Transfer of nitric oxide by blood from upstream to downstream resistance vessels causes microvascular dilation

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Submitted 20 February 2009; accepted in final form 6 August 2009

Bohlen HG, Zhou X, Unthank JL, Miller SJ, Bills R. Transfer of nitric oxide by blood from upstream to downstream resistance vessels causes microvascular dilation. Am J Physiol Heart Circ Physiol 297: H1337–H1346, 2009. First published August 7, 2009; doi:10.1152/ajpheart.00171.2009.—The discovery that hemoglobin, albumin, and glutathione carry and release nitric oxide (NO) may have consequences for movement of NO by blood within microvessels. We hypothesize that NO in plasma or bound to proteins likely survives to downstream locations. To confirm this hypothesis, there must be a finite NO concentration ([NO]) in arteriolar blood, and upstream resistance vessels must be able to increase the vessel wall [NO] of downstream arterioles. Arteriolar blood NO was measured with NO-sensitive microelectrodes, and vessel wall [NO] was consistently 25–40% higher than blood [NO]. Localized suppression of NO production in large arterioles over 500–1,000 μm with L-nitroarginine reduced the [NO] ~40%, indicating as much as 60% of the wall NO was from blood transfer. Flow in mesenteric arteries was elevated by occlusion of adjacent arteries to induce a flow-mediated increase in arterial NO production. Both arterial wall and downstream arteriolar [NO] increased and the arterioles dilated as the blood [NO] was increased. To study receptor-mediated NO generation, bradykinin was locally applied to upstream large arterioles and NO measured there and in downstream arterioles. At both sites, [NO] increased and both sets of vessels dilated. When isoproterenol was applied to the upstream vessels, they dilated, but neither the [NO] or diameter downstream arterioles increased. These observations indicate that NO can move in blood from upstream to downstream resistance vessels. This mechanism allows larger vessels that generate large [NO] to influence vascular tone in downstream vessels in response to both flow and receptor stimuli.

artery; arteriole dilation; S-nitrosoglutathione

THE PRESERVATION OF NITRIC oxide (NO) in the arterial blood by transiently complexing with glutathione, albumin, and hemoglobin, and likely other compounds, as a nitrosothiol (23, 35, 36, 39) has raised the distinct possibility that NO generated in one part of the cardiovascular system reaches distant locations. The evidence for and physiological importance of transient nitrosothiols in blood plasma and hemoglobin on vascular regulation and disease has been both extensively researched and reviewed by Stamler and colleagues (19, 37, 40, 41) since 1992. A key issue in this area is whether formation of nitrosothiols, particularly with hemoglobin, are so rapid and pervasive that NO made in vessel walls would be rapidly removed to and released at downstream locations. Direct measurements of in vivo vessel wall NO concentration ([NO]) by multiple laboratories indicate [NO] in the range of 300–1,000 nM in a variety of tissues and small mesenteric arteries, of which a few are now referenced (1, 2, 10, 25, 46, 47). In these studies, activation of endothelial nitric oxide synthase (eNOS) by appropriate receptor agonists increased the measured [NO], and suppression of nitric oxide synthases with nitroarginine compounds dramatically lowered the measured [NO], giving credence that a form of NO is measured by the microelectrode. The paradox of the vessel wall [NO] being in the mid- to high nanomolar range and the potential for hemoglobin to rapidly remove the NO may be partially resolved if NO diffusion in red blood cells is a rate-limited step plus plasma albumin and glutathione act as intermediary steps in NO sequestration, as proposed by Heuil et al. (23). Both nitrosothiols of glutathione and albumin are well documented to exist in arteriolar blood, with albumin being the highest concentration and nitrosothiols the fastest-known reaction with NO (23, 33, 39). Total nitrosothiol concentration in plasma is a controversial issue as Stamler and colleagues (19, 39) have reviewed because of technical issues, but could be as much as a low micromolar concentration (22). Slower than expected transfer of NO into red blood cells is supported both empirically and by disappearance of NO from solution in the presence of red blood cells. Liao et al. (30) found using isolated arterioles that NO-dependent vasodilation is not compromised by flowing red blood cells. The basis of this observation may be the diffusion situation generated by the plasma sleeve developed in arterioles by the flow stream. Another perspective is Liu et al.’s (31) observation that NO added to a red blood cell suspension has a half-life of >4 s in dilute red blood cells but was calculated to be of the order of 1.8 ms in whole blood. They propose this observation can occur if relatively slow diffusion of NO in the red blood cell limits the very rapid sequestration or destruction of NO by hemoglobin within the red blood cell.

The evolving concept is that blood has multiple reservoirs of NO that are in some equilibrium with NO produced by the endothelium; consequently, there is possibly an NO blood-vascular interaction that influences the [NO] in various parts of and near the vessel wall, as mathematical models developed by Lancaster and others (12, 28, 43) have predicted. Assuming this concept is partially correct, NO diffusion to and from blood to the vessel wall could influence vascular tone by altering the local vessel wall [NO]. For example, Diesen et al. (16) have proposed that NO released as hemoglobin desaturates could be of importance in hypoxic vascular regulation. Another potential source of NO from blood is nitrite in the red blood cells. Lauer et al. (29) and Dejam et al. (15) report that nitrite accumulates in the red blood cell, and Crawford et al. (14) found that this nitrite can be converted back to functional NO by a nitrite reductase ability of partially desaturated hemoglobin beginning at saturation levels of the order of 60% and perhaps even higher.

