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Rapid and Slow Nitric Oxide Responses During Conducted Vasodilation in the In Vivo Intestine and Brain Cortex Microvasculatures

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Abstract

Conduction of arteriolar vasodilation is initiated by activation of nitric oxide (NO) mechanisms, but dependent on conduction of hyperpolarization. Most studies have used brief (<1 sec) activation of the initial vasodilation to evaluate the fast conduction processes. However, most arteriolar mechanisms involving NO production persist for minutes. In this study, fast and slower components of arteriolar conduction in the in vivo rat brain and small intestine were compared using 3 minute stimulation of NO dependent vasodilation and measurement of [NO] at the distal sites. Within 10-15 seconds, both vasculatures had a rapidly conducted vasodilation and dilation at distance had a fast but small [NO] component. A slower but larger distal vasodilation occurred after 60-90 seconds in the intestine, but not the brain, and was associated with a substantial increase in [NO]. This slowly developed dilation appeared to be caused by flow mediated responses of larger arterioles as smaller arterioles dilated to lower downstream resistance. These results indicate while the intestinal and cerebral arterioles have a fast conducted vasodilation system, the intestinal arterioles also have a slower but larger dilation of major arterioles that is NO related and dependent on the conduction of vasodilation between small arterioles.

Keywords

Nitric Oxide; Intestine; Brain; Arteriole; Conducted Vasodilation

INTRODUCTION

Transfer of vasodilation along arterioles has been established as a significant mechanism to communicate along a given arteriole and even between successive generations of arterioles. Studies from the laboratories of Duling and Segal, both together and separately in many studies, of which only a portion are referenced {8; 14; 16; 17; 29; 34; 35}, have shown the conducted vasodilation mechanism is dependent on activation of localized generation of nitric oxide (NO) for ideal communication. However, application of nitroprusside as a source molecule for NO and other vasodilators whose mechanisms are not strongly dependent on generation of NO only cause localized vasodilation with little or no conducted vasodilation{1; 8; 13; 16}. Observations of this type have lead to the general consensus that for the spread of dilation to occur, electrical communication between arteriolar wall cells following the onset of NO production by endothelial cells is essential {18; 19; 26; 33}.

Both the small intestine and cerebral cortical microvasculatures are known to have well developed vascular nitric oxide regulatory systems and as such, the potential for cell to cell communication initiated by the generation of nitric oxide might be well developed. In the intestine, increased nitric oxide generation occurs in response to reduced oxygen tension {47; 49} , elevated sodium hyperosmolarity during villus nutrient absorption{4; 5; 47}, and

flow shear dependent NO mechanisms {6; 7}. In the cerebral circulation, NO mechanisms are more complex due to both endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase systems of the arterioles. Studies by Bauser-Heaton et al {2; 3} have shown that each NOS system has unique duties; nNOS is involved in the mechanism to sense and respond to localized reductions in oxygen tension as well as neurotransmitters and eNOS can respond to receptor mediated NO production and flow shear mechanisms. A study of isolated cerebral arterioles by Dacey and Duling {11} indicated that conducted vasodilation is possible and studies of isolated retinal arterioles by Dalsgaard et al {12} have also demonstrated conducted vasodilation. However, to what extent either in vivo intestinal or cerebral microvasculatures actually use NO generation as a mechanism to activate conducted vasodilation has not been evaluated.

The current study evaluated the hypothesis that the combined nNOS and eNOS systems of the brain could allow exceptionally well developed conducted vasodilation. Furthermore, it was also suspected that neurons containing nNOS in this vasculature could cause apparent conducted vasodilation which in fact could be due to neuronal mechanisms. In the peripheral microvasculature, neuronal mechanisms have little to do with causing conducted dilation {36; 37}. However, neuronal activity in an area of brain might communicate over considerable distances and in doing so, cause vasodilation over large distances through combined neuronal and vessel wall interaction. Within the intestinal microvasculature after preliminary studies, the hypothesis was developed that while conducted vasodilation rapidly initiates dilation over long distances, increased release of NO at distant locations is a major but relatively slowly developed mechanism to sustain and increase upstream dilation of major arterioles. In all of the studies to be presented, sustained point release of a NO dependent vasodilator will be used rather than very brief release of compounds as has been the normal approach for studies of conducted vasodilation. The sustained release approach allowed both fast and slowly developed arteriolar communication events to be studied in terms of both dilation and increases in perivascular [NO].

METHODS

All procedures were reviewed and approved by the Indiana University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 200-250 grams were used for brain vascular preparations and 250-300 gram rats were used for intestinal preparations. All animals were obtained from Harlan Labs. (Indianapolis, IN). The animals were anesthetized with 200 mg/kg sodium thiopental (Abbott, Chicago, IL) given in four subcutaneous locations over both thighs and the lower back. This provided a very long acting and stable level of anesthesia such that supplemental doses of anesthetic were seldom needed, as judged by no corneal reflexes and withdrawal to forepaw pinch. The animal was placed on a water circulated heated (37°C) matt as soon as it lost sensibility and the trachea was cannulated for mechanical ventilation. The ventilator tidal volume was based on the Harvard Apparatus pneumogram plus dead space of the tracheal cannula for a ventilation rate of 70 breathes per minute, a typical ventilation rate for conscious rats in the size range used. The percent saturation of hemoglobin with oxygen was measured over a forepaw "palm" and kept at ~90% by adjusting the tidal volume. The fore paw location has a lower percent saturation than the ear by 2-4 % units, but the ear was not available once the head surgery began. The right femoral artery was used to measure the arterial pressure and provide 0.5 ml saline /100 gram body weight / hour to support the cardiovascular system.

