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Obligatory role of nitric oxide in platelet-activating factor-induced microvascular leakage

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Abstract

We examined the independent and interdependent effects of platelet-activating factor (PAF) and nitric oxide (NO) on microvascular leakage of fluorescein isothiocyanate (FITC)-dextran in the cheek pouch microcirculation of anesthetized hamsters. Superfusing the cheek pouch microcirculation with 100-nM PAF elicited rapid leakage of FITC-dextran that was markedly inhibited by prior treatment with a nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine (L-NA; 1 μM). This inhibition by L-NA was completely reversed by application of a NO donor (*S*-nitroso-*N*-acetylpenicillamine, SNAP; 10 μM) at the same time PAF was applied. SNAP alone, however, did not cause leakage of FITC-dextran; neither did it enhance PAF-induced leakage. PAF-induced leakage was completely inhibited by prior treatment with the guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM). 8-bromoguanosine 3′,5′-cyclic monophosphate (8-br-cGMP) did not reverse this inhibition by ODQ although this cell permeable cGMP analog was able to completely reverse arteriolar vasoconstriction produced by ODQ. These results indicate that PAF-induced leakage of FITC-dextran in the hamster cheek pouch microcirculation requires an intact NO/cGMP pathway, although NO production does not cause PAF-induced leakage. This supports the hypothesis that NO plays an obligatory role in PAF-induced leakage.

Keywords: FITC-dextran; Arteriole; Permeability; Nitric oxide (NO); Platelet-activating factor (PAF); Nitric oxide synthase (NOS) inhibitor; Microcirculation

1. Introduction

Microvascular leakage of fluid and macromolecules is an early event during inflammatory responses, including those caused by acute endotoxemia (Matsuda et al., 1991a,b; Kurose et al., 1993; Klabunde and Calvello, 1995; Laniyonu et al., 1997). Many factors have been proposed to play a role in microvascular leakage during inflammation, including cytokines, leukotrienes, prostaglandins, histamine, platelet-activating factor (PAF), and nitric oxide (NO) (Glauser et al., 1991; Parrillo, 1993; Stoclet et al., 1993). We have previously shown that a PAF receptor antagonist and nitric oxide synthase (NOS) inhibitor prevent endotoxin-induced leakage of fluorescein isothiocyanate (FITC)-dextran in the hamster cheek pouch microcirculation, suggesting that both PAF and NO promote leakage during endotoxemia (Laniyonu et al., 1997). These findings regarding PAF were consistent with many studies, which have shown that application of PAF to the microcirculation induces microvascular leakage (Bjork and Smedegard, 1983; Dillon and Duran, 1988; Bekker et al., 1988; Kubes et al., 1990; Tomeo and Duran, 1991). The role for NO in leakage, however, is less clear. Some studies have suggested that NO promotes leakage by increasing vascular permeability (Mayhan, 1992, 1994; Yuan et al., 1993; Ramirez et al., 1995; Laniyonu et al., 1997; He et al., 1997a; Gimeno et al., 1998a,b). This has been demonstrated either by application of NO donors to the microcirculation or to isolated, perfused microvessels and inducing an increase in permeability, or by showing that NOS inhibition reduces leakage caused by pro-inflammatory mediators. On the other hand, there are studies suggesting that NO inhibits leakage (Hutcheson et al., 1990; Filep and Foldes-Filep, 1993; Kubes, 1993; Filep et al., 1993; Kubes et al., 1995; Gaboury et al., 1996; Harris, 1997; Yoshikawa et al., 1997; He et al., 1997b; Gimeno et al., 1998b) or has no effect on leakage other than by altering microvascular hemodynamics (Feletou et al.,

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1996). The reason for these conflicting results is not readily apparent, although a review by Kubes (1995) attempted to sort out the disparate data by noting differences in species, organ, leukocyte involvement, hemodynamic factors, and inflammatory versus non-inflammatory conditions. Other studies have shown that NO produced by constitutive, endothelial NOS (ecNOS) inhibits leakage while NO produced after inducible NOS (iNOS) activation increases microvascular permeability (Filep et al., 1997; Bernareggi et al., 1997).