Whether nitrosothiols or nitrite converted back to NO is the dominate mechanism for at least some carriage of a functional
form of NO is controversial. However, there is functional evidence that some storage or protected form of NO survives sufficiently in blood to be vasoactive at distal locations based on multiple types of studies using NO gas. For example, inhalation of 80 ppm NO gas should increase plasma NO ~120 nM and both Fox-Robichaud et al. (21) in cats and Cannon et al. (11) in humans found in vivo evidence of NO-induced peripheral vasodilation. However, both of these studies were done after pharmacological suppression of eNOS to evaluate the influence of exogenous NO. In normal humans with intact eNOS function, Rassaf et al. (35, 36) found both brachial artery dilation and increased forearm blood flow during infusion of NO dissolved in saline both in the brachial artery and, of particular interest, given intravenously. It is their opinion, based on measurement of extremely rapid removal of dissolved NO added to blood, that the original NO only partially survived per se to the site of vasodilation, but vasoactive plasma nitrosothiols were very rapidly formed, as evidenced by the increased concentration of nitrosothiols in plasma.

In all of the studies of exogenous NO mentioned thus far on distal vascular effects of exogenous NO, the delay from the source of exogenous NO to arrival in the vasculature was many seconds, often tens of seconds. But, what if the time frame of NO movement from one site to another within a vasculature was of the order of less than 2 s? Our focus in this study is not NO exchange between vascular beds, such as the lung to the peripheral organs, but within vascular beds, from larger to smaller resistance vessels. Within organs, even of large mammals, the time delay from the entry of arterial blood in an organ to the capillaries is of the order of ~2 s given the velocity of blood and distances traveled. The short time frames are important because larger resistance vessels operate at higher vessel wall [NO] than their successive branches. We recently reported that mesenteric arteries during in vivo conditions generate [NO] about two times that of even the largest intestinal arterioles; also, larger arterioles have higher [NO] than do smaller resistance vessels (34, 44, 45, 47). The high [NO] in small arteries could be because the incoming arterial blood contains NO storage forms that are being released. However, these small arteries are quite capable of locally changing their NO concentration, which is the topic of this study. To test this hypothesis, we used both increased flow shear and receptor-mediated activation of eNOS in upstream resistance vessels to evaluate whether it is possible for NO to be transported by blood in some form from larger to smaller resistance vessels.

To appreciate what fraction of vessel wall [NO] might be from blood in large arterioles, the [NO] was measured before and just after L-nitroarginine was used to suppress NO synthase over a 500- to 1,000-μm length, and the completeness of blockade was tested with bradykinin. We also evaluated NO release from S-nitrosoglutathione (GSNO) to determine if NO-sensitive microelectrodes interact with the nitrosothiol molecule as well as the NO released by nitrosothiol compounds during their degradation.

**METHODS**

**Surgical Preparation**

The procedures used in this study were reviewed and approved by the Indiana University Medical School Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN) and used at a weight range of 350–400 g. The animals were anesthetized with sodium thiopental (Abbott, Chicago, IL) given at 200 mg/kg using four subcutaneous injections over the lower back and thighs. This approach was necessary to avoid any damage of the small intestine vasculature by direct exposure to anesthetic agents. The animals were placed on a heating mat (35–36°C) as soon as they lost their righting reflex and a body temperature of 37–38°C measured in the abdomen (stomach) was maintained. The trachea was cannulated, and the rat was mechanically ventilated with a small mammal ventilator (Harvard Apparatus, Holliston, MA) at 70 breaths/min and an initial tidal volume based on the Harvard Apparatus pneumograph. The tidal volume was increased to compensate for tubing dead space and adjusted to generate an ear oximetry hemoglobin saturation of 90–95%. The right femoral artery was cannulated to measure the arterial pressure and administer 0.5 ml·h⁻¹·100 g⁻¹ of physiological saline to maintain stable cardiovascular parameters. The small intestine was prepared for in vivo microscopy with a well-established technique (4) and protected from atmospheric oxygen by using 5% oxygen, 5% carbon dioxide, and balance nitrogen bicarbonate-buffered physiological saline perfused at 5 ml/min in a tissue bath volume of ~7 ml. Intestinal motility was suppressed with 0.2–0.5 μM isoproterenol, which has only minor effects on vascular tone at this low concentration.
Optical Measurements

The mesenteric arteries and intestinal microvasculature were observed with a combination of both epi- and transillumination approaches as best suited visualizing the blood vessel and placement of microelectrodes. The light was filtered of infrared and ultraviolet wave lengths. An Olympus BHMJ microscope head (Hyde Park, NY) was used to obtain images that were collected with a closed-circuit video camera and Metamorph Imaging software (Molecular Devices, Sunnyvale, CA). Digital images were collected by time lapse at 5-s intervals plus VHS continuous video recording in case some aspect of the time lapse failed and image capture needed to be repeated. The vessel diameters were measured using the digital caliper feature of the image analysis software and calibrated to a stage micrometer for each magnification used. In some phases of the experiments, vasomotion of the microvessels was greatly accentuated and had a substantial impact on the average diameter. To provide a mean diameter during vasomotion of the arterioles, the vessel diameter was measured at each 5-s interval for 3 min both during control and steady-state responses. The results of each time period were averaged.