The parietal cortex was bilaterally exposed by removing both parietal bones across the midline of the skull. This requires a delicate removal of bone over the sagittal sinus vein, but bleeding is the exception. Most arterioles on the brain surface were visible through the dura mater membrane and the membrane was gently opened just over the vessel regions selected

with a 30 gauge hypodermic needle used as a micro knife. Vessel regions were selected on the basis of extensive distances of arterioles up to 2-3 mm long available to evaluate arteriole to arteriole conduction of vasodilation. A stainless steel, water heated fluid support was lowered over the head and the support was coated with stopcock grease to form a seal to the exposed skull. The chamber was perfused with 5 ml/min of heated bicarbonate buffered complete physiological solution equilibrated with 5% carbon dioxide, 5% oxygen, and balance nitrogen {2; 3}. The fluid passed over the exposed tissues and then was wicked away. The temperature of the fluid pool was measured and kept at $37.5 \pm 0.2^\circ\text{C}$ by regulating the temperature of the circulating hot water and by heating the incoming suffusion fluid before it passed over the brain surface.

The intestinal preparation was based on a standardized approach {7} in which a loop of jejunum was exposed and slit with a microcautery before being spread over a translucent rectangular pedestal. As with the cerebral preparation, a flow though well was placed above the tissue for suffusion with 5 ml/min of heated bicarbonate buffered complete physiological solution equilibrated with 5% carbon dioxide, 5% oxygen, and balance nitrogen {2; 3}. To minimize motility of the bowel, 1 mg/l of isoproterenol (Sigma-Aldrich, St. Louis, MO) was added to the bath and had trivial effects on arteriolar diameters.

Vessel images for both preparations were time lapse recorded at a rate of 1 image per second using a video camera and Metamorph Imaging software (Molecular Devices, Sunnyvale, CA). Images were taken for three minutes, but most vascular responses reached a new steady state within 90 seconds after the start of releasing a vasodilator chemical. The vessel diameters were measured by the operator using previously determined magnification parameters for the images based on a stage micrometer marked in 10 and 100 μm units.

Nitric oxide was measured with 7-8 μm diameter carbon fiber, open tip microelectrodes with the carbon either flush with the beveled tip of the glass envelope or slightly recessed by 2-4 μm {4}. This design is based on the approaches originally developed by Buerk {10} and Friedemann et al. {20} for NO sensitive microelectrodes. The tip outer diameter of the microelectrode with a glass envelope was $\sim 12 \mu\text{m}$ after the tip had been sharpened at a $\sim 35^\circ$ angle. Nafion (Sigma Chemical, St. Louis, MO) was electroplated at +0.7 V for ~ 15 -20 minutes to form a barrier that limits negatively charged organic molecules {30}, such as arginine and lysine, and nitrite/nitrate at physiological concentrations {20} from interacting with the carbon fiber. The microelectrodes were calibrated before each experiment at 0, 600, and 1200 nM NO gas in saline at 37.5° using a Keithley Model 6517A Electrometer (Cleveland, OH) to both polarize the electrode at +0.7V and measure currents typically in the range of 2-15 picoamperes.

All electrodes were calibrated with precision ($\pm 2\%$) NO gas concentrations (Praxair Distribution, Inc, Bethlehem, PA) in nitrogen at [NO] dissolved equivalents of 0, 600, and 1200 nM in a 80 ml glass tissue bath heated by circulating water and gassed with a small bubbler. NO microelectrodes consistently have linear current versus [NO], as described by {20}.

A “0” concentration of NO can be obtained during experiments by simply raising the electrode tip about 200 μm above the tissue surface in a total fluid depth of 2000 μm beneath a water immersion objective. The calibration of NO in nM per millivolt output of the Keithley electrometer was used to calculate the [NO]. The experiments were long in duration and only exceptionally stable electrodes were used for all studies. However, any drift in baseline was compensated by computing a virtual baseline between times that the “0” equivalent NO concentration was obtained in the bath after tissue measurements. The voltage generated by the electrometer and arterial blood pressure were recorded at 1 second

intervals with a PowerLab analog to digital chart recorder system (AD Instruments, Inc, Colorado Springs, CO).

