradient, and thus to increased lymph flow. In addition to the increased infusion rate, the amount of fluorescent solution near the interstitial injection site appeared less in the ligated animals. This suggests that, in conjunction with higher lymph fluid velocity and wider lymphatic vessel diameter, overall lymph formation must be augmented. Although establishing the exact mechanistic relationship between collecting and initial lymphatics warrants further investigation and may be revealed by mathematical modeling, these data represent strong evidence that collecting lymphatics act as regulators of microlymphatic lymph flow.

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soratory flow in the l-NMMA-treated animals. Moreover, we did not observe a reciprocal increase in relative resistance of the superficial network in terms of smaller mean lymphatic vessel diameter or incomplete network staining. Our data imply that an overall more-constricted state of the collecting lymphatics under eNOS inhibition leads to decreased total lymph flow. Whereas baseline NO production is needed for active lymph propulsion via collecting lymphatic smooth muscle contractility, NO may act, in states of high passive lymph flow. Interstitial fluid drainage, to lower overall lymphatic resistance.

We found that eNOS is insignificant in affecting initial lymphatic function. This is in agreement with the structural properties and draining role of the initial lymphatics, because these are mechanically coupled to the interstitial space, which intrinsically regulates initial lymphatic permeability.1,2 Clinically, these findings raise the question of the role of eNOS in lymphatic pathologies, such as edema. Whether NO donors could be used to specifically target the collecting lymphatics for the treatment of (noninflammatory) edema or influencing the function of epicardial lymphatics,26 warrants further investigation. In cancer, it has not been elucidated if tumors, shown to exhibit an abnormal lymphatic drainage pattern, can influence peritumor lymphatic function via eNOS.

In conclusion, we show that mice treated with eNOS inhibitors and eNOS−/− mice exhibit decreased lymphatic fluid velocity in the microlymphatic network and that this effect can be eliminated by functionally removing the collecting lymphatics. As a mechanism for the effect of eNOS inhibition on overall lymph flow, we show that the collecting lymphatics respond to NO and provide outflow resistance to the initial lymphatics.

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