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Is The Real In Vivo Nitric Oxide Concentration Pico or Nano Molar? Influence of Electrode Size on Unstirred Layers and NO Consumption

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Abstract

Objective—There is a debate if the nitric oxide concentration ([NO]) required to influence vascular smooth muscle is below 50 nM or much higher. 30 μ m and larger diameter electrodes report [NO] below 50 nM, whereas diameters of < 10–12 μ m report hundreds of nM. This study examined how size of electrodes influenced [NO] measurement due to NO consumption and unstirred layer issues.

Methods—Electrodes were 2 mm disk, 30 μ m X 2 mm carbon fiber, and single 7 μ m diameter carbon fiber within open tip microelectrode, and exposed 7 μ m carbon fiber of ~15 μ m to 2 mm length.

Results—All electrodes demonstrated linear calibrations with sufficient stirring. As stirring slowed, 30 μ m and 2 mm electrodes reported much lower [NO] due to unstirred layers and high NO consumption. The three 7 μ m microelectrodes had minor stirring issues. With limited stirring with NO present, 7 μ m open tip microelectrodes advanced toward 30 μ m and 2 mm electrodes experienced dramatically decreased current within 10–50 μ m of the larger electrodes due to high NO consumption. None of the 7 μ m microelectrodes interacted.

Conclusions—The data indicate large electrodes underestimate [NO] due to excessive NO consumption under conditions where unstirred layers are unavoidable and true microelectrodes are required for valid measurements.

Keywords

Nitric Oxide; Microelectrodes; Arteriole

Introduction

Measurement of nitric oxide (NO) with electrodes has been used to provide real time data on how the NO concentration ([NO]) changes with naturally occurring and pathophysiological stimuli. The vast majority of measurements are for vascular tissue, in particular microvascular tissues where NO undoubtedly has its greatest influence on blood flow regulation. However, there is concern that these measurements are so flawed as to be of little value. This issue was most notably raised in reviews by Hall and Garthwaite in 2009(17) and Chen et al (12) in which they questioned why NO measurements are over such a large range. The validity of in vivo measurements was particularly questioned as to whether nonspecific sensing of compounds in the in vivo environment was the cause of such high

[NO]. However, by using appropriate permeability barriers on the electrode surface, other investigators have found minimal reactivity issues at physiological to supraphysiological concentrations of L-arginine(19; 26), nitrite (1; 16; 23), ascorbic acid(1; 5), cysteine(1), nitrate(1), H₂O₂ (39), oxygen (2), glutathione(9), lysine (38), dopamine (16), norepinephrine (5), glucose (7; 21), and bradykinin (8). There appear to be other factors than inappropriate sensing of tissue chemicals tested thus far. As a frame of reference for the review by Hall and Garthwaite, in their Table 1 they list the measured [NO] in 49 studies. They did not take into account whether the study was done with large or small electrodes in the in vitro and in vivo preparation. For the references cited, if the electrode diameter was 30 μ m or larger, a [NO] below 50 nM was indicated. If the electrode diameter was below $\sim$ 15 μ m, the [NO] was indicated to be 100–1000 nM. Therefore, electrode size may have more to do with what [NO] is measured than any other factor, including the in vivo or in vitro status.

The dichotomy of [NO] ranges measured with larger versus smaller NO sensors is a matter of two overarching considerations. First, large electrode surfaces will develop a relatively thick unstirred layer if the fluid around the electrode is not sufficiently stirred. In general, the unstirred layer thickness is in proportion to the physical size of the surface divided by the velocity of flow near the surface (27). If the stirring conditions for the electrode calibration and those during fluid measurements are not essentially identical, the concentration at slower flow velocities may be seriously underestimated. The unstirred layer issue for oxygen sensitive electrodes has been studied in detail by Fatt (15) and by Pohl et al.(27) for ion selective microelectrodes. Both studies indicate reduced stirring can dramatically decrease what is thought to be the measured concentration and these physics apply to NO electrodes. When using recessed tip microelectrodes, stirring issues due to convective transport by diffusion are mitigated by the protected sensor environment formed inside the recess. This has been mathematically modeled by Schneiderman and Goldstick(30) and confirmed by experimental studies by Crawford and Cole(13). These studies are for oxygen sensitive microelectrodes, but the physics would apply to NO. For polarographic electrodes with exposed surfaces, making the covering membrane very thick could mitigate stirring issues, but unless the working current of the electrodes was dramatically decreased by the barrier to the same range of change of current per change in [NO] of microelectrodes, the larger electrodes would still excessively consume the entity they measure. This is the second overarching issue in that polarographic electrodes consume the entity they measure and there is a risk of lowering the actual concentration near the electrode sensor. Large polarographic electrodes consume a great deal of NO compared to true microscopic sensors simply due to size considerations. The Whalen laboratory (34; 35) was instrumental in showing that very small, true microelectrodes were best for tissue oxygen tension measurements because of very low oxygen consumption and stirring artifacts. Measurement of NO is even more of an issue because the [NO] is much lower than the tissue oxygen concentration. The purpose of this study is to demonstrate that overly large electrodes with significant unstirred layer issues and large consumption of NO will consistently underestimate [NO] in slow moving solutions and, even worse, when touched to tissue, they can consume a large fraction of the NO generated by tissue.

