Physiological roles of endogenous nitric oxide in lymphatic pump activity of rat mesentery in vivo

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Shirasawa, Yuichi, Fumitaka Ikomi, and Toshio Ohhashi. Physiological roles of endogenous nitric oxide in lymphatic pump activity of rat mesentery in vivo. Am J Physiol Gastrointest Liver Physiol 278: G551–G556, 2000.—Physiological roles of endogenous nitric oxide (NO) in the lymphatic pump activity of rat mesenteries in vivo were evaluated using an intravital video microscope system. Changes in the pumping frequency (F), the end diastolic diameter (EDD), and the end systolic diameter (ESD) of the mesenteric lymph microvessels were measured with the microscope system and then the pump flow index (PFI) was calculated. A 15-min superfusion of 30 µM N^•-nitro-L-arginine methyl ester (L-NAME) in the mesenteries caused significant increases of F and PFI and a significant decrease of the EDD and ESD. Simultaneous superfusion of 1 mM L-arginine with 30 µM L-NAME produced a significant reversal of the L-NAME-mediated increase of F and decrease of ESD. A 15-min superfusion of 100 µM aminoguanidine caused no significant effects on F, EDD, and ESD of the mesenteric lymph vessels in vivo. These findings suggest that endogenous NO has physiologically modulated the lymphatic pump activity in rat mesentery in vivo and that the production and release of NO may be mediated by constitutive NO synthase but not by inducible NO synthase.

lymph pump activity; vital microscope

THE LYMPHATIC SYSTEM RETURNS excess tissue fluid and protein to the systemic blood circulation and is important in the maintenance of a constant plasma volume and protein. The lymphatic system also plays an important role in the absorption of fluid and fat from the lumen of the small intestine. The central lacteals (lymph capillaries) begin in the intestinal villi. These lacteals converge to form submucosal collecting vessels, which emerge from the mesenteric border as mesenteric lymphatics. During fat absorption, they appear as fine white channels containing milky lymph (13).

The transport of lymph depends on passive and active driving forces as well as on the rate of lymph production in organs and tissues (19, 21). The active driving mechanism may also play a significant role in the centripetal propulsion of lymph, which is due to the intrinsic contractility of the lymph vessels (4, 7, 15). The rhythm and amplitude of the intrinsic contractions in bovine and murine mesenteric lymphatics are modified by neural and mechanical factors as well as hormonal ones (2, 9, 16, 17, 20, 29).

Recently, we and other groups (6, 20, 23, 28) have demonstrated that lymphatic endothelium-derived nitric oxide (NO) causes a significant reduction of the rhythm and amplitude of intrinsic contractions in mesenteric lymphatics in vitro (23, 28) and a relaxation of lymphatic smooth muscle in isolated dog thoracic ducts (20) and porcine tracheobronchial lymph vessels (6). We also reported recently that rat-exuded macrophages cultured with low concentrations of L-arginine were automatically activated to produce and release a large amount of NO (25) and that the exuded macrophages that migrated into lymph vessels also caused a relaxation of lymphatic smooth muscles through corelease of NO and vasodilative prostaglandins when the cells were activated by bacterial lipopolysaccharides (24).

Little information exists, however, regarding potential effects of endogenous NO on the lymphatic pump activity of mesenteries in vivo. Thus we have attempted to examine effects of N^•-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on the lymphatic pump activity of rat mesenteries in vivo by using a vital video microscope.

MATERIALS AND METHODS

Sixteen male Sprague-Dawley rats weighing 139 ± 3 g were used for the present studies. The rats were housed in an environmentally controlled vivarium and fed a standard pellet diet and water ad libitum. Access to food but not water was discontinued 18–24 h before the experiments.

Surgical preparation. The rats were anesthetized by subcutaneous injection of a mixture of 2% α-chloralose and 10% urethan (0.7 ml/100 g body wt). Additional anesthetics (0.2 ml/100 g body wt sc) was supplemented as needed. A tracheostomy was performed to ensure a patent airway. The right femoral artery was cannulated for monitoring the arterial blood pressure. The right femoral vein was also cannulated for continuous intravenous administration of isotonic saline solution (30 µl·min^{−1}·100 g body wt^{−1}) throughout the experiments. Experiments were terminated if the mean arterial blood pressure fell below 80 mmHg.

