fMLP-stimulated neutrophils increase endothelial $[Ca^{2+}]_i$ and microvessel permeability in the absence of adhesion: role of reactive oxygen species

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Zhu, Longkun, Vince Castranova, and Pingnian He. fMLP-stimulated neutrophils increase endothelial $[Ca^{2+}]_i$ and microvessel permeability in the absence of adhesion: role of reactive oxygen species. Am J Physiol Heart Circ Physiol 288: H1331–H1338, 2005. First published October 21, 2004; doi:10.1152/ajpheart.00802.2004.—Our previous study demonstrated that firm attachment of leukocytes to microvessel walls does not necessarily increase microvessel permeability (Am J Physiol Heart Circ Physiol 283: H2420–H2430, 2002). To further understand the mechanisms of the permeability increase associated with leukocyte accumulation during acute inflammation, we investigated the direct relation of reactive oxygen species (ROS) release during neutrophil respiratory burst to changes in microvessel permeability and endothelial intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in intact microvessels. ROS release from activated neutrophils was quantified by measuring changes in chemiluminescence. When isolated rat neutrophils ($2 \times 10^6$/ml) were exposed to formyl-Met-Leu-Phe-OH (fMLP, 10 µM), chemiluminescence transiently increased from 1.2 ± 0.2 x 10^4 to a peak value of 6.7 ± 1 x 10^4 cpm/min ($n = 12$). Correlatively, perfusing individual microvessels with fMLP-stimulated neutrophils in suspension ($2 \times 10^7$/ml) increased hydraulic conductivity ($L_p$) to 3.7 ± 0.4 times the control value ($n = 5$) and increased endothelial $[Ca^{2+}]_i$, from 84 ± 7 nM to a mean peak value of 170 ± 7 nM. In contrast, perfusing vessels with fMLP alone did not affect basal $L_p$. Application of antioxidant agents, superoxide dismutase, vitamin C, or an iron chelator, defereroxamine mesylate, attenuated ROS release in fMLP-stimulated neutrophils and abolished increases in $L_p$. These results indicate that release of ROS from fMLP-stimulated neutrophils increases microvessel permeability and endothelial $[Ca^{2+}]_i$, independently from leukocyte adhesion and the migration process.

ACUTE INFLAMMATION INVOLVES the initial release of proinflammatory mediators followed by the activation and mobilization of circulating leukocytes to the inflammatory sites. Although leukocyte recruitment provides the first line of defense in protecting the host from invading pathogens, it also causes host tissue injury and microvascular dysfunction. Extensive investigations have focused on the mechanisms of neutrophil-induced endothelial injury and protein leakage from microvessel walls. However, because of the complex nature of the process, the critical step leading to protein leakage and tissue injury remains obscure. Leukocyte adhesion is the first step in granulocyte migration from the vascular lumen to the interstitial space and has been implicated as a prerequisite for leukocyte-induced endothelial injury (6, 23). Some in vitro studies reported that firm adhesion is necessary to trigger the respiratory burst of neutrophils to release free radicals and/or proteases, resulting in endothelial injury (11, 25, 26). In contrast, other studies demonstrated that leukocyte adhesion did not occur at exactly the same sites as plasma leakage (2, 3, 20) and that activated leukocytes could increase vascular permeability in the absence of adhesion (29). Our previous study demonstrated that TNF-α-induced firm attachment of leukocytes to microvessel walls did not cause an increase in microvessel permeability (32). Because the direct physical contact between leukocytes and endothelium is not the critical step resulting in vascular protein leakage, we hypothesize that the generation/release of respiratory burst products upon leukocyte activation increases microvessel permeability independently from leukocyte adhesion.

Among multiple products generated or released during the neutrophil respiratory burst, reactive oxygen species (ROS) generated by a membrane-bound NADPH oxidase are considered to be important components (1, 9, 31). ROS-mediated injury to host tissues has been implicated in a number of animal models and human diseases (7, 10, 12, 14, 28). However, the direct relation of ROS production by activated neutrophils in the absence of adhesion to microvessel permeability has not been well defined. Therefore, the objective of this study is to provide a quantitative correlation between ROS production upon neutrophil activation and the changes in microvessel permeability and endothelial intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in the absence of leukocyte adhesion in intact microvessels. The effect of fMLP-stimulated neutrophils on microvessel permeability was investigated by measuring changes in chemiluminescence. The changes in endothelial $[Ca^{2+}]_i$ were measured with a fluorescent $Ca^{2+}$ indicator, fura 2, under the same experimental conditions applied for $L_p$ measurements.