Microelectrode measurements of NO have been questioned because of possible interactions of fluid flow or tissue motion with the sensor to generate a vibration related current or change the unstirred layer about the tip of the sensor{22}. The same arguments against reliable measures of PO₂ can be made. Electrodes with a long exposed sensor element beyond the insulating cover of glass or other non-conductive material are particularly prone to these artifacts due to the large surface area of the sensor element. However, Whalen et al. {46} found for oxygen sensing microelectrodes that have a sensor recessed slightly within the glass envelope of a true microelectrode, fluid motion issues were minimal with reasonable stirring. To check for possible artifacts due to movement of fluids about the microelectrode tip, two tests must be performed. First, the current of the electrode must not respond to the fluid stirring by nitrogen bubbles or a mechanical stirrer, other than with a small increase in electrode noise about a constant mean that likely reflects vibration issues. A reasonable test is the response of an electrode to a stream of ~0.5- 1mm diameter nitrogen bubbles rising at about 60-70 mm/sec through saline about the microelectrode tip. Stirring in this environment far exceeds any possible motion issues on the outside of arterioles. Electrodes that respond to fluid motion in an inert chemical environment with a sustained increase in current and should not be used if tissue motion or flow of fluid exceeds the motion artifact characteristics of the electrode. Secondly, for a given [NO] generated by bubbling a known gas concentration, the microelectrode current at a fluid depth of 1.5-2 cm should not decline for at least 30 seconds after stirring by bubbling has abruptly stopped. If the current did fall, this would indicate that the electrode measurement current was sensitive to rapid formation of an unstirred layer formed about the sensor element. Such electrodes would underestimate the [NO] in a micro-environment. In actual practice, the measured current at a given [NO] is nearly constant for upwards of 5 minutes after gas bubbling stopped at a depth of ~2 cm.

Glutamate and bradykinin (Sigma-Aldrich, St. Louis, MO) were iontophoretically released from microelectrodes sharpened to a tip diameter of ~8 µm to facilitate tissue penetration of the dura mater or intestinal wall. The sharpening also avoided problems with exceptionally high electrode resistance during iontophoresis when very small or unsharpened microelectrodes are pushed into tissue and likely are somewhat plugged by tissue. Microiontophoresis has the distinct advantage of allowing a near point source of a vasoactive compound to be applied to a very small region of a microvessel. A World Precision Instruments Model Iontophoresis Dual Current Generator Model 260P (Sarasota, FL) was used to control the electrical current passed through the microelectrode. To prevent diffusion of either glutamate or bradykinin from leaking from the microelectrode during resting conditions, a ~10 picoamperes current to attract the ionic species was used to retain the vasoactive ions. Currents of 25-400 picoamperes of the same polarity as the dissolved molecule were used to drive the vasoactive species from the microelectrode tip onto the vessel wall. Extracellular currents of this magnitude do not have energy sufficient to affect vessels and this was tested using carbon fiber electrodes polarized to generate identical range currents. Also, the iontophoresis unit and NO measurement system did not interact at either positive (bradykinin) or negative (glutamate) polarization of the iontophoresis unit at these iontophoresis currents. This was tested by having the NO and iontophoresis microelectrode tips essentially touch each other in saline over the tissue and increase the iontophoresis current to 400 picoamperes. To achieve the relatively low currents used, the concentration of sodium glutamate had to be 50 mM and 20 mM for bradykinin.

Protocols

Conducted Vasodilation Protocol—The following protocol was used for the data collection in all the figures. The goals of these measurements were to (1), evaluate the relative efficiency of bradykinin and glutamate to stimulate local NO production and vasodilation from eNOS and nNOS {2; 3}, and (2) to determine the ability of in vivo cerebral and intestinal arterioles to demonstrate conducted vasodilation both in magnitude and distance along the vessel and possibly increase the [NO] at distance away from the site of drug release. In the intestinal vasculature, only bradykinin was used because the vessels did not respond to glutamate, as this laboratory has previously shown {2}. On the wall of the small arteriole, the iontophoresis and NO microelectrodes were placed on the vessel outer surface in as close proximity as visual acuity allowed. After placement of two microelectrodes, the vessel allowed to equilibrate because with two microelectrodes touching a vessel, some slight dilation can initially occur. The drug used was released at the currents mentioned and each current release lasted 3 minutes to make sure steady state events had occurred, which they did. Pilot studies of the cerebral microvasculature indicated that for both bradykinin and glutamine, the conducted upstream and downstream dilations were quite limited compared to what occurred in the small intestinal vasculature. Therefore, the NO was only measured 250 μ m upstream from the drug release site and occasionally, the same was done for the 250 μ m downstream location. In the intestinal vasculature, the conducted dilation was so extraordinary that the upstream parent arteriole up to 2-3 mm distant would dilate. For most experiments on the small intestine, a conduction distance of ~ 1 mm was chosen to facilitate movement of the microscope stage between vessels so micropipette placement could be easily adjusted. The diameters of the intestinal vessels upstream and at the site of drug release could not be simultaneously monitored because of the great distance of conduction. Therefore, separate drug release applications were made for site of drug release and distal sites. In the brain studies, sections of vessels were chosen with no side branches for at least 250 μ m upstream and downstream of the agonist drug release were chosen. This selection process was used to avoid side branch arterioles because they might interact with conducted vasodilation. However, for the intestinal vasculature, the conducted vasodilation was so well developed that it was impossible to not have many side branches of the primary vessel studied.

Tissue versus Perivascular Sources of NO in the Brain

Glutamate is a routine neurotransmitter in the brain and could cause a widespread release of NO as it interacts with the N-methyl-D-aspartate (NMDA) receptor that allows the release of nNOS for NO production {27}. Therefore, glutamate and bradykinin were separately released onto the vessel wall and 250 μ m from the vessel wall in brain tissue to determine if either agent could increase vessel wall and general tissue [NO] and thereby affect vessel diameter. These types of tests were unnecessary in the intestinal vasculature because the arterioles did not respond to glutamate. In the intestine, the vasculature did not respond to a supramaximal bradykinin dosage unless the iontophoresis microelectrode tip was within ~75-100 μ m of the vessel wall. This indicated that both very little drug was being released and therefore the microelectrode tip must achieve intimate contact with the vessel wall for ideal responses. Consequently, maintaining consistent touching of the iontophoresis micropipette tip to the vessel wall was essential and the electrode tip had to be adjusted to the vessel wall as the vessel dilated during drug release.