To further illustrate that [NO] in the mid nanomolar range is needed to activate in vivo vascular smooth muscle relaxation, in vivo measurement of [NO] on the outer wall of large arterioles in the rat small intestine was accomplished with the smallest practical NO sensors. The [NO] in the tissue bath was increased with both NO gas and NO released from the breakdown of nitrosogluthathione (GSNO). The perivascular [NO] was increased sufficiently to cause dilation up to $\sim$ 40%, which is typical of intestinal arteriolar dilation during absorptive hyperemia and responses to moderate reductions in oxygen tension, both of which are highly dependent on NO mechanisms(37; 38). These studies established the range

of increased [NO] from both NO gas and decomposition of a nitroso compound that is required to cause physiological vasodilation in an in vivo arteriolar environment.

Methods

Nitric Oxide Electrodes

Three different sized electrodes from a commercial vendor, World Precision Instruments (Sarasota, FL), that are widely used in NO experimentation were evaluated. The electrodes were a 7 μm X ~2 mm carbon fiber, 30 μm X ~2 mm carbon fiber, and a 2 mm diameter metal surface electrode covered with a physical semipermeable membrane. The electrodes were coupled to an Apollo 4000 Radical Analyzer made by World Precision Instruments and the default settings for NO polarization were used. The electrodes were polarized in the test chamber for a minimum of 12 hours prior to use. All of the electrodes functioned flawlessly in calibration chambers so long as adequate stirring was present and had linear current/[NO] relationships with NO gas at 0, 600, and 1200 nM.

Microelectrodes fabricated by the author's laboratory based on designs by Friedemann et al. (16) and described in detail (8) were used as a representative of an open tip microelectrode containing a ~7 μm diameter carbon fiber. The carbon fiber was sealed both by the glass envelope mechanically adhering to the carbon fiber surface during pulling of the microelectrode and epoxy cement introduced down the microelectrode bore. The conductive epoxy cement (Grade PX, Dylon Industries, Cleveland, OH) was heat cured after being forced into the barrel of the micropipette; a small amount of acetone added to the cement made filling easier and some of the cement was carried to the tip down the glass microfiber over ~12 hours at room temperature before heat curing. This approach greatly strengthened and sealed the carbon-glass interface. After sharpening the tip of the electrode at a 30° angle to expose the carbon fiber and electrolytically forming a 2–3 μm recess, the open tip-carbon fiber surface was either electroplated with Nafion (+0.7V for 15–20 minutes) or repeatedly dipped into Nafion and heated to 210°C for 5 minutes after each dip to form a semipermeable membrane to exclude negatively charged ions. Prior studies using this approach have shown the carbon fiber microelectrodes electroplated or dip/heat application with Nafion are insensitive to biological concentrations of nitrite(8), nitrate(8), hydrogen peroxide(39), arginine(26), lysine(38), glucose (8), bradykinin(9) and oxygen tension(3; 38). The microelectrodes were polarized at +0.7 V for 10–12 hours before calibration with a Keithley Model 6517A Electrometer. The microelectrodes can also be used at +0.9V and the polarization voltage had little or no effect on the results. With either polarization voltage, these electrodes rarely have currents higher than 30 picoamperes when exposed to 1200 nM NO.

Kation Carbostar-1 microelectrodes (Kation Scientific, Minneapolis, MN) were modified to serve as NO sensitive microelectrodes based in part on their use by Tsai et al (33). These electrodes are a glass envelope pulled over a ~7 μm carbon fiber, which is exposed as a 12–15 μm tapered cone formed by the manufacturer. The glass to carbon fiber seal was problematic in many electrodes and bathing solution entered the electrode, particularly if one attempted to penetrate tissue. This caused a progressive increase in current and loss of sensitivity to NO as the current increased due to the greater “wetted” surface area inside the lumen of the glass microelectrode that reacted slowly or not at all to NO. This problem was resolved by placing the tip of the microelectrode in low viscosity, fresh cyanoacrylic glue for 15–20 minutes followed by a rapid dip in absolute ethanol to remove the glue on the carbon fiber surface. The glue required about 48 hours to “set” at room temperature and maintained a water tight barrier for at least 48 hours of use. These electrodes were electroplated with Nafion to form a selective barrier. The electrodes were polarized for 12

hours prior to use. Any electrode with a current higher than 50 picoamperes at 1200 nM NO was not used because it consumed too much NO.