MATERIALS AND METHODS

Animal Preparation

Experiments were carried out in venular microvessels in rat mesentery. All procedures and animal use were approved by the Animal Care and Use Committees at West Virginia University. Female Sprague-Dawley rats (2–3 mo old, 220–250 g body wt; Hilltop Laboratory Animal, Scottsdale, PA) were anesthetized with pentobarbital sodium given subcutaneously. The initial dosage was 65 mg/kg

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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 rat was then transferred to a tray and kept warm on a heating pad. The mesentery was gently removed from the abdominal cavity and spread over a pillar for measurements of \(L_p\) or over a glass coverslip for measurements of endothelial \([Ca^{2+}]_i\). The upper surface of the mesentery was continuously superfused with mammalian Ringer solution during preparation and experimentation. The temperature of the superfusate was maintained at 37°C and was monitored by a thermometer probe and regulated by a digital controlled water bath. All experiments were carried out in venular microvessels, which are classified as segments where there is convergent flow, two to four branches distal from true capillaries. Blood flow was brisk in all vessels selected for experiments, and there were no more than two adherent leukocytes per 100 \(\mu m\) of the vessel wall.

Isolation of Rat Neutrophils

Blood was collected from male (350–400 g) and female (250–300 g) Sprague-Dawley rats by catheterization through the carotid artery and anticoagulated with 3.8% sodium citrate (9:1 vol/vol). Neutrophils were isolated from whole blood by means of a two-step discontinuous Percoll gradient centrifugation method. The densities of the Percoll gradient were 1.083 and 1.102 g/ml. Whole blood (4 ml) was gently layered on the Percoll gradient (3 ml of each density) and centrifuged at 1,000 \(g\) for 30 min at 20°C. The polymorphonuclear leukocyte (PMN) band plus all volume between the lower band and the red blood cell layer was collected. Residual erythrocytes were lysed using a lysing reagent (PharM Lyse). PMNs were then washed twice with \(Ca^{2+}/Mg^{2+}\)-free phosphate-buffered saline (pH 7.4). The pellets (>95% neutrophils) were resuspended in albumin-Ringer solution (10 mg/ml) and placed on ice until use. The viability, evaluated with trypan blue exclusion test, was >97%.

Measurement of Chemiluminescence

ROS production from fMLP-stimulated neutrophils was determined by measuring cellular chemiluminescence using a luminometer (AutoLumat LB953). Neutrophils were preincubated at 37°C for 10 min before the chemiluminescence measurement. Each sample (0.25 ml) contained 0.5 \(\times \) 10° neutrophils with 0.8 \(\mu\)M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or 10 \(\mu\)M isoluminol in the presence of 4 \(\mu\)l horseradish peroxidase (24). For measurements involving an antioxidant agent or an ion chelator, each agent was added before the incubation. Because of the transient nature of the response, the reaction to fMLP was initiated after incubation and immediately before the measurement. Chemiluminescence was measured sequentially in a group of samples and continuously recorded for 10 min. The peak maximum counts per minute (cpm/min per 0.5 \(\times \) 10° neutrophils) was used to compare the chemiluminescence changes from the baseline sample.

Measurement of \(L_p\)