Statistics

Comparisons of events were by one way repeated measures ANOVA for a given location exposed directly or at distance to a specific drug. Comparisons between locations or drugs were made by two way repeated measures ANOVA (Drug X Location or Drug A vs. Drug

B). When significant effects were found, a post hoc test using Least Significant Difference testing was applied. All statistical tests used Statistica Software (Statsoft, Tulsa, OK).

RESULTS

Intestinal Vascular Responses

Responses of the intestinal vasculature to bradykinin will be presented first to illustrate that the technical protocol did cause well developed conducted vasodilation, which by comparison was poorly developed in the cerebral vasculature. The upper panel of Figure 1 presents the diameter responses to bradykinin of the intestinal small diameter arterioles, which will be called 2nd order arterioles, and the large arterioles that are 1st order arterioles. First order arterioles are the largest in the intestinal wall and are the first generation or 1A of arterioles after the mesenteric arterial vessels and course radially around the intestinal wall. The 2A are collateral vessels between the 1A and are themselves the parent vessels for the smallest arterioles to the muscle layer, submucosal crypts, and individual villi. In 15 rats, pairs of 1A and 2A measured with the same NO microelectrode. The 1A had an average resting [NO] of 381 ± 74 nM and the smaller 2A had an average [NO] of 436 ± 41.7 nM. The actual diameters and their responses of the 1A and 2A are shown in the inset figure of Figure 1. The inset data illustrate the large disparity of diameters of the two sets of vessels. However, on a percent of control basis shown as the main issue of Figure 1, the 1A and 2A dilated proportionately equally for the respective dosages of bradykinin applied to just the 2A. The 1A dilated because of conducted vasodilation over a distance of at least 2000 μ m from the site of bradykinin release on the 2A plus a likely component of dilation caused by flow mediated dilation, as will be discussed. The dilation of the 1A was centered about the branch point where the 2A being subjected to bradykinin joined the larger arteriole. The dilation extending out from the branch point was decremental and essentially absent by 500-750 μ m upstream and downstream. It would be impossible for bradykinin to have reached the 1A branch point because (1) the flow of blood was from the 1A toward the 2A, (2), 2A were chosen with no immediately paired venule to possibly absorb some of the bradykinin and through retrograde flow transmit the drug to the 1A, and (3), when bradykinin was released ~ 100 μ m from the wall of the 2A, the dilation of 2A for all dosages was substantially curtailed or absent.

The lower panel of Figure 1 presents the [NO] measured at the wall of the 1A and 2A during bradykinin release on the 2A. Only two current dosages of bradykinin routinely were routinely tested, 25 nanoamperes as the threshold response dosage, and 100 nanoamperes which caused essentially maximum dilation (Fig. 1, upper panel). The data in Figure 1 for diameter and [NO] responses represent the final steady state events that occurred after at least 60-90 seconds of downstream bradykinin release. The increase in [NO] at the large arteriole after time for all events to develop was proportionately equivalent to that at the location of bradykinin release on the smaller arteriole. As shown in Figure 2, the increase in [NO] during the response was a two stage event for the large 1A. First, there was a rapid rise in [NO] during the first 15-30 seconds of downstream bradykinin release followed by a slower but larger increase in [NO] over the next 60-90 seconds. For the 2A, the increase in [NO] reached steady state within 20-30 seconds after the release of bradykinin and was stable thereafter. The data in Figure 1 are based on studies to 12 sets of vessels (small and large arterioles) in 12 rats.

Figure 2 provides representative timing events of 1A for both diameter and [NO], both of which are presented in percent of control format for sake of comparison, during responses to 25 and 100 nanoampere bradykinin release on the downstream 2A. The fast conducted response and slower developed responses to downstream 25 nanoampere bradykinin were typically small and in some cases, the slower developed response did not occur, as shown in

Panel A, because the conducted vasodilation decremented before reaching the 1A. There were two types of responses to 100 nanoampere bradykinin as seen on the distal 1A branch point: a gradual increase in [NO] that developed slowly within 60 seconds, as shown in the upper two panels, and a larger increase in [NO] that developed rapidly in the first minute of downstream bradykinin release. The two examples shown for large responses in the lower two panels are the largest responses found in the study. Most of the responses, about 70%, were intermediate responses similar to those in panels A and B for 20 large arterioles in 15 animals were studied. In attempting to understand why occasional very large responses occurred, the issue was a few 2A are interconnected with several other 2A and the conducted dilation occurred over three 2A rather than just one 2A. Most 2A extend from two adjacent 1A and have either no or only one small connection to other 2A. A few 2A have multiple interconnections that are not apparent due to constriction until conducted vasodilation reveals the set of interconnections.

