Improved measurements of intracellular nitric oxide in intact microvessels using 4,5-diaminofluorescein diacetate

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Zhou X, He P. Improved measurements of intracellular nitric oxide in intact microvessels using 4,5-diaminofluorescein diacetate. Am J Physiol Heart Circ Physiol 301: H108–H114, 2011. First published May 13, 2011; doi:10.1152/ajpheart.00195.2011.—4,5-Diaminofluorescein diacetate (DAF-2 DA) has been widely used for the measurement of nitric oxide (NO) in living cells and tissues. We previously established a method that demonstrated platelet activating factor (PAF)-induced endothelial NO production in intact venules using DAF-2 DA. In previous applications, the loading dye was removed from the extracellular space before NO measurements. However, in high permeability vessels, endothelial cells quickly released the accumulated intracellular DAF-2 after the washout, which compromises the NO measurement. The objective of this study was to investigate if the presence of DAF-2 DA during NO measurements could overcome the dye retention problem and enhance the sensitivity of NO detection. Experiments were conducted in individually perfused rat venules, and endothelial NO was measured using fluorescence imaging under basal and stimulated conditions with continuous perfusion of DAF-2 DA. Continuous dye perfusion was found to promote a relatively constant endothelial dye concentration in both normal and high permeability vessels throughout the experiment. With the use of this method, the basal and stimulated NO was quantified after endothelial DAF-2 concentrations reached a steady state. Our results showed enhanced sensitivity of detecting PAF-stimulated NO compared with a previous method. We also found that the hydrolyzed intracellular DAF-2, the precursor of DAF-2 triazole, contributed significantly to the measured fluorescence and that an appropriate subtraction of non-NO-dependent intracellular DAF-2 fluorescence is critical for the assessment of NO in living tissues. This method overcame the dye leakage problem, enhanced the sensitivity of NO detection, and improved NO quantification, demonstrating significant advantages over existing methodologies using DAF-2.

NITRIC OXIDE (NO) IS INVOLVED in the regulation of various physiological and pathological processes in the cardiovascular system (14, 15, 19). A better understanding of the functional roles played by NO largely depends on reliable approaches to the measurements of NO, especially in living tissues. However, this highly diffusive gas with its well-known short half-life makes such measurements particularly difficult.

Currently, the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2) has been widely used as a specific and sensitive probe for NO measurement in cultured cells (1, 3, 12, 16, 21, 25), isolated vessel segments (27), and intact vessels (5, 9, 29, 30). Using this fluorescent indicator, we have quantitatively measured platelet activating factor (PAF)-induced NO production in endothelial cells lining intact microvessel walls. Results from the previous work (29, 30) provided important in vivo evidence for the roles of NO in the regulation of microvessel permeability under inflammatory conditions. However, in some experiments, following washout of the dye from the vessel lumen, we observed a marked decrease in DAF-2 fluorescence intensity (FIDAF), and the low fluorescence frequently led to a failed experiment. We considered the decrease in FIDAF to be mainly a result of dye leakage from endothelial cells, a phenomenon associated with increased microvessel permeability.

In the present study, we investigated if the use of continuous DAF-2 diacetate (DA) perfusion could overcome the dye leakage problem and also provide valid NO measurements. We first examined the relationship between dye retention by endothelial cells and microvessel permeability. We then examined whether continuous DAF-2 DA perfusion would be able to provide a relatively constant endothelial DAF-2 concentration in vessels having normal or increased permeability. We also evaluated the relative contributions of intracellular DAF-2 and DAF-2 triazole (DAF-2T; a product formed by the interaction of DAF-2 with NO) to the measured FIDAF. PAF-induced NO production was further examined with continuous dye perfusion to provide a direct comparison between the current and previous method.

