Platelet-activating factor increases endothelial \([\text{Ca}^{2+}]_i\) and NO production in individually perfused intact microvessels

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Zhu, Longkun, and Pingnian He. Platelet-activating factor increases endothelial \([\text{Ca}^{2+}]_i\), and NO production in individually perfused intact microvessels. Am J Physiol Heart Circ Physiol 288: H2869–H2877, 2005—We have demonstrated that inhibition of NO synthase (NOS) in endothelial cells by either the NOS inhibitor \(\text{N}^\bullet\)-monomethyl-\(\text{L}\)-arginine (L-NMMA) or the internalization of caveolin-1 scaffolding domain attenuated platelet-activating factor (PAF)-induced increases in microvessel permeability (Am J Physiol Heart Circ Physiol 286: H195–H201, 2004) indicating the involvement of an NO-dependent signaling pathway. To investigate whether an increase in endothelial cytoplasmic \([\text{Ca}^{2+}]_i\) is the initiating event and \([\text{Ca}^{2+}]_i\) and NO production were measured in individually perfused rat mesenteric venular microvessels via fluorescence microscopy. When venular microvessels were exposed to PAF, endothelial \([\text{Ca}^{2+}]_i\), increased from 69 ± 8 nM to a peak value of 374 ± 26 nM within 3 min and then declined to a sustained level at 190 ± 12 nM after 15 min. Inhibition of NOS did not modify PAF-induced increases in endothelial \([\text{Ca}^{2+}]_i\). PAF-induced NO production was visualized and quantified at cellular levels in individually perfused microvessels using 4,5-diaminofluorescein diacetate and fluorescence imaging. Increased fluorescence intensity (FI), which is an indication of increased NO production, occurred in 75 ± 7% of endothelial cells in each vessel. The mean maximum FI increase was 140 ± 7% of baseline value. This increased FI was abolished by pretreatment of the vessel with L-NMMA and attenuated in the absence of extracellular \(\text{Ca}^{2+}\). These results provide direct evidence from intact microvessels that increased endothelial \([\text{Ca}^{2+}]_i\), is the initial signal that activates endothelial NOS, and the subsequent increased NO production contributes to PAF-induced increases in microvessel permeability.

nitric oxide; permeability; fluorescence imaging; 4,5-diaminofluorescein diacetate; endothelial cytoplasmic \([\text{Ca}^{2+}]_i\) concentration

AN INCREASE IN PERMEABILITY of microvessels to water and macromolecules is the hallmark of acute inflammation, which is a critical event that results in edema formation and organ dysfunction. Platelet-activating factor (PAF), produced by a variety of cells that participate in the inflammatory reaction, has been identified as a potent mediator that increases microvessel permeability (27). The action of PAF is via a G protein-coupled receptor. Therefore, it has often been used as the representative agonist for the investigation of mechanisms or signaling pathways of receptor-mediated inflammatory responses.

We and other investigators have demonstrated that the inhibition of nitric oxide (NO) synthase (NOS) in endothelial cells through either the application of a pharmacological agent such as \(\text{N}^\bullet\)-monomethyl-\(\text{L}\)-arginine (L-NMMA) (11, 30) or the internalization of caveolin-1 scaffolding domain (36) attenuated PAF-induced increases in microvessel permeability, indicating the involvement of endothelial NOS (eNOS) activation and an NO-dependent signaling pathway. In most of the in vitro studies, an increase in intracellular \([\text{Ca}^{2+}]_i\) concentration was considered necessary for certain agonists such as bradykinin or ATP-induced eNOS activation (3, 5), whereas under other circumstances, such as studies of shear stress or the effects of estrogen or insulin, a \(\text{Ca}^{2+}\)-independent mechanism was reported for the regulation of eNOS activity (6, 8, 26, 31). Although eNOS is a constitutively expressed enzyme, its activity appears to be regulated by different mechanisms to control different cell functions. Presently, limited in vivo information is available for the mechanisms of eNOS activation and its relationship with endothelial cytoplasmic \([\text{Ca}^{2+}]_i\) especially with regard to its role in the regulation of microvessel permeability.