The studies of Duling and Segal have noted that conduction of ascending vasodilation is rapid {13; 14; 16; 25}. The conduction speed of the 2A to the 1A was estimated by the distance involved and the time required to increase the diameter of the 1A by 5%, which is greater than the limited vasomotion variations in diameter of these vessels. The data calculations were based on 10 arterioles in 9 rats. Over an average distance of $901.1 \pm 48.5 \mu\text{m}$, the average velocity to 5% dilation was $115.8 \pm 7.4 \mu\text{m/s}$ at 25 nanoampere bradykinin, the threshold dosage, and $121.1 \pm 12.8 \mu\text{m/s}$ for 200 nanoampere bradykinin, a supramaximal dosage. The equivalent velocities despite quite different stimulus strengths to the smaller arteriole was interpreted as evidence of conduction independent of initiating dilation magnitudes of the small arterioles (Fig. 1). These events occurred within 8-11 seconds and represent the first stage of dilation. There was a small increase in perivascular [NO] associated with the initial 5% dilation. The percent of control [NO] at the threshold dosage was $103.3 \pm 1.5\%$ ($p > 0.07$) and for the supramaximal dosage was $109.4 \pm 1.9\%$ ($p < 0.05$) at the times of 5% dilation. By way of comparison, the final slowly developed increase in [NO] of 1A for threshold was $111.8 \pm 5.7\%$ and for supramaximal bradykinin was $139.7 \pm 12.3\%$ percent of control.

To verify that the slower but larger dilation of the parent arterioles was highly dependent upon localized NO production, the dilation and NO responses of the branch point portion of the larger arteriole were compared before and after localized blockade of eNOS with L-nitro arginine methyl ester (L-NAME). The 1 mM L-NAME was applied at $0.5 \mu\text{l/min}$ from a large micropipette ($\sim 100 \mu\text{m}$ diameter) directly over the branch point and essentially touching the tissue. The fluid contained 1 mM lissamine green as a dye to track the movement of the column of drug solution. Lissamine green did not stain living tissue although it did temporarily permeate the tissue to allow confirmation that L-NAME was likely also diffusing around the branch point. The dyed solution flowed away from the region of the smaller downstream arteriole and quickly dissipated in the 5 ml/min bath flow. The response of the large and small arteriole to direct bradykinin release at 400 nanoampere, a supramaximal dosage, was compared before and after L-NAME application for 20-30 minutes. Successful blockade was first evident by localized constriction at the branch point and a sustained decrease in [NO] of about 50% within usually 20 minutes. As shown in Figure 3, responses of the small arteriole were fully preserved after L-NAME exposure to the branch point. However, the large arteriole response to direct bradykinin challenge was lost in terms of dilation and increased [NO] and the conducted response did not cause either dilation or increased [NO] at the branch point. The data set is based on 4 arterioles studied in 4 rats.

Cerebral Arteriolar Responses

The conducted vascular responses of the cerebral arterioles were so limited that the technical aspects of the protocol were questioned and in part, this was the justification for the studies of the intestinal arterioles. However, as just presented, the well developed conducted dilation responses of the intestinal arterioles with the same techniques used to evaluate the cerebral arterioles would argue that the technical approach was more than sufficient for the study of in vivo conducted dilation in the cerebral vessels. The results of glutamate and bradykinin iontophoretically released for three minutes onto the wall of a cerebral arteriole at currents up to 400 nanoamperes are shown in Figure 4 for diameter responses and simultaneous [NO] events in Figure 5. Vessel diameters and nitric oxide were measured at the drug release location and also on the arteriolar wall at 250 μ m upstream and downstream. The average resting [NO] of the cerebral arterioles studied was 942 ± 62.9 nM based on measurements of 32 vessels in 18 rats. The brain vessels are equivalent in anatomy and location in the microvascular tree as the 2A in the study of intestinal vessels in that they interconnect the largest arterioles and directly feed the small arterioles to discrete columns of brain cortex. As shown in Figure 4, both glutamate and bradykinin caused comparable dilation at the site of drug release with lesser but equivalent dilation at the 250 μ m up and down stream locations. The distance of 250 μ m upstream and downstream of the drug release location was chosen for presentation in Figure 4 because about 70% of the original dilation was preserved for the upstream location: the downstream location consistently dilated less for both bradykinin and glutamate activation. Only a 3-5% dilation was detectable at about 500 μ m upstream from the site of agonist release. Over this distance in the brain, there were side branches of the parent arteriole and these smaller vessels did dilate near their origin. Data in Figure 4 are based on the same 14 arterioles in 14 rats for glutamate and 14 arterioles in 14 different rats for bradykinin.

Even though glutamate and bradykinin caused equivalent dilations at the sites monitored, there were very major differences in the [NO] responses measured on the outside of the arteriolar wall. As shown in Figure 5, glutamate caused a very large increase in [NO], consistently larger than occurred with bradykinin. At a site 250 μ m upstream from the glutamate or bradykinin release site at 400 nanoamperes, the [NO] did not increase with either drug and similar results were found for tested downstream sites. Therefore, the upstream dilation in cerebral cortical surface arterioles was due to a conducted mechanism which did not cause a localized increase in [NO]. The data are based on 14 arterioles in 14 rats for glutamate and 14 in 14 different rats for bradykinin and are the same vessels and animals used for diameter responses in Figure 4.