NO Measurement

NO-sensitive microelectrodes were made from 6- to 8-µm-diameter carbon fibers (Fibre Glast, Brookville, OH) sealed in glass micropipettes, calibrated the same as published recently (2, 34), and tested for reactions with all chemicals to be used. Only the open tip of the microelectrode can respond to NO because of the insulation provided by the glass envelope. The microelectrodes were beveled to a 30–35° angle on a surface of optical glass covered by a thin layer of 5-angstrom alumina polishing compound to form a final tip diameter of ~10–12 µm. Many of the microelectrode tips were capable of penetrating the wall of arterioles with little mechanical dimpling of the vessel wall and complete recovery of the 0 [NO] current after being within the vessel lumen and returned to the tissue bath. If recovery did not occur, the electrode was assumed damaged or altered by interaction with blood, and the data were not accepted. Just before penetration, the animal was given heparin (~25 U/100 g) to avoid having platelet adhesion to the polarized tip and glass envelope of the microelectrode. Narishige MO-203 micromanipulators were used to move the microelectrode to the vessel surface through the overburden of intestinal tissue, cause a slight dimple of the vessel wall, and then slightly withdraw the microelectrode tip until the dimple was resolved. In some cases, the vessels react to the microelectrode with a small dilation or constriction, so we waited at least 2 min for the issue to be resolved before proceeding to obtain a stable [NO] measurement. Thereafter, the vessel was gently penetrated to place the electrode tip near the center of the lumen and allowed to recover from the slight constriction associated with vessel penetration. Once a stable lumen [NO] was recorded with no evidence of clot formation, the microelectrode tip was moved about in the lumen to determine if a gradient of NO could be found across the lumen. We did notice the [NO] was higher very near the endothelial layer, as might be expected.

The NO microelectrodes were polarized at 0.7 volts and calibrated at [NO] of 0, 600, and 1,200 nM generated with precision mixtures of NO in nitrogen at 37°C. The current recorded by a Keithley Electrometer model 6517A was output as voltage proportional to the [NO] and recorded by an analog-to-digital chart recorder (PowerLab; AD Instruments). During experiments, the baseline or “zero” [NO] was determined by elevating the microelectrode tip ~200 µm above the tissue in an area where there were no large vessels and in the full flow stream of the incoming bathing media. Raising or lowering the electrode tip 50 µm from this location had no effect on the electrode current. A signal was detected ~50 µm above the tissue if large arterioles, venules, or arteries are nearby. Slight, essentially linear drift of the microelectrode base current always occurred, and a virtual baseline was calculated for the measurement periods. Because of the long periods of time required for NO measurements, often >45 min/vessel because of various perturbations, only microelectrodes with minimal electronic drift were used.

Protocols

Shear-dependent stimulation of arterial endothelial cells. We have recently shown that mesenteric arteries preceding the small intestinal microvasculature have a well-developed ability to increase NO generation as blood flow is increased (47). To elevate the blood flow, nontraumatic microclamps are placed on one and then both collateral arteries adjacent to the center vessel, as shown in Fig. 1. The [NO] at rest and during elevation of flow by one and then two arterial occlusions was measured on the artery and also on a large arteriole directly perfused by the center artery. The goal was to increase the [NO] in the blood of the artery by its response to increased blood flow and have this elevated NO in blood enter the microvascular region of the bowel wall not influenced by collateral artery occlusions. After stable responses to occlusions were recorded, the microclamps on collateral arteries were removed, and normal flow and [NO] conditions resumed.

Receptor-mediated stimulation of eNOS in large arterioles. As shown in Fig. 1, there is a section of the largest intestinal arterioles just outside the intestinal wall that is readily accessible for application of pharmacological agents. In this experiment, either 400 nM bradykinin or 10 µM isoproterenol was applied to the surface of the large arteriole from a ~50-µm-diameter micropipette at the rate of 50 µl/min using a syringe pump (model 100; KD Scientific, Holliston, MA). This very small flow was restricted to the area of the large arteriole and then removed by the 5 ml/min bath stream based on using lissamine green dye to evaluate the area of influence of the ejected media. Bradykinin was used to activate eNOS in a localized area of a large arteriole to locally raise the [NO] and potentially elevate the blood [NO] in that vessel. Isoproterenol was used to cause a non-endothelial-dependent dilation and avoid appreciably altering the local blood or vascular [NO]. In both cases, the goal was to locally Fig. 1. Schematic drawing of the mesenteric and intestinal circulation used for various protocols. The preparation is three mesenteric arteries leading to a section of bowel. The left and right small arteries could be sequentially occluded to force a higher blood flow in the central artery as greater bowel wall is perfused by the remaining vessel. This protocol was used for the data in Figs. 4 and 5. For data in Fig. 6, bradykinin and isoproterenol were applied to a small artery before it penetrated the bowel wall to become a large arteriole. The drug solution was released at a flow of 50 µl/min and contained lissamine green to verify that the solution was dissipated by the 5 ml/min suffusion flow before reaching downstream arterioles. Suffusion flow direction is shown to illustrate that the arterioles studied were upstream of the sites of bradykinin and isoproterenol release to help avoid as much as possible these agents reaching the arterioles. After the suffusion solution moved over the tissue, it was wicked away to minimize possible stirring of drugs in the solution.

