Transport of extracellular l-arginine via cationic amino acid transporter is required during in vivo endothelial nitric oxide production

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Zani, Brett G., and H. Glenn Bohlen. Transport of extracellular l-arginine via cationic amino acid transporter is required during in vivo endothelial nitric oxide production. Am J Physiol Heart Circ Physiol 289: H1381–H1390, 2005. First published April 22, 2005; doi:10.1152/ajpheart.01231.2004.—In cultured endothelial cells, 70–95% of extracellular L-arginine uptake has been attributed to the cationic amino acid transporter-1 protein (CAT-1). We tested the hypothesis that extracellular l-arginine entry into endothelial cells via CAT-1 plays a crucial role in endothelial nitric oxide (NO) production during in vivo conditions. Using L-lysine, the preferred amino acid transported by CAT-1, we competitively inhibited extracellular l-arginine transport into endothelial cells during conditions of NaCl hyperosmolality, low oxygen, and flow increase. Our prior studies indicate that each of these perturbations causes NO-dependent vasodilation. The perivascular NO concentration ([NO]) and blood flow were determined in the in vivo rat intestinal microvasculature. Suppression of extracellular l-arginine transport significantly and strongly inhibited increases in vascular [NO] and intestinal blood flow during NaCl hyperosmolality, lowered oxygen tension, and increased flow. These results suggest that l-arginine from the extracellular space is accumulated by CAT-1. When CAT-1-mediated transport of extracellular l-arginine into endothelial cells was suppressed, the endothelial cell NO response to a wide range of physiological stimuli was strongly depressed.

Within caveolar regions of endothelial cells, the endothelial isoform of nitric oxide synthase (eNOS) is localized with the cationic amino acid transporter protein CAT-1 (45). CAT-1 is part of a family of transport proteins that also includes CAT-2b and CAT-3. These transporters possess transport properties characteristic of system y+, which interacts with cationic amino acids, such as l-arginine and l-lysine (20). In endothelial cells, CAT-1 has been shown to be predominantly expressed and accounts for 70–95% of extracellular l-arginine uptake in cultured endothelial cells (31, 63). To what extent eNOS uses l-arginine from the extracellular space or from the intracellular stores has not been determined in the in vivo state. The half-saturating arginine concentration for eNOS is in the range of 1–10 μM (49, 52). Physiological l-arginine concentrations in the extracellular space are 50–200 μM (60). Within cultured endothelial cells, the l-arginine concentration range is 100–800 μM (4, 6, 30, 35) and even higher in freshly isolated endothelial cells (35). From the intracellular concentration, it would seem eNOS operates under a saturating intracellular arginine concentration and would not require any arginine from the extracellular space. However, basal NO production in vascular endothelial cells declines when the extracellular concentration of l-arginine is below typical plasma concentrations (33, 44). Our laboratory has shown that the in vivo NO concentration ([NO]) can be almost doubled with 1 mM l-arginine (12). The observation that extracellular l-arginine can enhance endothelial NO production, despite a saturating intracellular arginine concentration, has been termed the “arginine paradox” (41). A possible answer to the arginine paradox was proposed by McDonald et al. (45), who showed the existence of a caveolar complex between eNOS and CAT-1 in pulmonary artery endothelial cells. From these results it has been suggested that caveolar eNOS may be functionally isolated from the large internal store of l-arginine, potentially allowing the eNOS-CAT-1 complex to influence NO production through directed delivery of l-arginine to eNOS (1, 45). In this context, Closs et al. (23) found human endothelial cells have two l-arginine pools: pool I that is and pool II that is not freely exchangeable with the extracellular space via CAT-1.

In vivo and in vitro vascular studies (12, 26, 28, 38, 42, 53, 56) have shown that extracellular l-arginine administration above plasma concentrations improves either NO formation or vascular relaxation attributable to NO. This might be taken as evidence that the supply of L-arginine is somehow limiting to NO formation. Hardy and May (33), using cultured bovine aortic endothelial cells, found stimulation of eNOS with bradykinin and acetylcholine, along with other endothelium-dependent dilators, was associated with increased l-arginine uptake. In contrast, using the same cell source, Arnal et al. (2) showed that the presence of L-arginine in cell culture medium did not significantly increase bradykinin-stimulated NO release. In a similar context, MacKenzie and Wadsworth (44) found only a small increase in aortic relaxation to methacholine in the presence of 100 mM l-arginine vs. no l-arginine. Even though the l-arginine sources for eNOS are controversial, there is enough in vitro and in vivo evidence to support the notion that extracellular l-arginine entry into endothelial cells via CAT-1 may have a role in endothelial NO production. However, the relevance of this system to in vivo conditions has not been determined by direct measurement of changes in vascular wall [NO] and blood flow as the CAT-1 transport of l-arginine is suppressed. Therefore, in these studies l-lysine, a competitive inhibitor of l-arginine entry via CAT-1 (21, 22, 33), was suffused over the rat intestinal microvasculature to suppress CAT-1 transport of l-arginine during conditions of NaCl hyperosmolality, lowered oxygen availability, and increased blood flow. Prior studies from our laboratory (8, 12, 48) indicate that each of these physiological conditions results in NO-dependent vasodilation. For each perturbation, arteriolar

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diameter, blood flow, and arteriolar wall [NO] were measured before and after the suppression of CAT-1 L-arginine transport by t-lysine. For all conditions tested, the results suggest that CAT-1 transport of extracellular L-arginine into endothelial cells was essential for increased NO production with subsequent increases in arteriolar diameter and blood flow. Given the wide range of physiological stimuli we tested, CAT-1 transport of extracellular L-arginine into endothelial cells is a crucial component of in vivo vascular regulation.