The higher [NO] caused by glutamate could be because nNOS in brain tissue some distance from the vessel wall could be influencing the vessel wall [NO]. This was tested and the data are shown in Figure 6. Both glutamate and bradykinin were released at the vessel wall at 50 and 400 nanoamperes and then at 250 μ m from the vessel wall at 400 nanoamperes. In addition, the vessel diameter for each site of drug release and the vessel wall [NO] were measured. Glutamate, which acted on nNOS, was able to moderately increase both vessel [NO] and diameter when released at 250 μ m from the vessel wall, but nothing occurred with bradykinin release. The tissue effect of glutamate at 400 nanoampere on [NO] and diameter were equivalent to that of glutamate at 50 nanoampere directly released onto the vessel wall. The elevated [NO] at the vessel wall caused by the distal release of glutamate was caused by a generalized increase in [NO] in tissue surrounding the vessel. However, most of the NO generation during release of glutamate on the vessel wall at dosages above 50 nanoamperes was from the immediate vessel wall area. Bradykinin had trivial effects on [NO] and vessel diameters when released 250 μ m from the vessel wall. Bradykinin is predominately an eNOS dependent vasodilator with trivial, if any effects, on nNOS in our evaluations {2; 3}. This data set is based on 4 arterioles in 4 rats, each vessel being studied for both drugs.

Asterisks indicate significant change from control. Number symbols indicate the glutamate response was significantly greater than that for bradykinin at equivalent conditions.

Discussion

For the sake of comparison of conducted vasodilation along arterioles in the cerebral and intestinal microvasculatures, second generation order arterioles were used. These medium diameter arterioles (30-50 μm , id) are both collaterals between large arterioles coursing through the tissue and the parent arterioles of individual small arterioles to specific tissue regions. The cerebral and intestinal arterioles had very different resting [NO], 942.5 ± 62.9 nM in the brain and 436.4 ± 41.8 nM in the small intestine. These [NO] for brain and intestinal arterioles are similar to those previously reported for each vasculature and appropriate local blockade of or interference with NOS functions lowered the resting [NO] by at least 50% {2; 3; 48; 49}. The much higher [NO] in the brain than intestinal vessels occurred because cerebral vessels have NO contributions from the nNOS and eNOS systems, whereas the intestinal system has only a measurable contribution of NO from eNOS because the vessels do not respond to glutamate at supraphysiological concentration {2; 3}. The in vivo perivascular [NO] measured in this and many other studies, of which only a partial listing is referenced, by the Malinski laboratory {42; 45; 50}, Buerk and colleagues {9; 10; 41} and this laboratory {2; 3; 7; 48; 49} have consistently shown [NO] in the range of 200-1000 nM on the outer surface of microvessels. However, in vitro evaluation of NO activation of guanylate cyclase requires [NO] in only the tens of nM, as reviewed by Hall and Garthwaite in 2009 {22} and a more recent contribution {21}. Perhaps there is a large loss of NO by consumption or destruction in the in vivo environment as NO enters the cells or the in vivo cells are less sensitive to NO than are cultured cells. There is independent evidence that in vivo concentration of NO is much higher than is needed to activate guanylate cyclase as judged by the in vitro studies cited {22}. This evidence is based on the concentration of NO in storage forms within arterial blood. NO within blood presumably is donated by endothelial cells and NOS within the formed elements, particularly the red blood cells due to their overwhelming volume {24} and their ability to increase NO production during shear stress {44}. Functional hemoglobin carries NO in a nitrosylated releasable form, as does albumin, cysteine, and glutathione in plasma {23; 28; 31; 32; 38; 39}. Measurements of nitrosylated amino acids, albumin and hemoglobin within arterial blood by methods that do not use microelectrodes in the studies listed have indicated concentrations of at least 300-1000 μM , which spans the concentration range from microelectrode measurements of in vivo vessel wall [NO]. The key support of measurement of a reliable index of how periarteriolar [NO] changes in the current study is that 1) blockade of eNOS with L-NAME resulted in an approximate 50% localized decrease in [NO] that was associated with a 15% constriction (Fig.3, Panel B), and 2) stimulation of eNOS with bradykinin and nNOS with glutamate caused a dose dependent increase in [NO] and vessel diameter (Figs. 1-6), both of which fully recovered when drug release was stopped. Due to the content of NO in releasable forms in arterial blood, including that in the intestinal microvasculature from an earlier study {7}, the localized block of eNOS in the current study was unable to decrease the [NO] more than 50%, but all sensitivity to bradykinin of the treated vessel was lost (Fig. 3).

In the context of prior studies of conducted vasodilation to agents which initiated conducted dilation, of which only a few of the many are cited {14; 17; 18; 26}, the intestinal microvasculature behaved much more like microvessels in those prior studies than did the cerebral circulation. The conducted dilation of intestinal 2A arterioles from its midpoint occurred over the entire length to the nearest 1A, which can be up to 2.5 mm, in a fast phase within 10-15 seconds followed by a slowly developed but much larger dilation that required 60-90 seconds to reach steady state (Figs. 1 and 2). The fast phase of conduction, judged