MATERIALS AND METHODS

Animal preparation. Female Sprague-Dawley rats (2–3 mo old, 220–250 g; Hilltop Laboratory Animal, Scottsdale, PA) were anesthetized with pentobarbital sodium (65 mg/kg of body wt) given subcutaneously. A midline surgical incision (1.5–2 cm) was made in the abdominal wall, and the mesentery was gently removed from the abdominal cavity and spread over a pillar and a glass coverslip attached to an animal tray for measurements of microvessel hydraulic conductivity, Lp, and endothelial NO, respectively. The upper surface of the mesentery was continuously superfused with mammalian Ringer solution at 37°C. The mesentery when spread over a coverslip was exposed to atmospheric oxygen. Therefore, the tissue PO2 was predicted to be relatively constant and close to the atmospheric PO2. All experiments were carried out on venular microvessels with diameters ranging from 35 to 50 μm. Each experiment was performed on one microvessel per animal to avoid the potential effect of the applied inflammatory mediator on subsequent vessel studies. All procedures and animal use were approved by the Animal Care and Use Committee at West Virginia University.

Measurement of Lp. All measurements were based on the modified Landis technique, which measures the volume flux of water across the microvessel wall. The assumptions and limitations of the original method and its application to mammalian microvessels have been evaluated in detail (4, 10). Briefly, a single venular microvessel was cannulated with a glass micropipette and perfused with albumin-Ringer solution (control) that contained red blood cells as markers. A hydrostatic pressure (range 40–70 cm H2O) controlled by a water manometer, was applied through the micropipette to the microvessel...
lumen. For each measurement, the perfused vessel was briefly occluded downstream with a glass rod. The initial water flux per unit area of microvessel wall \([J/(S)_0]\), where \(J\) was the water flux and \(S\) was the unit area of the microvessel wall] was calculated from the velocity of the marker cell after the vessel was occluded, the vessel radius, and the distance between the marker cell and the occlusion site. Microvessel Lp was calculated as the slope of the relation between \([J/(S)_0]\) and the pressure difference across the vessel wall.

**Measurement of endothelial NO using fluorescent indicator DAF-2.** Endothelial NO levels were visualized and quantified at the cellular level in individually perfused venules using DAF-2 DA, a membrane-permeable fluorescent indicator for NO, and fluorescence imaging. Experiments were performed on a Nikon Diaphot 300 microscope equipped with a 12-bit digital CCD camera (ORCA; Hamamatsu) and a computer-controlled shutter (Lambda 10–2; Sutter Instrument; Novato, CA). A 75-W xenon lamp was used as the light source. The excitation wavelength for DAF-2 was selected by an interference filter (480/40 nm), and emission was separated by a dichroic mirror (505/50 nm). All the images were acquired and analyzed using MetaFluor software (Universal Imaging). To minimize photobleaching, a neutral density filter (0.5 N) was positioned in front of the interference filter and the exposure time was minimized to 0.12 s at 1-min intervals. Identical instrumental settings were used for all measurements including the fluorescence measurements of DAF-2 and DAF-2 DA in solutions and all vessel experiments.

For experiments on intact venules, each vessel was first cannulated and perfused with albumin-Ringer solution containing DAF-2 DA (1 or 5 \(\mu\)M). Then, instead of removing DAF-2 DA from the vessel lumen before collecting images as done in previous experiments (29, 30), DAF-2 DA was present in the perfusate throughout the experimental duration. All images were collected from a group of endothelial cells located in the same focal plane of the vessel wall using a Nikon Fluor lens (×20, numerical aperture, 0.75). A steady state of endothelial DAF-2 fluorescence with continuous perfusion of DAF-2 DA was indicated by the rate change of the increased FI (see Fig. 3 for details).

Data analysis was conducted at the individual endothelial cell level using manually selected regions of interest (ROIs) along the vessel wall. Each ROI covered the area of one individual cell as indicated by the fluorescence outline. The tissue autofluorescence was subtracted from all of the measured Fls. The basal NO production rate was calculated from the slope of the FI increase during albumin-Ringer perfusion after DAF-2 loading reached the steady state. The changes in FlDAF on PAF stimulation were expressed as the net changes in FI (ΔFI) caused by DAF-2T formation, which was the difference between the maximum FI before the increased FI rate fell back to the basal level and the FI right before the application of PAF. The percent change in FI was expressed as ΔFI/FI0 * 100, where FI0 was the initial steady-state FI of DAF-2. FI was expressed in arbitrary units (AU) measured with identical instrumental settings. The rate of FlDAF change was derived by first differential conversion of cumulative FlDAF over time. Details have been described in a previous method (30).