Electrode Calibration and Stirring Effects on Electrode Function

Each electrode was calibrated in bathing solution at a temperature of 37.5° C using 100% N₂, 400 ppm NO in N₂ and 800 ppm NO gas in N₂. A ~35 ml Radnotti tissue bath with tubular bubbler (Radnotti LLC, Monrovia, CA) was the test chamber. The opening of the test chamber was covered with a plastic sheet with openings for the sensing electrodes and reference electrode. The plastic barrier separated the bubbled gas from the atmosphere over the fluid and minimizes water vapor effects on the surface of electrodes. The fritted bubbler was to a far edge of the lumen base to prevent bubbles from directly interacting with the electrode surfaces and to provide stirring. The level of stirring with gas flow was determined empirically in that the concentration of NO would rise ~600 nM in about 3 minutes for ~35 ml of bathing solution. This is a mild level of stirring when watching silk fibers in the solution and has trivial effects on the current generated by true microelectrodes for a given nitrogen or [NO]. Using gas to provide NO and stir solutions requires that the fluid motion not induce a current or noise yet reach a stable [NO] that is insensitive to ~20% increases or decreases in gas flow. Having a somewhat sealed gas chamber above the fluid greatly facilitates using a low gas flow and mild stirring conditions.

WPI proposes using chemical NO donors in their calibration procedure and requires a stirred environment. Therefore, stirring with very small gas bubbles rather than a mechanical stirring device are somewhat different. However, the WPI electrodes consistently performed flawlessly with linear current/[NO] calibrations similar in the current range provided by the company for each purchased electrode. The calculated [NO] was ~600 and ~1200 nM, depending on ambient atmospheric pressure on the day of measure (14) and assuming saline and distilled water have similar solubility properties for NO at 37.55° C. Studies of NO solubility in physiological salt solutions suggest a slight diminution of solubility (36) if measured at a lower temperature and extrapolated to 37.5° C, but the effect is very small for measurements below 1 μM.

After electrode calibration, the bubbling at a particular [NO] or nitrogen flow was stopped by closing the inflow gas line with a stopcock (airway resistance was constant and all gas tanks were at equal outflow pressure of 4 psi). The bubbling slowed to a stop in about 30 seconds. Because the chamber is somewhat sealed, the [NO] measured by 7 μm diameter carbon fibers in the open tip microelectrode format only declined significantly for the first 30 seconds and thereafter, a slow decline developed as NO gas dissipated from the bath. Examples of these events are shown in Figures 1 and 2. The actual stoppage of gas flow versus bubbling of solution is used to show the extremes of stirring effects on the [NO] estimated by the various electrodes. The moment to moment events during electrode tests and animal experiments were recorded with PowerLab Software (ADInstruments, Inc, Colorado Springs, CO) and stored for later analysis.

Testing of Local Area Consumption of NO by Electrodes

William Whalen proposed to the author (personal communication) a simple means to determine if an oxygen sensor consumed so much oxygen in the local environment that it might lower the oxygen tension measured in tissues was to oppose two oxygen electrodes in a stable bath at a fixed oxygen tension. Brian Duling (personal communication) has also used this approach in his work with oxygen sensitive microelectrodes. This procedure has the advantage that it closely mimics conditions of an electrode in physical contact with cells and consuming NO made by the cells. Using this approach, a WPI or Carbostar electrode was directly opposed with an open tip 7 μm carbon fiber microelectrode in the tissue bath

used for in vivo intestinal vascular studies. This bath has a volume of ~10 ml and a flow rate of 5 ml/min of physiological solution through the bath. The dimensions of the bath over the tissue surface are height 3 mm, width 2 cm, and length of 6 cm. The interaction of the two electrodes was controlled by microscopy with the electrodes far apart (1 mm), almost touching, and withdrawn from each other by a precision micromanipulator by 10 or 50 μm increments in the presence of N_2 and various concentrations of NO.

Resolution of NO Electrodes

The temperature of calibration cells and measurement chambers must be identical for electrodes and the polarization voltage must be checked in both to be sure stray currents from other electronics or ground faults have not offset the polarization voltage. This is quite easy to do with the Keithley electrometer but not feasible for the WPI electrometer. The resolution of the electrodes begins by knowing the [NO]/current relationship slope, but the true resolution must also take into account electronic noise and drift. All the electrodes tested, both made by the author and purchased, seldom had the true resolution to detect less than a ± 10 nM change in [NO] due to random noise. The resolution is definitely not equivalent with the slope of the [NO]/current change. Based on noise and drift issues, none of the microelectrodes or larger electrodes, whether made by the author or purchased, could have detected an actual difference in 0 nM vs. ~25 nM under the best of conditions. This is a crucial issue for measurements at what are presumed to be very low [NO] in this range.

