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.1 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.2 The initial lymphatics are dynamically coupled to collagen fibers of the interstitium via anchoring filaments,3 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 layer4 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.5 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.6 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.7–10 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.11 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.
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 assess lymphatic fluid velocity and mean lymphatic vessel diameter, and to confirm that lymphatic fluid velocities were consistent with our previous data and C57BL/6 mice (n = 4) without pump implantation.

**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<sup>−/−</sup> 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 were used for these experiments: 24 for the inhibition experiments, 15 for the 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-D-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<sup>−/−</sup> mice; group 4 (n = 4), wild-type mice; group 5 (n = 4), cavtratin 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 H<sub>2</sub>O 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 x 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 vessels 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 polyethylene...
We performed lymphatic function measurements in mice that had received 3 days of L-NMMA treatment for NOS inhibition, and found that overall lymphatic fluid velocity in the dermal lymphatic network was decreased by 42% compared with controls that had received D-NMMA (5.1±0.6 versus 8.7±0.4 μm/s, respectively; *P<0.05) (Figure 2A). The lymphatic fluid velocity in the control group was comparable to that in mice without an infusion pump (8.7±0.4 versus 8.6±1.2 μm/s, respectively) as well as to that in humans. 23 Injection rate of the fluorescent tracer into the interstitium was not significantly different between L-NMMA–treated animals and controls (11.3±1.2 versus 15.7±1.5 nL/min, respectively; *P=0.14) (Figure 2B). In addition, there was no difference in mean lymphatic vessel diameter (61.5±0.7 versus 61.6±1.6 μm, respectively; NS) (Figure 2C). To exclude a confounding effect of blood pressure at the time point studied, we measured MAP in a separate group of mice, and found no difference between mice that had received L-NMMA and controls (71.7±1.4 versus 72.7±3.1 mm Hg, respectively; NS). These data show that NOS inhibition decreases initial lymphatic fluid velocity without affecting mean lymphatic vessel diameter in the superficial network.

The absence of a significant effect on injection rate should be interpreted with caution, because this is only an indirect indicator of lymphatic uptake. Although the collecting lymphatics are not directly functionally evaluated in this experiment, the regular connections between the initial and collecting lymphatics make it reasonable to assume that the decrease in lymphatic filling in the deep collecting lymphatics mirrors that in the superficial initial network. 27 Taken together, these data suggest that NOS inhibition decreases overall lymph flow.

**eNOS 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 cavatrin, an eNOS inhibitor that does not have any effect on iNOS. 24 The used dose of cavatrin 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 cavatrin, 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 Cavatrin prevented an optimally effective concentration from reaching the lymphatic system. Taken together, these data show that eNOS inhibition decreases lymphatic fluid 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-
lymphatic function of the nonligated and ligated control groups. In the ligated mice, the lymphatic fluid velocity was significantly higher than in non-ligated mice (11.2 ± 0.5 versus 8.7 ± 0.4 μm/s, respectively; *P < 0.05) (Figure 2A). In addition, the injection rate was increased (21.8 ± 1.9 versus 15.7 ± 1.5 nL/min, respectively; *P < 0.05) (Figure 2B), as was the mean lymphatic diameter (78.1 ± 2.3 versus 61.6 ± 1.5 μm, respectively; *P < 0.05) (Figure 2C). The mean lymphatic vessel diameter is inversely proportional to initial lymphatic network resistance, which is thus decreased in the ligated group. These data indicate that, in an intact microlymphatic network, the collecting lymphatics provide outflow resistance to the initial network and regulate overall lymph flow.

Discussion

We have shown that pharmacological blockade or genetic deletion of eNOS decreases lymphatic fluid velocity in the skin microlymphatic network and that this is mediated via the collecting lymphatics. These data provide new mechanistic and molecular insights into the regulation of microlymphatic function.

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, ie, 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.23 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.17 The collecting lymphatics can constrict, resulting in increased outflow resistance for the entire network (Figure 4). 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.

What is the role of eNOS expression and NO in these mechanistic processes? Our data show that lymph flow is decreased in the initial lymphatic network under eNOS inhibition and in eNOS−/− mice. Because the collecting lymphatics are not visualized in this model, we cannot exclude shunting of flow to the deeper network. If, however, the collecting lymphatics are calculated to equal 35% of the cross-sectional area of the total lymphatic network, a simple mass balance implies that a ~75% increase in velocity would be necessary in the collecting lymphatics for fully compen-

Figure 3. eNOS is expressed in collecting lymphatics. Cross-sections through mouse tail prepared after ferritin lymphangiography. A, Functional initial lymphatic vessels (arrows) containing ferritin are highlighted green. A collecting lymphatic vessel (asterisk) can be identified as a larger ferritin containing structure adjacent to the tail vein (V). Scale bar=100 μm. B, eNOS is expression (arrows) is localized to the wall of collecting lymphatics containing ferritin (asterisk). The expression pattern resembles that of the tail vein (V). Scale bar=15 μm.

Initial Lymphatic Resistance Is Decreased After Ligation of the Collecting Lymphatics

To further examine the functional interaction between the initial and collecting lymphatic networks, we compared the
satory 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 lymphatics under eNOS inhibition leads to decreased total lymph flow, we show that the collecting lymphatics. As a mechanism for the effect of eNOS inhibition on overall lymph flow, we show that the collecting lymphatics. The latter have a muscular wall and intraluminal valves. C, A constricted state of the collecting lymphatics under eNOS inhibition increases resistance and decreases fluid velocity in the lymphatic network. D, Proximal ligation of the collecting lymphatics leaves the initial lymphatics as the only route for fluid flow. Loss of control of lymph fluid transport and decrease in total resistance, to which lymph vessel diameter is inversely proportional, leads to increased fluid velocity and injection flow rate.

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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References

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