All measurements were based on the modified Landis technique, which measures the volume flux of water across the microvessel wall (8). The assumptions and limitations of the original method and its application in mammalian microvessels have been evaluated in detail elsewhere (8, 21). Briefly, a single venular microvessel was cannulated with a glass micropipette and perfused with albumin-Ringer solution (control) containing 1% (vol/vol) rat red blood cells as markers. A hydrostatic pressure (range 50–80 cmH\(_2\)O), controlled by a water manometer, was applied through the micropipette to the microvessel lumen. A charge-coupled device camera was connected to the microscope, and a video was continuously recorded from a segment of the perfused microvessel (400 \(\mu m\) long) throughout each experiment for data analysis. The initial water flow per unit area of microvessel wall \((J_w/S)_0\), where \(J_w\) is water flux and \(S\) is 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 length between the marker cell and the occlusion site. Any vessel diameter changes can be precisely measured from the video images, which are incorporated into the \(L_p\) calculation. To avoid the effect of vessel compliance on the marker cell movement, the velocity of the marker cell was calculated after 2 s of occlusion. Microvessel \(L_p\) was calculated from the Starling equation: \(L_p = (J_w/S)/\Delta P\), where \(\Delta P\) is the effective hydrostatic and oncotic pressure difference across the microvessel wall. On the basis of experimental data and theoretical estimations, the tissue hydrostatic and oncotic pressures are assumed to be negligible (19, 21). \(\Delta P\) represents the pressure difference between the hydrostatic pressure applied to the microvessel and the effective oncotic pressure generated from the albumin in the perfusate (BSA, at 10 mg/ml, has effective oncotic pressure of 3.6 cmH\(_2\)O). In each experiment, baseline \(L_p\) and the stimulus-induced \(L_p\) changes were measured in the same vessel, which allows the changes to be compared with its own control. It minimizes the variations between vessels and between animals and is a more powerful experimental design than statistics where comparisons are conducted between two groups of vessels or animals. If \(L_p\) was relatively constant throughout the time course, the mean \(L_p\) for each perfusate was calculated from all the occlusions during that perfusion period. If a transient increase in \(L_p\) was observed, \(L_p\) is reported as the means of peak and sustained values.

Measurements of Endothelial \([Ca^{2+}]_i\)

The fluorescent \(Ca^{2+}\)-indicator fura 2-AM was used to measure endothelial \([Ca^{2+}]_i\), in individually perfused microvessels. Experiments were carried out on a Nikon Diaphot 300 microscope equipped with a Nikon photometry system. Details have been described elsewhere (18). Briefly, a venular microvessel in rat mesentery was cannulated and perfused with albumin-Ringer solution containing 10 \(\mu\)M fura 2-AM for 45 min in the dark. Then the vessel was recannulated and perfused with albumin-Ringer solution for 10 min to remove all the fura 2-AM from the vessel lumen and tissue. Fluorescence intensity (FI) was collected by a dry Nikon Fluor lens \((\times 20\), numerical aperture 0.75) from a measuring window \((150 \times 50 \mu m)\) placed \(~100 \mu m\) downstream from the cannulation site of the vessel. The excitation wavelengths for fura 2 were selected by two narrow-band interference filters (Oriel, 340 ± 5 and 380 ± 5 nm), and the emission was separated with a dichroic mirror (DM400) and a wide-band interference filter (Oriel, 500 ± 35 nm). We estimated that the fluorescent signals collected in the measuring window were from ~50 endothelial cells forming the segment of the vessel wall. The excitation wavelength alternated between 340 and 380 nm, and FI340 and FI380 were collected with a 0.25-s exposure at each wavelength. At the end of each experiment, the microvessel was superfused with a modified Ringer solution \((5 \text{mM} \text{Mn}^{2+}\) without \(Ca^{2+}\)) during perfusion with the same solution containing ionomycin \((10 \mu\)M). This procedure bleaches the \(Ca^{2+}\)-sensitive form of fura 2. The background FI due to unconverted fura 2-AM and other \(Ca^{2+}\)-insensitive forms of fura 2 were recorded at both wavelengths and subtracted from FI340 and FI380. The ratios \((R)\) of the two FIs were calculated and normalized with \(R_{\text{min}}\), i.e., \(R = (\text{FI340} - \text{FI380})/(\text{FI380} - \text{B380})\). This in vitro ratio of FI340 to FI380 at zero \(Ca^{2+}\) concentration after correction of the background fluorescence \((\text{FI340 or B380})\), was measured at the end of each experiment to compensate for daily variations of lamp intensity. The normalized ratios were converted to \(Ca^{2+}\) concentrations with an in vitro calibration curve. Detailed procedures for calibration have been described previously (18).