Our previous observation in the hamster cheek pouch microcirculation that both PAF and NO inhibition independently abolished leakage caused by endotoxin suggested that PAF may increase NO production, which leads to microvascular leakage (Laniyonu et al., 1997). Others have shown that lipopolysaccharide (LPS) stimulates PAF production that in turn stimulates ecNOS and iNOS production of NO (Szabo et al., 1993). If PAF causes increased formation of NO, and if NO causes leakage, then blocking either PAF or NO would diminish leakage by endotoxin as we have previously shown (Laniyonu et al., 1997). Alternatively, NO may not directly cause leakage but rather serve an obligatory or permissive role in PAF-mediated leakage. That is, a background level of NO may be required for PAF to cause leakage.

The purpose of this study was to examine the independent and interdependent actions of NO and PAF on microvascular leakage of FITC-dextran in the hamster cheek pouch microcirculation. These interactions were examined by treating the hamster cheek pouch microcirculation with a NOS inhibitor and a guanylyl cyclase inhibitor to determine the role of the NO/cGMP pathway in PAF-induced microvascular leakage.

2. Materials and methods

2.1. Animal preparation

The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Ohio University. Male Golden Syrian hamsters (120 ± 2 g; $n = 90$) were anesthetized with pentobarbital sodium (65 mg/kg, i.p.). The right carotid artery was cannulated for administration of FITC-dextran (150,000 MW; initial dose of 100 mg/kg followed by continuous infusion of 5 mg kg^{-1} h^{-1}), for supplemental anesthetic (25 mg kg^{-1} h^{-1}), and for continuous monitoring of mean arterial pressure. These drugs were delivered through the carotid cannula at a rate of 10 ml kg^{-1} h^{-1}. Animals were included in the study if their initial mean arterial pressure was greater than 85 mm Hg.

The left cheek pouch was cleared of food and debris, rinsed with 0.9% saline solution, exteriorized, and spread out over a Plexiglas microscope stage. The upper layer of the pouch was carefully opened to expose the serosal surface and its edges pulled back, spread out, and tied to the stage using four to five ligatures. Once the stage was mounted onto the microscope, the cheek pouch was superfused with Krebs–Henseleit buffer (containing in mM: NaCl, 118; $NaHCO_3$, 25; D-glucose, 11.1; Mg_2SO_4, 1.17; KCl, 4.7; $CaCl_2$, 2.0; KH_2PO_4, 1.2; pH 7.4; 37°C; bubbled with room air containing 5% CO_2) at a rate of 1 ml min^{-1}. The core body temperature and arterial pressure were displayed using a personal computer running MP100WS for Windows (BioPac Systems, Goleta, CA). The temperature of the water-heated Plexiglas stage was maintained between 35°C and 37°C.

2.2. Intravital microscopy

The cheek pouch was observed with a microscope (Nikon Optiphot) through the $5\times$ objective and the image was projected onto a high resolution CCD camera (Paultek). A $1.0\times$ camera projector lens was mounted between the microscope and the camera sensor. The camera output was controlled by a Paultek video controller, that was linked to a FOR.A date/time generator (VGT-10) to project the date and time onto a video monitor (Sony PVM-1343MD). Transmitted light was supplied by a 100-W halogen light source. Epi-illumination of the microvessels following the injection of FITC-dextran was supplied by a 100-W mercury lamp through a FITC filter set (excitation wavelength 450–490 nm; emission wavelength 520 nm). The tissue was excited with the light only long enough to digitize a video frame. For measurement of arteriolar diameter, a $40\times$ objective was used. Arteriolar internal diameters were measured on-line using a video caliper (Microcirculation Research Institute, College Station, TX). When a vasoactive agent was applied to an arteriole, the maximal change in diameter was measured and compared to baseline diameter.

2.3. Image analysis

When an image was acquired from the video signal, we assigned an area of interest on the monitor that corresponded to 2.56 mm^2 of the cheek pouch using the $5\times$ objective. This area of interest contained 640×422 (270,080 pixels). Each pixel had an intensity value between 0 and 255 (0 = black; 255 = white). Using Image-Pro Plus, a histogram was plotted from the control AOI showing the frequency distribution of number of pixels versus pixel intensity. A Threshold Pixel Intensity (TPI) was found, which represented the value at which 80% of the total number of pixels fell below. The 20% of pixels eliminated from analysis represented, to large extent, intravascular fluorescence. In subsequent images from the same experiment, the percentage of pixels within the area of interest that were sub-threshold in the control image, but attained TPI following drug treatment, was quantified. Therefore, in the control image, 0% of pixels had attained

TPI. With leakage, the percentage of pixels attaining the TPI would approach a maximum of 100%. Microvascular leakage of FITC-dextran, therefore, was expressed as %TPI. This image analysis technique was similar to what we have previously used (Laniyonu et al., 1997) except that software algorithms were used to eliminate the inclusion of fluorescence from the vessels, thereby minimizing the effects of changes in intravascular fluorescence in the analysis.

