Nitric oxide permits hypoxia-induced lymphatic perfusion by controlling arterial-lymphatic conduits in zebrafish and glass catfish

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Editors by Robert Langer, Massachusetts Institute of Technology, Cambridge, MA, and approved September 4, 2009 (received for review July 9, 2009)

The blood and lymphatic vasculatures are structurally and functionally coupled in controlling tissue perfusion, extracellular interstitial fluids, and immune surveillance. Little is known, however, about the molecular mechanisms that underlie the regulation of blood-lymphatic vessel connections and lymphatic perfusion. Here we show in the adult zebrafish and glass catfish (Kryptopterus bicirrhis) that blood-lymphatic conduits directly connect arterial vessels to the lymphatic system. Under hypoxic conditions, arterial-lymphatic conduits (ALCs) became highly dilated and linearized by NO-induced vascular relaxation, which led to blood perfusion into the lymphatic system. NO blockade almost completely abrogated hypoxia-induced ALC relaxation and lymphatic perfusion. These findings uncover mechanisms underlying hypoxia-induced oxygen compensation by perfusion of existing lymphatics in fish. Our results might also imply that the hypoxia-induced NO pathway contributes to development of progression of pathologies, including promotion of lymphatic metastasis by modulating arterial-lymphatic conduits, in the mammalian system.

Results

Characterization of the Thoracic Duct Lymphatic Vessel in Adult Zebrafish and K. bicirrhis. To characterize the lymphatic system in both zebrafish and K. bicirrhis, the tail regions of adult fish were histologically analyzed. Thoracic ducts (TDs) were readily detectable in both species (Fig. S1). Coexistence of the dorsal aorta, the cardinal vein, and the TD in zebrafish was further validated using fli1:EGFP (enhanced green fluorescent protein) transgenic zebrafish (15), in which both blood vessel endothelial cells (BVECs) and lymphatic endothelial cells (LECs) express EGFP (Fig. S1 B and D). Electron microscopy examination further showed that the TD consisted of a single thin layer of LECs (Fig. S1C). TD also exhibited positive staining for prox-1, which is specifically expressed in LECs (16) (Fig. S1D). To further characterize the lymphatic system in fish, we chose K. bicirrhis (17), which is completely transparent and allowed us to non-invasively study lymphatic perfusion in adult fish (Fig. S1E). Similar to zebrafish, K. bicirrhis also had TD that specifically expressed prox-1 (Fig. S1 F–H) and lacked blood cells (Fig. S1H). The anatomical relationships of the dorsal aorta (DA), posterior cardinal vein (PCV), TD, and the descending branches in K. bicirrhis are schematically shown (Fig. S1J). These findings demonstrate that similar lymphatic systems exist in zebrafish and K. bicirrhis.

Hypoxia Induces Blood Perfusion in the Lymphatics of the Zebrafish Tailfin. The functional relationship between the blood vasculature and the lymphatic system has not been previously studied. Tissue hypoxia is known to alter angiogenesis, vascular architecture, vascular permeability, and blood perfusion (18–20). To study blood perfusion and the relationship between the microvessels of blood and lymphatic vasculatures, we chose the zebrafish tailfin for further characterization. Similar to fli1:EGFP fish strain, the VEGFR2:EGFP fish strain was generated to express EGFP under VEGFR2 promoter and vascular endothelial cells primarily express EGFP (21). Intriguingly, fli1:EGFP and VEGFR2:EGFP transgenic strains of zebrafish had overlapping but distinct patterns of EGFP expression in the vasculature (Fig. L4). The VEGFR2:EGFP strain displayed a relatively restricted staining pattern, consistent with the restricted expression of VEGFR2 in BVECs but not in LECs. In this study, we examined the physical connections between the lymphatic system and arterial vessels in zebrafish and Kryptopterus bicirrhis. We assessed the effects of hypoxia on these connections and considered how these connections may play a role in cancer-related metastasis.

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The authors declare no conflict of interest.


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This article contains supporting information online at www.pnas.org/cgi/content/full/0907608106/DCSupplemental.


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addition, prox-1-positive lymphatic vessels in the tailfin were also EGFP positive in the fli1:EGFP strain (Fig. 1B).