**Solutions and reagents.** Mammalian Ringer solution was used for dissecting mesenteries, superfusing tissues, and preparing the perfusion solutions. The composition of the mammalian Ringer solution has been described in detail (7). DAF-2 DA, DAF-2 DA, N\(^{2}\)-monomethyl-L-arginine (L-NMMA), 1-ethyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), and sodium nitroprusside were all purchased from Sigma. The stock solutions of DAF-2 DA and DAF-2 were prepared in DMSO saturated with 95% \(N_2\) to prevent auto-oxidation. PAF was initially dissolved in 95% ethyl alcohol, and L-NMMA was in Ringer solution. The final solution of each agent was prepared by dilution of the stock with albumin-Ringer solution, and more than 1:1,000 dilutions were made from DMSO and ethyl alcohol prepared stock solutions. All the perfusates containing the test agents were freshly prepared before each cannulation.

**RESULTS**

**Potential problems of NO measurement using DAF-2 DA in high permeability vessels.** Previously, we developed a method that enabled us to quantitatively measure endothelial NO using DAF-2 DA in intact venules (29, 30). In those experiments, after each vessel was perfused with DAF-2 DA (5 \(\mu\)M) for 45 min, the dye was washed out from the vessel lumen before the control images were collected. The control images showed a slight linear decline in FlDAF during 30 min of albumin-Ringer perfusion. The mean Fl decline rate was 0.7 ± 0.02% of the initial Fl per minute after removal of the loading dye (\(n = 8\)), which was considered as the balance between the increased FI due to basal NO production, the dye leakage from the cell, and the excitation-caused photobleaching. The PAF-induced increases in FlDAF were then corrected with the Fl decline rate measured under control conditions. Although effort was made to minimize photobleaching by reducing the excitation light intensity, exposure time, and frequency, in some of the experiments, we experienced a higher FlDAF decline rate after loading and the low fluorescence signals prevented valid NO measurements. To test if the fast decline in FlDAF was associated with increased microvessel permeability, we investigated the relationship between the decline rate of FlDAF and microvessel Lp. Experiments were conducted in nine vessels that have either normal (2.1 ± 0.20 × 10\(^{-7}\) \(\text{cm} \cdot \text{s}^{-1} \cdot \text{cm}^{-2} \cdot \text{O}_{2}^{-1}\)) or high (4.9 ± 0.54 × 10\(^{-7}\) \(\text{cm} \cdot \text{s}^{-1} \cdot \text{cm}^{-2} \cdot \text{O}_{2}^{-1}\)) baseline Lp. In the high Lp vessel group (\(n = 4\)), the mean FlDAF decline rate was 3.0 ± 0.12%/min, which was more than four times higher than 0.7 ± 0.04%/min measured in normal Lp vessels (\(n = 5\); \(P < 0.001\); Fig. 1A). The fluorescence images in Fig. 1B, left, illustrate that after 30 min of albumin-Ringer perfusion following DAF-2 DA loading the DAF-2 retention is much less in a high Lp vessel than that in a normal Lp vessel.

**Continuous perfusion of DAF-2 DA overcame the dye retention problem in high permeability vessels.** To overcome the dye retention problem in high permeability vessels, we first examined whether continuous perfusion of DAF-2 DA would provide a relatively constant dye concentration in cells regardless of their permeability states. Experiments were conducted in both high and normal Lp vessels. After each vessel was perfused with DAF-2 DA (5 \(\mu\)M) for 45 min, the control images were collected with the continuous perfusion of DAF-2 DA. The representative images are shown in Fig. 1B, right. In contrast to a marked reduction of FlDAF observed in high Lp vessels after DAF-2 DA was removed from the vessel lumen (Fig. 1B, left), the continuous perfusion of DAF-2 DA in both high and normal Lp vessels resulted in a small constant increase in FlDAF after 40–45 min of the dye loading period. These results indicate that the continuous perfusion of DAF-2 DA enabled the endothelial cells to maintain a relatively constant DAF-2 concentration, which overcame the dye loss, especially in vessels with increased permeability. The small increase in FlDAF may be attributed to the Fl of DAF-2T due to basal NO production. Further validations were conducted in the following studies.
same range of concentrations. Figure 2 presents the FIs measured with DAF-2 and DAF-2 DA at different concentrations. These results indicate that with continuous perfusion of DAF-2 DA at a concentration $\approx 5 \, \mu M$, the FI of DAF-2 DA in the vessel lumen was negligible, whereas the accumulated DAF-2 in endothelial cells may contribute significantly to the measured $F_{DAF}$.