METHODS

All animal procedures were submitted to, independently reviewed, and performed in accordance with the Institutional Animal Care and Use Committee Guidelines of Indiana University Medical School.

Animal and tissue preparation. Adult male Sprague-Dawley rats (250–350 g) (Harlan, Indianapolis, IN) were anesthetized with sodium thiopental (200 mg/kg, subcutaneous; Abbott, Chicago, IL). A rectal temperature of 37°C was maintained by placing each rat on a heating pad (35–36°C). The trachea was intubated with polyethylene tubing (PE-240) and mechanically ventilated (70 breaths/min) to have a percent saturation of hemoglobin with oxygen of ~95%. The left femoral artery was cannulated to measure arterial blood pressure.

An established technique (7) was used to prepare the small intestine for observation. An ~10-cm loop of the jejunal region was exteriorized and slit ~2.5 cm with a microsurgery along the antimesenteric border. The intestine was restored to physiological dimensions with small sutures tied to the edges of the intestinal incisions. After being heated to 37.5 ± 0.5°C, a 5 ml/min flow of bicarbonate-buffered physiological saline solution (118 mM NaCl, 6 mM KCl, 25 mM NaHCO3, and 3.5 mM CaCl2) was passed through the chamber. Equilibration of the physiological saline was done with 90% nitrogen, 5% carbon dioxide, and 5% oxygen. To partially suppress intestinal motility to allow micropipette placement, we added norepinephrine (Sigma Chemical, St. Louis, MO) to the bathing fluid. The concentration of norepinephrine was in the range of 10–100 nM and had minor effects on vascular resistance.

Microvascular observation and flow velocity measurements. Using a ×10 or ×20 Nikon water-immersion lens, we observed microvessels and small arteries through the microscope (model BHMJ; Olympus, Hyde Park, NY). A Video Scope camera (model CCD 200E; VideoScope International, Washington, DC) joined with a computerized digitizing and image analysis system (MetaMorph; Universal Imaging, Downingtown, PA) was used to record all images and measure vessel inner diameter. An Optical Doppler Velocimeter (Microcirculation Research Institution, Texas A&M, TX) was used to measure the red blood cell flow velocity. Red blood cells on a rotating disk for velocities of ~100–600 μm/s were used to evaluate the linearity of the velocity versus signal output. The following equation, which assumes a circular diameter of arterioles, was used to calculate linearity of the velocity versus signal output. The following equation, which assumes a circular diameter of arterioles, was used to calculate linearity of the velocity versus signal output.

Perivascular NO measurements. A polarographic technique, using carbon fiber, recessed-tip glass microelectrodes, was implemented to measure [NO] in the intestine. In past studies (8, 12, 48), NO-sensitive microelectrodes have been used by our laboratory. Our experience and techniques, developed by Buerk et al. (18) and Freidemann et al. (29), were the basis for the production and calibration of the NO-sensitive microelectrodes. The microelectrodes had a sharpened outer tip diameter of 7–10 μm. The NO-sensitive microelectrodes were polarized at +0.7 or +0.9 V relative to a World Precision Instruments carbon fiber reference electrode (Sarasota, FL) or a silver-silver chloride electrode. The polarization voltage at which a given electrode was most sensitive to [NO] was used, as measured with a Keithley model 6517A electrometer (Cleveland, OH). The currents generated were typically in the range of 0–20 pA. A calibration curve was established by measurement of the microelectrode current at [NO] of 0, ~600, and ~1,200 nM, based on the composition of the NO-N2 precision calibration gases (Matheson, Joliet, IL) in saline at 37.5°C. The working resolution of the microelectrodes was typically <10 nM, allowing for random noise and current drift. As checked in past studies (8, 62), the sensitivity of the microelectrodes to NO is retained when the NaCl osmolarity of the bathing fluid is altered. The microelectrodes are completely insensitive to oxygen when positively polarized. The current generated when the microelectrode was placed 200 μm above the tissue was used as the 0 nM reference immediately before and after each tissue measurement to obtain a baseline measurement. The rate of drift and an interpolated baseline current for any given time was calculated from these data. The interpolated baseline current was subtracted from the current during the time period when tissue [NO] was measured to yield a value representing the current equivalent of the [NO].

Perivascular Po2 measurements. Carbon fiber, recessed-tip glass microelectrodes with a sharpened outer tip diameter of 7–10 μm were used to measure perivascular Po2. The electrodes were polarized at −0.7 V relative to the ground electrode, and current was measured with a Keithley 6517 electrometer. A tonometer was used to calibrate and test the electrodes for a linear current-Po2 relationship at Po2 of 0, 40, and 144 mmHg at 37°C.