Under hypoxic conditions, the prox-1/H11001/VEGFR2 lymphatic vessels in both proximal and distal regions of the tailfin became perfused with blood cells (Fig. 1C and Movies S1 and S2). The hypoxia-induced lymphatic perfusion was further demonstrated by intra-cardiac injection of fluorescence-labeled dextran (Fig. 1D). Lymphatic flow and the number of circulating cells were markedly increased in the lymphatics under tissue hypoxia (Fig. 2B and C). The hypoxia-induced blood perfusion into the lymphatic system was further demonstrated by crossing two transgenic fish lines carrying fli1:EGFP and gata-1:dsRed, an early marker of the erythroid lineage. In agreement with our data showing increased blood flow as revealed by light microscopy and rhodamine-labeled dextran, fli1:EGFP and gata-1:dsRed double-transgenic zebrafish exhibited erythrocyte perfusion into the lymphatics under hypoxic conditions (Fig. 2A).

In addition to tissue hypoxia, exposure of zebrafish to chelating agents such as CoCl2 also significantly increased blood perfusion into the lymphatics (Fig. 2B and C). Hypoxia-induced lymphatic perfusion appeared to be separated from stress-induced responses. For example, theophylline, a pan-adenosine receptor antagonist (22), did not block hypoxia-induced blood perfusion (Fig. 2B and C). These findings demonstrate that tissue hypoxia controls lymphatic perfusion via an adenosine-independent mechanism.

**Hypoxia-Induced Dilation and Alteration of the Architecture of ALCs.**

The increase in blood perfusion and erythrocytes into the lymphatics under hypoxic conditions led us to further study the molecular mechanism underlying the regulation of this transition. To identify the structural basis for hypoxia-induced lymphatic perfusion, we carefully examined both zebrafish and K. bicirrhis. Under normoxia, a significant number of ALCs were present in the intersegmental vasculature (Fig. 3B). ALCs appeared as tightly interwoven vascular clusters that budded from the segmental arteries and collected into the segmental lymphatics (Fig. 3A and B). Similar to K. bicirrhis, considerable numbers of ALCs were also found in the tail region of fli1:EGFP zebrafish (Fig. 3C). Again, dilated and less-interwoven ALCs were found under hypoxic conditions (Fig. 3C, F, and G). Consistent with structural and diameter changes in the ALCs, the collecting lymphatic vessel (CLV), which is perfused with few blood cells under physiological normoxic conditions, was tightly packed with blood cells when tissue was exposed to hypoxia (Fig. 3D and Movie S4). Interestingly, the TD

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**Fig. 1.** Hypoxia-induced lymphatic perfusion in the tailfin. (A) EGFP-positive signals in the tailfin of fli1:EGFP fish were compared with those in VEGFR2:EGFP fish. The tailfin of VEGFR2:EGFP zebrafish shows a subset of EGFP-positive structures as compared with those of fli1:EGFP zebrafish. Schematic presentation indicates the proximal (P) and distal (D) regions of the tailfin. Blue represents veins, red represents arteries, and green indicates lymphatic vessels. (B) Prox-1 immunohistochemical staining shows that a portion of the fli1:EGFP-positive vasculature is stained with prox-1 (red color, arrows). (Scale bar, 50 μm.) (C) Bright-field microscopic examination of proximal and distal regions of the tailfin under conditions of tissue normoxia and hypoxia. In both distal and proximal regions, hypoxia increases lymphatic dilation (diameters of vessels marked by blue dashed lines) and blood perfusion (blood cells in proximal region, marked with red dots in the distal region). FR, fin ray; M, melanocyte. (Scale bar, 50 μm.) (D) Hypoxia-induced rhodamine (Rh)-dextran perfusion in the lymphatic system of both proximal and distal regions. The bottom row of figures shows merged images of fli1:EGFP expression and Rh-dextran fluorescence. Arrows indicate lymphatic vessels. (Scale bar, 50 μm.)
also became dilated and filled with blood cells under hypoxic conditions (Fig. 3E). These findings demonstrate that the ALC is the structural basis involved in the switch that permits blood perfusion into the lymphatic system in both zebrafish and K. bicirrhis.