when a 5% dilation of the larger arteriole occurred, indicated a transmission velocity of dilation along small arterioles in the range of 100-120 $\mu\text{m/s}$ (Results). This velocity of transmission is similar to reports from similar sized arterioles in multiple vasculatures {8; 14-17; 29; 34; 35; 40; 43}. The velocity of conduction was equivalent for threshold and supramaximal dosages of bradykinin on the downstream small arterioles. This is satisfactory evidence that initiation of conduction was independent of the initial local dilation (Fig. 1) and very likely an electrical conducted process. At the time of 5% dilation at a distal location, the increase in [NO] of the upstream parent arteriole (Results) was small, even during maximal downstream stimulation with bradykinin. Consequently, the fast component of conduction in the small intestine did activate a small increase in [NO], perhaps secondary to a wave of increased endothelial $[\text{Ca}^{+2}]$, based on the data of Uhrenholt and Segal{43}. The same situation occurred for the cerebral circulation, conduction was of the order of 100-120 $\mu\text{m/s}$ with no appreciable increase in distal [NO] at 250 μm from the site of drug release (Fig. 5). However, only about half of the original dilation was conducted downstream at 250 μm and 70% of the dilation was conducted upstream at 250 μm (Fig. 3). The up and downstream dilations were not reliably detectable at 500 μm from the site of drug release. Consequently, the up and downstream dilation of the cerebral arterioles are considered examples of rapid cell to cell conduction which decayed over a relatively small distance. As both activation of eNOS in endothelial cells and nNOS in neural and neural support cells was used in the current study, the source of NO did not appear to matter in the limited conducted dilation of cerebral arterioles, as shown in Figures 4 and 5.

The well developed conducted dilation over the vessel length in the intestine gradually lead to a dilation of entire 2A and all of its smaller downstream vessels within ~30 seconds. The net result was flow into the vascular region would increase dramatically over time and thereby increase the blood flow in the immediate upstream large arterioles, the 1A. As shown in Figure 2, the larger arterioles' diameter and [NO] responses required at least 60 seconds to fully develop and thereafter were stable. At threshold to maximal dosages of bradykinin on the smaller arterioles, the final relative increase in [NO] at distance on upstream 1A equaled the NO events at the downstream site of bradykinin release (Fig. 1). It is impossible for bradykinin released downstream to have reached the larger arterioles because 2A vessels do not respond unless the microelectrode tip almost touches the vessel wall and locations were chosen where venules in the area would not have carried blood from the dilated region past the region of 1A measurement. Therefore, the slowly developed but larger increase in [NO] and dilation by larger arterioles is likely due to flow mediated vasodilation. In prior studies of flow dependent dilation of the largest intestinal arterioles when the smaller arterioles are dilated, the [NO] gradually increased in the large arterioles over a 60-90 second interval as the downstream vessels dilated {6; 49}. In those studies, one of two large arterioles that perfuse a set of interconnecting intermediate diameter 2A arterioles was occluded while the flow and [NO] of the open large vessel that now perfused all the tissue was recorded. In all cases, the increase in flow and [NO] required 60-90 seconds to fully develop, which is similar to the time course of large arteriole dilation and increased [NO] in the current study. The combined data from the past and current studies is interpreted to indicate that conducted vasodilation clearly serves the purposes of rapidly coordinating the dilation of small and terminal arterioles, conducting a fast but modest dilation to upstream larger arterioles, and when allowed to cause sustained dilation to increase the overall blood flow over time, activated flow dependent formation of additional NO to augment dilation of the larger arterioles.

At the outset of this study, the combined eNOS and in particular nNOS systems of the brain vasculature were assumed to be of advantage to conducted vasodilation in this organ system. The assumption was that nNOS in neurons might be able to influence vast lengths of microvessels through nerves approaching vessels from the brain tissue. Dacey and Duling

{11} have reported some degree of conducted vasodilation in isolated cerebral arterioles and Dalsgaard et al {12} using in vitro retinal arterioles also reported conducted vasodilation. However, as discussed earlier, conducted vasodilation by the in vivo brain vessels while quite rapid was unable to sustain dilation over appreciable distances compared to other vascular beds, including the small intestine in this study. Furthermore, the dilation of cerebral arterioles that did occur at distance simply was not related to an increase in perivascular [NO] at the distal site (Figs. 5 and 6). Therefore, to the extent that dilation at distance occurred in the cortical arterioles, the communication was likely electrotonic, which is also supported by the fast conduction velocity observed in this study (Results). The absence of a well developed conducted dilation over large distances is not due to an absence of local vascular activation. Both eNOS and nNOS activation caused much larger increases in [NO] in the brain vasculature than occurred with eNOS activation in the small intestinal vasculature (Fig. 5). Plus, the cerebral vessels dilated at the site of drug release as much or more than occurred for intestinal vessels over the dose response range for eNOS activation (Fig. 1). Therefore, the stimulus to start conducted vasodilation which appears to be the overall process related to eNOS activation {8; 14; 16; 17; 29; 34; 35}, and the magnitude of initial dilation was not limiting in the cerebral vasculature. In the case of nNOS activation by glutamate, NO is produced outside the vessel wall and should be viewed as NO donated by an external source. This point is mentioned because application of an NO donor to arterioles in other peripheral vascular beds caused local vasodilation but limited conducted vasodilation {1; 8; 13; 16}. Therefore, limited conducted vasodilation by nNOS mechanisms perhaps should not be well developed from the view point of earlier studies. Why the production of NO by the vessel wall during bradykinin stimulation of eNOS did not produce a well developed conducted vasodilation by the cerebral vessels remains speculative at this point. However, the results with both eNOS and nNOS activation are clear in that local generation of NO from brain cell activity that stimulates either NOS provides a highly localized conducted arteriolar dilation which may be unique among organ system vasculatures.