PAF-induced increases in microvessel permeability have been investigated in different experimental models in vivo (1, 30); however, there has been no correlative information on PAF-induced changes in endothelial \([\text{Ca}^{2+}]_i\). Furthermore, no study is available to quantify and visualize the agonist-stimulated NO production with temporal and spatial resolution at cellular levels in intact microvessels. Therefore, the objective of this study was to further test the hypothesis that a receptor-mediated increase in endothelial \([\text{Ca}^{2+}]_i\) is responsible for the activation of eNOS, and the subsequent increased NO production plays a key role for PAF-induced increases in microvessel permeability. The experiments were designed to provide direct evidence of PAF-induced changes in endothelial \([\text{Ca}^{2+}]_i\), and NO production in individually perfused microvessels. Endothelial \([\text{Ca}^{2+}]_i\), was measured using the fluorescent \(\text{Ca}^{2+}\) indicator fura 2-AM and a photometry system. NO production was delineated with fluorescence imaging using the fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2 DA). Both measurements were conducted under the same experimental conditions as those applied for studies of permeability in intact microvessels. To further elucidate the relationship between \([\text{Ca}^{2+}]_i\) and NO, endothelial \([\text{Ca}^{2+}]_i\), and NO production were also measured in the presence of the NOS inhibitor \(\text{L}\)-NMMA and in the absence of extracellular \(\text{Ca}^{2+}\).

MATERIALS AND METHODS

Animal preparation. Experiments were carried out on venular microvessels in rat mesenteries. All procedures and animal use protocols were approved by the Animal Care and Use Committee at West...
Virginia University. Female Sprague-Dawley rats (2–3 mo old; body wt, 220–250 g; Hilltop Laboratory Animals; Scottsdale, PA) were anesthetized with pentobarbital sodium given subcutaneously. The initial dosage was 65 mg/kg of body wt with an additional 3-mg dose given as needed. The trachea was intubated, and a midline surgical incision (1.5–2 cm) was made in the abdominal wall. The mesentery was gently removed from the abdominal cavity and spread over a glass coverslip attached to an animal tray. The upper surface of the mesentry was continuously superfused with mammalian Ringer solution. The temperature of the superfusate was maintained at 37°C. All experiments were carried out on venular microvessels with diameters ranging between 40 and 50 μm. Each experiment was performed on a single microvessel with one experiment per animal.

**Measurements of endothelial \([\text{Ca}^{2+}]_e\).** Endothelial \([\text{Ca}^{2+}]_e\) was measured in individually perfused microvessels using the fluorescent Ca²⁺ indicator fura 2-AM. Experiments were performed on a Nikon Diaphot 300 microscope equipped with a Nikon photometry system including photometer head and finder (P101), computer-controlled shutter, and filter changer (Lambda 10-2; Sutter Instrument; Novato, CA). A rectangular variable diaphragm located in the photometer finder determined the size of the measuring window through which the fluorescence intensity (FI) was collected. In each experiment, a venular microvessel in rat mesentery was cannulated and perfused first with albumin-Ringer solution that contained 10 μM fura 2-AM for 45 min. The vessel was then recannulated and perfused with albumin-Ringer solution for 10 min to remove fura 2-AM from the vessel lumen. A segment of fura 2-AM-loaded vessel at least 100 μm away from the cannulation site was then positioned within the field of view of the measuring window. The size of the window was adjusted to ~150 × 50 μm, which covered ~50 endothelial cells forming the vessel wall. A Nikon Fluor lens (×20, numerical aperture, 0.75) was used to collect FI values. The excitation wavelengths for fura 2-AM were selected by two narrow-band interference filters (340 ± 5 and 380 ± 5 nm; Oriel), and the emission was separated with a dichroic mirror (DM400) and a wide-band interference filter (500 ± 35 nm; Oriel). The excitation wavelength alternated between 340 and 380 nm, and corresponding FI values (FI₃₄₀ and FI₃₈₀, respectively) were collected with a 0.25-s exposure at each wavelength. At the end of the experiment, the microvessel was superfused with a modified Ringer solution (5 mM Mn²⁺) to bleach the Ca²⁺-insensitive forms of fura 2 while being perfused with the same solution that contained ionomycin (10 μM) to bleach the Ca²⁺-sensitive form of fura 2. The background FI due to unconverted fura 2-AM and other Ca²⁺-insensitive forms of fura 2 were subtracted from FI₃₄₀ and FI₃₈₀ values. The ratios of the two FI values were converted to Ca²⁺ concentrations using an in vitro calibration curve (14).