**NO Controls Lymphatic Blood Perfusion.** We next studied the molecular players that control lymphatic blood perfusion. NO relaxes and dilates arterial vessels by acting on vascular smooth muscle cells (VSMCs) (23). Because of the unique structural features and specific location of ALCs as the continuation of arteries, we hypothesized that as in arteries, the wall of ALCs might contain numerous VSMCs that might respond similarly to NO. Intriguingly, the addition of sodium nitroprusside (SNP), an NO donor (24), recapitulated the hypoxic effect by increasing blood cell perfusion into the lymphatics (Fig. 4A, B, and I, and Movie S5). To determine whether NO mediated this hypoxia-induced lymphatic perfusion, zebrafish were exposed to hypoxic conditions in the presence or absence of the NO synthase (NOS) inhibitor NG-monomethyl L-arginine (l-NMMA) (25). Remarkably, l-NMMA almost completely blocked the hypoxia-induced lymphatic perfusion (Fig. 4C, D, and I, and Movie S5). To further validate the role of NO in mediating hypoxia-induced lymphatic perfusion, the inhibitor of the soluble guanylyl cyclase (sGC) 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) was used (26). As expected, ODQ was also sufficiently potent in blocking hypoxia-induced lymphatic perfusion (Fig. 4E and I). Similarly, NO-scavenger carboxy-PTIO (cPTIO), a specific NO scavenger (27), almost completely inhibited hypoxia-induced lymphatic perfusion (Fig. 4F and I). Additionally, we performed two NO rescue experiments to determine the role of the NO system in the regulation of lymphatic perfusion. First, l-NMMA and SNP were simultaneously added to hypoxia-exposed zebrafish, and lymphatic perfusion was monitored. SNP significantly restored lymphatic perfusion under the l-NMMA-mediated blockade because l-NMMA acts upstream of the NO signaling pathway and the actions of SNP were not affected by this inhibitor (Fig. 4G and I). Also, ODQ blocked hypoxia- and SNP-induced lymphatic perfusion (Fig. 4H and I), indicating that the effects of NO are primarily mediated by cGMP via activation of sGC.

Taken together, these results provide compelling evidence that hypoxia-induced lymphatic blood perfusion is dependent on activation of the NO signaling pathway.

**Discussion**

The vascular and lymphatic systems are anatomically and functionally coupled in both vertebrate and zebrafish embryos and adults (6, 9, 10). However, the functional relationship between blood and lymphatic vessels that coordinate and contribute to pathogenesis under conditions such as tissue hypoxia remains unknown. In this study, we provide compelling evidence that hypoxia induces a switch that allows lymphatic vessels to become perfused with blood via NO-regulated ALCs in zebrafish and K. bicirrhis. This mechanism seems to be triggered by an acute response to hypoxia. In this system, the compensatory circulatory system is switched on, allowing maintenance of physiological functions in the body and survival of the organism. This system is suited for response to severe hypoxia, because it is rapid and practical to use existing lymphatics as blood vessels rather than growing new vessels and generating new blood cells.

Hypoxia-inducible factor-1 alpha (HIF-1α) is known to mediate hypoxia-induced transcriptional activation of vessel dilation, angiogenesis and erythropoiesis via upregulation of e-NOS, VEGF, and erythropoietin (EPO) (28, 29). Although HIF-1α is also expressed in zebrafish (30), and hypoxia could lead to an increase in transcriptional activation and stabilization of mRNA as seen in mammalian cells (31), HIF-1α does not seem to be involved in switching from lymphatic to blood vessels in our models. Instead, the HIF-1α-mediated hypoxic responses are probably involved in chronic hypoxia, which leads to upregulation of VEGF to stimulate angiogenesis (18) and EPO to
increase the production of erythrocytes (32). Both VEGF- and EPO-induced compensatory responses are delayed relative to the acute response observed in our models because of the time required for transcriptional activation. In our zebrafish and K. bicirrhis models, hypoxia triggered lymphatic perfusion within minutes, suggesting that mechanisms other than those mediated by HIF-1α are responsible.

The arterial-like features of ALCs, which often appear as tangled corkscrew vascular structures, suggest that these vascular segments contain VSMCs. There are, however, no reliable markers for detecting fish VSMCs. NO induces immediate vascular dilatation by relaxing VSMCs (33, 34). Using various NO donors and antagonists, we provide compelling evidence that NO controls the switch of hypoxia-induced lymphatic perfusion. ALCs seem to act as a gatekeeper for lymphatic perfusion, and hypoxia-induced eNOS might switch the functional identity of these vessels from lymphatic to blood by relaxation or constriction.