2.4. Experimental protocol

A region of the cheek pouch was selected using a 5× objective. An important criterion was that the microcirculatory region had brisk flow throughout. The cheek pouch was then allowed to equilibrate for at least 30 min. In experiments evaluating microvascular leakage, the protocol began at −20 min with a bolus injection of FITC-dextran. Thereafter, digital images were obtained every 5 min for 80 min, then the experiment was ended. In animals treated with either N^{ω}-nitro-L-arginine (L-NA) or 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), these were applied to the cheek pouch in the superfusate at −20 min. PAF, *S*-nitroso-*N*-acetylpenicillamine (SNAP), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-br-cGMP), either alone or in combination, were added to the superfusate at 0 min. PAF was applied for 10 min; SNAP and 8-br-cGMP were superfused for 60 min.

In experiments where changes in arteriolar diameter were measured, an arteriole was selected using a 40× objective. In many experiments, adenosine (10^{-4} M) was added to the superfusate to verify that the vessel had good tone. Various vasodilators (adenosine, SNAP, 8-br-cGMP) and vasoconstrictors (PAF, L-NA, ODQ) were then added to the superfusate to evaluate vascular reactivity. If the study involved L-NA or ODQ, these were added to the superfusate for 20 min before vascular reactivity was reevaluated. Superfusion with adenosine, SNAP, 8-br-cGMP, and PAF was maintained until the maximal change in arteriolar diameter was achieved (typically < 3 min).

2.5. Drugs

All drugs used in this study were purchased from Sigma, St. Louis, MO. PAF (> 50% C16 in a chloroform solution) was diluted into Krebs–Henseleit buffer containing 1.5% bovine serum albumin to a concentration of 7.87×10^{-7} M. L-NA was dissolved into 0.9% saline, acidified with 1 N HCl, then further diluted to 10^{-4} M. ODQ (5 mg) was first solubilized in 100 μl of dimethyl sulfoxide, then further diluted with non-gassed Krebs–Henseleit buffer. The final concentration of dimethyl sulfoxide that the tissue was exposed to was 0.00375%. SNAP (2.20 mg) was dissolved into 100 μl of ethanol, then further diluted in non-gassed Krebs–Henseleit buffer to 10^{-4} M and protected from light. The final concentration of ethanol that the tissue was exposed to was 0.01%. Adenosine and 8-br-cGMP were dissolved into distilled water. Drug solutions were then added directly to the Krebs–Henseleit superfusate reservoir (L-NA and ODQ) or infused using a syringe pump or roller pump into the outflow line of the Krebs–Henseleit buffer reservoir (adenosine, SNAP, 8-br-cGMP) at a rate required to achieve the desired final concentration.

2.6. Statistics

Data were transferred to an Excel spreadsheet for calculation of descriptive statistics (mean ± S.E.). Comparisons between multiple groups were conducted using one-way analysis of variance followed by a Tukey–Kramer test to compare differences among groups. All statistical evaluations were conducted using InStat® software (GraphPad, San Diego, CA). Statistical significance was determined at the 0.05 level. Data were expressed and plotted in graphical form as mean ± S.E.

3. Results

Topical application of 100-nM PAF caused rapid leakage of FITC-dextran from the vascular space into the interstitium of the hamster cheek pouch (Fig. 1). The leakage began during the 10 min of PAF superfusion and continued after the superfusion ceased, reaching a maximal value at about 45 min after the initial exposure to PAF. Time controls showed no significant leakage over the same 60-min time period.