**Fluorescence imaging of endothelial NO production.** Endothelial NO levels were visualized and quantified at cellular levels in individually perfused microvessels using DAF-2 DA, a membrane-permeable fluorescent indicator for NO, and a fluorescence imaging system. The experimental rig was the same as that used for Ca²⁺ measurements, except that a 12-bit digital, cooled, charge-couple device camera (ORCA; Hamamatsu) was used for image acquisition. A 75-W xenon lamp provided the light source. The excitation wavelength for DAF-2 DA was selected by an interference filter (480/40 nm), and emission was separated by a dichroic mirror (505 nm) and a band-pass barrier (535/50 nm). In each experiment, a venular microvessel was cannulated and perfused with albumin-Ringer solution that contained DAF-2 DA (5 μM) for 15 min. After dye was loaded, it was washed out from the vessel lumen with albumin-Ringer perfusate for 10 min before control images were acquired. Under control conditions, individual endothelial cells forming the vessel wall were slightly visible. An objective (×20, 0.75 numerical aperture) was focused on a group of endothelial cells that were located at the same focal plane of the microvessel wall. In each experiment, images under control conditions and after exposure to PAF were acquired from the same group of endothelial cells in each vessel using identical instrument settings. To minimize photobleaching, a neutral-density filter (0.5) was positioned in front of the excitation filter, and the exposure time was 50 ms at 10-s intervals. At the end of each experiment, the NO donor sodium nitroprusside (SNP, 50 mM) was applied to the superfusate to examine the loading status of endothelial cells in the vessel wall. This procedure was used to ensure that the dye was loaded into all of the cells in the perfused vessel (see Fig. 3A, right).

**Data analysis and statistics.** All values are means ± SE except where otherwise noted. For statistical comparisons, a normality test was conducted in each data set to determine whether the use of a parametric test was appropriate; otherwise, a nonparametric test was...
applied. A paired $t$-test or nonparametric Wilcoxon signed rank test was used for paired data analysis such as the mean values obtained before and after stimulation from the same vessel. An unpaired $t$-test or Mann-Whitney $U$-test was used to compare data between groups. A probability value of $P < 0.05$ was considered statistically significant.

To evaluate the significance of NO-DAF-2 FI changes before and after stimulation in each ROI, the mean values and standard deviations of measured FIs in individual ROIs were calculated from images collected in a regular time interval under control conditions and after the stimulated FI reached a plateau. The maximum stimulated FI (at plateau level) was compared with the control FI measured in the same ROI, and a significant increase in FI was the criterion for a responsive cell.

RESULTS

PAF increases endothelial [Ca$^{2+}$], in intact microvessels. Experiments were conducted on nine microvessels from nine different rats. Endothelial [Ca$^{2+}$], was measured under control conditions and after each vessel was exposed to 10 nM PAF. The mean baseline value of endothelial [Ca$^{2+}$], of nine microvessels was $69 \pm 8$ nM. After vessels were exposed to perfusate that contained 10 nM PAF, [Ca$^{2+}$], increased to a mean peak value of $374 \pm 26$ nM at 1.3 min and then fell to a sustained level of $190 \pm 12$ nM after 15 min. The mean [Ca$^{2+}$], returned to the control level within 2 min after the PAF was washed out with albumin-Ringer solution. Figure 1A shows the time course of the changes in endothelial [Ca$^{2+}$], of an individual experiment, and Fig. 1B summarizes the mean changes in [Ca$^{2+}$], from nine vessels.

Effects of extracellular Ca$^{2+}$ on PAF-induced increases in endothelial [Ca$^{2+}$]. To investigate the sources of Ca$^{2+}$ ions that contribute to PAF-induced increases in endothelial [Ca$^{2+}$], paired measurements were conducted in each vessel in the absence and presence of extracellular Ca$^{2+}$ in a single vessel. The mean baseline [Ca$^{2+}$], of six microvessels was $66 \pm 8$ nM. Removal of extracellular Ca$^{2+}$ by perfusion and superfusion of the vessel with Ca$^{2+}$-free Ringer solutions for 6–8 min slightly lowered the [Ca$^{2+}$], to $58 \pm 15$ nM, which was not significantly different from the baseline value. When each vessel was exposed to PAF in the absence of extracellular Ca$^{2+}$, the mean peak level of [Ca$^{2+}$], was $370 \pm 26$ nM. The time course was similar to that reported in Fig. 1A. When extracellular Ca$^{2+}$ was removed, the small-magnitude increase in [Ca$^{2+}$], in response to PAF was a significant reduction from that mea-
Fig. 3. Representative fluorescent images of venular microvessels loaded with 4,5-diamino-fluorescein diacetate (DAF-2 DA). A: PAF-induced significant increases in nitric oxide (NO)-DAF-2 fluorescence intensity (FI) relative to control levels (left) in endothelial cells that form the microvessel wall (middle). FI was further increased with the application of the NO donor sodium nitroprusside (SNP) to the superfusate (right). B: NO synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NMMA) completely abolished PAF-induced increases in NO-DAF-2 FI (middle). When photobleaching was minimized by increasing the excitation interval from 10 to 40 s, NOS inhibition showed no effect on SNP-induced increases in NO-DAF-2 FI (right).
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The regions of higher FI correspond to the perinuclear area of the endothelial cell that is thicker in the region of the nucleus, also showed that the dye was distributed into both cytoplasm well loaded into cells that form microvessel walls. The image SNP was added to the superfusate. It indicated that DAF-2 was cellular structure of the vessel wall was well delineated when observed after the vessel was exposed to PAF (10 nM). The sum of the five experiments.