The mesentery was prepared for intravital video microscopic observation as described by Benoit et al. (3). Briefly, a midline incision was made and the mesentery adjacent to a segment of small intestine was exteriorized. Then the exteriorized intestinal segment was positioned on a 37°C saline solution-soaked cotton layer in a semicircular channel (inner diameter 10 mm, depth 3 mm) that surrounded an optical
window for observing lymphatic pump activity in the mesentery. Special care was taken not to twist or occlude the lymph and blood vessels. A muscular collecting lymph vessel (~80 µm in diameter) in the mesentery was identified and centered over the heated (37–38°C) window. The collecting lymph vessels chosen for the study were located ~10 mm from the intestinal wall. A plastic plate was placed over the intestinal semicircular channel with Dow Corning silicone grease in a manner that produced a seal between the intestine and mesentery but did not obstruct blood or lymph flow. By covering the intestine in this way, we were able to prevent direct contact of drugs and solutions with the serous surface of the intestine. The exposed mesentery was continuously superfused (6.45 ml/min) with 37–38°C bicarbonate-buffered salt solution containing (in mM) 131.9 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, and 20 NaHCO3. The solution was also equilibrated with a mixture of 5% CO2-95% N2 to maintain pH at 7.4. The preparation was transferred to a video microscope system, by which the lymphatic pump activity in rat mesentery was continuously monitored and recorded.

Analysis of lymphatic pump activity. Lymphatic vessels were observed through a microscope (BHS-323, Olympus) using a x10 water-immersion optic lens (CFW, Nikon). A charge-coupled device camera (KP-M, Hitachi) was used to obtain bright-field images. All continuous images were observed using a monitor (Argus 50, Hamamatsu) and stored using a video recorder (BR-S800DX, Victor) with a video timer (VTG-33, FOR-A) for subsequent analysis. Playing back the stored images in slow motion (1/6 of real time), the frequency of the lymphatic pump activity was counted. Compared with the frame-by-frame images around each pump activity, the end diastolic and end systolic diameters were decided and measured manually on the corresponding images by using a video caliper incorporated with MacLab Chart v.3.4.3 data acquisition system (AD Instruments, Castle Hill, Australia). The other parameters of the lymphatic pump activity, i.e., stroke volume index and pump flow index, were calculated as reported by Benoit et al. (3). For comparison between animals, all values have been normalized to control (baseline) values before superfusion of drugs and are reported as time controls. Only one lymphangion per rat was studied.

Experimental protocol. Once the surgical procedure was completed, the mesenteric preparation was allowed to stabilize for 30 min or until lymphatic rhythmic beatings became constant. The last 5 min of the stabilizing period was defined as the baseline period. The experiment was discarded if the microvasculature, including lymph vessels, showed any signs of obstruction, leakage, or inflammation during the baseline period.

We separated the rats into four groups. Group 1 (n = 4 as control) was established as time control. In this group, the mesenteric lymphatic pump activity was evaluated during at least 15 min after the stabilizing period when the salt solution containing no drug superfused the mesentery. Thus the timing of the control group was preliminarily confirmed to be enough to give a comparison of the stability of this group compared with the others. After the full stabilization, the superfusing salt solution containing 30 µM L-NAME (group 2), 30 µM L-NAME + 1 mM L-arginine (group 3), or 100 µM aminoguanidine (group 4) was started on the mesentery, and then the mesenteric lymphatic pump activity was evaluated during at least 15 min. We decided on a 15-min time period through preliminary experiments mainly to evaluate the direct effect of the drugs on the lymph pump activity with minimized modification of the indirect effects of altering their vascular exchange and thus lymph formation. The contraction frequency and end diastolic and end systolic diameters were averaged for three parts of the experimental time courses, i.e., 0–5 min, 5–10 min, and 10–15 min after starting the experiments with or without the superfusion of drugs. These concentrations of L-NAME, L-arginine, and aminoguanidine chosen in the present study are well known to inhibit NO production, L-NAME-mediated reduction of NO production (27), and inducible NO synthase (NOS) activity (26), respectively. The inhibitory effect of aminoguanidine on inducible NOS (iNOS) is also known to be observed at <10 min after the application of the drug (26). The perfusate containing L-NAME, L-NAME + L-arginine, or aminoguanidine did not flow into the peritoneum or onto exteriorized intestine.

Drugs. The following drugs were used: α-chloralose, urethane ethyl carbamate, L-NAME ester hydrochloride, L-arginine hydrochloride, and aminoguanidine hemisulfate (Sigma Chemical, St. Louis, MO). The doses of drugs are reported as the final concentrations used for each experiment.

Statistical analysis. All lymphatic pump parameters were normalized to the values in the baseline period and expressed as means ± SE. Statistical comparisons were made using one- or two-way ANOVA followed by Bonferroni or Dunnett’s test. Differences in means were considered statistically significant when statistical significance was observed at P < 0.05 or P < 0.01.