Evidence suggests that ALCs might be found in mammals. For example, inter-connective structures between mesentery blood vessels and lymphatic vessels have been reported (10). Several recent studies using genetic deletion of mouse lymphangiogenesis-related genes including mucin-type O-glycans, Syk, and SLP-76 show a similar phenotype of blood perfusion into the lymphatics (7, 35, 36). Collectively, these findings demonstrate the presence of intimate structural and functional connections between the two systems. In addition, fast-growing malignant tissues usually experience tissue hypoxia, which promotes cancer cell invasion and metastasis (37–40). Tumor lymphatics are often filled with malignant and blood cells, although the underlying mechanisms that lead to this accumulation remain unknown (41–43). Given our present findings, it is possible that tumor hypoxia could also permit blood perfusion into the lymphatic system. If so, this could be a mechanism by which tumor cells further spread from the bloodstream into the lymphatic system. Thus, our findings provide further support for the idea that tumor hypoxia is crucial for metastasis.

Taken together, our findings in zebrafish and K. bicirrhis have uncovered a function of the lymphatic system under hypoxic conditions. Our results provide information about the molecular mechanisms by which hypoxia-induced NO functions as a switch for permitting lymphatic perfusion. Our findings could be extended to better understand the mechanism of lymphatic perfusion in hypoxia-associated pathological settings such as cancer and metastasis. On the other hand, hypoxia-triggered lymphatic perfusion could be beneficial for improving tissue and blood perfusion in tissues such as the ischemic myocardium. It is plausible that in those patients whose myocardia are poorly protected from the ischemic insult due to a failure of lymphatic perfusion in muscle tissues.

Materials and Methods

Zebrafish and K. bicirrhis Strains and Husbandry. Zebrafish (Danio rerio) strains used in this study include transgenic fli1:EGFP1 (ZFIN), transgenic VEGFR2:EGFP, and double-transgenic fli1:EGFP1 x gata1:dsRed (both kindly provided by Dr. Tim Chico, University of Sheffield). K. bicirrhis were obtained from a local pet store. All fish were kept at the Marine Biological Laboratory, University of Copenhagen, Denmark; Sorø University, Huddinge, Sweden; or the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden, as previously described (44). All experiments were performed in accordance with ethical permissions granted by the ethical councils in Copenhagen, Denmark and Stockholm, Sweden. Adult fish used in these experiments were 6 months or older.

Fig. 3. Hypoxia-induced linearization of arterial-lymphatic conduits (ALCs), lymphatic dilation, and blood perfusion in zebrafish and K. bicirrhis. (A) Schematic presentation of the ALC in the adult K. bicirrhis, with an expanded view of an ALC and its associated segmental artery (SA). DA, dorsal aorta; CLV, collecting lymphatic vessel; PCV, posterior cardinal vein; SL, segmental lymphatic, TD, thoracic duct. (B) Bright-field micrographs of ALCs in K. bicirrhis under normoxia and hypoxia. Black boxes show the ALCs. Dashed lines mark the SA and the lymphatic vessel (LV), as shown by EGFP-positive structures in fli1:EGFP zebrafish. ALCs budded from SAs and appeared as “tangled” compact corkscrew vascular plexuses (red dashed lines). Under hypoxic conditions, the compact, tangled architecture became linearized. (Scale bar, 20 μm.) (C) Hypoxia-induced dilation and blood perfusion in the CLV. Dashed blue lines mark the border of the CLV. B, bone; P, pigment; FR, fin ray. (Scale bar, 100 μm.) (D) Dilation of the TD under hypoxic conditions. Dashed blue lines mark the border of the TD, which became perfused under hypoxic conditions. (F) Quantification of averages of vessel diameters of the ALC, lymphatic vessel, and segmental artery under normoxia and hypoxia. *** P < 0.001. NS, not significant. The open bars presents the values under normoxia and filled bars indicate the values under hypoxia. (G) Quantification of linearization of ALCs under normoxia and hypoxia. ** P < 0.01.
Treatment Protocol for the Pharmacological Study. Exposure of fish to hypoxia was carried out using our previously described protocols (44). Fish were individually placed into a 50-mL tube connected to a reservoir where water oxygen tension was tightly controlled by an oxygen regulatory system and to which L-NMMA, ODQ, CoCl2, c-PTIO, or SNP (all from Sigma) were added as indicated. Water flow was driven by a pump at approximately 0.7 mL/s from the reservoir to the tube and back again in a closed circuit. Fish were incubated under these conditions for approximately 30 min before microscopic examination.