Arteriolar Responses to Exogenous NO

The animal studies were reviewed and approved by the Indiana University School of Medicine IACUC. Male rats (Harlan Industries, Indianapolis, IN) in the weight range of 225–300 gm were anesthetized with either sodium thiopental or Inactin at 200 mg/kg given in 4 equal volumes under the skin of both thighs. The rats were mechanically ventilated and the arterial pressure was measured through a femoral artery catheter. The intestinal surface arterioles were exposed using a well established technique (4). The largest arterioles to enter the small intestine were selected because they are only covered by a thin layer of mesentery such that the bathing solution can interact with their gaseous environment and penetration of the microelectrode into tissue is unnecessary. The latter issue is of importance because carbon fibers in a glass envelope have piezo electric properties and bending caused transient increases in current for 3–5 minutes. Fortunately, piezo electric effects are very obvious as rapid increases in current that dissipate even if the electrode is not moved to relieve slight bending.

The inner diameter of the arteriole was observed with a microscope-closed circuit video system coupled to a computer running Metamorph Image Analysis (Universal Imaging). Images of the vessel were recorded and later measured based on calibration from a stage micrometer. The bathing solution was bicarbonate buffered physiological saline with calcium added (4) after adjusting pH with 95% N_2 /5% CO_2 . A bath oxygen tension below ~20 mmHg was essential to allow increases in [NO] from both NO gas and decomposition of GSNO. This caused a problem because reduced oxygen tension increased [NO] and caused dilation, as previously reported (24). To reverse these problems, norepinephrine (~1 μM) was used to restore most of the vasculature tone lost in the low oxygen tension environment. The norepinephrine also suppressed intestinal smooth muscle activity to quiet bowel motility for microelectrode measurements.

Prior to measurements, the microelectrode tip was placed ~200 μm above the tissue surface to obtain a current equivalent to 0 [NO]. The calibration of nM [NO]/picoampere current is used to determine subsequent values for the bath or tissue [NO]. As there is a slow negative drift of virtually all electrodes, the essentially linear drift of the baseline current was

compensated by calculating the virtual baseline of 0 [NO] for any given time point using the baseline current before and after all tissue measurements. In the course of a three hour experiment, drift can affect current equivalent to about a 100 nM offset for most microelectrodes and must be compensated multiple times during an experiment: larger electrodes have this same issue. During the experiment, the arteriole was observed as the microelectrode tip was placed on the mesentery surface adhered to the outer wall of the arteriole. The arterioles did not react to the light touching of their outer surface. After steady state measurements of NO and diameter were made, the [NO] of the bathing solution was increased by addition of NO equilibrated solution (1200 nM) to the 95% N₂/5% CO₂ equilibrated solution reservoir. Multiple concentrations of NO in the solution were used and then the solution was restored to just 95% N₂/5% CO₂ to allow the vessel to recover to baseline.

As an alternative source of NO and to check if nitrosoglutathione (GSNO) has vasoactive properties of its own, 10 μ M stock solution was added with a syringe pump at various flows into the bathing solution just before it entered the tissue chamber bath. The stock GSNO was made in ice cold saline equilibrated with nitrogen and the stock was transferred to ice chilled glass vials, filled nearly completely, and immediately frozen. During the experiment, the GSNO glass container was covered with aluminum foil and thawed in ice water. The GSNO solution was placed in an aluminum foil covered syringe that was constantly chilled by a frozen cold pack over the syringe. The solution line to the tissue bath was PE 50 tubing wrapped in aluminum foil and the transit time to the bath was of the order of one minute or less. As the bathing solution contained very little oxygen, the GSNO infusion was able to maintain a quite stable [NO] up to ~800 nM if required. However, increases in [NO] of < 300 nM were sufficient for the purposes of this study.

Results

Stirring Effects on Electrode Measurement of NO

Stirring of the solution decreases the unstirred layer thickness at the electrode tip such that the highest possible [NO] can be measured. Slowing or stopping most of the stirring allows a larger unstirred layer to form, which limits by diffusion the amount of NO the electrode can consume, and thereby underestimates the solution [NO]. Figures 1 and 2 demonstrate the actual events when mild stirring by bubbling stopped within about 30 seconds. The simultaneous measurements with a given commercial electrode and an open tip 7 μ m microelectrode provide information on both stirring issues and the decline in [NO] with time due to NO dissipation to the atmosphere. In these figures and all other figures, electronic damping of the 7 μ m open tip and Kation microelectrode currents was avoided to show typical “noise” variations with the true microelectrodes. Figure 3 presents a comparison of changes in [NO] for paired electrodes of different size when the stirring had been stopped and near stable records were obtained. In general, N₂ bubbling and stoppage was not a significant issue for the electrodes, even the large 2 mm (Fig. 1, Panel A) and 30 μ m WPI electrodes (Fig. 1, Panel B). Therefore, stirring of the solution per se was not a significant source of artifact. When NO was bubbled, there could be two effects, a reduction in NO as bubbling stopped due to loss of NO versus secondly, a decline in current related to stirring artifacts. The approximate reduction in actual [NO] when bubbling NO gas was stopped was considered by estimating how the change would have occurred by loss of NO based on extrapolation back from the decay of signal during the second and third minute after gas flow stopped. For the 7 μ m microelectrodes and the inherent assumptions of this analysis, about half of the apparent drop in [NO] is due to loss of NO with the remainder due to their small stirring artifact. With the larger electrodes, the decrease in current due to the drop in [NO] should have been small, but the large apparent decline was the stirring artifact issue related to both unstirred layer formation as stir velocity declined and the high consumption