Competitive inhibition of cationic amino acid transporter during NaCl hyperosmolality. The effects of L-lysine, a competitive inhibitor of organic transport through the cationic amino acid transporter (21), on endothelial NO production and arteriolar blood flow during NaCl hyperosmolality were determined. The concentration of L-lysine used in these studies, 200 μM, was based on previously published reports (33, 44). L-Lysine did not have access to the epithelial cells of the mucosa to avoid absorptive hyperemia. At the control osmolarity of 300 mosM and at 360 mosM before and after the administration of L-lysine (200 μM), we measured the periarteriolar [NO], arteriolar diameter, and blood flow velocity for an individual arteriole in the intestine. L-Lysine was exposed to the tissue for 30 min to assure diffusion and bath turnover in this and all the other protocols.

Competitive inhibition of cationic amino acid transporter during low-oxygen conditions. During low-oxygen conditions, the effects of L-lysine competition on endothelial NO production and arteriolar blood flow were determined. At the control condition (90% nitrogen, 5% carbon dioxide, and 5% oxygen) and low-oxygen condition (95% nitrogen and 5% carbon dioxide, we measured the periarteriolar [NO], arteriolar diameter, and blood flow velocity for an individual intestinal arteriole before and after L-lysine (200 μM) was applied. Before and after the addition of L-lysine, all measurements were made 5–10 min after the oxygen percentage in the bathing solution was changed. The typical bath partial pressure of oxygen is 40–45 mmHg with 5% oxygen and below 10 mmHg when 0% oxygen is used.

Competitive inhibition of Na+/Ca2+ exchanger during low-oxygen conditions. We found in a prior study (62) that the Na+/Ca2+ exchanger (NCX) is very active in vivo endothelial cells. As sodium ions increase their leakage into endothelial cells at reduced oxygen tension (32, 61), this pump system might be activated to remove sodium ions in exchange for calcium ions. This would elevate intracellular calcium and possibly explain our prior findings that decreased vessel wall oxygen tension caused an increase in [NO] (9, 48). The effects of KB-R7943, a selective inhibitor of the NCX, on periarteriolar Po2 and arteriolar blood flow were determined during low-oxygen conditions. On the basis of previously published reports (3, 24), 50 μM of KB-R7943 was used in these studies. We measured
ing collateral flow to an occluded arteriole before and after L-lysine
diameter, and blood flow velocity for an individual 1A or 2A provid-
eutral extracellular arginine, we measured periarteriolar [NO], arteriolar
ossion of a 1A or 2A, L-lysine (400
was applied. After measurements were made with and without occlu-
response; natural vs. pharmacological blockade), with post hoc
parisons were made using repeated-measures, two-way ANOVA (rest
the endothelium-dependent vasodilator bradykinin was used to
determine whether the concentration of KB-R7943 used in our experi-
ments had any nonspecific effect on arterial vasodilation. Known
concentrations of bradykinin were suffused over the entire vasculature
before and after KB-R7943 was applied, and the arteriole dilatory
responses were directly compared.

Competitive inhibition of cationic amino acid transporter during
collateral perfusion. To increase blood flow in a given vessel, we
nontraumatically occluded either a large arteriole [first-order arteriole
(1A)] or an intermediate-sized arteriole [second-order arteriole (2A)]
in anatomical hemodynamic parallel using a glass micropipette, as
previously reported (12). This procedure resulted in an increase in
resting blood flow in 1As and 2As, because these vessels became
collateral arterioles to perfuse the tissue normally perfused by the
occluded vessels in the bowel wall. The blood flow in the selected
vessels typically increased 50–100% during the occlusion of their
parallel neighbor vessel. The venous drainage of the venule beside the
occluded 1A or 2A was not impeded. Therefore, in flow-deficient
areas of tissue, the normal venous outflow from that area precluded
vasoactive materials in venous blood from causing vasodilation of the
collateral perfusing 1A or 2A. The major stimulus to the collateral 1A
or 2A should be flow-mediated vasodilation as the shear rate is
increased. To study the effects of blocking CAT-1 transport of
extracellular arginine, we measured periarteriolar [NO], arteriolar
diameter, and blood flow velocity for an individual 1A or 2A provid-
ing collateral flow to an occluded arteriole before and after l-lysine
was applied. After measurements were made with and without occlu-
sion of a 1A or 2A, L-lysine (400 μM) was added to the bicarbonate
solution, and this solution suffused the intestine for 30 min before
measurements were made with and without the 1A or 2A occlusion.
Responses to increased shear rate were not consistently suppressed by
200 μM L-lysine. However, 400 μM L-lysine was very effective in
suppressing dilatory and [NO] responses. We believe this situation
occurs because of the near doubling of blood flow involved to deliver
plasma arginine to the tissue during the collateral perfusion protocol.

Data and statistical analysis. All data are expressed as means ±
SE. Statistica 6.0 software was used for all statistical analysis.
Comparisons were made using repeated-measures, two-way ANOVA (rest
vs. response; natural vs. pharmacological blockade), with post hoc
analysis via the Fishers least significant difference (LSD) procedure to
assess differences in each variable.