AJP-Heart Circ Physiol • VOL 297 • OCTOBER 2009 • www.ajpheart.org
cause maximum responses of the large arteriole to illustrate that even elevation of [NO] over an ∼0.5-mm length of large arteriole could influence blood and downstream vascular [NO]. There was some conducted vasodilation from the large arteriole to downstream locations with both agents. To minimize this problem at the downstream site, the location of the microvascular measurement was ∼3–4 mm distant from the release site of drugs.

Microelectrode response to GSNO. GSNO (Sigma-Aldrich, St. Louis, MO) was made in stock solution (500 μM) in ice-cold saline that had been equilibrated with nitrogen to remove oxygen and then immediately frozen at −20°C in 2-ml vials until needed. If these precautions were not used, the GSNO is grossly decomposed within 10 min at room temperature and oxygen tensions. For experiments, the stock solution was warmed to 37°C as quickly as possible and immediately used. Various volumes of the stock solution were added to nitrogen-equilibrated saline to produce 100 nM, 500 nM, 1 μM, 2 μM, and 4 μM GSNO solution. The electrodes did not respond to decomposed nitrosothiolute to 50 μM after overnight storage at room temperature. Therefore, the electrodes are insensitive to the amino acid.

To avoid oxygen destruction of NO generated by GSNO in the solutions, the glass beaker was covered with Plexiglas that had an opening to admit the microelectrode and an injection port. The samples were mechanically stirred with each addition so as to avoid contamination by atmospheric oxygen. Nitrogen gas was above the solution to minimize atmospheric oxygen contamination, and light levels were just adequate to find the injection port and stirring device. The NO microelectrode current was continuously measured during the various additions of GSNO. For concentrations in the 0.1- to 4-μM range, the signal generated by each concentration of GSNO was allowed to dissipate before adding the next higher concentration. To quickly remove the NO gas produced by GSNO, the solution could be brieﬂy bubbled with nitrogen to restore the baseline current. To determine if the electrode was sensitive to GSNO, per se, a response to GSNO was run at 1, 2, or 4 μM and then repeated, but, when the NO signal peaked during the second test, a burst of bubbling with nitrogen was used to transiently and quickly wash out NO gas to show any residual response to remaining intact GSNO.

Residual periarteriolar [NO] after localized eNOS blockade. To estimate the contribution of bloodborne NO to arteriolar wall [NO], a section of 500–1,000 μm of a large arteriole was exposed to 100 μM nitroarginine released from a micropipette with a tip diameter of ∼50 μm. A small syringe pump was used to generate a pipette flow of 5–10 μl/min that was directed immediately over a superficial arteriole and moved up and down the vessel axis to apply nitroarginine. The bath flow was 5 ml/min, and this would quickly dilute the nitroarginine to minimize impact on nearby vessels. The release of nitroarginine was continued until the [NO] did not decrease further, which usually required ∼20 min. We have previously reported that nitroarginine can quickly suppress in vivo microvascular NO production (5) and is reported to be transported independent of arginine (3). To evaluate the quality of the eNOS blockade, the dilation and increased [NO] responses to locally applied 500 nM bradykinin from another micropipette near the center of the blocked vessel region were measured before and after nitroarginine application. During the second application of bradykinin, nitroarginine continued to be released because in vivo arterioles can recover within 15–30 min after short-term nitroarginine exposure, and exposure to bradykinin appeared to hasten the recovery in pilot studies.

Statistics

One-way ANOVA was used for events in which a simple control and response pattern occurred, and two-way ANOVA repeated measures was used when multiple tests of responses were used. In both cases, where significant differences were indicated by ANOVA, a least-significant difference post hoc test was used for individual comparisons. P ≤ 0.05 was accepted as indication of a significant change. All data analysis was done with Statistica Software (StatSoft, Tulsa, OK).