Experimental Protocols

Measurements of chemiluminescence. Neutrophils obtained from 12 rats were used for 12 chemiluminescence assays (1 assay per rat). Each assay included five or six samples obtained from the same rat but
were treated under different experimental conditions (control, fMLP-stimulated neutrophils, and fMLP-stimulated neutrophils that were pretreated with an antioxidant agent). Each experimental condition was repeated 12 times in samples obtained from 12 different animals. The donor rats comprised 6 males and 6 females. There was no significant difference in neutrophil responses to fMLP between male and female rats.

**Measurements of Lp.** Lp was measured in 40 microvessels from 40 rats (1 vessel per rat). In each experiment, the control Lp was measured with albumin-Ringer perfusate. Then the same vessel was recannulated with another pipette containing the testing solution (e.g., the suspension of fMLP-stimulated neutrophils), and the changes in Lp were measured immediately. The fMLP reaction with neutrophils was initiated immediately before each recannulation, the same procedure used for chemiluminescence measurements. No neutrophil adhesion occurred during neutrophil perfusion, which was verified by direct visualization under the microscope and video recordings. The effect of fMLP-stimulated neutrophils on microvessel Lp was investigated in 26 microvessels, in which the effect of fMLP alone was studied in 7 vessels, and fMLP-stimulated neutrophils at 4 different concentrations (see Results) were studied in another 19 microvessels. The effects of antioxidant agents, superoxide dismutase (SOD, n = 4), vitamin C (n = 5), or an iron chelator, deferoxamine mesylate (DFO, n = 5), were studied in another 14 microvessels.

**Measurements of endothelial [Ca2+]i.** Ca2+ measurements were conducted in eight microvessels from eight animals (1 vessel per animal). The experimental conditions were the same as those described for Lp measurements. The fMLP-stimulated neutrophil-induced [Ca2+]i changes and the effect of fMLP alone on endothelial [Ca2+]i were studied in four of the microvessels, and the paired measurements with fMLP-stimulated neutrophils in the presence and absence of SOD were studied in another four microvessels.

**Solutions and Reagents**

Mammalian Ringer solution was used for dissecting mesenteries, superfusing tissue, and preparing the perfusion solutions. The composition of the mammalian Ringer solution was (in mM) 132 NaCl, 4.6 KCl, 2 CaCl2, 1.2 MgSO4, 5.5 glucose, 5.0 NaHCO3, and 20 HEPES and Na-HEPES; pH of the Ringer solution was maintained at 7.40–7.45 by adjustment of the ratio of Na-HEPES to HEPES. All perfusates used for control and test perfusion contained BSA (10 mg/ml).

The chemotactic peptide fMLP was purchased from Calbiochem (San Diego, CA), Percoll from ICN Biomedicals (Costa Mesa, CA), and all other reagents from Sigma. The stock solutions of fMLP (10 mM) and fura 2-AM (10 mM; Molecular Probes) were prepared with 100% dry dimethyl sulfoxide (DMSO). The final concentration of fMLP (10 μM) or fura 2-AM (10 μM) was achieved by 1:1,000 dilution of the stock with albumin-Ringer solution. All perfusates containing test agent were freshly prepared before each cannulation.

**Data Analysis and Statistics**

Values are means ± SE, except where noted otherwise. Changes in Lp are expressed as the ratio of testing Lp to control Lp (Lp/test/Lp/control). The mean values of Lp (control and test) measured in the same vessel were used as paired data. The significance of the differences within or between groups was evaluated by paired r-test and analysis of variance. *P < 0.05* was considered statistically significant.

**RESULTS**

**ROS Production From fMLP-Stimulated Neutrophils**

ROS production from fMLP-stimulated neutrophils was examined by measuring the generation of chemiluminescence. Figure 1A shows the time course of changes in chemilumines-