RESULTS
For these studies, a total of 22 male Sprague-Dawley rats
(315.1 ± 8.9 g) was used with a mean arterial pressure of
123.2 ± 2.5 mmHg. The recorded measurements were not used
if during any part of the experiment the mean arterial pressure
was not relatively constant or fell below 90 mmHg for an
extended period of time. Under control conditions the arteri-
oles used in this study had a baseline diameter of 53.1 ± 3.4
μm, whereas the measured red blood cell velocities were
35.5 ± 2.7 m/s. The baseline blood flows were calculated to
be 0.098 ± 0.021 mM/s. Also, the average periarteriolar
[NO], measured on the lateral flank of the arterioles, was
545.8 ± 144.7 nM under control conditions.

Figure 1A presents the averaged blood flow and NO re-
sponses to 200 μM L-lysine from experiments to be described
in Figs. 2 and 4. After L-lysine was applied, the blood flow
significantly decreased to 79.7 ± 5.7% of control and [NO]
decreased to 90.8 ± 3.1% of control. We found a similar
reduction in blood flow with 400 μM L-lysine (Fig. 3). We
were requested to determine whether a component of the
vasoconstriction during L-lysine exposure is independent of an
NO mechanism. We compared resting blood flow to conditions
during 0.5 mM Nω-nitro-L-arginine methyl ester (L-NAME)
and the combination of L-NAME and 200 μM L-lysine. In
addition, the ability of 200 nM bradykinin to increase intestinal
blood flow after each type of suppression was evaluated. These
data based on studies of four rats are shown in Fig. 1B.
L-NAME lowered blood flow to 63.3 ± 4.6% of control and
strongly limited the vasodilation to bradykinin. The combina-
tion of L-NAME and L-lysine did not significantly alter the flow
relative to that at rest or during bradykinin exposure with
L-NAME alone. Therefore, for these conditions, L-lysine did not
cause constriction over that induced by a strong suppres-
sion of eNOS with L-NAME. In addition, both 200 and 400
μM L-lysine reduced basal flow about one-half as much as did
L-NAME, yet subsequent data in Figs. 2–5 show that these
concentrations of L-lysine strongly suppressed endothelium-
dependent vasodilation and generation of NO to a wide variety of mechanisms.

Effect of competitive inhibition of arginine transport via cationic amino acid transporter by L-lysine during NaCl hyperosmolarity. Five male Sprague-Dawley rats (322 ± 38.2 g) were used in these studies, and all values are percentages of control. Figure 2A shows a significant reduction in blood flow to 75.6 ± 10.1% of control after the application of L-lysine. Also, when the osmolarity was increased to 360 mosM, there was a significant difference between natural blood flow of 129.9 ± 9.2% and blood flow after L-lysine was administered of 83.2 ± 8.8%. Relative to the L-lysine resting blood flow, hyperosmolarity did not increase flow.

For periarteriolar [NO], there was a consistent decline in the mean concentration with L-lysine that did not achieve a significant difference at the resting condition of 300 mosM due to variability between vessels (Fig. 2B). At 360 mosM, periarteriolar [NO] was increased significantly to 139.1 ± 10.2% during the natural state, but the periarteriolar [NO] did not increase (99.1 ± 6.5%) in response to NaCl hyperosmolarity in the presence of L-lysine. These results suggest that the cationic amino acid transporter may play an important role in regulating blood flow and periarteriolar [NO] during exposure of NaCl hyperosmolarity to the intestinal vasculature.

Effect of competitive inhibition of arginine transport via cationic amino acid transporter by L-lysine during collateral perfusion. Five male Sprague-Dawley rats (282.2 ± 7.1 g) were used in these studies, and all values are percentages of control. During natural conditions, blood flow decreased significantly compared with the control after the adjacent arteriole was occluded. There was a significantly diminished increase in blood flow compared with the natural paired condition during occlusion after L-lysine (400 μM) was applied. Compared with the natural paired condition, when flow was elevated, there was a significantly diminished increase in periarteriolar [NO] after an adjacent arteriole was occluded. Data are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. natural paired condition.

Fig. 2. Effect of L-lysine on intestinal arteriolar blood flow and periarteriolar [NO] during NaCl hyperosmolarity. A: during natural conditions, blood flow increased significantly compared with the control as the osmolarity of the bathing fluid was increased. At 300 mosM (rest) and at 360 mosM (hyperosmotic) there was a significant reduction in blood flow compared with the natural paired condition after the application of L-lysine (200 μM). B: periarteriolar [NO] increased significantly compared with the control as the bathing fluid osmolarity was increased during natural conditions. At 360 mosM (hyperosmotic), application of L-lysine (200 μM) resulted in a significant reduction in periarteriolar [NO] compared with the natural paired condition.