$\text{PAF increases endothelial NO production in intact microvessels.}$ Fluorescence imaging was used to directly visualize and quantify endothelial NO levels before and after PAF stimulation in each individually perfused microvessel. Experiments were conducted on five microvessels from five different animals. Figure 3A shows representative images selected from an individual experiment. A significant increase in FI was observed after the vessel was exposed to PAF (10 nM). The cellular structure of the vessel wall was well delineated when SNP was added to the superfusate. It indicated that DAF-2 was well loaded into cells that form microvessel walls. The image also showed that the dye was distributed into both cytoplasm and nucleus within each cell. However, due to the geometry of the endothelial cell that is thicker in the region of the nucleus, the two-dimensional image shows nonuniform FI distribution. The regions of higher FI correspond to the perinuclear area of the cell (Fig. 3A, right). To quantify the PAF-induced FI changes, ROIs were selected in each vessel for FI measurements. The number of ROIs selected from each vessel varied from 4 to 15 cells, which depended on the available cells that were located on the same focal plane of the vessel wall. Although all of the cells in each vessel were well loaded with DAF-2 as verified by the addition of SNP at the end of each experiment, the significant increases in FI after PAF stimulation in each ROI, which we considered as responsive, occurred only in 75 ± 7% of the cells in the focal plane of each vessel. The mean maximum FI (measured at plateau) from the responsive cells in five vessels was 141 ± 7% of the baseline level. The mean FI of the rest of the unresponsive cells (no significant increases in FI after PAF stimulation) in five vessels was 99.2 ± 1.1% of the baseline level. Figure 4 shows the fitted curve of the time-dependent FI changes relative to control and the calculated NO production rate in response to PAF in an individual experiment. The converted NO production rate indicated that PAF-induced NO production peaked at 2 min and returned to the baseline level at 6 min. Figure 5 (top curve) shows the time-dependent FI changes as well as the variations of FI measured in five endothelial cells from the same vessel. Summary results of five microvessels are shown in Fig. 6.

$\text{PAF-induced NO production was inhibited by NOS inhibitor l-NMMA.}$ To test whether the increased DAF-2 fluorescence was due to increased NO production, the NOS inhibitor l-NMMA was applied to the perfusate throughout the experiment including the period of DAF-2 loading and washout. In five microvessels, the mean basal FI in the presence of l-NMMA was 58 ± 11% of that measured in the absence of the NOS inhibitor ($n = 5$). In addition, the basal FI decline rate increased from 0.25 ± 0.2% per minute under normal control conditions to 1.5 ± 0.7% per minute. PAF-induced increases in NO-DAF-2 FI (after the correction for photobleaching) were completely blocked by the application of l-NMMA in all ROIs of five microvessels. The time course of an individual experiment is shown (see Fig. 5, bottom curve), which is the mean value of 11 cells selected from the same vessel. Figure 6 summarizes the results of five microvessels.

In this group of study, we also noted that when SNP was added to the superfusate at the end of each experiment, although we observed a significant increase in FI relative to basal FI, the magnitude of the FI increase was less than that observed without the application of an NOS inhibitor. To examine whether the low FI due to photobleaching and low basal FI under these experimental conditions contributed to the reduced effect of SNP, two additional experiments were conducted. To minimize the photobleaching effect, the excitation interval was increased from 10 to 40 s, which reduced the FI decline rate to 0.12% per minute and improved the FI level at the end of the experiment. These two experiments showed consistent evidence of an inhibitory effect of l-NMMA on PAF-induced NO production.