cence after neutrophils were exposed to fMLP in the absence or presence of an antioxidant agent from a single assay. Results of 12 assays from 12 different animals showed that exposure of neutrophils to fMLP transiently increased chemiluminescence from a resting level of 1.2 ± 0.2 × 104 cpm/min to a peak value of 6.7 ± 1.0 × 104 cpm/min. The chemiluminescence generation peaked 1–1.25 min after exposure to fMLP and then fell toward baseline. The increased chemiluminescence was only twice the baseline level after 6 min. When samples were preincubated with SOD (1,500 U/ml), vitamin C (1 mM), or DFO (1 mM), the peak values of chemiluminescence in response to fMLP were significantly reduced, which were 1.9 ± 0.5 × 104, 3.3 ± 0.6 × 104, and 3.3 ± 0.6 × 104 cpm/min, respectively. Figure 1B summarizes the results. To distinguish the chemiluminescence generated extracellularly from that generated intracellularly, five assays were conducted with a cell-impermeant amplifier, isoluminol. The chemiluminescence produced upon neutrophil activation increased from 10.8 ± 0.2 × 104 cpm/min to a peak of 64.1 ± 1.3 × 104 cpm/min. The magnitude of chemiluminescence increase was not significantly different from that measured with luminol. In
addition, the application of SOD completely inhibited the chemiluminescence increase measured with isoluminol.

Effect of fMLP-Stimulated Neutrophils on Microvessel Permeability

To examine whether the release of ROS from fMLP-stimulated neutrophils causes a permeability increase in the absence of neutrophil adhesion, \( L_p \) was measured when individually cannulated microvessels were perfused with fMLP-stimulated suspended neutrophils at \( 2 \times 10^5 \text{ to } 2 \times 10^7 \text{ cells/ml} \). The mean control \( L_p \) values of five different series of experiments (total 26 vessels) were not significantly different from each other: 1.8 – 2.2 \( \times \) 10\(^{-7} \) cm\( \cdot \)s\(^{-1} \) \( \cdot \)cmH\(_2\)O\(^{-1} \). No significant changes in \( L_p \) were observed when microvessels were perfused with fMLP (10 \( \mu \)M) alone (in the absence of neutrophils, \( n = 7 \)) or with 2 \( \times \) 10\(^5 \) cells/ml fMLP-stimulated neutrophils (\( n = 5 \)). Significant increases in \( L_p \) were observed when vessels were exposed to fMLP-stimulated neutrophils at \( 2 \times 10^6 \) cells/ml. Figure 2A shows a typical time course of \( L_p \) changes in a single experiment. All peak increases in \( L_p \) occurred within 2 min of exposure to activated neutrophils. The magnitude of the peak increase in \( L_p \) was neutrophil concentration dependent (Fig. 2B).

The mean peak \( L_p \) was 2.5 \( \pm \) 0.1 times the control level when microvessels were exposed to 2 \( \times \) 10\(^6 \) activated neutrophils/ml (\( n = 5 \)). It increased to 3.7 \( \pm \) 0.4 and 3.8 \( \pm \) 0.4 times the control value when microvessels were exposed to 2 \( \times \) 10\(^7 \) (\( n = 5 \)) and 2 \( \times \) 10\(^8 \) activated neutrophils/ml (\( n = 4 \)), respectively.

Effect of Antioxidant Agents on Activated Neutrophil-Induced Increases in Microvessel Permeability

Measurements of chemiluminescence demonstrated that SOD, DFO, or vitamin C significantly inhibited the ROS production from fMLP-stimulated neutrophils (Fig. 1). To investigate whether the release of ROS is responsible for the \( L_p \) increase after perfusion of the vessels with fMLP-stimulated neutrophils, we measured the changes in \( L_p \) after perfusing microvessels with fMLP-stimulated neutrophils that were pretreated with DFO (1 \( \text{mM} \)), SOD (15,000 U/ml), or vitamin C (1 \( \text{mM} \)) before exposure to fMLP (10 \( \mu \)M). A: individual experiments. B: summary results. *Significant increase from negative control (\( P < 0.05 \)). †Significant decrease from positive controls (\( P < 0.05 \)).
fMLP-Stimulated Neutrophils Increase Endothelial [Ca$^{2+}$]$_{i}$