Fig. 3. Effect of L-lysine on intestinal arteriolar blood flow and periarteriolar [NO] during collateral perfusion. A: blood flow during natural conditions significantly increased compared with the control after the adjacent arteriole was occluded. There was a significantly diminished increase in blood flow compared with the natural paired condition during occlusion after L-lysine (400 μM) was applied. Compared with the natural paired condition, when flow was elevated, there was a significantly diminished increase in periarteriolar [NO] after an adjacent arteriole was occluded. Data are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. natural paired condition.
As shown in Fig. 3B, there was no significant difference compared with control in periarteriolar [NO] during natural conditions after 400 μM L-lysine was applied. The periarteriolar [NO] significantly increased to 161.1 ± 10.0% after an adjacent 1A was occluded during natural conditions. In the presence of L-lysine during the occlusion, there was a significantly diminished increase in [NO] of 114.1 ± 5.6%, or a response of about one-fourth of normal. These results suggest that during collateral perfusion, the interference with transport of extracellular arginine by the cationic amino acid transporter may play a significant role in regulating flow-mediated increases in periarteriolar NO production.

**Effect of competitive inhibition of arginine transport via the cationic amino acid transporter by L-lysine during low-oxygen conditions.** Seven male Sprague-Dawley rats (323.3 ± 9.2 g) were used in these studies, and all values are percentages of control. At the resting condition of 5% oxygen, there was a significant difference in blood flow to 82.7 ± 7.7% after L-lysine was applied compared with control, as shown in Fig. 4A. At 0% oxygen, there was no significant increase in blood flow (86.9 ± 7.2%) after the application of L-lysine, whereas the natural paired condition significantly increased flow to 125.3 ± 2.7% of control.

As shown in Fig. 4B, there was no significant difference in periarteriolar [NO] at 5% oxygen after the application of L-lysine compared with control, although the average [NO] was reduced to 90.1 ± 4.6% of normal. During natural conditions at 0% oxygen, periarteriolar [NO] was significantly increased to 132.7 ± 10.4%. After L-lysine was applied, the [NO] response (92.5 ± 5.4%) was absent compared with that during the natural condition at 0% oxygen. These results indicate the transport of arginine through the cationic amino acid transporter has a significant role in regulating blood flow and periarteriolar NO production when oxygen availability is decreased. As shown below, 0% oxygen in the bathing medium decreases the periarteriolar oxygen tension <10%. However, the increase in blood flow of ~25% and increase in [NO] of ~33% in normal conditions indicate the oxygen regulatory system of vessels has been activated to increase NO production and associated dilation.

**Effect of NCX inhibition by KB-R7943 during low-oxygen conditions.** In these studies, five male Sprague-Dawley rats (329.8 ± 7.8 g) were used and all values are percentage of control. There was a significant reduction in blood flow to 53.3 ± 4.7% after KB-R7943 was applied compared with control flow at 5% oxygen, as shown in Fig. 5A. At 0% oxygen, there was a significant increase in blood flow to 132.5 ± 8.23% compared with control under natural conditions. After KB-R7943 was administered, exposure to 0% oxygen media did significantly increase the blood flow to 65.6 ± 3.7% versus that of 53.3 ± 4.7% during exposure to 5% oxygen medium with KB-R7943. These data should be considered in the context of how the periarteriolar PO2 is influenced when these flow events occur. There was a significant reduction in periarteriolar PO2 at 5% oxygen after the application of KB-R7943 to 70.5 ± 5.2% compared with control, as shown in Fig. 5B. During natural conditions at 0% oxygen, periarteriolar PO2 was diminished to 91.6 ± 3.4%. After KB-R7943 was applied, there was a significant PO2 decrease to 61.2 ± 4.6% compared with control with KB-R7943 and the natural condition at 0% oxygen. Therefore, a much larger decrease in vessel wall oxygen tension occurred after suppression of the NCX at both oxygen tensions used. These results suggests that the transport of calcium ions into caveolar regions of endothelial cells via the NCX has a significant role in regulating blood flow and periarteriolar PO2 levels during normal and low-oxygen conditions.

Because of the substantial constrictor effect KB-R7943 had on arteriole behavior, we were concerned that KB-R7943 may have nonspecific effects on the intestinal vasculature. We used the endothelium-dependent vasodilator bradykinin to determine whether the arteriole dilatory response was still intact. As shown in Fig. 6, KB-R7943 only significantly diminished the arteriole dilatory response at 20 nM bradykinin, which is a threshold dosage under normal conditions. However, KB-R7943 had no significant effect on the ability of arterioles to dilate in response to the application of 100 or 200 nM bradykinin. These results suggest that the observed responses to KB-R7943 are not a result of nonspecific effects on endothelium-dependent dilation but result from the specific inhibition of the NCX.
stressors. L-Lysine is the preferred transported molecule for dilatory responses were strongly attenuated to each of the stressors. CAT-1 and limits the transport of L-arginine in the caveolar regions (21, 22, 33). Our observations from in vivo conditions predict that L-arginine is transported into vascular endothelial cells and that interference with this process suppresses the production of NO both at rest and during major endothelium-dependent vasodilatory mechanisms. The remainder of the DISCUSSION is devoted to how each of the endothelial stressors interacts with the cells to both require CAT-1 transport of L-arginine and an increase in NO production due to the stimulation of eNOS. We include Fig. 7 to illustrate how multiple mechanisms that lead to an increase in microvascular endothelial NO production are linked to transport of extracellular L-arginine through the CAT-1 system.