Microangiography. Vascular perfusion of macromolecules was performed by injection of approximately 1 μL rhodamine-conjugated dextran (70 kDa, Sigma) into the ventricle of each adult zebrafish. Before injection, fish were anesthetized with 0.02% tricaine (MS-222, Sigma) until their gills stopped moving, and a small incision was made on the ventral side, slightly posterior to the gills. The injection was done under a stereomicroscope using a standard microinjection setup equipped with a pump and a micromanipulator (Eppendorf). Fish were examined immediately after the injection for a maximum of 10 min. The entire procedure was finished within 15 min, and the fish recovered within 5 min of being transferred back to tank water. No adverse effects were seen in the fish during the measurement procedure.

Histology and Immunohistochemistry. For histological analysis, fish were anesthetized with 0.03% tricaine for at least 20 min after gill activity had stopped, and their brains were crushed with sharp forceps. For frozen sections, fish tissues were snap frozen in OCT Tissue Tec and cut into 10-μm-thick sections with a cryostat. For other staining procedures, tissues were fixed with 4% paraformaldehyde (Sigma) for 24 h at 4 °C. Fixed fish tissues were decalcified with 0.5 M EDTA for 7 days at room temperature and were dissected. Following fixation, some tissues were embedded in paraffin and others were used for whole-mount staining or were mounted directly onto glass slides in Vectashield (Vector Laboratories). Paraffin-embedded tissues were sectioned into 5-μm-thick sections and stained with hematoxylin and eosin (H&E) or were used for immunohistochemical staining. Immunohistochemical staining was performed with the primary rabbit anti-human prox-1 antibody (a kind gift from Dr. S. Baxendale, University of Sheffield) at a dilution of 1:200 and the secondary antibody, Cy3-conjugated goat anti-rabbit IgG (1:200; Chemicon); sections were then mounted in Vectashield.

Electron Microscopy. Fish tissues were immersed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate-HCl buffer (pH 7.3) with 0.05 M sucrose. A few hours later, tissues were cut into small pieces and placed into fresh fixitative. After rinsing with buffer, the specimens were postfixed in 1.5% osmium tetroxide in 0.1 mol/L cacodylate buffer (pH 7.3) with 0.7% potassium ferrocyanate for 1 h at 4 °C, dehydrated in ethanol (70, 95, and 100%), stained with 2% uranyl acetate in ethanol, and embedded in Spurr low-viscosity epoxy resin. Thin sections were examined in a Philips CM120 electron microscope at 80 kV. The specimens were photographed using a Kodak MegaPlus CCD camera.

Fig. 4. Hypoxia-induced lymphatic perfusion is dependent on NO. (A) Tailfin lymphatic perfusion under normoxia. (Scale bar, 50 μm.) (B) Tailfin lymphatic perfusion under normoxia in zebrafish treated with 100 μM sodium nitroprusside (SNP). Under hypoxic conditions, tailfin lymphatic perfusion of (C) control zebrafish (vehicle), and zebrafish treated with (D) 1 μM L-NMMA, (E) 1 μM ODQ, (F) 10 μM c-PTIO, (G) 1 μM L-NMMA + 100 μM SNP, and (H) 1 μM ODQ + 100 μM SNP. Dashed blue lines mark the border of the lymphatics, and red dots indicate blood cells. (I) Quantification of blood cells in tailfin lymphatics under different conditions. ***, P < 0.001; NS, not significant.
Microscopy and Imaging. Images of immunostained and fixed tissues were recorded with a fluorescence microscope equipped with a high-resolution color camera (Nikon).

Photos of living fish, as well as video sequences, were taken following anesthesia. The fish were examined for a maximum of 10 min (zebrafish) or 45 min (K. bicirrhis) and then were transferred to tank water where they fully recovered within 5 min. Videos were analyzed with a Vernier Logger Pro 3.4.5 program (Vernier Software and Technology). Blood flow rates (the speed of a non-rolling red cell in pixels per second), vessel diameters, and the number of cells passing a particular vessel cross section per second were recorded.