The first series of experiments examined the effects of NOS inhibition on leakage of FITC-dextran induced by PAF. Leakage responses were compared at 40 min among the different treatment groups (Fig. 2). At 40 min, PAF-in-

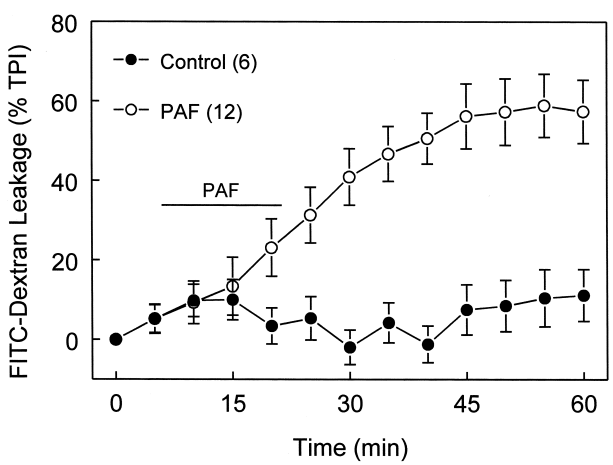

Fig. 1. PAF-induced leakage of FITC-dextran in the hamster cheek pouch microcirculation. There was a 6-min time delay (from 0 min) before for the cheek pouch was exposed to PAF. Vertical bars represent S.E.; number in parentheses is number of animals.

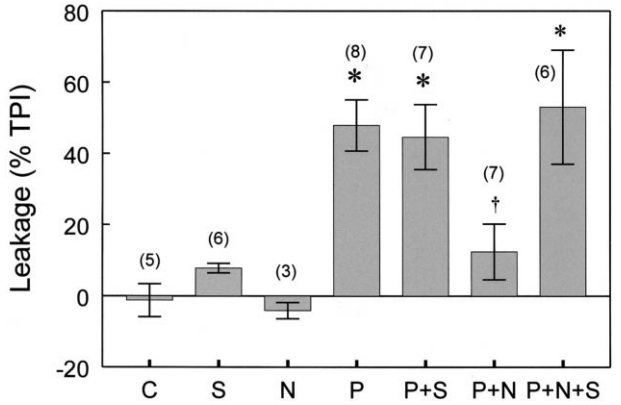

Fig. 2. Effects of NOS inhibition on PAF-induced leakage. C, control; S, SNAP (10 μM); N, L-NA (1 μM); P, PAF (100 nM). Vertical bars represent S.E.; number in parentheses is number of animals; * Indicates $P < 0.05$ related to control; † Indicates $P < 0.05$ relative to P, P + S, and P + N + S treatment groups.

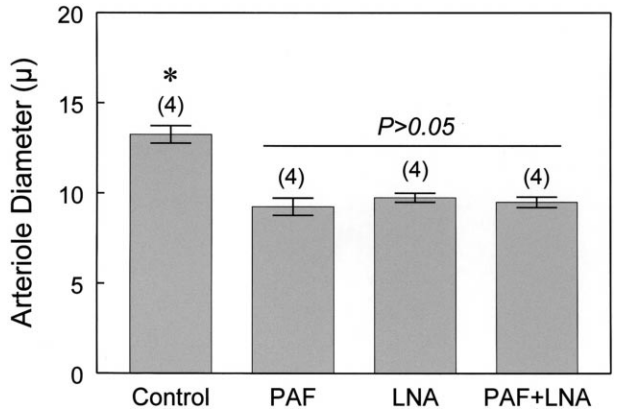

Fig. 4. Arteriolar vasoconstriction induced by PAF + L-NA was no greater than by either PAF (100 nM) or L-NA (1 μM) alone. Vertical bars represent S.E.; number in parentheses is number of animals. * Indicates $P < 0.05$ compared to all other groups. There was no significant difference ($P > 0.05$) among PAF, L-NA, and PAF + L-NA-treated arterioles.

duced leakage was 48 ± 7% TPI. This compared to −1 ± 5 for the control group. In separate experiments ($n = 7$), this concentration of PAF decreased arteriolar diameters from 17.7 ± 2.8 to 9.3 ± 0.6 μm, a 43 ± 6% reduction (Fig. 3). Therefore, 100-nM PAF caused pronounced arteriolar vasoconstriction, yet at the same time, caused a large leakage of FITC-dextran. A lower concentration of PAF (10 nM) did not cause reproducible leakage, although it reduced arteriolar diameters by 45 ± 7% ($n = 3$).

PAF-induced leakage (48 ± 7% TPI) was significantly reduced to 12 ± 8% TPI by treatment with L-NA (1 μM) 20 min prior to the application of PAF (Fig. 2). L-NA alone, however, had no effect on baseline leakage. In separate experiments ($n = 12$), 1 μM L-NA reduced arteriolar diameters from 17.5 ± 2.1 to 10.5 ± 0.9 μm, a 37 ± 4% diameter reduction (Fig. 3).