of NO immediately around the electrode. In the presence of NO based on this analysis, all of the electrodes had stirring artifacts, including the open tip 7 μm microelectrodes shown in each panel of Figures 1 and 2. However, both the 2 mm (Fig. 1, Panel A) and 30 μm WPI electrodes (Fig. 1, Panel B) did demonstrate much greater reductions in “measured” [NO] with low stirring than other electrodes. This is easier to appreciate in the summary data in Figure 3. WPI 7 μm diameter microelectrodes in Panel A of Figure 2 had only a slightly greater stir effect in NO solution than the tapered 7 μm Kation Carbostar microelectrodes in Panels B and C of Figure 2, and open tip 7 μm microelectrodes in all panels.

Electrode Consumption of NO Effects

When a very small microelectrode enters the unstirred layer of another electrode, the NO in that layer may have been partially consumed and the smaller electrode could sense an abruptly lower [NO]. The larger the electrode, the greater will be the reduction of [NO] due to both a larger unstirred layer and higher NO consumption, as explained in the Introduction. Figures 4 and 5 present real time events when an open tip 7 μm microelectrode approached and nearly touched the surface of the other types of electrodes. The term “touch” means that at 125X magnification, the tip of the microelectrode had a finite gap of $<5 \mu\text{m}$ from the surface of the opposing electrode. As the microelectrodes did not show any response in N_2 equilibrated fluid when approaching any of the other electrodes, interaction of the electrical polarization of the two electrodes was not the cause of events when NO was present in the bath (Figs. 4 and 5). As mentioned in Methods, there was a constant flow through the tissue bath and the entire bath was replenished approximately every 12 minutes. These conditions represent a low stirring environment to simulate electrodes approaching a tissue surface or measuring NO generated by cells in slow moving fluid. During both N_2 and NO tests, currents of the commercial electrodes are not shown because they did not change within measurement error by the proximity of the open tip 7 μm microelectrodes. When NO was present, the [NO] measured by the open tip 7 μm microelectrodes near 2 mm (Fig. 4, Panels A and B) and 30 μm WPI electrodes (Fig. 4, Panels C and D) was decreased by as much as half the prevailing [NO]. In both cases, this is hundreds of nM of NO. The distance of influence of the 2 mm electrodes on NO consumption in Panels A and B of Figure 4 can be appreciated by the loss of NO signal even at 50 μm from the large electrode surface. The distance of effect for 30 μm WPI electrodes was usually about a 10 μm separation distance, as shown in panels C and D of Figure 4. The 7 μm WPI electrode had no appreciable indication of excessive consumption of NO, as shown in Panels A and B of Figure 5, and would be suitable for measurement of NO in slow moving fluid and when touched to tissue along the entire length of the exposed carbon fiber. However, for tissue measurements, the entire 2 mm length of the electrode should touch the tissue or the [NO] will be underestimated to the extent of active electrode length not touching the source of NO. The opposition of the Kation and 7 μm open tip microelectrodes was problematic due to their small size. However, very little interaction under N_2 or NO conditions could be verified as the electrode tips nearly touched or were withdrawn in 10 or 50 μm steps.

During the NO consumption tests, the [NO] measured in the tissue flow chamber by the open tip 7 mm microelectrode was compared to that of the 2 mm and 30 μm WPI electrodes. The [NO] estimated by these WPI electrodes was typically below 30% of the [NO] measured simultaneously by the open tip 7 mm microelectrode with a mean and SEM of $20.7 \pm 3.7\%$ at identical fluid depths and within $\sim 1 \text{ mm}$ distance of each other. The low indicated [NO] by the larger electrodes was due to their unstirred layer issues in slowly moving fluid and their high NO consumption. The 7 μm WPI and 7 μm Carbostar Kation microelectrodes faithfully replicated the [NO] measured by the open tip 7 mm microelectrodes.