RESULTS

Periarteriolar and Blood [NO]

The perivascular [NO] of intestinal arterioles and their respective luminal [NO] are shown for resting conditions in Fig. 2. Each vessel is from one of seven animals, and these vessels are the largest arterioles in the bowel wall. In all cases, there was a decline in [NO] after the microelectrode tip pierced the wall of the microvessel, but a substantial signal did remain. The tip of the microelectrode could be moved about in these large arterioles with inner diameter in the 50- to 70-μm range without appreciably changing the measured [NO] within resolution limits (±20 nM) of the microelectrode system. In all the data presented, there was no visual evidence of clotting or platelet adhesions to the microelectrode. However, when these events did occur in recordings not presented, the recorded NO signal substantially decreased as the clotting process developed. After successful luminal penetrations, the electrode tips were removed to just outside the vessel wall to verify NO was still being generated, which it was in all cases so long as clots did not form.

Residual Periarteriolar [NO] After eNOS Blockade

To determine what, if any, amount of bloodborne NO reached the periarteriolar location, the [NO] of 11 arterioles in 8 rats was measured before and after localized suppression of eNOS with L-nitroarginine. Pilot studies indicated an ∼500- to 1,000-μm length of arteriole needed to be treated to fully abolish vasodilation and increased [NO] in response to localized application of a supraphysiological concentration of bradykinin (7). For these studies, the microelectrode tip was placed on the surface of the bowel just above the arteriolar wall, a distance of 20–30 μm, because having both an infusion microelectrode and NO sensor physically impaling the tissue was obviously traumatic to the arteriole and caused sustained dilation. Consequently, the [NO] measured will be lower than those reported in the other figures because of diffusional losses.

![Graph](Fig. 2. Paired measurements of periarteriolar nitric oxide concentration ([NO]) and intravascular blood [NO] for large arterioles of the intestinal wall. The periarteriolar measurement is with the open tip of the microelectrode touching the outer surface of the vessel. Intravascular measurements were only accepted if no clot or platelet activation occurred at the microelectrode tip during lumen impalement. Each vessel was from 1 of the 7 animals tested.)
For the experimental conditions, the resting [NO] of these vessels was 380 nM and declined to 219 nM during nitroarginine application, plus the vessel diameter decreased from 48.1 to 40.6 m. The absence of both dilatory and increased [NO] responses to bradykinin after nitroarginine application in Fig. 3 indicates that, with eNOS functionally eliminated, the arteriolar [NO] decreased to 58.6% of the resting state when NO synthase in the region was blocked. The only source of the NO would have to be blood because tissue [NO] away from the vessel wall was much lower than that at the vessel wall.

**GSNO NO Generation**

The signal interpreted as NO within the microvascular blood could be from many sources, as outlined in the Introduction. Both nitrosothiols of glutathione and albumin are well documented to exist in arterial blood, with albumin being the highest concentration (23, 33, 39). GSNO was chosen as a representative nitrosothiol because it withstands shipment from a commercial source (Sigma-Aldrich) and is known to be a potent endothelial independent vasodilator in the sub-1 M range (32, 36, 38). Heuil et al. (23) found that GSNO is one of the first nitrosothiols formed in the presence of added NO and subsequently appears to transfer NO to albumin and hemoglobin. In addition, as a small molecule, GSNO could also diffuse through vessel walls and potentially contribute to the NO signal measured by electrodes on the exterior of the arteriolar wall. This would be a particularly troublesome situation if the microelectrodes reacted to GSNO independent of NO dissolved in the tissue water, as is assumed. As shown by a typical experiment in Fig. 4A, the lower limit of detection for NO generated by GSNO was at a GSNO concentration of 100 nM for the assay conditions used of low oxygen tension and simple saline in a glass container. All of the tests were done in nitrogen-equilibrated saline because use of room air-equilibrated saline dramatically decreased the signal recorded and duration of the signal for any concentration of GSNO used. Consequently, for these types of studies, oxygen content of solutions does deteriorate the results because of NO-oxygen destructive interactions. To illustrate the sensitivity of the

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assay at [NO] encountered in the various protocols of the study; the majority of calibrations were confined to the range of 100 nM to 4 μM GSNO. As shown in 4A, the signal generated by GSNO in nitrogen-equilibrated saline increased in proportion to the GSNO concentration and then slowly dissipated over 10–20 min. The changes in [NO] reflect decomposition of GSNO and subsequent loss of NO because the electrodes are capable of responding to an ∼1,000-nM increase in NO within ∼15 s. In every test, there was a linear relationship of measured [NO] (or current) vs. GSNO concentration; using the area under the curve for each concentration was also linearly related to the GSNO concentration. The peak [NO] achieved in nitrogen-equilibrated saline was about one-tenth the 0.1- to 4-μM concentration range of GSNO. This observation was in keeping with prior studies of this type by Heuil et al. (23). After exposure of the microelectrodes to 0.1–4 μM GSNO and its decomposition to glutathione and probably nitrate, the recorded current returned to baseline. We also used 100–400 μM GSNO to determine both the usefulness of the compound as an NO donor and reaction of the electrodes to the residual glutathione at supraphysiological concentrations after full decomposition. These high concentrations of GSNO do generate stable [NO] for periods of up to 30 min in low oxygen content media; thereafter, the baseline current was eventually restored in ∼1 h. Therefore, it is not likely that the microelectrodes responded to glutathione per se in the test chamber or at blood concentrations (<50 μM) (42). Studies with both supraphysiological concentrations of arginine and lysine that alter NO production also indicate that, when properly prepared, the NO microelectrode is insensitive to these amino acids as well (34, 45).