In these studies, the entire intestinal vasculature was exposed to various, but separate, physiological perturbations while arteriolar diameter, blood flow, and arteriolar wall [NO] were measured. Figure 1A combines data for responses to 200 μM L-lysine at rest from Figs. 2 and 4. The data demonstrate that after L-lysine was applied, the blood flow diminished to ~80% of control. The [NO] measured on larger arterioles was depressed to ~91% of control. This relatively small reduction in NO was questioned as the cause of the reduced blood flow. Perhaps L-lysine at supraphysiological concentrations has a constrictor effect separate from an NO mechanism. To test this possibility, we measured the effects of L-lysine on blood flow after L-NAME had been used to strongly suppress eNOS. As a frame of reference, the effects of 0.5 mM L-NAME on blood flow at rest and during bradykinin challenge are compared with control conditions and those during combined L-NAME and 200 μM L-lysine. The data indicate that after eNOS is suppressed by L-NAME, L-lysine has no significant effect on basal blood flow or that during bradykinin stimulation, L-NAME reduced resting blood flow to ~63% of control (Fig. 1B), compared with ~80% during just 200 μM L-lysine (Fig. 1A) as well as 400 μM L-lysine (Fig. 3). Using various arginine analogs to block eNOS in past studies of the small intestine, we typically found that if dilatory responses to locally applied acetylcholine or bradykinin were strongly suppressed, intestinal blood flow declined by ~40% (10–12, 54). L-lysine was about one-half as effective as L-NAME to reduce resting blood flow, yet the elevated L-lysine concentrations essentially eliminated functional activation of the eNOS system to NaCl hyperosmolarity, increased flow shear, and decreased oxygen tension, as shown in Figs. 2–5. Also, in Figs. 2–4, which show that the various perturbations caused large increases in [NO] during natural conditions, the NO responses were markedly suppressed during L-lysine exposure. This would support our argument that L-lysine can interfere with NO generation by limiting the availability of L-arginine transported by CAT-1.

![Graph A](image)

**Fig. 5.** Effect of KB-R7943 on intestinal arteriolar blood flow and periar-teriolar PO2 during low-oxygen conditions. A: at 0% oxygen compared with the control at 5% oxygen during natural conditions blood flow increased significantly. After KB-R7943 (50 μM) was applied, there was a significant decrease in blood flow compared with the natural paired condition at 0% and 5% oxygen. B: there was a significant reduction in periarteriolar PO2 at 5% oxygen after KB-R7943 (50 μM) was applied compared with the control, as well as a significant reduction in periarteriolar PO2 at 0% oxygen compared with the control and the natural paired condition. Data are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. natural paired condition.

**DISCUSSION**

The focus of the present study was to determine whether CAT-1-mediated transport of extracellular L-arginine into endothelial cells is important for the normal in vivo microvascular responses to a wide range of physiological stimuli. When the small intestinal arterioles were exposed to NaCl hyperosmolarity, low-oxygen conditions, or increased flow shear, the results were an increase in [NO] and vasodilation (Figs. 2–4). However, when we limited L-arginine transport by providing excess L-lysine for the CAT-1 transporter, the [NO] and vasodilatory responses were strongly attenuated to each of the stressors. L-Lysine is the preferred transported molecule for CAT-1 and limits the transport of L-arginine in the caveolar regions (21, 22, 33). Our observations from in vivo conditions predict that L-arginine is transported into vascular endothelial cells and that interference with this process suppresses the production of NO both at rest and during major endothelium-dependent vasodilatory mechanisms. The remainder of the DISCUSSION is devoted to how each of the endothelial stressors interacts with the cells to both require CAT-1 transport of L-arginine and an increase in NO production due to the stimulation of eNOS. We include Fig. 7 to illustrate how multiple mechanisms that lead to an increase in microvascular endothelial NO production are linked to transport of extracellular L-arginine through the CAT-1 system.

In these studies, the entire intestinal vasculature was exposed to various, but separate, physiological perturbations while arteriolar diameter, blood flow, and arteriolar wall [NO] were measured. Figure 1A combines data for responses to 200 μM L-lysine at rest from Figs. 2 and 4. The data demonstrate that after L-lysine was applied, the blood flow diminished to ~80% of control. The [NO] measured on larger arterioles was depressed to ~91% of control. This relatively small reduction in NO was questioned as the cause of the reduced blood flow. Perhaps L-lysine at supraphysiological concentrations has a constrictor effect separate from an NO mechanism. To test this possibility, we measured the effects of L-lysine on blood flow after L-NAME had been used to strongly suppress eNOS. As a frame of reference, the effects of 0.5 mM L-NAME on blood flow at rest and during bradykinin challenge are compared with control conditions and those during combined L-NAME and 200 μM L-lysine. The data indicate that after eNOS is suppressed by L-NAME, L-lysine has no significant effect on basal blood flow or that during bradykinin stimulation, L-NAME reduced resting blood flow to ~63% of control (Fig. 1B), compared with ~80% during just 200 μM L-lysine (Fig. 1A) as well as 400 μM L-lysine (Fig. 3). Using various arginine analogs to block eNOS in past studies of the small intestine, we typically found that if dilatory responses to locally applied acetylcholine or bradykinin were strongly suppressed, intestinal blood flow declined by ~40% (10–12, 54). L-lysine was about one-half as effective as L-NAME to reduce resting blood flow, yet the elevated L-lysine concentrations essentially eliminated functional activation of the eNOS system to NaCl hyperosmolarity, increased flow shear, and decreased oxygen tension, as shown in Figs. 2–5. Also, in Figs. 2–4, which show that the various perturbations caused large increases in [NO] during natural conditions, the NO responses were markedly suppressed during L-lysine exposure. This would support our argument that L-lysine can interfere with NO generation by limiting the availability of L-arginine transported by CAT-1.