We then examined the dynamic changes in DAF-2 fluorescence in endothelial cells when the vessel was continuously perfused with DAF-2 DA. To exclude the DAF-2T formation caused by endothelial basal NO, each vessel was first perfused with the NO synthase (NOS) inhibitor L-NMMA ($500 \, \mu M$) for 30 min followed by DAF-2 DA perfusion at 1 or 5 $\mu M$ in the presence of L-NMMA for 80 min ($n = 4$ per group). Image acquisition started at the beginning of DAF-2 DA perfusion. In vessels perfused with DAF-2 DA at 1 $\mu M$, we observed an initial increase in FI at a mean rate of $0.2 \pm 0.01 \, AU/min followed by a plateau FI at 4.4 $\pm 0.1 \, AU$ after 21 $\pm 1.4 \, min$ of perfusion (Fig. 3A). In 5 $\mu M$ DAF-2 DA-perfused vessels, the mean rate of the initial FI increase was $0.3 \pm 0.01 \, AU/min and a plateau FI of 13.2 $\pm 0.31 \, AU$ occurred 41 $\pm 1.1 \, min$ after the start of the perfusion. To ensure a complete inhibition of basal NO production, L-NMMA concentration was increased to 1 mM in two of the experiments. No further reduction of steady-state FI was observed, indicating that 500 $\mu M$ L-NMMA was sufficient in inhibition of basal NO under our experimental conditions (Fig. 3B). These results indicate that, in the absence of basal NO, the initial FI increase was mainly from the accumulation of DAF-2 in endothelial cells and the plateau level of FI indicates a steady state of endothelial DAF-2 concentration. The time needed to reach steady state and the steady-state FI levels varied directly with DAF-2 DA concentration in the vessel lumen. Even the driving force for DAF-2 DA diffusion into the cells is higher when a higher DAF-2 DA concentration is used; the cytosolic esterase can be a limiting factor for the rate of hydrolyzing DAF-2 DA to DAF-2. This explains the longer time needed to reach steady state with higher concentration of DAF-2 DA. We also found that the continuous DAF-2 DA perfusion reduced the variations of individual endothelial DAF-2 FI in each vessel. The

**Fig. 1.** Increased microvessel permeability causes higher leakage of intracellular 4,5-diaminofluorescein (DAF-2) in intact venules. A: summary results of nine microvessels show a positive correlation between the baseline microvessel hydraulic conductivity (Lp) and the decline rate of DAF-2 fluorescence intensity ($F_{DAF}$). B: representative DAF-2 fluorescence images from normal (top) and high (bottom) baseline Lp vessels. Images in the left two columns were collected after the loading DAF-2 diacetate (DA) was washed away for 0 and 30 min. After 30 min of albumin-Ringer perfusion, only a slight decrease in $F_{DAF}$ occurred in the vessel with Lp at $2.2 \times 10^{-7} \, \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_{2} \text{O}^{-1}$, whereas a marked $F_{DAF}$ decrease occurred in the vessel with basal Lp at $4.5 \times 10^{-7} \, \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_{2} \text{O}^{-1}$. Images in the right two columns were collected with continuous perfusion of DAF-2 DA at 5 $\mu M$. After 40–45 min of DAF-2 DA loading period, a constant increase in $F_{DAF}$ was observed in both normal ($1.5 \times 10^{-7} \, \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_{2} \text{O}^{-1}$) and high ($4.4 \times 10^{-7} \, \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_{2} \text{O}^{-1}$) Lp vessels, representing basal NO production.

**Fig. 2.** Autofluorescence of DAF-2 and DAF-2 DA at different concentrations. FI of DAF-2 and DAF-2 DA in Ringer-albumin solution was measured with identical instrumental settings to that used in intact microvessels. ○: Negligible and very low FI with DAF-2 DA concentrations at 5 and 10 $\mu M$; ●: DAF-2 in the same concentration range has much higher FI. Each data point is the means $\pm$ SE of 3 measurements.
individual cell FI varied at 10.0% of the mean FI with the continuous dye perfusion compared with 21.8% of the mean FI using the previous method with the dye removal.