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Figure 1.

Intestinal arteriolar diameter and [NO] responses to bradykinin iontophoretically released onto small arterioles and subsequent communication to large arterioles is shown. The responses of small arterioles to direct application of bradykinin occurred within 15 seconds after drug release commenced, but the combined conducted and presumably flow mediated responses of larger arterioles required up to 90 seconds to reach steady state. Only final steady state events are shown in the figure. The inset figure in the upper panel presents the actual diameters of the small and large arterioles during bradykinin exposure and the relative dilation of each is in the larger panel. These data indicate essentially identical diameter responses by small and large arterioles once all events over three minute exposure period

had occurred. The relative increases in [NO] of the small and large arterioles, shown in the lower panel, were similar at 25 nanoampere release and for 100 nanoampere, which caused maximum dilation, a greater relative increase in [NO] of large than small arterioles. The data sets are based on the study of 12 rats, one set of arterioles per rat.

Figure 2.

Representative examples of the temporal diameter and [NO] responses of large arterioles to 25 and 100 nanoampere bradykinin released onto the small arterioles is shown. The dilations to 25 nanoampere downstream vasodilation were generally accompanied by a small, slowly developed increase in [NO] and diameter, as shown in all panels. Responses to supramaximal bradykinin (100 nAmp) generated two major patterns of behavior. In the upper two panels A and B, a slowly developed dilation and increase in [NO] occurred over about 60 seconds. This was the typical pattern of behavior. Very large responses occurred in some vessels, of which two examples are shown in panels C and D. These vessels displayed a rapid and larger increase in both [NO] and diameter that began to develop within 10 seconds or less after downstream drug release began and was essentially fully developed by 60 seconds with 100 nanoampere bradykinin. These high response vessels always had exceptionally large downstream dilations to bradykinin.

Figure 3.

The response of a small arteriole conducting dilation to a larger parent arteriole were compared before and after L-NAME was applied very locally to the branch point of the large to small arteriole. L-NAME could not have reached the downstream portions of the small arteriole. The [NO] and diameter at the branch point was measured at rest, during direct bradykinin release at the branch point, and during bradykinin release on the small arteriole to cause conducted dilation. This was repeated after L-NAME was applied for 20-30 minutes. The data set are based on 4 vessel pairs in 4 rats. The [NO] (Panel A) and diameter (Panel B) of the large arteriole increased with direct and distal bradykinin release. Application of L-NAME decreased the [NO] at the branch point by almost 50% (Panel A)

and the large arteriole constricted ~15% in the area treated (Panel B). After L-NAME, neither diameter nor [NO] responses of the large arteriole occurred with direct or conducted bradykinin stimulation, however, the small arteriole dilated normally (Panel C). All responses during various bradykinin responses prior to L-NAME were significantly different from control ($p < 0.05$) and none of the larger arteriole responses were significant after L-NAME.

Figure 4.

Bradykinin and glutamate were iontophoretically released for three minutes onto the wall of a cerebral arteriole at currents up to 400 nanoamperes. The arteriole's inner diameter was measured at the site of bradykinin release, as well as 250 μ m upstream and downstream of that location. The current dosage/concentration of drugs were set such that maximum dilation with bradykinin and glutamate occurred at 200-400 nanoamperes. For equivalent currents, dilations of the arterioles were similar for bradykinin and glutamate at the site of drug release. Dilations up and downstream from the release site occurred for both drugs but were consistently smaller than that at the site of drug release. Data based on 14 arterioles in

14 rats for glutamate and 14 arterioles in 14 different rats for bradykinin. Asterisks indicate significant change ($p < 0.05$) from control.

Figure 5.

For the same vessels used in Figure 3 with glutamate and bradykinin, nitric oxide was measured beside the vessel at the same location and also at 250 μm upstream. Glutamate is used to primarily activate neuronal nitric oxide synthase in neurons and brain support cells just outside the vessel wall. Bradykinin is generally presumed to primarily activate endothelial nitric oxide synthase. Glutamate was capable of causing a very large increase in [NO], consistently larger than occurred with bradykinin. At a site 250 μm upstream from the glutamate or bradykinin release site at 400 nanoamperes, the [NO] did not increase with either drug and similar results were found for tested downstream sites. Data based on 14 arterioles in 14 rats for glutamate and 14 in 14 different rats for bradykinin. Asterisks indicate significant change from control.

Figure 6.

To determine if tissue release of NO away from the vessel wall by either bradykinin or glutamate caused increased NO or vasodilation, either agent was released at the vessel wall at 50 and 400 nanoampere and 250 μm from the vessel wall at 400 nanoampere. In addition, the vessel diameter at the site of drug release was measured. Glutamate, which acts on nNOS, was able to increase both vessel [NO] and diameter when released at 250 μm from the vessel wall, but nothing occurred with bradykinin. The tissue effect of 400 nanoampere glutamate on [NO] and diameter were equivalent to that of glutamate at 50 nanoampere released onto the vessel wall. Data set based on 4 arterioles in 4 rats. Asterisks indicate

significant change from control. Number symbols indicate the glutamate response was significantly greater than that for bradykinin at equivalent conditions.