![Graph B](image)

**Fig. 6.** Effect of KB-R7943 on arteriole dilation stimulated by bradykinin. At rest and at 20 nM bradykinin, there was a significant reduction in arteriole dilation compared with the control after KB-R7943 (50 μM) was applied to the intestinal vasculature. At 100 and 200 nM bradykinin, there was a significant increase in arteriole dilation compared with the control before and after KB-R7943 was administered. Data are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. natural paired condition.
Fig. 7. Hypothetical scheme of NO production in vascular endothelial cells during different physiological stimuli. A: during NaCl hyperosmolarity, Na\(^+\) enters endothelial cells predominantly through the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter. Na\(^+\) then extruded for extracellular Ca\(^{2+}\) via the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), which in turn increases the subplasmalemmal Ca\(^{2+}\) concentration. Ca\(^{2+}\) then complexes with calmodulin and eNOS, dissociating eNOS from the eNOS-inhibitory protein caveolin-1 (Cav-1). Some of the l-arginine that is oxidized by eNOS to form NO is transported into the cell by the cationic amino acid transporter CAT-1. B: acute shear increase stimulates an increase in intracellular Ca\(^{2+}\) through either the transport of extracellular Ca\(^{2+}\) or internal Ca\(^{2+}\) stores. Calmodulin and Ca\(^{2+}\) complex with and activate eNOS, which oxidizes l-arginine (some of which is transported into the cell by CAT-1) to form NO. C: low oxygen initiates a Na\(^+\) influx, possibly through the Na\(^+\)-glucose cotransport and/or the Na\(^+\)/H\(^+\) exchanger. Na\(^+\) is then exchanged for extracellular Ca\(^{2+}\) by the NCX, thereby increasing subplasmalemmal Ca\(^{2+}\), which then complexes with calmodulin to activate eNOS. Extracellular l-arginine transported into the caveolar region by CAT-1 is an important l-arginine source for NO production during low-oxygen conditions.

In the small intestine, the physiological form of hyperosmolarity routinely developed is NaCl hyperosmolarity during luminal nutrient absorption. In this study, the entire intestinal vasculature was exposed to a NaCl concentration comparable to concentrations that occur naturally in the submucosal layer during nutrient absorption (8, 13). Limiting the CAT-1 transport of extracellular l-arginine strongly suppressed the increase in intestinal blood flow and elevation in arteriolar wall [NO] during NaCl hyperosmolarity (Fig. 2). Our laboratory previously showed (62) that during NaCl hyperosmolarity, sodium ions enter intestinal endothelial cells mainly through Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter channels, as shown in Fig. 7. The sodium ion is then extruded out of the cell in exchange for extracellular calcium ion into the cell by the NCX (62). The NCX transporter is localized in caveolae (57), along with eNOS and the CAT-1 transporter, as diagrammed in Fig. 7. During NaCl hyperosmolarity, the increase in eNOS activity is partly due to the NCX increasing endothelial calcium, which in concert with calmodulin binds to and activates eNOS by dissociating eNOS from caveolin-1 (46). The final step in the hyperosmolarity pathway is the transport of extracellular l-arginine into caveolar regions of intestinal endothelial cells via CAT-1 for increased NO production, as the data in Fig. 2 support.