To determine if the NO donor SNAP would reverse the effects of L-NA on PAF-induced leakage, 10 μM SNAP was applied to the cheek pouch at the same time as PAF (20 min after L-NA treatment). The addition of SNAP to the PAF restored PAF-induced leakage in the presence of L-NA, thereby effectively reversing the effects on L-NA (Fig. 2). By itself, SNAP had no significant effect on baseline leakage, nor did it alter PAF-induced leakage (Fig. 2). In separate experiments ($n = 3$), 10 μM SNAP was found dilate arterioles from 11.7 ± 1.9 to 17.7 ± 2.0 μm, a 55 ± 11% increase in diameter (Fig. 3). This was similar to the vasodilation produced by 100-μM adenosine, which increased diameters by 51 ± 12% in the same arterioles. SNAP was also able to completely reverse vasoconstriction caused by L-NA and achieve the same magnitude of arteriolar vasodilation in the presence of L-NA as it did in the absence of L-NA (SNAP-induced change in diameter was 6.3 ± 0.5 μm pre-L-NA versus 7.0 ± 0.8 μm post-L-NA; $n = 4$) (Fig. 3).

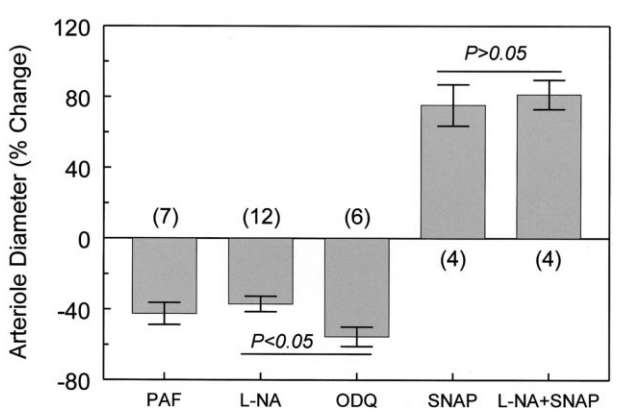

Fig. 3. Effects of PAF (100 nM), L-NA (1 μM), ODQ (10 μM) and SNAP (10 μM) on arteriolar diameters. The percentage change for the L-NA + SNAP is expressed relative to pre-L-NA diameter, the same as for SNAP alone. Vertical bars represent S.E.; number in parentheses is number of animals.

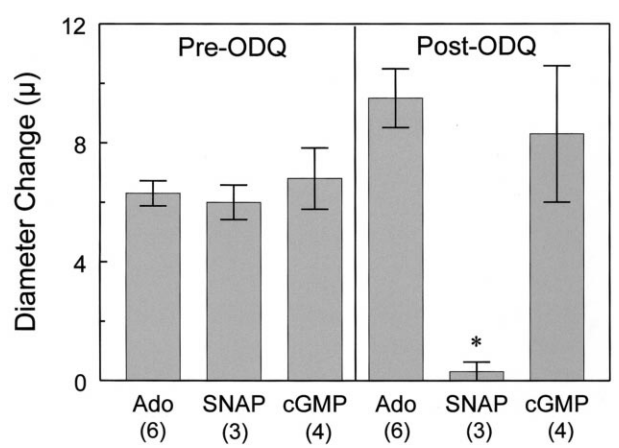

Fig. 5. Effects of guanylyl cyclase inhibition by ODQ (10 μM) on adenosine (100 μM), SNAP (10 μM), and 8-br-cGMP (10 μM)-induced vasodilation. Vertical bars represent S.E.; number in parentheses is number of animals. * Indicates $P < 0.05$ compared to Pre-ODQ.

Because both PAF and L-NA caused significant arteriolar vasoconstriction, it was important to determine if the effects of these two vasoconstrictors were additive at the concentrations tested. In separate experiments ($n = 4$), we measured arteriolar responses to PAF (100 nM), followed by 20 min of L-NA superfusion (1 μM), then reapplication of PAF. In these experiments, arteriolar vasoconstriction was the same for PAF, L-NA and PAF + L-NA (Fig. 4). Therefore, L-NA and PAF together did not constrict arterioles beyond that which was observe with either PAF or L-NA alone.