To determine if the majority of signal recorded by the microelectrodes was NO gas or an electrochemical reaction with the nitrosothiol, we proposed that purging the solution with nitrogen would quickly wash out the NO gas but not an organic molecule, such as GSNO, reacting with the microelectrode. Figure 4B shows a typical test with the addition of 2 μM GSNO and then exactly repeating the addition with nitrogen bubbling to remove NO gas just as, or before, the signal peaked and then measuring the remaining signal. The nitrogen bubbling rapidly decreased the signal generation compared with a standard test run with just mechanical stirring, indicating that GSNO decomposed rapidly to give off gaseous NO. The NO signal for high GSNO concentrations (100–400 μM) would also decrease quickly with nitrogen bubbling. Based on these observations, the vast majority of signal generated by GSNO was gaseous NO rather than the electrode interacting with GSNO per se. As additional tests to support the view that the electrodes have a minimal interaction with GSNO, we compared the washout curve of NO gas starting at ∼600 nM to equivalent [NO] generated by decomposing GSNO with a caveat; we used sufficient GSNO that it should have reached ∼1,000 and 2,000 nM in Fig. 4C and ∼2,000 and 4,000 nM Fig. 4D. In effect, GSNO continued to be present and decomposing during washout. Note that the washout curves from the start of time relative to equal-flow nitrogen bubbling for gaseous NO or NO from GSNO are identical for practical purposes for each electrode test. The washout times were intentionally prolonged to provide ample time for the electrodes to demonstrate significant interaction with GSNO, if of appreciable importance. Had the electrodes responded to GSNO, the washout curves would be much prolonged compared with washout of simple NO gas.

Flow-Induced Arterial NO Effects

Figure 5 presents the effect of increasing blood flow through a mesenteric artery on the [NO] on that vessel’s wall as well as the perivascular [NO] of the first large arteriole the artery’s blood would enter, as diagrammatically depicted in Fig. 1.
Common-shaped symbols in the lines for arteries and arterioles in Fig. 5A represent measurements in the same animal. In all cases, occlusion of one and then two parallel mesenteric arteries increased the absolute perivascular [NO] of the open artery and immediate downstream arteriole. The arteriole studied received blood from its parent artery and was at least 1 cm from nearby tissue receiving the collateral blood flow from the parent artery.

Figure 5B presents the NO data of Fig. 5A as a percentage of control changes in arterial and arteriolar [NO], and Fig. 5C shows the percentage of control diameter of the arteriole where [NO] was measured. As can be seen in Fig. 5B, the arteriolar [NO] increased by the same proportion as the relative increase in arterial [NO] during collateral occlusions. As the arteriolar [NO] increased, all of the arterioles from large to small being observed in the region were dilated, but data in Fig. 5C are only shown for the larger arterioles where NO was measured.

Receptor-Mediated Arterial NO Effects

This protocol determined if application of bradykinin to activate eNOS in a short section of small artery/large arteriole could influence the blood [NO] sufficiently to influence downstream [NO] and vascular diameters. In pilot studies, we attempted to use point source iontophoresis of bradykinin to locally increase [NO] generation. Iontophoresis is the typical method used to study conducted vasodilation to eNOS-dependent vasodilators (8, 17, 18), and we assumed it might serve our purposes. However, such localized bradykinin release only caused conducted vasodilation downstream for ~1 mm on the small artery/large arterioles just outside the bowel wall and had no influence on downstream arteriolar [NO]. Measurement of NO at the site of bradykinin release indicated a localized increase in [NO], within 20–30 μm of the release site, and the increased [NO] could not be detected downstream at ~100 μm from the bradykinin release site using iontophoresis. Therefore, we resorted to releasing bradykinin in solution over an ~500-μm length of small artery to generate the results. As shown in Fig. 1, localized, low-flow drug solution was used to avoid any possibility of bradykinin or isoproterenol reaching any downstream arterioles, and the distance from drug site to point of arteriolar measurement was ~3–4 mm. The data in Fig. 6 indicate that upstream application of bradykinin over a large enough length of large arteriole did cause dilation of the downstream arteriole associated with a large increase in the average perivascular [NO] at the downstream location. As with earlier protocols that increased blood [NO], greater-amplitude vasomotion excursions were noted while bradykinin was being released at an upstream site. To confirm that NO generation of the upstream vessel was reaching the distal arteriole to cause dilation and the dilation was not conducted vasodilation during bradykinin application, we caused comparable maximum dilation of the upstream artery segment with isoproterenol. Isoproterenol, a β-receptor agonist with no reported eNOS relationship, did not influence either distal arteriolar [NO] or vessel diameter. Isoproterenol is a much smaller molecule than the peptide bradykinin and would be more likely to diffuse in blood than bradykinin. The complete lack of distal effects of isoproterenol argues that neither drug in this test would influence downstream conditions, and the increased downstream [NO] with bradykinin was the result of upstream generation of NO in blood.