Because they are in direct contact with moving blood, vascular endothelial cells constantly encounter shear stress (14). In both cultured cells (40) and our studies of in vivo vessels (9, 12), changes in shear stress have been shown to very quickly alter endothelial NO production. In our in vivo studies, the [NO] changes essentially as rapidly as the flow alteration, whether increased or decreased (9, 12). Multiple mechanisms have been implicated in increased eNOS activity during elevated shear, including endothelial calcium-activated potassium channels (55), and AKT activation of eNOS (15, 39). In vitro studies of vessels, such as those by Bryan et al. (17) and Jen et al. (37), report an increased endothelial calcium concentration as shear stress or flow is increased, although other in vitro studies do not find a shear-calcium relationship (58) or, in the case of Worthen and Nollert (59), a shear-calcium relationship depends on the presence of either thrombin or histamine. An intermediate position found by Muller et al. (47) for isolated coronary arterioles is both calcium-dependent and -independent components of shear-dependent vasodilation. An in vivo study by Duza and Sarelius (27) found that low flow is associated with a reduction in the frequency of endothelial calcium concentration transients. Given that a calcium-shear relationship is more probable than not, we included calcium and arginine transport as common components of the cellular shear mechanism in our overview model of endothelial function in Fig. 7. Our evidence of the arginine linkage is shown in Fig. 3. These data show that intestinal blood flow and arteriolar wall [NO] were significantly suppressed during elevated shear flow after the application of l-lysine to suppress the availability of l-arginine. During natural conditions, the forcing of increased flow in larger arterioles elevated flow to ~190% of control and the [NO] increased to ~161% of control. These are major responses compared with those during hyperosmosmolarity and reduced oxygen stresses (Figs. 2 and 4). During l-lysine exposure, the increase in flow was from the resting state of ~80% of control to ~116% during the increased flow stage, and the [NO] increased ~17%. These are minor responses compared with those during the natural state. The limited blood flow response during forced collateral perfusion is the inability of arterioles to appropriately dilate, presumably because the NO mechanism is suppressed. We interpret these results to support a role for CAT-1-mediated transport of extracellular l-arginine in shear stress-induced endothelial NO production. What we do not know is whether the shear mechanism increases CAT-1 activity or whether a caveolar mechanism linked with eNOS activation increases the transport of l-arginine. It has been previously reported (25) that during the initial phase of increased NO production by shear stress, there appears to be a transient increase in intracellular free Ca\(^{2+}\).
This phase lasts from seconds to roughly 30 min and is Ca\(^{2+}\)/calmodulin dependent (14). Because our measurements were over this time frame, the Ca\(^{2+}\)/calmodulin-dependent pathway seems a likely possibility to activate CAT-1 transport.

In many vascular beds, an important regulator of microvascular tone is oxygen. Previous studies have shown that NO release from endothelial cells, isolated vessels, and in vivo arterioles is increased during reduced oxygen availability (19, 48, 51, 51). Data from these types of studies indicate the possibility that endothelial cells are somehow an oxygen sensor and that when reduced oxygen is sensed, the NO mechanism is activated. This possibility is supported by our results in Fig. 4. When the intestinal vasculature is exposed to lower oxygen availability, there is a significant increase in arteriole [NO] and blood flow. These vascular responses to reduced oxygen availability are strongly suppressed when the transport of extracellular l-arginine by CAT-1 into arteriole endothelial cells is reduced. This suppression of the vasculature to both increase blood flow and elevate NO was substantially suppressed.

We suspect that reduced oxygen tension initiates increased NO production by the entry of sodium ions into endothelial cells followed by exchange for extracellular calcium ions by NCX for Na\(^{+}/Ca\(^{2+}\) exchange. During hypoxia, previous studies of cultured endothelial cells (34, 36) have implicated the activation of the Na\(^{+}/H\(^{+}\) exchanger (NHE), causing an increase in intracellular sodium ions, which results in an increase in intracellular calcium ions via NCXs (43). In endothelial cells, localized increases in caveolae endothelial subplasmalemmal calcium concentration may be all that is required for eNOS activation and subsequent NO production (50, 60). This scenario is possible because Teubl et al. (57) have shown the presence of the NCX in endothelial caveolae and that calcium entry via reverse mode of the NCX is required for sodium-dependent facilitation of eNOS activation. A study on human umbilical vein endothelial cells suggested that under hypoxic conditions, glycolysis is activated, leading to the stimulation of sodium-glucose cotransport, which results in an influx of sodium. This sodium influx, in turn, activates the NCX causing a net influx of Ca\(^{2+}\) (5). There are also studies that reduced oxygen availability results in loading of sodium into endothelial cells (32, 61), with activation of the NCX thereafter. As shown in Fig. 5, after NCX blockade to suppress the exchange of intracellular sodium ions for extracellular calcium ions, the increase in blood flow to reduced oxygen tension was strongly suppressed. Furthermore, without a mechanism to allow normal vasodilation the vessel oxygen tension was lowered more by reduced oxygen availability than occurred during normal conditions. In effect, a greater oxygen deficit occurred because the vasculature was unable to use NO to defend itself. What we did not expect to happen was vasoconstriction at rest during NCX blockade with an attendant decrease in resting blood flow and vessel wall oxygen tension. We believe this indicates that NCX transporters in endothelial cells are removing sodium ions at rest and replacing them with calcium ions that influence resting NO generation. In times of reduced oxygen availability, this exchange process is even greater. The presumed effects on intracellular calcium concentration are to provide a higher rate of eNOS activity. This would explain the increase in [NO] we find during reduced oxygen tension during normal conditions in this (Fig. 4) and prior studies (9, 48). In reference to the role of CAT-1 in the endothelial response to decreased oxygen, as shown schematically in Fig. 7, the initiating event is likely in part an increase in sodium entry followed by Na\(^{+}/Ca\(^{2+}\) exchange to activate eNOS, and as part of the overall activation of the cell, CAT-1 provides the l-arginine required for elevated NO production.

It is clear that in vascular endothelial cells, eNOS production of NO can result from a variety of mechanisms depending on the physiological stimulus. In this study we have shown that a common factor in the NO-eNOS mechanisms for NaCl hyperosmolarity, low oxygen, and increased flow shear is the transport of extracellular l-arginine into vascular endothelial cells via CAT-1. These studies further elucidate the mechanisms by which three physiologically relevant stimuli induce an increase in vascular endothelial NO production.

GRANTS

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REFERENCES