![Fig. 4. PAF-induced increase in NO-DAF-2 FI, which is an indication of increased NO production in endothelial cells that form an intact venular microvessel. PAF-induced cumulative NO production (solid line) is shown as a function of time (left y-axis). F, measured FI after stimulation; $F_0$, control FI level. Differential conversion of the cumulative FI changes ($df/dt$, dotted line) represents the NO production rate (right y-axis). After the vessel was exposed to PAF, NO production reached its peak at 2 min and terminated at 5 min when the cumulative amount of NO reached a maximum and formed a plateau.](http://ajpheart.physiology.org/)

![Fig. 5. Three individual experiments show the magnitudes of PAF-induced FI changes in the presence and absence of extracellular $\text{Ca}^{2+}$ or NOS inhibitor. Representative PAF-induced FI changes with normal Ringer solutions are shown in the top curve (the same curve as in Fig. 4, except here the variations of FI changes measured in five different endothelial cells in the same vessel are also included). Significant attenuation of PAF-induced increase in FI occurred after removal of extracellular $\text{Ca}^{2+}$ (middle curve). This curve represents the mean FI changes measured in 11 endothelial cells of the same vessel. Complete inhibition of PAF-induced NO production by l-NMMA is shown in the bottom curve.](http://ajpheart.physiology.org/)
production and also demonstrated a comparable magnitude of SNP responses to those observed without NOS inhibition. These results indicated that NOS inhibition did not modify SNP-derived NO and its interaction with DAF-2. Images from one of these experiments are presented in Fig. 3B.

Removal of extracellular Ca\(^{2+}\) attenuated PAF-induced NO production. PAF-induced increases in endothelial [Ca\(^{2+}\)]\(_{i}\) were significantly attenuated in the absence of extracellular Ca\(^{2+}\). The mean peak value was reduced from 370 to 111 nM (see Fig. 2B). To investigate whether PAF-induced NO production is Ca\(^{2+}\)-influx dependent and whether the magnitude of endothelial [Ca\(^{2+}\)]\(_{i}\) determines the magnitude of NO production, PAF-induced NO production in the absence of extracellular Ca\(^{2+}\) was measured under the same experimental conditions as those used to study endothelial [Ca\(^{2+}\)]\(_{i}\). In five microvessels, the Ca\(^{2+}\)-free Ringer solutions were applied for 6–8 min after DAF-2 loading. During this period, the mean FI decline rate was 1.0 ± 0.8% per minute, which was higher than that when Ca\(^{2+}\) was present but lower than that when NOS was inhibited. When each vessel was exposed to PAF, the magnitude increase in NO-DAF-2 FI was significantly attenuated from that when Ca\(^{2+}\) was present but was not completely inhibited. The mean NO-DAF-2 FI was reduced from 140 ± 7 to 115 ± 1%. PAF-induced time-dependent changes in FI from a single vessel (five endothelial cells) are shown (see Fig. 5, middle curve). The results of five experiments are summarized in Fig. 6.

Effects of NOS inhibitor on PAF-induced increases in endothelial [Ca\(^{2+}\)]\(_{i}\). To investigate whether the action of the NOS inhibitor in attenuating PAF-induced permeability increases is due to the reduction of the PAF-induced increase in endothelial [Ca\(^{2+}\)]\(_{i}\), paired measurements were conducted in each vessel to compare PAF-induced changes in endothelial [Ca\(^{2+}\)]\(_{i}\) in the absence and presence of the NOS inhibitor l-NMMA. No significant changes in [Ca\(^{2+}\)]\(_{i}\) were observed when vessels were perfused with l-NMMA (100 μM) alone. The results also showed that l-NMMA did not affect the magnitude and time course of PAF-induced increases in endothelial [Ca\(^{2+}\)]\(_{i}\). Figure 7A shows the paired measurements of endothelial [Ca\(^{2+}\)]\(_{i}\) in response to PAF (10 nM) in the absence and presence of l-NMMA (100 μM) in a single vessel. In a total of four such experiments, the mean peak increases in [Ca\(^{2+}\)]\(_{i}\) induced by PAF (10 nM) before and after application of l-NMMA were 432 ± 52 and 428 ± 52 nM, respectively. These results are summarized in Fig. 7B.
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DISCUSSION