RESULTS

Lymphatic pump activity at the baseline period. Figure 1A shows a representative video image of rat...
mesentery. A part of the collecting lymph vessel with a 85- to 95-µm diameter was observed as a white channel. Figure 1B demonstrates a representative rhythmic change in the diameter of the segment of one lymphangion. It shows a sequence of nine images recorded every 0.2 s at the same area of one lymphangion, which is shown as a white square in Fig. 1A. The lymphatic images labeled 5 and 9 (Fig. 1B) demonstrate a maximal constriction and a maximal dilatation in one pump beating, respectively. At the baseline period of the control group (n = 4), the pumping frequency, the end diastolic diameter, and the end systolic diameter were 4.5 ± 0.7/min, 82 ± 6 µm, and 45 ± 3 µm, respectively (Table 1). The mean body weight and mean arterial blood pressure in the control group were also 133 ± 6 g and 110 ± 7 mmHg, respectively (Table 1). The parameters of lymphatic pump activity at the baseline period, the mean body weight, and the mean arterial blood pressure in groups 2, 3, and 4 are also summarized in Table 1. There was no significant difference in the lymphatic pumping parameters at the baseline period among the four groups.

Effects of L-NAME on lymphatic pump activity. Figure 2 demonstrates effects of 30 µM L-NAME on time-dependent changes in the pumping frequency (Fig. 2A), and the end diastolic (Fig. 2B) and end systolic diameters (Fig. 2C) of one lymphangion in rat mesenteries. In the control group, there were no significant time-dependent changes in the pumping frequency, the end diastolic diameter, or the end systolic diameter of the mesenteric lymph vessels. In contrast, in group 2, superfusion of the salt solution containing 30 µM L-NAME rapidly caused a significant increase of the pumping frequency, and then simultaneously produced a significant decrease of the end systolic and end diastolic diameters of the lymph vessels. Figure 3 also shows the effects of 30 µM L-NAME on the pump flow index of the mesenteric lymph vessels in vivo. The pump flow index was calculated during the 5-min period of the baseline or the experimental period as [total numbers of the rhythmic beatings during the period] × [the corresponding stroke volume index]. The stroke volume index is defined as [(π(end diastolic diameter/2)²) − (π(end systolic diameter/2)²)] (3). Treatment with 30 µM L-NAME caused a significant increase of the pump flow index calculated from the first experimental period of 0–5 min (P < 0.05 vs. the control group).

Effects of L-NAME and L-arginine on lymphatic pump activity. Figure 4 summarizes effects of 30 µM L-NAME and 30 µM L-NAME + 1 mM L-arginine on the pump frequency (Fig. 4A), the end diastolic diameter (Fig.
4B), and the end systolic diameter (Fig. 4C) of one lymphangion in rat mesenteries measured during the 10–15 min after starting the superfusion of these drugs. Pretreatment with 30 µM L-NAME (group 2) only produced a significant increase of the pumping frequency in the mesenteric lymph vessels (Fig. 4A). The treatment with L-NAME only also caused a significant reduction of the end diastolic (Fig. 4B) and end systolic (Fig. 4C) diameters of the lymph vessels. In contrast, the simultaneous treatment with 30 µM L-NAME and 1 mM L-arginine caused no significant changes in the pumping frequency or the end diastolic and end systolic diameters of the mesenteric lymph vessels (Fig. 4).

Effects of aminoguanidine on lymphatic pump activity. Figure 5 summarizes the effects of 100 µM aminoguanidine, a concentration that is known to inhibit iNOS activity (26), on time-dependent changes in the pumping frequency and the end diastolic and end systolic diameters of the segment of one lymphangion in rat mesenteries. There is no significant difference in the time-dependent changes in the pumping frequency or the end diastolic and end systolic diameters of the mesenteric lymph vessels between the aminoguanidine-treated group (group 4) and the control group (group 1).