In Vivo NO Reactivity Tests

If the [NO] required to dilate arterioles is very low, then adding a low nM concentration of NO gas to the bathing solution and thereby the vessel wall should cause substantial vasodilation. However, if the resting [NO] is in the hundreds of nM as microelectrode data indicate, a much larger increase in [NO] will be required to cause significant dilation. Figure 6 presents the change in periarteriolar [NO] in nM and percent of control arteriole diameter during exposure to NO either from NO gas or NO formed by decomposition of GSNO. Separate animals were used for the NO gas and GSNO studies. The data sets are based on 13 arterioles in 8 rats for NO gas studies and 7 arterioles in 5 rats for GSNO. With both NO gas and GSNO, the [NO] was only increased sufficiently to cause at most ~40% dilation because intestinal vessels rarely dilate more than this amount in studies of intestinal absorption hyperemia(5), increased arteriolar flow velocity(6), and physiologically decreased perivascular oxygen tension(24). As the [NO] was progressively increased by up to ~300 nM, the vessels dilated in dose dependent fashion. The data for NO gas and GSNO effects on dilation are not different as judged by linear regression analysis of the slope and intercept of the relationships. An interesting issue of this study was after the vessels had been dilated by NO for many minutes (~30 minutes), when the NO source was removed, the arterioles throughout the bowel wall would constrict and the locally generated [NO] would decline. These data are to the left side of the graph where the NO change is negative. This period of constriction lasted about 10–15 minutes and then the vessel diameter and resting [NO] fully recovered.

Discussion

Electrode Size Issues

The current study emphasizes that NO electrochemical sensors of 30 μm in diameter or larger are very flow sensitive and consume too much NO in a microenvironment to make reliable [NO] measurements. Consequently, these electrodes are subject to major underestimation of [NO] in fluids at low stir velocity if calibrated at a higher stir velocity (Figs. 1–3). A much worse problem is their high NO consumption which would make them unreliable to measure the [NO] of tissue (Figs 4 and 5). As shown in Figures 4 and 5 for NO consumption studies, electrodes with a diameter of 30 μm or larger demonstrate a major decline in [NO] within their unstirred layer due to high NO consumption. The practical consequence is these large electrodes would indicate a very low [NO], perhaps not measurable, if the actual [NO] was below ~100 nM in low stirring conditions or if approaching tissue. It bears repeating that as a frame of reference for the review by Hall and Garthwaithe questioning why reported in vivo [NO] are high, in their Table 1 they list the measured [NO] in 49 studies. Whether the study was done in vitro or in vivo, if the electrode diameter was 30 μm or larger, a [NO] below 50 nM was indicated. If the electrode diameter was below ~15 μm , the [NO] was measured to be 100–1000 nM. The current evaluation of high NO consumption and unstirred layer issues explains why large electrodes have consistently indicated in vitro and in vivo tissue [NO], particularly vascular tissue, below 50 nM and often, much lower. Fortunately, small microelectrodes, including the 7 μm carbon fiber open tip, 7 μm Kation Carbostar, and 7 μm WPI electrodes, have much smaller stirring artifacts, as shown in Figures 2 and 3, and essentially no issue of excessive NO consumption at their tip (Fig. 5). This was particularly interesting for the 7 μm WPI electrodes because they are 2 mm long. These long length, but small diameter microelectrodes made by WPI and similar to those developed by Friedemann et al (16) and Malinski and Taha (23), present a very small surface area per volume of surrounding fluid, just as do much smaller length Carbostar microelectrodes and the open tip NO microelectrode. Such microelectrodes can make valid tissue measurements of [NO] so long as virtually all of their long length touches the NO source.

The effects of unstirred layers influenced by fluid motion and NO consumption by electrodes has a special bearing on measurement of [NO] of in vivo arterioles perfused with blood. Is it possible that the measured [NO] near in vivo arterioles in the mid to high nM range in in vivo preparations of the retina(10; 11), small intestine(6; 8; 26), skeletal muscle(24), cheek pouch of hamsters(20), and cerebral cortex(2; 3), and in many more studies, is an artifact caused by flow inside the vessel causing some stir effect? In the case of an in vivo arteriole, the electrode sensor is positioned touching the outer smooth muscle layer of the vessel wall or perhaps some overlying connective tissue with blood flow on the endothelial surface. The vessel wall acts as a gas permeable structural barrier that eliminates any unstirred layer caused by fluid motion because the sensor is enveloped by the tissue. In this microenvironment, it is imperative the electrode consume as little NO as possible. However, there is a distinct possibility that even the very low NO consumption by true microelectrodes likely underestimates the [NO] in this microenvironment. If the [NO] within the lumen of the vessel in the flowing blood needs to be measured, stirring artifacts of the electrode are of paramount importance. When NO in blood inside flowing vessels is measured, as has been done by this author(9), microelectrodes with essentially irrelevant flow stir issues must be used and this can be achieved only with open tip microelectrodes with a recess of at least 5 μm . Unfortunately, such microelectrodes operate at very low currents and measurements are difficult.