DISCUSSION

We are concerned about the movement of some vasoactive form of NO through blood from larger to smaller resistance vessels as a potential blood-dependent mechanism to assist in regulation of organ blood flow. Large arterioles and small arteries of the mesenteric and intestinal vasculatures generate higher in vivo NO concentrations than do smaller arterioles at rest and during increased activation of eNOS by both elevated blood flow shear and endothelial-dependent vasodilator mechanisms (6, 44, 45, 47). If some of this upstream NO reached downstream locations within the blood, the diffusion of NO out of the downstream vessels in blood would be lessened and, in effect, raise the distal vessel wall [NO]. Although there is substantial in vitro evidence and mathematical modeling that hemoglobin extremely rapidly interacts with NO within the red blood cell, as reviewed by Kim-Shapiro et al. (26), there has to be an NO signal in blood plasma within the vessel lumen during the transition. The key issue is whether this signal is very small or of some vascular relevance. The results shown in
Fig. 2 indicate that there is indeed a signal interpreted by the microelectrode at a vasoactive [NO] within the vessel lumen as judged by in vivo studies by various laboratories (1, 2, 9, 10, 25, 44, 45). In Fig. 3, the residual vessel wall [NO] is shown after localized eNOS blockade of a ~1,000-μm length of a large arteriole of the type studied in Fig. 2. These results indicate the bloodborne NO can provide a residual [NO] of ~60% of the intact concentration. These studies with nitroarginine to suppress eNOS also have a bearing on the issue of what the microelectrode is detecting. In test chambers, neither nitrite nor nitrate at physiological concentrations influence the NO-sensitive microelectrode (5), nor do the electrodes respond to arginine and lysine at physiological and vasoactive supraphysiological concentrations (34, 45) and do not detect signals from frozen and rewarmed intestinal tissue (5). In this study, as well as others using nitroarginine analogues and lysine to suppress \( \text{L-arginine transport} \) (1, 34, 45), the measured signal interpreted as NO is substantially decreased, but not driven to nearly undetectable as is possible with in vitro preparations (13, 24).

We have suspected for some time that the residual [NO] after localized suppression of eNOS is due to NO from blood. The results of Fig. 3 support this possibility.

A potential source of NO measured immediately outside the vessel wall and the NO signal measured in blood in Fig. 2 and all the other figures in this study could be GSNO if the electrodes directly interact with this molecule. Nitrosothiols of glutathione and albumin occur in arterial blood, with albumin being the highest concentration (23, 33, 39). Total nitrosothiol concentration in plasma is a controversial issue, as Stamler’s group (19, 39) has reviewed, but could be at a low micromolar concentration. This is cause for concern because direct measurements of NO vessel wall [NO] predict a range of 300–1,000 nM [NO] in a variety of tissues (10), arterioles (1, 2, 46), and small mesenteric arteries (47). (25). Exactly what the microelectrode senses, NO, nitrosothiol, or both, will substantially influence the understanding of NO physiology. The studies with the nitrosothiol GSNO shown in Fig. 4 provide evidence that the electrodes adequately coated with Nafion to exclude negatively charged molecules were predominately interacting with NO gas rather than GSNO as a representative nitrosothiol. Figure 4A presents the NO signal generated by adding GSNO to the bath followed by the natural decay because the bath was open to the atmosphere. In Fig. 4B, natural-loss NO generated by GSNO was compared with identical conditions but with nitrogen purging of the solution to remove NO. The washout time was much faster, indicating loss of NO because an organic molecule such as GSNO would not be purged. To pursue this theme, the decay of comparable [NO] generated by NO gas or excessive GSNO that would have reached much higher [NO] was compared with the onset of nitrogen purging. As shown in Fig. 4, C and D, the washout curves are very similar even though GSNO would have been in the media. The washout kinetics would have to be prolonged if the electrodes responded to GSNO or the rate-limiting step was decomposition of GSNO to NO and glutathione. These observations helped us predict that plasma nitrosothiols generated in upstream vessels with high [NO] would likely be releasing NO gas in distal blood vessels, given the rapidity of their decomposition in our studies using saline. The decomposition is even faster in blood plasma. Heuil et al. (23) found that the NO signal recorded by an NO electrode after 10 μM GSNO decreased by ~50% in 2 min in the presence of human plasma, whereas a prolonged signal was measured in saline solution. The faster disappearance of what was likely NO gas in the presence of albumin was proposed to be transfer of NO from GSNO to albumin. However, the fact that NO was detected electrochemically during decomposition of nitrosodiol does indicate that NO was in solution during transfer, and such a release of NO in in vivo conditions could influence blood vessels. Our results in Fig. 3B clearly demonstrate that the NO released by GSNO in simple saline was gaseous because the signal could be so quickly dissipated by nitrogen bubbling.