The FITC-leakage experiments demonstrated that NOS inhibition by L-NA greatly attenuated PAF-induced leakage. In another series of experiments, we examined further the role of the NO pathway in PAF-induced leakage by using an inhibitor of guanylyl cyclase. We reasoned that guanylyl cyclase inhibition should give the same results as NOS inhibition. We found in preliminary experiments that the guanylyl cyclase inhibitor, ODQ (10 μM), constricted arterioles from 15.0 ± 2.7 to 7.0 ± 1.1 μm ($n = 4$), which was significantly greater than L-NA alone (55 ± 6% versus 37 ± 4% decrease) (Fig. 3). It is important to note that ODQ completely abolished SNAP-induced vasodilation, but not adenosine-induced vasodilation (Fig. 5). Furthermore, 8-br-cGMP reversed the vasoconstrictor effects of ODQ. These experiments with SNAP and ODQ demonstrated the specificity of ODQ for blocking the NO pathway.

When the cheek pouch microcirculation was exposed to 10 μM ODQ for 20 min, PAF-induced leakage was completely inhibited (Fig. 6). Neither ODQ nor its vehicle caused leakage. The effects of ODQ on PAF-induced leakage were not reversed by topical application of 8-br-cGMP (10 μM) (Fig. 5).

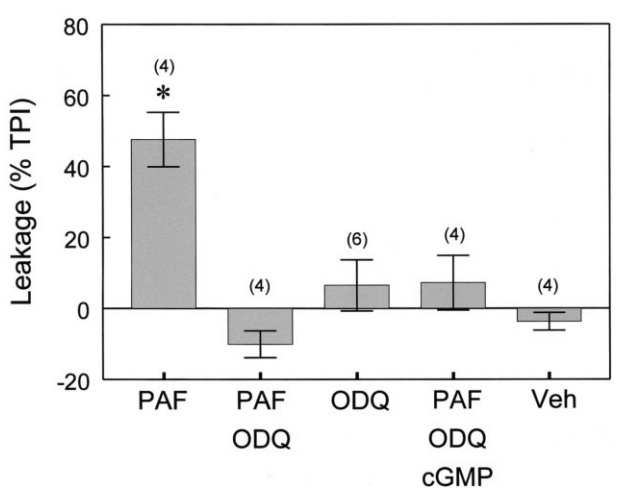

Fig. 6. Effects of guanylyl cyclase inhibition by ODQ (10 μM) on PAF (100 nM)-induced leakage. The 8-br-cGMP (10 μM) did not reverse the effects of ODQ. Vertical bars represent S.E.; number in parentheses is number of animals. * Indicates $P < 0.05$ compared to all other groups.

4. Discussion

This study was designed to examine how NO and PAF interact to cause microvascular leakage of FITC-dextran in the hamster cheek pouch microcirculation. Specifically, we wanted to determine if NO directly causes microvascular leakage or indirectly promotes leakage by serving an obligatory role in PAF-induced leakage. We found that PAF-induced leakage was significantly attenuated by pretreatment with a NOS inhibitor (L-NA). Furthermore, the addition of a NO donor (SNAP) to the PAF reversed the inhibitory effects of L-NA. Pretreatment with an inhibitor of guanylyl cyclase (ODQ) also abolished PAF-induced leakage, thereby supporting the results with the NOS inhibitor. Therefore, an intact NO/cGMP pathway is necessary for PAF to cause microvascular leakage, but NO does not appear to be the mediator of PAF-induced leakage because SNAP alone did not cause leakage of FITC-dextran.

This study was conducted because we had previously shown that both a PAF receptor antagonist (A-87648) and NOS inhibitor (L-NAME) abolished FITC-dextran leakage in the hamster cheek pouch microcirculation following systemic administration of endotoxin (LPS) (Laniyonu et al., 1997). Two possible explanations for this finding were (1) LPS released PAF, which in turn stimulated NOS production of NO that caused microvascular leakage, or (2) that a background level of NO was necessary (obligatory) for PAF to cause leakage. To investigate these possible mechanisms, we needed to determine the independent and interdependent effects of PAF and NO on leakage.