Measurement of basal NO production rate with continuous DAF-2 DA perfusion in intact venules. After characterizing the contribution of non-NO-dependent FI to the total measured FIDAF, we evaluated if basal NO could be directly measured with continuous perfusion of DAF-2 DA. Experiments were conducted in eight vessels. DAF-2 DA was perfused at 1 μM in four of the vessels. The initial rate of FI increase during the first 20 ± 0.5 min of perfusion was 0.3 ± 0.01 AU/min. Then, instead of showing a plateau phase as seen in the absence of basal NO production, the FI showed a slow increase at a constant rate of 0.09 ± 0.005 AU/min. In the other four vessels perfused with 5 μM DAF-2 DA, the initial FI increased at a rate of 0.4 ± 0.03 AU/min and lasted for 40 ± 1.8 min before changing to a slower rate at 0.11 ± 0.006 AU/min. The rate change of FIDAF represented the transition from the loading phase to the steady state of DAF-2 in endothelial cells, which was illustrated in Fig. 3 with the pooled FIDAF curves. The mean loading phase period was consistent with the time reaching the plateau when basal NO was inhibited by l-NMMA. The FI increase after the dye loading phase was attributed to the basal NO-dependent DAF-2T accumulation, and the slope of the FI increase is the basal NO production rate under control conditions. We obtained similar basal NO production rate in both 1 and 5 μM DAF-2 DA-perfused vessels, indicating that the basal NO production rate did not vary within the concentration range we applied.

Measurement of PAF-induced NO production with continuous DAF-2 DA perfusion in intact venules. PAF-induced NO production was measured in eight microvessels with continuous DAF-2 DA perfusion at 1 and 5 μM (2 vessels per group). After the loading phase, the basal NO-induced increase in FIDAF was consistent with that reported above (Fig. 3). When PAF (10 nM) was added to the perfusate in the presence of DAF-2 DA, 100% of endothelial cells in both group of vessels showed an immediate increase in FIDAF. This is significantly higher than the 83 ± 2% of responsive cells reported in our previous studies (P < 0.001; Refs. 29, 30). The peak FI increase occurred within the first 1 min of PAF addition, which was 1.7 ± 0.10 and 1.5 ± 0.13 AU/min in vessels perfused with 1 and 5 μM of DAF-2 DA, respectively. The increased FI returned to the control level after 20–25 min of PAF exposure. Data were derived from 8 vessels with a total of 78 ROIs and 7 to 12 ROIs per vessel. Figure 4A shows the time-dependent changes in FIDAF from two representative experiments. The changes in slopes of the FI curve indicate different NO production rates before and after PAF stimulation. Both 1 and 5 μM DAF-2 DA-perfused vessels have similar ΔFIs, indicating that the PAF-induced net change in FIDAF (ΔFI, area under the NO production rate curve) did not vary with the steady-state FI levels. However, should the changes of FI be calculated as the percentage relative to the steady-state level, the value would be varied inversely with the intracellular dye concentration. For example, the PAF-induced maximum increase in FIDAF relative to steady-state FI level (ΔFI/FI0 * 100) was 163 ± 15.1% in 1 μM DAF-2 DA-perfused vessels but only 88 ± 2.8% in 5 μM DAF-2 DA-perfused vessels. Images in Fig. 4B illustrate the PAF-induced cumulated endothelial NO production from a representative experiment.