This study provided direct evidence of the involvement of a $Ca^{2+}$-NO-dependent pathway in the PAF-induced permeability increase in intact venular microvessels. Our results demonstrate that PAF induces a transient increase in endothelial $[Ca^{2+}]_i$, that is followed by a transient increase in NO production in endothelial cells forming microvessel walls. The inhibition of NOS completely abolishes PAF-induced NO production but showed no effect on PAF-induced increases in endothelial $[Ca^{2+}]_i$, which suggests that the activation of NOS in endothelial cells occurred downstream from PAF-induced initial increases in endothelial $[Ca^{2+}]_i$. In addition, our results demonstrate that the attenuated increase in $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$ results in a significant reduction in NO production indicating the important role of endothelial $[Ca^{2+}]_i$ in the regulation of eNOS activity. Fluorescent images of DAF-2-loaded microvessels provide a direct visualization and quantification of NO production in endothelial cells with temporal and spatial resolutions in intact microvessels. These results support the hypothesis that increased endothelial $[Ca^{2+}]_i$ is responsible for activation of eNOS, and the subsequent increased NO production plays a key role in PAF-induced increases in microvessel permeability. This new information will contribute to a better understanding of the mechanisms of $Ca^{2+}$-NO-dependent regulation of microvessel permeability.

PAF causes transient increases in endothelial $[Ca^{2+}]_i$, in intact microvessels, a pattern similar to that induced by other inflammatory mediators. The role of $Ca^{2+}$ signaling in the regulation of permeability has been reported in cultured endothelial monolayers and intact microvessels (23, 32, 33). Our previous studies (10, 14) in frog, hamster, and rat mesenteric venular microvessels demonstrated a close relationship between the magnitude of the initial increase in endothelial $[Ca^{2+}]_i$, and that of the initial increase in microvessel permeability. The agonists that were investigated include $Ca^{2+}$-ionophore, ATP, and bradykinin. In those studies, we found that agonist-induced $Ca^{2+}$ release from internal stores contributed a small portion (~30%) of the initial transient increase in $[Ca^{2+}]_i$, whereas 70% of the initial peak was due to $Ca^{2+}$ influx through a passive conductance pathway (14). These observations are different from those reported in cultured endothelial cells, in which the agonist (including PAF)-induced initial peak increase in $[Ca^{2+}]_i$ was due mainly to the release of intracellular pools (4, 17).

Our present study of PAF-induced increases in endothelial $[Ca^{2+}]_i$, was conducted under the same experimental conditions as those applied for the permeability measurements (36). Our results demonstrate that the pattern of PAF-induced changes in endothelial $[Ca^{2+}]_i$ is similar to that induced by other inflammatory mediators such as ATP, bradykinin, VEGF, etc. (2, 12, 14). In the absence of extracellular $Ca^{2+}$, the magnitude of $[Ca^{2+}]_i$ increase in response to PAF was significantly attenuated, which suggests that $Ca^{2+}$ influx was the main source for the PAF-induced peak increase in $[Ca^{2+}]_i$, in intact microvessels. The small magnitude increases in endothelial $[Ca^{2+}]_i$, were attributed to PAF-induced $Ca^{2+}$ release from internal stores. To avoid the depletion of internal $Ca^{2+}$ stores by the removal of external $Ca^{2+}$ from bathing solutions, the EGTA concentrations in $Ca^{2+}$-free Ringer solutions were kept at a minimum (in the micromolar range), and the period of $Ca^{2+}$ removal was limited to 5–10 min. Our previous study demonstrated that the application of $Ca^{2+}$-free Ringer solutions for 5–10 min, the magnitude of the ATP-induced increase in $[Ca^{2+}]_i$ was comparable to that when $Ca^{2+}$ was present but $Ca^{2+}$ influx was inhibited by membrane depolarization that reduced the electrochemical driving force for $Ca^{2+}$ entry (14). Those results indicated that the internal $Ca^{2+}$ stores were relatively intact under those defined experimental conditions.