Exogenous [NO] Required for In Vivo Vasodilation

If very low concentrations of NO in the low nM range are capable of causing cGMP reactions, then addition of a small amount of NO in the low nM range to the wall of an in vivo arteriole should cause extensive dilation. This was tested by providing exogenous NO in the bathing solution either from NO gas or NO generated from the decomposition of GSNO. As this study was done in vivo, any S-nitrosothiol compounds that are made by NO complexing with various molecules and then causing cGMP activation would be present. The data in Figure 6 present the results as change in nanomoles of [NO] versus dilation of the vessels for both sources of NO. A range of [NO] were used to reproduce [NO] changes measured during intestinal arteriolar responses to absorption hyperemia(5), increased arteriolar flow velocity(6), and decreased perivascular oxygen tension(24). To cause a 5–40% dilation, an increase in [NO] of 30–200 nM was required for both NO gas and NO from GSNO. The NO gas and NO from GSNO had the same the relationship of the change in [NO] versus the diameter responses. Therefore, these data demonstrate that appreciable increases in exogenous [NO] of 30–200 nM from NO gas or GSNO decomposition are required in an in vivo environment to activate the dilatory mechanisms seen with the intestinal microvascular responses listed above. One of the most interesting aspects of the effects of exogenous NO was what happened after the NO from gas or GSNO was abruptly removed. The previously dilated vessels constricted with a decline in [NO] (left side of Fig. 6). The vessels fully recovered after 10–15 minutes of rest, indicating that the vasculature had not been harmed by the exogenous NO. The presumed explanation for the constriction is that other mechanisms are compensating for excessive blood flow during NO exposure and the resulting temporary constriction would have lowered flow velocity in the vessels and less flow mediated NO would have been formed. Evidence that a reduction in intestinal perivascular [NO] and constriction occurred with a mechanically induced decline in flow velocity has been provided by this laboratory (6).

As the resting [NO] at the outer wall of the vessels studied were in the mid 300 nM range, when exogenous NO was applied, the highest concentrations were in the mid 500 nM range with maximum dilation occurring in the 800 nM range for intestinal vessels. By the standards of NO measurements with large NO sensitive microelectrodes, these are extraordinarily high [NO], but with true microelectrodes, these [NO] data fit the routinely

measured ranges for ~ 7–12 μm diameter sensors as mentioned previously. There are other types of in vivo studies that indicate vascular [NO] well into the nM range. There is a very extensive literature on S-nitrosothiol compounds formed in blood as NO reacts reversibly and unharmed with hemoglobin, cysteine, glutathione, and albumin. Measurements of S-nitrosothiol compounds have used light and chemical measurement assays rather than NO sensitive electrodes (18; 25; 28; 29; 31; 32). The concentration of S-nitrosothiol compounds are typically 300–1000 nM and higher for arterial hemoglobin, albumin, cysteine, and glutathione in plasma (18; 25; 28; 29; 31; 32). It is very important to state that at some point in the life of the nitrosothiol compounds, the [NO] in plasma or the red blood cell cytoplasm had to be as high as or higher than the molar concentration of the complexed S-nitrosothiol compound. This argues there is a vascular endothelial or red blood cell NO source concentration of hundreds of nM. A separate line of data on mid nanomolar [NO] to cause vascular dilation comes from infusion studies with GSNO and nitroglycerin into human arm vessels. Quite remarkably, single digit nanomoles per minute infusion caused 50–100% increases in blood flow (10; 15; 22). However, if one calculates the plasma flow, not whole blood flow, in milliliters per minute into which the nanomoles per min infusions occurred, the effective plasma concentrations initially reached into the multiple hundreds of nanomolar concentration. These data and those on naturally occurring plasma S-nitrosothiol concentrations indicate much higher [NO] and support the proposal that in vivo [NO] for resistance vessels are quite substantial and well into the nanomolar range.

Perspectives

In most studies reporting what is presumed to be low [NO] in tissue or to activate cGMP, one or both of the following problems can occur based on the results of this study: (1), the [NO] in low stirred solution of data collection will be seriously underestimated from calibrations using NO in well stirred solutions and (2), large electrodes used to monitor in vivo or in vitro tissue [NO] will excessively consume tissue generated NO and report very low [NO]. While microelectrodes with sensors less than 10 μm in diameter have their many problems with electronic noise, fragility, and the difficulties of very low electrical current measurement, their very low NO consumption and relatively minor stirring issues can provide reasonably reliable resolution of the true nature of the physiological concentration of NO, which appears to be in the hundreds of nanomolar range in the in vivo microcirculation.

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List of Abbreviations

NO	Nitric Oxide
[NO]	Nitric Oxide Concentration
μm	micron
NM	nanomolar
μM	micro molar
CGMP	cyclic granulate monophosphate
P_{O2}	Partial pressure of oxygen

GSNO S-nitrosoglutathione

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Figure 1.

Measurements of NO by 2 mm metallic disk in Panel A and 30 μm X~2 mm diameter carbon fiber WPI electrodes in Panel B with simultaneous measurements by 7 μm open tip carbon fiber microelectrodes during bubbling with various concentration of NO gas and stopping gas flow over about 30 seconds. These large electrodes had a substantial decline in estimated [NO] as bubbling stopped and should not have decreased the measured [NO] by more than did 7 μm microelectrodes as NO was lost through dissipation. The decline in reported NO indicates the larger electrodes consumed too much NO within a larger unstirred layer formed with the slowing and stoppage of stirring by bubbling.