Supporting Information

Fig. S1. The lymphatic system in zebrafish and K. bicirrhis. Cross-sections of the fltl:EGFP zebrafish trunk region were (A) stained with H&E or (B) examined by fluorescence microscopy for EGFP expression (green). (C) Electron microscopic examination of the thoracic duct (TD). Arrow indicates a lymphatic valve of the TD. (D) A cross-section of fltl:EGFP zebrafish trunk tissue was immunohistochemically stained with anti-prox-1 (red), demonstrating overlapping positive signals with EGFP (arrows). (E) The boxed region of transparent K. bicirrhis was used to examine blood flow and the TD. (F) Whereas blood flow and red blood cells were easily detectable in the dorsal aorta (DA) and portal cardinal vein (PCV), the TD lacked blood perfusion. (G) H&E staining showed the location of the TD next to the PCV. (H) Immunostaining of K. bicirrhis trunk tissue with anti-prox-1 (red) and with DAPI to show erythrocyte nuclei (blue) showed that the TD was positive for prox-1 (arrows) and lacked erythrocytes (encircled with dashed lines). (I) Schematic presentation of the anatomy of the arterial, venous, and lymphatic systems [adapted from Steffensen JF, Lomholt JP, Vogel WOP (1986) In vivo observations on a specialized microvasculature, the primary and secondary vessels in fishes. Acta Zoologica (Stockh.) 67:193–200]. SA, segmental artery; SV, segmental vein; FR, fin ray; CLV, collecting lymphatic vessel; AFCN, anal fin capillary network. (Scale bar, 50 μm.)
Movie S1. Hypoxia-induced lymphatic perfusion in the proximal tailfin. Bright field microscopic videos of the proximal region of zebrafish tailfins (also shown in Fig. 2C) recorded at 25 fps. Hypoxia induced high rates of blood flow containing high number blood cells in lymphatic vessels.
Movie S2. Hypoxia-induced lymphatic perfusion in the distal tailfin. Bright field microscopic videos of the distal region of zebrafish tailfins (also shown in Fig. 2C) recorded at 25 fps. Hypoxia induced high rates of blood flow containing high number blood cells in lymphatic vessels.
Movie S3. Hypoxia induced linearization of ALCs, lymphatic dilation and blood perfusion in *Kryptopterus bicirrhis*. Bright field microscopic videos of representative ALCs close to segmental arteries in the region shown in Fig. 4 A and B. Videos were recorded at 25 fps. Hypoxia induced both dilation and linearization of the ALC, and high rates of blood perfusion in the lymphatics.

[Movie S3 (MOV)]
Movie S4. Hypoxia induced dilation and blood perfusion in the CLV in Kryptopterus bicirrhis. Bright field microscopic videos of the collecting lymphatic vessel (CLV) in the region shown in Fig. 4 A and D. Videos were recorded at 25 fps. Hypoxia induced high rates blood perfusion in CLV.
Movie S5. Hypoxia induced NO dependent lymphatic perfusion. Bright field microscopic videos of the proximal or distal region of zebrafish tailfins (distal regions also shown in Fig. 5 A–F) recorded at 25 fps. Sequential orders are indicated as follows: Clips 1 and 2 show lymphatic perfusion under normoxia; Clips 3 and 4 show lymphatic perfusion under normoxia treated with 100 μM sodium nitroprusside (SNP); Clips 5 and 6 show hypoxia-induced lymphatic perfusion in tailfin of zebrafish receiving vehicle (DMSO 1:1,000); Clips 7 and 8 show hypoxia-induced lymphatic perfusion in tailfin of zebrafish receiving 1 μM L-NMMA; Clips 9 and 10 show hypoxia-induced lymphatic perfusion in tailfin of zebrafish receiving 10 μM c-PTIO; and Clips 11 and 12 show hypoxia-induced lymphatic perfusion in tailfin of zebrafish receiving 1 μM ODQ. NOS-produced NO triggered a hypoxia-induced increase of blood flow in lymphatic vessels via the NO receptor-mediated signaling.

Movie S5 (MOV)