Visualization of PAF-induced NO production in endothelial cells with DAF-2-loaded intact microvessels. Our previous studies demonstrated that NOS inhibitors (either l-NMMA or nitro-l-arginine methyl ester) attenuated the permeability increases induced by ATP, bradykinin, and ionomycin in individually perfused mesenteric venular microvessels (11, 12). Similar results have also been reported for VEGF-, histamine-, leukotriene $C_4$-, and PAF-induced permeability increases in different types of vessels and animal species (18–21, 30, 34). However, due to the nonspecificity of NOS inhibitors, these studies cannot distinguish the isoforms of NOS that are activated during stimulation and are therefore responsible for the increased microvessel permeability. Recently we developed a method that successfully internalized caveolin-1 (Cav-1) scaffolding domain, the specific endogenous inhibitor of eNOS, to endothelial cells that form microvessels in vivo. The results that internalization of Cav-1 scaffolding domain prevented PAF-induced permeability increase indicated the role of eNOS in permeability increases in intact venular microvessels (36). However, due to the short biological half-life of NO, it is a real challenge to quantify the NO production, the direct evidence of the eNOS activation, with spatial and temporal resolutions in vivo, especially under the same experimental conditions as those applied for the permeability measurements. A study in hamster cheek pouches reported an increase in NO production with PAF stimulation by measuring chemiluminescence from superfusate samples (7), but the method has limitations in its ability to distinguish the types of cells and NOS that are responsible for the increased NO production.

DAF-2 has been introduced as a specific fluorescent indicator of NO (16). Its fluorescent chemical transformation is based on the reactivity of the aromatic vicinal diamines with NO in the presence of dioxygen. Its reaction with NO produces a green-fluorescent triazole form that results in an NO concentration-dependent enhancement of fluorescence. DAF-2 diacetate (the membrane-permeable form) has been used for real-time imaging of NO in cultured cells, isolated coronary arterial segments, and the whole rat mesentery vasculature (15, 16, 28, 35). Our present study using DAF-2 DA in individually perfused intact microvessels provides further evidence that DAF-2 is a valid fluorescent indicator of NO. The fluorescent images of DAF-2-loaded microvessels enabled a real-time assessment of NO production at the cellular level with detailed spatial resolution. Our results demonstrated that a PAF-induced transient increase in endothelial $[Ca^{2+}]_i$, was immediately followed by a transient increase in NO production. The complete blockage of NO production with l-NMMA indicated that the activation of NOS in endothelial cells is the mechanism. Under control conditions, the low basal FI (58% of basal FI in the absence of l-NMMA) and the high FI decline rate occurred in the presence of l-NMMA indicated a correlation between NO-DAF-2 FI and basal NO production. Under normal control...
conditions, the FI decline rate was six times lower than that when NOS was inhibited. Since the rate of the FI decline was the balance between the increased FI due to basal NO production and the decreased FI due to photobleaching, there was an inverse relationship between FI decline rate and basal NO production. The higher FI decline rate in the absence of extracellular Ca\(^{2+}\) was an indication of attenuated basal NO production even though the endothelial [Ca\(^{2+}\)]\(\text{i}\) was only slightly declined (not significantly different) when extracellular Ca\(^{2+}\) was removed.

**Relationship between Ca\(^{2+}\) and NO in regulation of microvessel permeability.** Considerable evidence indicates that eNOS is a Ca\(^{2+}\)/calmodulin-dependent enzyme, and increased intracellular Ca\(^{2+}\) increases eNOS activity (9, 24, 25). To identify the temporal relationship between Ca\(^{2+}\) and NO, we first compared the timing of these two events by superimposing the mean time course of PAF-induced increases in endothelial [Ca\(^{2+}\)]\(\text{i}\) (n = 9) to the PAF-induced NO production curve (n = 5). Figure 8 shows that the NO production curve shifts to the right of the Ca\(^{2+}\) curve for ~0.7 min. If we compare peak values, the mean peak increase in endothelial [Ca\(^{2+}\)]\(\text{i}\) occurred at 1.3 min of PAF exposure, whereas the mean peak increase in NO production occurred at 2 min of PAF exposure. These two sequential events with a 0.7-min time lag indicate a potential causal link between the initial peak increase in endothelial [Ca\(^{2+}\)]\(\text{i}\), and the subsequently increased NO. These results provide a better understanding of how eNOS is activated after exposure to PAF. Comparing the two time courses in Fig. 8, we note that NO production rapidly declined back to basal levels while Ca\(^{2+}\) remained significantly elevated. This suggests that the decline in [Ca\(^{2+}\)]\(\text{i}\) after the initial peak was not the mechanism for the inactivation of eNOS. Regulatory mechanisms different from Ca\(^{2+}\) reduction might be responsible for the inactivation of eNOS after stimulation and need to be further investigated.