DISCUSSION

This study improved and validated a methodology that uses DAF-2 DA to measure intracellular NO in intact microvessels. The results demonstrated that the continuous perfusion of DAF-2 DA during NO measurement permitted a relatively constant endothelial dye concentration throughout the experiment. This technique overcame the dye retention problem, especially in vessels with increased permeability. The relatively constant intracellular DAF-2 concentration enhanced the sensitivity of NO detection under both basal and stimulated conditions. Additionally, the differentiation of NO-dependent and non-NO-dependent FI in DAF-2 DA-perfused vessels provided a more accurate assessment of NO production. This improved technique and data analysis demonstrated advantages over existing methodologies of using DAF-2 DA to measure intracellular NO in live tissues. More importantly, it is applicable not only to individually perfused microvessels but also to cell culture and other in vivo vascular applications.
DAF-2 DA is membrane permeable and diffuses freely into the cells driven by concentration gradient and is then hydrolyzed by cytosolic esterase to form DAF-2. Intracellular DAF-2 is less membrane permeable due to its polarity and is designed to be trapped inside the cells. When DAF-2 DA was first introduced as an NO-specific indicator, it was used to measure intracellular NO in rat aortic smooth muscle cells and macrophages after the loading dye was washed out from the media, and DAF-2 efflux was not reported to be an issue (12). Following similar procedures, DAF-2 DA has been used to measure intracellular NO in a wide range of biological systems (1, 3, 5, 9, 16, 21, 25, 27, 29, 30). The method we previously established enabled us to demonstrate the inflammatory mediator-induced temporal and spatial changes in NO at the individual endothelial cell level (30) and to elucidate the molecular mechanisms that regulate endothelial NOS activity in intact microvessels (29). In those studies, experiments were conducted in vessels having normal basal permeability and stimuli were applied acutely. A small but constant decline in FI during albumin-Ringer perfusion was observed after the loading DAF-2 DA was washed away from the vessel lumen. Upon PAF stimulation, significant increases in FI, DAF were detected from an average of 83% of the endothelial cells in each vessel. During data analysis, the magnitude of PAF-induced FI increase was corrected with the DAF-2 FI decline rate measured under control conditions. However, when the same method was applied to vessels with elevated basal permeability, i.e., with existing inflammatory conditions, a fast reduction in FI occurred after DAF-2 DA was washed out from the vessel lumen and the low fluorescence signal compromised the NO measurements. It has been documented in the literature (23) that the intracellular retention of fluorescent dye depended on the integrity of the cell membrane and an instantaneous dye release occurred in artificially damaged cells. Our study by measuring the FI decline rate in vessels with normal and elevated basal Lp provided quantitative evidence that the retention of intracellular DAF-2 by microvessel endothelial cells is correlated with the level of microvessel permeability. This correlation indicates that vessels with elevated Lp are also involved in the increases in transmembrane transport for DAF-2, even though it was dominated by increased fluid transport through paracellular pathways.

In the present study, we demonstrated that a relatively constant level of intracellular DAF-2 could be achieved via the continuous perfusion of DAF-2 DA. The dye loading variations among individual cells and vessels are much smaller than the previous methods. Most importantly, this new approach enabled us to assess endothelial NO in intact microvessels regardless of their permeability states. We predict that the dye leakage problem must also exist when DAF-2 DA is used for measuring NO in cultured endothelial cells that usually have baseline permeability one to two orders of magnitude higher than that in intact microvessels (4). In many cell culture studies, the dye leakage factor was not adequately corrected (1, 3, 11, 12, 16), which is likely to result in an underestimation of NO production.

Under our experimental conditions, the DAF-2 DA loading was achieved via the luminal side perfusion. In this way, only the endothelium lining the microvessel wall was loaded, which minimized NO contributions from other sources. Additionally, the absence of blood in our perfused vessels avoided the potential effects from circulating erythrocytes on endothelial NO measurement (20, 24). Based on the DAF-2 fluorescence level we detected in solutions, we consider the plateau level of FI in the presence of L-NMMA to be mainly from the accumulated intracellular DAF-2, not DAF-2T. The plateau FI in the absence of basal NO production in 1 and 5 μM DAF-2 DA-perfused vessels was equivalent to the FI level measured with 5 and 15 μM of DAF-2 in solution, respectively (Fig. 2). This is a reasonable range of intracellular concentration for...
DA-mediated fluorescence dye accumulation (2), although large variations exist among different cell types. Concerns regarding DAF-2 autofluorescence have been raised when DAF-2 was used to measure the released NO from cultured endothelial cells (22). In systems with a high NO production rate, the contribution of DAF-2 autofluorescence may be negligible due to the much higher quantum yield of DAF-2T (12). However, in endothelial cells with a low output of NO (100 pM to 5 nM; Refs. 6, 26), the autofluorescence of intracellular DAF-2 may contribute significantly to the measured Fl_{DAF}. Therefore, a careful calibration of the DAF-2 autofluorescence for each specific system with adequate subtraction from the measured Fl_{DAF} is critical to achieve a more accurate estimation of NO levels. This information is not only critical to perfused vessels but also important to all systems that use DAF-2 to measure NO production.