DISCUSSION

Lymphatic endothelial cells, as well as arterial and venous endothelial cells, have an ability to produce and release NO (6, 17, 20, 23, 28). Endothelial constitutive NO synthase (eNOS) and iNOS are also demonstrated immunohistochemically in cultured lymphatic endothelial cells (12, 14, 18). Thus in isolated dog thoracic ducts, acetylcholine (ACh) caused a high-affinity muscarinic receptor-mediated and endothelium-dependent relaxation (20). The ACh-induced relaxation was significantly suppressed by pretreatment with oxyhemoglobin (NO scavenger), N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA; an inhibitor of NO biosynthesis), and methylene blue (an inhibitor of guanylate cyclase) but not by aspirin (an inhibitor of cyclooxygenase) (20). NO released from lymphatic endothelial cells produces endothelium-dependent negative chronotropic and inotropic effects on spontaneous contractions in isolated bovine mesenteric lymph vessels through activation of low-affinity muscarinic receptors (28).
The physiological roles of lymphatic endothelial cells in the regulation of the lymphatic pump activity in vivo remain unclear. The lymphatic endothelial cells may play little or no role in the regulation of pump activity because mechanical rubbing of the endothelium or an addition of L-NMMA (3 \times 10^{-5} \text{ M}) caused no significant effect on the rhythm and amplitude of spontaneous contractions of the isolated lymphatic smooth muscles (28). The hypothesis is strongly supported by the recent study in which the pumping activity of bovine mesenteric lymph vessels containing several lymphangions (5) or that endothelial NO is an important modulator of lymphatic pumping activity in rat cannulated lymph vessels (17). Recently, von der Weid et al. (23) demonstrated that the lymphatic endothelium in guinea pig mesenteric lymph vessels produced and released NO endogenously to decrease the efficacy of spontaneous transient depolarizations, which were related to generation of action potentials of the pacemaker cells. Thus endothelium-derived NO may be the signal that leads to the observed rhythmic oscillations in lymphatic smooth muscles. The discrepancy in the physiological roles of endothelium-derived NO in the lymphatic system may be mainly related to differences of lymphatic preparations used, i.e., tissue strips or cannulated vessels, or to regional and animal differences of lymph vessels used.

In the present study with vital microscopic observation, we have demonstrated that endogenous NO seemed to physiologically regulate lymphatic pump activity in rat mesenteries in vivo. Thus the superfusion of bathing salt solution containing 30 µM L-NAME into rat mesenteries caused a significant increase of the pumping frequency and a significant decrease of the end diastolic and systolic diameters of the lymph microvessels. Simultaneous superfusion of 1 mM L-arginine with 30 µM L-NAME produced a significant reversal of the L-NAME-mediated increase of the pumping frequency and decrease of the end systolic diameter (Fig. 4). In addition, the same concentration of L-NAME significantly increased the pump flow index calculated by using total numbers of the lymphatic pumping activity and the stroke volume index (Fig. 3). These experimental findings enabled us to reach our conclusion. The origin of endogenous NO in the mesenteric lymph microvessels may be, in part, related to flow-mediated production and release of NO from the lymphatic endothelial cells. The suggestion may be compatible with our preliminary report that flow stimulation induced production and release of NO from the lymphatic endothelial cells and resulted in a significant relaxation of the cannulated dog thoracic ducts in vitro (22).

Another important aspect of the present study is that aminoguanidine, a highly selective inhibitor of iNOS, had no significant effect on the pumping frequency and diameters in the rat mesenteric lymph microvessels in vivo. The concentration and incubation time of aminoguanidine used in the present study is known to significantly reduce iNOS activity in tissues and macrophages (26). Thus we concluded that endogenous NO produced by activation of the iNOS in rat mesentery did not contribute to physiological modulation of the endogenous NO of the lymphatic pumping activity.

Finally, there is also the possibility that indirect increasing effects of L-NAME on vascular exchange and lymph formation (1, 10, 11) may be, in part, related to the L-NAME-mediated increase of the lymphatic pumping frequency in the present experiments. The increase of lymph formation produced by the indirect effects of
L-NAME may also induce an enlargement of the end
diastolic and end systolic diameters in rat mesenteric
lymph microvessels. If the L-NAME induced increase of
the vascular exchange and lymph formation begins <15
min after the superfusion of the drug in the present
study, we should take account of such L-NAME indirect
effects in the present conclusion. However, such enlarge-
ment of the diameters was not observed in the present
experiments. We also focused on rapid effects of L-NAME
on lymphatic pump activity just 0–15 min after the
superfusion of the drug. This time may be not enough to
evaluate indirect effects of L-NAME on the lymphatic
pump activity. We also took care to prevent direct
contact of the drugs and solutions with the serous
surface of the small intestine. Therefore, we finally
supposed that such possibility may be in part or
negligibly related to the effects of the drugs on the
lymphatic pump activity obtained with the present
experiments.

In conclusion, endogenous NO has physiologically
modulated the lymphatic pumping activity in rat
mesentery in vivo, and the production and release of NO
may be mediated by eNOS but not by iNOS in the
lymphatic endothelial cells.

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