The independent effects of PAF and NO were evaluated by superfusing the cheek pouch microcirculation with either PAF or a NO donor (SNAP). PAF (100 nM) produced leakage of FITC-dextran similar to that demonstrated in other laboratories (Bjork and Smedegard, 1983; Dillon and Duran, 1988; Bekker et al., 1988; Kubes et al., 1990; Ramirez et al., 1995; Tomeo and Duran, 1991). This concentration of PAF caused leakage despite intense arteriolar constriction. PAF-induced arteriolar constriction is commonly observed in the hamster cheek pouch circulation (Bjork and Smedegard, 1983; Ramirez et al., 1995; Tomeo and Duran, 1991); other microcirculation models also demonstrate PAF-induced vasoconstriction (Kubes et al., 1990; Schwappach et al., 1995). We chose to use 100-nM PAF because it produces maximal leakage in the hamster cheek pouch (Ramirez et al., 1995). Although PAF was applied for only 10 min, it caused rapid leakage that was maximal within 45 min after starting PAF superfusion. The time course of the leakage was similar to that observed when LPS was administered either systemically or superfused onto the hamster cheek pouch (Klabunde and Calvello, 1995; Laniyonu et al., 1997). PAF-induced leakage is due to an increase in capillary and venular permeability (Bjork and Smedegard, 1983; Humphrey et al., 1984; Bekker et al., 1988). Unlike PAF, superfusing

the cheek pouch with SNAP did not cause leakage of FITC-dextran. We know that SNAP (or NO released by the SNAP) was able to diffuse into the tissue from the superfusate solution because it elicited arteriolar vasodilation similar in magnitude to 100-μM adenosine. Therefore, because NO alone did not cause leakage, PAF-induced leakage is not mediated by NO as suggested by others (Ramirez et al., 1995).

Our finding that the NO donor SNAP does not cause leakage of FITC-dextran despite a large degree of vasodilation adds to the conflicting picture presented in the literature regarding the role of NO (and cGMP) in modulating vascular permeability (Kubes, 1995). There are studies suggesting that NO reduces permeability (Hutcheson et al., 1990; Filep and Foldes-Filep, 1993; Kubes, 1993; Filep et al., 1993; Kubes et al., 1995; Gaboury et al., 1996; Harris, 1997; Yoshikawa et al., 1997; He et al., 1997b; Gimeno et al., 1998b) and other studies suggesting that NO increases permeability (Mayhan, 1992, 1994; Yuan et al., 1993; Ramirez et al., 1995; Laniyonu et al., 1997; He et al., 1997a; Gimeno et al., 1998a,b) or has no effect (Feletou et al., 1996). As pointed out by Kubes (1995), the reason for these disparate results is not clear although differences in species, organ, isolated vessels versus in situ observations, leukocyte involvement, hemodynamic factors, and inflammatory versus non-inflammatory conditions should be considered. Other investigators have shown that NO produced by ecNOS inhibits leakage while NO produced after iNOS activation increases microvascular permeability (Filep et al., 1997; Bernareggi et al., 1997). In our study, the short duration of the experiments and the observation that PAF-induced leakage occurred within several minutes, suggests that NO was being produced by ecNOS. Although this NO was apparently produced by ecNOS, it served a permissive role in leakage caused by PAF.

The alternative hypothesis we tested was that NO played an obligatory role in PAF-induced leakage. To test this hypothesis, we used both NOS inhibition and guanylyl cyclase inhibition to determine if PAF-induced leakage was dependent upon an intact NO/cGMP pathway. It is well established that there is basal NOS activity in vascular endothelium (endothelial constitutive NOS, or ecNOS) that produces NO under basal conditions (Rees et al., 1990; Salter et al., 1991; Vargas et al., 1991). This NO has significant effects on vascular tone, and may affect baseline microvascular permeability (Kubes, 1995). NO activates guanylyl cyclase, which leads to the formation of cGMP, the primary second messenger system in NO-mediated vascular events (Rapoport et al., 1983). If an intact NO/cGMP pathway is required for PAF to cause leakage, then inhibiting this pathway at either ecNOS or guanylyl cyclase should prevent PAF from causing microvascular leakage.