To investigate whether an increase in endothelial [Ca$^{2+}$]$_{i}$, was associated with activated neutrophil-induced $L_p$ increases, endothelial [Ca$^{2+}$]$_{i}$ was measured under the same experimental conditions applied for $L_p$ measurements. In four microvessels, the mean baseline endothelial [Ca$^{2+}$]$_{i}$ was 84 ± 7 nM. When each vessel was perfused with fMLP-stimulated neutrophils in suspension (2 × 10$^7$ cells/ml), [Ca$^{2+}$]$_{i}$ transiently increased to a mean peak value of 170 ± 7 nM and declined to 131 ± 13 nM in 20 min. The effect of fMLP alone on endothelial [Ca$^{2+}$]$_{i}$ was also examined in the same four vessels. After washout of activated neutrophils with Ringer-albumin solution, the mean [Ca$^{2+}$]$_{i}$ was 91 ± 6 nM. Perfusion of fMLP alone induced a small brief increase in [Ca$^{2+}$]$_{i}$. The mean peak value was 106 ± 8 nM and returned to the control level within 5 min. This magnitude of [Ca$^{2+}$]$_{i}$ increase did not cause a significant permeability change as shown by $L_p$ measurement. Figure 4A shows the time course of a single experiment, and Fig. 4B summarizes the results from four microvessels.

Effect of ROS Scavenger on Activated Neutrophil-Induced Increases in Endothelial [Ca$^{2+}$]$_{i}$

To investigate whether the ROS released from fMLP-stimulated neutrophils account for the increases in endothelial [Ca$^{2+}$]$_{i}$, paired measurements were conducted in the same vessel to compare the magnitude changes in endothelial [Ca$^{2+}$]$_{i}$ when each vessel was exposed to fMLP-stimulated neutrophils in the presence and absence of SOD. In four microvessels, the mean baseline endothelial [Ca$^{2+}$]$_{i}$ was 73 ± 5 nM. When each vessel was perfused with fMLP-stimulated neutrophils that were pretreated with SOD (1,500 U/ml), [Ca$^{2+}$]$_{i}$ transiently increased to a mean peak value of 134 ± 5 nM within 5 min and fell to 94 ± 8 nM in 15 min. After washout of the vessel lumen with albumin-Ringer solution for 20 min, the mean [Ca$^{2+}$]$_{i}$ was 76 ± 7 nM. Then each vessel was recannulated with fMLP-stimulated neutrophils in the absence of SOD. [Ca$^{2+}$]$_{i}$ increased to a mean peak value of 192 ± 8 nM in 5 min and fell to 130 ± 10 nM in 15 min (Fig. 5). SOD significantly attenuated the [Ca$^{2+}$]$_{i}$ increase induced by fMLP-activated neutrophils. Part of the small increases in [Ca$^{2+}$]$_{i}$ in the presence of SOD should be attributed to the effect of fMLP alone on endothelial [Ca$^{2+}$]$_{i}$, (the mean peak value of fMLP alone was 106 ± 8 nM).

DISCUSSION

This study demonstrated a direct relation between neutrophil activation, ROS release during the respiratory burst, and cor-
relative changes in endothelial $[Ca^{2+}]$, and microvessel permeability in intact microvessels.

Many animal models and clinical evidence have implicated a role for neutrophils in tissue injury (16, 22, 27). Some studies indicated that the physical contact between leukocytes and endothelium is the critical step for endothelial barrier damage resulting in protein leakage and tissue edema (6, 23). Others reported that the adhesion process was the trigger for the resulting in protein leakage and tissue edema (6, 23). However, our previous study on TNF-α-induced leukocyte adhesion and microvessel permeability in vivo demonstrated that systemic application of TNF-α induced significant leukocyte adhesion without a measurable increase in $L_p$ or solute permeability (32). Those results indicated that leukocyte adhesion to microvessel walls does not necessarily induce increases in microvessel permeability. Thus the mechanisms that regulate the adhesion process may act independently from mechanisms of permeability increases associated with leukocyte accumulation. Our results are consistent with evidence reported by Baluk et al. (3) that 94% of the gap formations in rat tracheal mucosa were distinct from sites of leukocyte adhesion or migration in the leaky venules. These findings challenged the roles of leukocyte adhesion in vascular injury, indicating that the critical step for leukocyte-dependent permeability increases during acute inflammation remained undefined. A recent study reported that C5a-activated neutrophils increased permeability in isolated coronary venules in the absence of adhesion (29), but the direct cause of the permeability increase has not been further explored. Therefore, the goal of the present study was to elucidate the mechanisms of neutrophil-induced permeability increase.