Figure 2.

Measurements of NO by 7 μm X ~2mm carbon fiber WPI electrodes in Panel A and 7 μm diameter X ~12–15 μm long Kation Carbostar microelectrodes in Panels B and C with simultaneous measurements by 7 μm open tip carbon fiber microelectrodes during bubbling with various concentration of NO gas and stopping gas flow over about 30 seconds. The declines in [NO] with the stoppage of bubbling and subsequent loss of NO from solution were generally similar for all these electrodes with somewhat better performance by Kation than WPI electrodes. All the true microelectrodes did demonstrate an initial rapid but relatively small decline in reported [NO] that reflects some stirring effect.

Figure 3.

This figure compares all the various types of electrodes to simultaneous events with an open tip 7 μm diameter carbon fiber microelectrode when either NO or nitrogen gas was stopped. The worst performing electrode of a given type is shown in the left side of each panel and the best performing electrode is shown in the right side of each panel for a particular type of electrode. Typical electrodes in all categories are closer in performance to the best case electrode shown in this figure. The 7 μm diameter carbon fiber microelectrodes from WPI in Panel C and Kation in Panel D compared very favorably to open tip 7 μm diameter carbon fiber microelectrodes in terms of having the least effects of stirring/NO consumption interaction and at least half of the decline in reported [NO] is from NO dissipation based on the decline in [NO] reported by the open tip electrode. The larger electrodes, the 2 mm WPI in Panel A and 30 μm WPI in Panel B, had the largest net declines in measured [NO] compared to 7 μm open tip microelectrodes. The responses of all electrodes to nitrogen bubbling (~0 nM NO) are shown to illustrate that gas stirring per se was not a significant cause of artifact relative to responses with NO bubbling/stirring was stopped.

Figure 4.

Open tip 7 μm diameter carbon fiber microelectrodes approached and almost touched the surface of 2 mm metallic disk in Panels A and B and 30 μm X~2 mm diameter carbon fiber WPI electrodes in Panel C and D in the presence of NO solution flowing through a tissue chamber. The decline in measured [NO] by the open tip 7 μm diameter carbon fiber microelectrodes reflects NO consumed by the larger electrodes within their unstirred layer with slowly stirred conditions typical of tissue baths over in vitro or in vivo tissue layers. The [NO] change of larger electrodes is not shown because it was not altered by the microelectrode presence. As the open tip 7 μm microelectrode was withdrawn, the measured [NO] was restored at about 50 μm distance for 2 mm WPI electrodes and at about 10 μm for 30 μm WPI electrodes. These results give an approximate estimate of the unstirred layer/NO consumption issue distances for larger electrodes. During nitrogen equilibrated fluid, approach of the open tip 7 μm diameter carbon fiber microelectrodes did not result in any average change in current. This indicated no electrical interaction of the two types of electrodes.

Figure 5.

Open tip 7 μm diameter carbon fiber microelectrodes approached and almost touched the surface of 7 μm X ~2mm carbon fiber WPI electrodes in Panels A and B and 7 μm diameter X ~12–15 μm long Kation Carbostar microelectrodes in Panels C and D in the presence of NO solution flowing through a tissue chamber. The decline in measured [NO] by the open tip 7 μm diameter carbon fiber microelectrodes reflects NO consumed by the other microelectrodes within their unstirred layer with slowly stirred conditions typical of tissue baths over in vitro or in vivo tissue layers. The [NO] change of these microelectrodes is not shown because it was not altered by the presence of the approaching microelectrode. None of the 7 μm diameter carbon fiber electrodes, including the WPI format with a 2 mm shaft, had significant issues caused by unstirred layer/NO consumption interactions. During nitrogen equilibrated fluid, approach of the open tip 7 μm diameter carbon fiber microelectrodes did not result in any average change in current. This indicated no electrical interaction of the two types of electrodes.

Figure 6.

The [NO] and inner diameter was measured for in vivo rat intestinal large arterioles as they enter the bowel wall. The bathing fluid was mixed with 1200 nM NO gas equilibrated solution or GSNO was added to the incoming bathing solution just at it entered the chamber. The change in [NO] in nM is shown for various percent of control diameter responses. There were similar increases in diameter for equivalent increases in vessel wall [NO] with both NO gas and NO from GSNO. The equations and regression for the relationships are shown. After NO exposure stopped, there was a rebound vasoconstriction and simultaneous decline in [NO] as the vasculature compensated for a 20–40 minute period of elevated blood flow caused by increased [NO]. Within 15 minutes, all the vessels recovered their resting [NO] and diameter. The data are based on 13 arterioles in 8 rats for NO gas studies and 7 arterioles in 5 rats for GSNO.