The measurements of PAF-induced endothelial [Ca\(^{2+}\)]\(\text{i}\) and NO production in the absence of extracellular Ca\(^{2+}\) allowed us to evaluate whether there is a magnitude correlation between the increased endothelial [Ca\(^{2+}\)]\(\text{i}\), and the increased NO production and whether the NO production is Ca\(^{2+}\)-influx dependent. In the absence of extracellular Ca\(^{2+}\), the PAF-induced increase in endothelial [Ca\(^{2+}\)]\(\text{i}\) was significantly attenuated and correlated with a significant attenuation of NO production. These results suggest that the magnitude of the initial increase in endothelial [Ca\(^{2+}\)]\(\text{i}\), determines the magnitude of NO production regardless of the Ca\(^{2+}\) sources. However, in the absence of extracellular Ca\(^{2+}\), the NO production appears to be reduced to a smaller extent than the reduction in the peak Ca\(^{2+}\) response. This result suggests that a localized [Ca\(^{2+}\)]\(\text{i}\), (e.g., under the membrane or near caveolae) might be different in magnitude from that measured with our present photometric method which measures mean values of [Ca\(^{2+}\)]. The localized Ca\(^{2+}\) changes may be more important in the regulation of eNOS activity. The contribution of such local heterogeneity remains to be evaluated.

Previous Ca\(^{2+}\)-imaging studies in intact venular microvessels demonstrated that endothelial [Ca\(^{2+}\)]\(\text{i}\) does not increase uniformly in response to an inflammatory mediator (22, 29). The heterogeneous Ca\(^{2+}\) responses along the microvessel wall account for the formation of localized leaky sites during acute inflammation (29). Our fluorescence imaging of PAF-induced NO production demonstrate a similar heterogeneity pattern in endothelial cells that form microvessel walls. Our results show that the significant increase in FI, an indication of increased NO production, occurs in 75% of the endothelial cells in a vessel exposed to PAF. Within these responding endothelial cells, variations also existed (see Fig. 5). The variations in [Ca\(^{2+}\)]\(\text{i}\) responses might be directly linked to the variations in NO production of individual endothelial cells, which lead to localized leaky site formation along the vessel walls. Our present study provides the temporal correlation between endothelial [Ca\(^{2+}\)]\(\text{i}\), and NO production among groups of endothelial cells in different vessels. New approaches are under development, which will allow the spatial heterogeneity correlation between endothelial [Ca\(^{2+}\)]\(\text{i}\), and NO production to be evaluated at cellular levels. To accomplish that, both [Ca\(^{2+}\)]\(\text{i}\), and NO need to be measured in the same group of individual cells in the same vessel using a fluorescence-imaging approach.

In the present study, we also tested the possibility that the action of eNOS inhibition in the attenuation of PAF-induced permeability increase was due to the attenuation of PAF-induced Ca\(^{2+}\) entry. Our results show that both the magnitude and time course of PAF-induced increase in endothelial [Ca\(^{2+}\)]\(\text{i}\), is not modified by inhibition of NOS using l-NMMA (see Fig. 7). These results further support the hypothesis that the function of eNOS is exerted downstream from the initial Ca\(^{2+}\) entry, which are consistent with studies using other mediators (11, 12).

In regard to how increased NO increases microvessel permeability, the downstream mechanisms are not fully understood. Our previous studies (12, 13) demonstrated that the activation of cGMP was involved, and a reduction of cAMP level due to the activation of cGMP-stimulated phosphodiesterase might be one of the mechanisms. A recent electron microscopy study (1) confirmed that PAF induces paracellular inflammatory gaps in rat venular microvessels. Those results suggest that the mechanisms that modulate cell-to-cell adhesion appeared to be more important than active actin-myosin contraction to increase permeability. Further investigations on the steps between increased NO and the gap formation are important.

In summary, this study provides direct evidence that the PAF-induced increase in microvessel permeability is associated with a transient increase in endothelial [Ca\(^{2+}\)]\(\text{i}\), followed
by a transient increase in NO production. The attenuation of the PAF-induced permeability increase by inhibition of NOS is not due to the attenuation of PAF-induced increase in endothelial [Ca\(^{2+}\)]. Instead, the function of eNOS and PAF-induced increased NO production occurs downstream from the initial increase in endothelial [Ca\(^{2+}\)]. The magnitude of the initial increase in endothelial [Ca\(^{2+}\)] determines the magnitude of NO production in endothelial cells. This Ca\(^{2+}\)-dependent eNOS activation may represent a common mechanism for receptor-mediated permeability increase in vivo.

**GRANTS**

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**REFERENCES**