The use of continuously superfused DAF-2 DA in tissue to examine microvascular NO production in vivo was first developed by Kashiwagi et al. (9) and has been followed by others (5). In those studies all of the detected DAF-associated fluorescence was attributed to DAF-2T and the intracellular DAF-2 fluorescence was not taken into consideration. In Kashiwagi’s study (9) conducted in rat mesentery microvessels, a large fraction of DAF-associated fluorescence was found not to be abolished by a sufficient amount of NOS inhibitors and it was concluded that ~50% of the DAF-2T signals were derived from nonenzymatic sources of NO (9). However, it is noteworthy to reevaluate whether all of the DAF-associated fluorescence was due to DAF-2T formation. It is likely that the remaining DAF-associated fluorescence in the presence of NOS inhibitors was basically from the non-NO-dependent autofluorescence of DAF-2.

Our study demonstrated that the continuous perfusion of DAF-2 DA is a better approach to the measurements of basal and stimulated NO in vessels under either normal or high permeability state. Using the previous method, we had to rely on the calculation of the differences in FI decline rates obtained with and without the NOS inhibition to assess the basal NO production. Using the continuous DAF-2 DA perfusion, we can directly visualize the basal NO-induced steady increase in Fl_{DAF}. The rate of this Fl_{DAF} increase was found to be very constant between individual endothelial cells and vessels and was completely abolished by the NOS inhibitor L-NMMA. In PAF-stimulated vessels, the general pattern of PAF-induced NO production was similar between the current and previous method (29, 30). However, with the continuous DAF-2 DA perfusion, the number of endothelial cells that showed significant increases in Fl_{DAF} upon PAF stimulation was increased from previous 83 to 100%. This difference can be attributed to the improved dye loading status in all of the endothelial cells of the vessel wall that enhanced the sensitivity for NO detection. Our results also showed that, with continuous perfusion of DAF-2 DA at 5 μM, the magnitude of PAF-induced NO production was increased from 43% with previous method (29) to 88%. We consider that the lower PAF response obtained with the previous method can be the result of an undercorrected decline rate of Fl_{DAF}. Under those conditions, the Fl_{DAF} decline rate could only be measured under control conditions, which was expected to be smaller than that under PAF stimulation with increased microvessel permeability.

The commonly used DAF-2 DA concentrations in biological systems were between 3 and 10 μM (5, 9, 12, 16, 27, 30). Our study indicated that the continuous perfusion of vessels with 1–5 μM of DAF-2 DA enabled the endothelial cells to accumulate sufficient intracellular DAF-2 to interact with basal and stimulated NO production. Under these conditions, the NO-dependent net changes in Fl_{DAF} are independent of the steady-state FI level. However, the percent changes in Fl_{DAF} (ΔFl/Fl_{0} * 100) are inversely correlated with the steady-state FI level. Therefore, should the percentage changes of FI be used to compare the magnitude of NO production between tissues, a comparable dye loading is important. We are aware that some of the studies converted the DAF-associated fluorescence into actual value of DAF-2T based on the fluorescence calibration curve generated with known concentrations of DAF-2T in the solution. However, the conversion has to be based on a good estimation of FI that was contributed by DAF-2T and valid assumptions. It has been indicated that not all of the NO is captured by DAF-2 and the NO trapping efficiency by DAF-2 varies in tissue types (9, 12, 18). Due to many uncertainties, the advantage of using the absolute value of DAF-2T over the estimated NO production rate appears limited. Although DAF-2-NO reaction is a complex process and some oxidants and reducers have been reported to affect DAF-2 reaction with NO (8, 17, 28), it remains a relatively reliable and specific NO indicator. The probe has its limitations but also has unique advantages over some of the methodologies for NO measurements, especially in regard to cell specificity and real-time measurements in live tissues.

In summary, our study improved the measurements of intracellular NO in intact microvessels using DAF-2DA. This improved approach overcame the dye leakage problem that was commonly seen with increased permeability such as in cultured endothelial cells or in intact vessels under inflammatory conditions. The relatively constant DAF-2 concentration in all endothelial cells of the vessel wall enhanced the sensitivity for NO detection. The characterization of NO-dependent and non-NO-dependent DAF-2 fluorescence will benefit the assessment of NO production not only in perfused microvessels but also in other biological applications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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