ROS release upon neutrophil activation has been studied for a few decades. ROS-induced tissue injury has been linked to pathogenesis of many human diseases (7, 14, 28). However, a direct correlation between ROS released from activated neutrophils and increases in microvessel permeability has not been established. The present study is the first investigation to demonstrate in individually perfused intact microvessels that ROS released from fMLP-stimulated neutrophils is associated with increased endothelial $[Ca^{2+}]$, and microvessel permeability independently from neutrophil adhesion and migration.

To identify the direct cause of the permeability increases upon perfusion of vessels with fMLP-stimulated neutrophils in suspension, we first distinguished the effect of fMLP alone on endothelial cell $[Ca^{2+}]$, and microvessel $L_p$ from that of fMLP-stimulated neutrophils. Perfusing vessels with fMLP alone caused a short-lived small increase in endothelial $[Ca^{2+}]$, that was significantly smaller and less sustained than the $[Ca^{2+}]$, increase induced by fMLP-activated neutrophils. In addition, this small-magnitude increase in endothelial $[Ca^{2+}]$, in response to fMLP alone did not cause an increase in microvessel $L_p$. This is consistent with our previous finding that increased endothelial $[Ca^{2+}]$, needs to reach a threshold to elicit an increase in microvessel permeability (17). Because fMLP alone did not change the basal $L_p$, the permeability increases observed with perfusion of fMLP-stimulated neutrophils appear to be due to factors produced by activated neutrophils. Our results demonstrated that the time course of the $L_p$ increases correlated well with the time course of chemiluminescence generated from activated neutrophils. Inhibition of ROS production by ROS scavenger or antioxidant agents abolished or attenuated the $L_p$ increases, providing further evidence that the increases in microvessel permeability are directly linked with the release of ROS from activated neutrophils.

The respiratory burst upon neutrophil activation involves the generation or release of various ROS and their derivatives. Because chemiluminescence was measured as an index of ROS generation from activated neutrophils, it is important to understand which oxygen species are measured by the chemiluminescence reaction and which species are critical for the permeability increases. Superoxide anion has been reported as the initial product generated through the NADPH oxidase-mediated respiratory burst upon neutrophil activation by fMLP (4, 5, 9). The released superoxide anion may spontaneously disproportionate to $H_2O_2$, which further generates hydroxyl radicals and other toxic substances. There is also a possibility that hydroxyl radical is formed directly from superoxide anion through metal ion oxidation without $H_2O_2$ as a precursor (15). The chemiluminescence reaction measures the light generated during energy release from ROS. $H_2O_2$, the relatively stable reactive oxygen metabolite, was reported not to participate in the light-generating reaction (24). Our results that fMLP-induced chemiluminescence activity in neutrophils was almost completely inhibited by the superoxide scavenger SOD also indicate that the chemiluminescence activity is directly linked with the generation of superoxide, instead of $H_2O_2$. Because the magnitude and time course of fMLP-induced chemiluminescence is directly correlated with the changes in $L_p$, we conclude that superoxide anion and superoxide-derived oxidant radicals are the main components responsible for the permeability increases induced by fMLP-stimulated neutrophils.

Our experimental results also indicated that the extracellularly released oxygen species are responsible for the increases in microvessel permeability. Luminol, used as an amplifier in chemiluminescence measurement, is membrane permeable. Thus the chemiluminescence reaction with luminol is a measure of intracellularly and extracellularly generated reactive species. To distinguish the dominant source of the chemiluminescence generated upon neutrophil activation by fMLP, we examined the chemiluminescence reaction with another amplifier, isoluminol, which is considered impermeant and specifically measures extracellular light activity (24). The magnitude increase in chemiluminescence upon neutrophil activation measured with isoluminol was similar to that measured with luminol, which suggests that the extracellularly released ROS significantly contributed to the chemiluminescence response.