We found that both L-NA and ODQ independently inhibited (or abolished) PAF-induced leakage. L-NA and ODQ are potent inhibitors of ecNOS (Ishii et al., 1990) and guanylyl cyclase (Moro et al., 1996; Olson et al., 1997), respectively. Our findings using L-NA and ODQ support the hypothesis that the NO/cGMP pathway has an obligatory role in PAF-induced leakage. In the case of L-NA, when SNAP was infused along with PAF, leakage was restored. This suggests that some background level of NO is necessary for PAF to cause leakage, although the NO per se is not causing the leakage. We attempted an analogous experiment using 8-br-cGMP to reverse the effects of ODQ on PAF-induced leakage; however, 8-br-cGMP was unable to reverse the inhibitory effect of ODQ on PAF-induced leakage, yet it was able to reverse the arteriolar vasoconstriction caused by ODQ. If the obligatory role of NO acts through the cGMP pathway, then 8-br-cGMP should have reversed the effects of ODQ on leakage. We have no clear explanation for our findings regarding 8-br-cGMP and leakage. What the data might be telling us is that NO is necessary for PAF-induced leakage, but it does not involved the cGMP. This interpretation, however, is only reasonable if ODQ reduced PAF-induced leakage by a mechanism unrelated to its inhibitory effect on guanylyl cyclase.

If NO has an obligatory role in PAF-mediated microvascular leakage as indicated by this study, then by what mechanism does this occur? There are two general possibilities. First, NO may be acting to permit PAF-induced leakage because of hemodynamic effects. Changes in arteriolar and venular resistance could alter capillary and post-capillary venular hydrostatic pressures and thereby affect leakage. We were unable to measure hydrostatic pressure changes, however we did measure changes in arteriolar diameter. When NO (or cGMP) production was inhibited in our study, arteriolar vasoconstriction occurred. L-NA and ODQ decreased arteriolar diameters by $37 \pm 4\%$ and $55 \pm 6\%$, respectively. These decreases were not significantly different from PAF ($43 \pm 6\%$). An increase in pre-capillary resistance may reduce capillary and venular hydrostatic pressures thereby reducing leakage. However, this hemodynamic mechanism is unlikely to have played a significant role in our results because PAF-induced leakage occurred despite intense arteriolar vasoconstriction by PAF. Furthermore, L-NA added to PAF did not cause any more arteriolar constriction than PAF alone. This would argue against L-NA inhibiting PAF-induced leakage by causing further arteriolar constriction. If leakage of FITC-dextran were highly dependent upon the state of vascular tone (and hence capillary and venular pressures), then superfusion with SNAP, which caused significant arteriolar vasodilation, should have increased leakage of FITC-dextran; however, SNAP alone did not cause leakage. A second possibility is that a background level of NO (or cGMP) is necessary (obligatory) for PAF to increase microvascular permeability.

Other studies have shown that PAF-induced macromolecular leakage is inhibited by NOS inhibitors, implying

that PAF triggers NO synthesis, which then causes leakage (Ramirez et al., 1995). These studies, however, did not determine if NO is capable of causing leakage. Our results clearly show that NO does not cause leakage; however, an intact NO/cGMP pathway is necessary for PAF-induced leakage to occur. Other studies, using NOS inhibitors, have implicated NO in causing histamine-induced leakage (Yuan et al., 1993; Mayhan, 1994), and in leakage caused by ischemia and reperfusion (Noel et al., 1996), but these studies did not determine if NO per se can cause leakage.

We do not know the mechanism for the obligatory role of NO in PAF-induced microvascular leakage. In the hamster cheek pouch, PAF has been shown bind to specific receptors and activate protein kinase C, which then results in microvascular leakage (Kobayashi et al., 1994; Ramirez et al., 1996). Phorbol esters, which activate protein kinase C, also cause leakage in the cheek pouch (Kobayashi et al., 1994) that is inhibited by NOS inhibitors (Ramirez et al., 1996). Other studies in the cheek pouch have shown that PAF-induced leakage is also linked to activation of the tyrosine kinase pathway (Kim and Duran, 1995), and activation of L-type calcium channels (Oshiro et al., 1995). PAF also leads to formation of reactive oxygen species that may cause leakage (Schwappach et al., 1995; Kurose et al., 1996). The mechanism of PAF-induced leakage is complex, and therefore it is not clear how NO or distal products (e.g., cGMP, peroxynitrite) might interact with PAF.

In summary, these results indicate that PAF-induced leakage of FITC-dextran in the hamster cheek pouch microcirculation is dependent upon a functioning NO/cGMP pathway. NO production, however, does not cause PAF-induced leakage. Therefore, these results support the hypothesis that NO plays an obligatory role in PAF-induced leakage.

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