Evidence of distal dilution caused by increased upstream NO availability and survival of a vasoactive form of NO in flowing blood has been documented in humans (11) and animals (20, 21) during breathing of NO gas, as well as intravascular injection of dissolved NO gas (35, 36). On a miniature scale, we have duplicated this type of study by elevated NO generation by the resistance artery to a section of bowel wall in an attempt to raise the microvascular [NO]. The first approach, shown diagrammatically in Fig. 1, was to use flow-mediated increased NO generation in mesenteric arteries by selective, temporary clamping of adjacent arteries. Using this approach, we have reported a decline in [NO] when resting blood flow is reduced by clamping one of the two major downstream branches of the artery at rest and a well-developed ability of in vivo mesenteric artery to increase NO generation at elevated blood flow when adjacent arteries are nontraumatically occluded and the central artery perfuses about two times as much tissue (47). Using the scheme to increase blood flow by arterial occlusion procedures, the data in Fig. 5 show both arterial and downstream arteriolar [NO] increased in essentially identical proportion (A and B) during the occlusion of one and then both adjacent arteries to the parent artery, and, simultaneously, the downstream arterioles dilated (C). The elevation of the arterial wall [NO] at elevated flow was the source of additional NO to be added to the blood flow and reach downstream arterioles. The site of arteriolar [NO] measurement was the first arteriole perfused by the open artery, so there is little probability of a deficit of flow to this arteriole, and no venous blood from flow-deficient areas of the bowel wall could have possibly reached the arteriole studied. The typical increase in artery blood flow with one and then two collateral occlusions is 50–75 and 100–150% of control (47) and consequently are major stimuli to shear-dependent generation of NO in mesenteric arteries. The increases in [NO] in arterioles were quite large, ~60% with a single artery occlusion and ~100% with dual artery occlusion because of the simultaneous and even larger actual increases in arterial [NO] (Fig. 5, A and B). We believe the vast majority of the increased arteriolar [NO] was due to flow of NO-enriched blood from the parent artery to the arterioles. To place these responses in context, the elevation of periarteriolar [NO] of large arterioles during occlusion of a single artery was similar to that during intestinal absorptive hyperemia at about one-half maximal transport of glucose in our experience (5). In past studies of intestinal absorptive hyperemia (5), mesenteric arterial resistance decreased proportionately more than did microvascular resistance as the metabolic rate was progressively increased by exposing higher glucose concentrations to the villi. We assumed that flow-mediated vasodilation of the arteries was occurring as the microvasculature dilated to conditions in the bowel wall. The
arteries were not exposed to tissue conditions and had to dilate based on some form of communication with the microvessels, which we believe would be blood flow effects. The consequences of such event, based on the results of the current study, are the possibility that excess NO generation by arteries as flow is increased could have assisted microvascular dilation during absorptive hyperemia.

To offer a different approach to increasing upstream NO generation, bradykinin was very locally applied to the origin of large arterioles before they entered the bowel wall (Fig. 6). To simply cause dilation of these vessels independent of an NO mechanism, isoproterenol was similarly applied to the same vessels. In both cases, maximum dilation of the vessel origin was used, and the goal was to increase the downstream [NO] ~40–50%, which is consistent with responses during absorption hyperemia (5). The downstream measurement point was at minimum 3 mm from the site of drug application, and this distance is generally the limit for cell-to-cell communication of an eNOS-dependent dilation for arterioles (8, 17, 18), and isoproterenol did not cause downstream dilation for more than ~1 mm from the release point. The bradykinin must penetrate the vessel wall to locally activate the endothelial cells, and probably some small amount reaches blood where it will be diluted in the plasma fluid. This does raise the possibility that some fraction of the downstream increase in [NO] and vasodilation was directly caused by bradykinin. We evaluated this possibility by using isoproterenol as a non-endothelial-dependent vasodilator. Isoproterenol (247 D) is a much smaller molecule than bradykinin (1060 D) by a factor of about one-fourth and should readily penetrate the vessel wall. Yet, when applied to the surface of vessels just as with bradykinin, isoproterenol did nothing to downstream vessels in terms of dilation or [NO] (Fig. 6). This is good evidence that bradykinin increased localized NO generation of the upstream vessel, which in turn raised the blood [NO] at that site as well as in downstream arterioles, as shown in Fig. 6. The increase in downstream [NO] was sufficient to cause vasodilation. The results of this pharmacological study with bradykinin demonstrate that receptor-mediated activation of eNOS in upstream vessels can influence NO in downstream vessels through movement of a vasoactive form of NO in blood. However, for upstream vessels to generate sufficient NO to influence downstream vessels, a considerable length of the upstream vessel of the order of 1 mm must undergo increased NO generation. In vivo conditions, it is likely that vasoactive molecules generated by the tissue, and definitely increased blood flow, would indeed activate NO generation over long lengths of larger arterioles and small arteries and, in doing so, have the potential to augment downstream [NO] of the entire rank of arterioles from large to small. In effect, it is possible that larger arterioles and resistance arteries not only contribute to the flow regulation of an organ but also have the potential to influence the [NO] experienced by arterioles within the organ because of rapid, possibly chemically protected, carriage of NO in blood.

GRANTS

The study was supported by National Heart, Lung, and Blood Institute Grants HL-20605 and HL-42898.

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