Of course, in addition to neutrophil-derived oxygen metabolites, activated neutrophils may release other factors, such as proteolytic enzymes, which may also affect microvessel permeability. We are unable to completely eliminate their potential effects. However, if we consider the immediate, transient increases in $L_p$, it is unlikely that proteolytic enzymes play a dominant role. A delayed longer time course of the $L_p$ increases would be expected from such an enzyme effect. We reported previously that fMLP-induced leukocyte migration in intact microvessels does not cause a measurable increase in microvessel permeability (32). In that study, we focused on the effect of fMLP-induced leukocyte migration on microvessel permeability. We first induced leukocyte adhesion with systemic TNF-α application and then applied fMLP to the superfusate to induce the migration of adherent leukocytes across the microvessel wall. $L_p$ was measured after 10 min of fMLP...
application with a significant number of migrated leukocytes, and no changes in $L_p$ were found. These results indicate that leukocyte adhesion-associated local release of proteases and leukocyte migration-associated local mechanical disruption of endothelial barrier are not significant factors in increased microvessel permeability.

Although ROS-induced tissue damage is thought to contribute to a number of human diseases, the actual molecular mechanisms remain obscure. In recent years, accumulating evidence has suggested that ROS are no longer merely considered injurious by-products of metabolism but are also essential participants in cell signaling to regulate cellular functions (28). We believe that the transient increase in microvessel permeability induced by ROS release from activated neutrophils is more likely due to the role of ROS as a mediator activating signaling pathways in endothelial cells than as toxic by-products resulting in direct damage to the cell membrane or proteins. Even though detailed cellular mechanisms need to be explored further, the increases in endothelial [Ca$^{2+}$]i in response to activated neutrophils provide the first evidence to support this hypothesis.

ROS-involved changes in cellular Ca$^{2+}$ homeostasis have been reported by numerous studies, most of which were conducted in vitro with exogenously applied ROS as stimuli. It was reported that the toxicity of high levels of ROS could lead to a massive, steady influx of extracellular Ca$^{2+}$, whereas low concentrations of ROS induce only transient Ca$^{2+}$ changes, thus appearing to act as signaling agonists (30). The measurements of endothelial [Ca$^{2+}$]i, after perfusion of vessels with fMLP-stimulated neutrophils demonstrated that ROS-induced increases in microvessel permeability are associated with transient increases in endothelial [Ca$^{2+}$]i. The magnitude and time course of the increases in [Ca$^{2+}$]i are similar to responses to other inflammatory mediators that induce increases in microvessel permeability. The significant attenuation of the [Ca$^{2+}$]i increases by SOD (134 ± 5 nM with SOD vs. 192 ± 8 nM without SOD) suggested that the released superoxide upon neutrophil activation was the main cause for the [Ca$^{2+}$]i increases. When vessels were exposed to SOD-pretreated neutrophils with fMLP stimulation, the small-magnitude increase in [Ca$^{2+}$]i was slightly higher than the effect of fMLP alone (106 ± 8 nM), which might be caused by other releasing agents associated with neutrophil activation. However, this magnitude of [Ca$^{2+}$]i increase was not associated with a significant permeability change, as shown by $L_p$ measurement (Fig. 3A). Although further studies are needed to identify the exact sensing and signaling pathways that lead to increased microvessel permeability by ROS, these transient intracellular Ca$^{2+}$ changes suggest that ROS do not directly damage endothelial cells but, rather, may act as the mediator that initiates the Ca$^{2+}$-dependent signaling pathways, resulting in increases in microvessel permeability.

In summary, neutrophil activation by fMLP was associated with a transient release of ROS, which was abolished or inhibited by antioxidant agents or an iron chelator, such as SOD, vitamin C, or DFO. Perfusion of fMLP-stimulated suspended neutrophils caused a correlated increase in microvessel $L_p$, which was independent from the adhesion process. Pretreatment of neutrophils with DFO, SOD, or vitamin C before exposure to fMLP abolished or attenuated the $L_p$ increases, indicating that increases in microvessel permeability were directly related to the ROS release. Stimulated neutrophils also increased endothelial [Ca$^{2+}$], initiating Ca$^{2+}$-dependent signaling pathways, which may be responsible for increasing microvessel permeability. The antioxidant agents efficiently inhibited ROS release and prevented permeability increase and attenuated the increases in endothelial [Ca$^{2+}$]i. Our results suggested that these antioxidant agents may have therapeutic potential to prevent signaling-dependent increases in vascular permeability that may occur during acute inflammation.

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GRANTS

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REFERENCES


