Calcium influx-dependent differential actions of superoxide and hydrogen peroxide on microvessel permeability

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Zhou X, Wen K, Yuan D, Ai L, He P. Calcium influx-dependent differential actions of superoxide and hydrogen peroxide on microvessel permeability. Am J Physiol Heart Circ Physiol 296: H1096–H1107, 2009. First published February 6, 2009; doi:10.1152/ajpheart.01037.2008.—Our previous study demonstrated that reactive oxygen species (ROS) released from activated blood cells contribute significantly to the increased microvessel permeability during inflammation. This study aims to define the individual roles of hydrogen peroxide (H2O2) and superoxide in ROS-induced increases in permeability and endothelial intracellular Ca2+ concentration ([Ca2+]i) in individually perfused rat mesenteric venules. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). Endothelial [Ca2+]i was measured in fura-2 AM-loaded microvessels. Perfusing microvessels with superoxide generated by hypoxanthine and xanthine oxidase (HX/XO) induced immediate and transient increases in Lp. The mean peak value, which occurred within 5 min of HX/XO exposure, was 4.3 ± 0.6 times that of the control. In contrast, the perfusion of H2O2 (100 and 500 µM) caused no immediate increases in Lp, but a significant Lp increase, 3.6 ± 0.6 times the control value, occurred 30 min after the perfusion of H2O2 at 500 µM. The perfusion of H2O2 at 100 or 500 µM for 1 h increased Lp to 6.5 ± 0.9 and 11.3 ± 3.6 times the control value, respectively. The increased endothelial [Ca2+]i in HX/XO or H2O2 perfused vessels was correlated with the time course of the increased permeability. Inhibiting Ca2+ influx by LaCl3 prevented the permeability increase induced by HX/XO or H2O2. These results demonstrated differential actions of superoxide and H2O2 on microvessel permeability and endothelial [Ca2+]i. Superoxide-induced permeability increases were immediate and transient, whereas H2O2-induced permeability increases were progressive, demonstrating concentration and time dependence. Ca2+ influx plays an essential role in both superoxide and H2O2-induced permeability increases.

Reactive oxygen species (ROS) contribute to various pathological conditions such as inflammation and ischemic reperfusion as well as atherosclerosis. A major source of ROS in the vascular system is from activated blood cells during respiratory burst. Our previous studies demonstrated that the ROS released from formyl Met-Leu-Phe-OH (fMLP)-stimulated neutrophils, either in suspension or from adherent leukocytes, cause immediate and transient increases in microvessel permeability (37, 38). Prolonged permeability increases were observed with leukocyte/platelet aggregate adhesion to microvessel walls after initial endothelial cell activation by inflammatory mediators and endothelial gap formation (15). However, whether superoxide or hydrogen peroxide (H2O2) converted from superoxide is the main species causing the increases in permeability under such experimental conditions remains unknown. Currently, most ROS-induced signaling and cell dysfunction studies are conducted in cultured endothelial monolayers, with few investigations in vivo. The objective of this study is to characterize the individual contributions of H2O2 and superoxide to the increased microvessel permeability and define the role of Ca2+ influx in each oxygen species-induced permeability increase in intact microvessels.

Experiments were conducted in individually perfused microvessels in rat mesentery. Changes in microvessel permeability were determined by measuring hydraulic conductivity (Lp) before and after each vessel was exposed to superoxide, generated by hypoxanthine and xanthine oxidase (HX/XO) or H2O2. The endothelial intracellular Ca2+ concentration ([Ca2+]i) was measured in fura-2 AM-loaded microvessels with both photometry and imaging systems, and the experimental conditions were similar to those of the Lp measurements. The role of Ca2+ influx in superoxide and H2O2-induced permeability increases were examined in vessels pretreated with a Ca2+ channel blocker, LaCl3.

Material and Methods

Animal preparation. Experiments were carried out on female Sprague-Dawley rats (2 to 3 mo old; 220–250 g; Hilltop Laboratory Animals, Scottsdale, PA) anesthetized with pentobarbital sodium (65 mg/kg body wt) given subcutaneously. Additional 3-mg doses were given as needed to maintain anesthesia during the experiment. All procedures and animal use were approved by the Animal Care and Use Committee at West Virginia University. Each rat was first anesthetized, the trachea was then intubated. A midline surgical incision (1.5 to 2 cm) was made in the abdominal wall. The mesentery was gently moved out of the abdominal cavity and spread over a pillar for Lp measurements or over a glass coverslip attached to an animal tray for endothelial [Ca2+]i measurements. The animal was kept warm on a heating pad, and the upper surface of the mesentery was continuously superfused with warmed (37°C) mammalian Ringer’s solution. Venular microvessels free of firmly attached leukocytes and with brisk blood flow were selected for the experiments. The diameter of the selected vessels ranged between 40 and 50 µm. Each experiment was carried out on a single microvessel from each animal.

Measurement of Lp. All measurements were based on the modified Landis technique, which measures the volume flux of water across the microvessel wall. The assumptions and limitations of the original method and its application to mammalian microvessels have been evaluated in detail elsewhere (4, 19). Briefly, a single venular microvessel is cannulated with a glass micropipette and perfused with albumin-Ringer’s solution (control) that contains 1% (vol/vol) hammer red blood cells as markers. A hydrostatic pressure (ranged from

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containing ionomycin (10 μM) was subtracted from all of the FI measurements. To evaluate the degree of Ca2+ fluorescence outline. The autofluorescence measured from the sur-
ROI covers the area of one individual cell as indicated by the vessel wall. Metafluo software was used to quantify the FI. Each endothelial cell levels using selected regions of interest (ROIs) along and after exposure to testing perfusate using identical instrumental procedures have been described in detail elsewhere (14, 16). In brief, a venular microvessel in rat mesentery was first cannulated and after each cannulation.

Measurements of endothelial Ca2+ concentration were made with a photometer or Ca2+ imaging in individually perfused microvessels using the fluorescent Ca2+ indicator fura-2 MA. A Nikon Diaphot 300 microscope equipped with a Nikon photometry system and a digital cooled charge-coupled device camera (ORCA, Hamamatsu, 12 bit) was used for the experiments. The experimental procedures have been described in detail elsewhere (14, 16). In brief, a venular microvessel in rat mesentery was first cannulated and perfused with albumin-Ringer solution containing 10 μM fura-2 AM for 45 min in the dark. The vessel was then recannulated and perfused with albumin-Ringer solution for 10 min to remove fura-2 AM from the vessel lumen. The excitation wavelengths for fura-2 were selected by two narrow band interference filters (Oriel; 340 ± 5 nm and 380 ± 5 nm). The emission was separated with a dichroic mirror (DM400) and an interference filter (Oriel; 500 ± 20 nm). The excitation wavelength alternated between 340 and 380 nm, and values of fluorescence intensity (FI340 and FI380) were collected with 0.25-s exposure at each wavelength. A dry Nikon Fluor lens (×20, numerical aperture, 0.75) was used for collecting the fluorescence. At the end of each experiment, the microvessel was superfused with a modified Ringer solution (5 mM Na-HEPES to HEPES. All perfusates used for control and test perfusion contained 0.8 mM CaCl2, 1.2 mM MgSO4, 5.0 mM NaHCO3, 20 HEPES, and Na-HEPES. The pH of the Ringer solution was maintained at 7.40 to 7.45 by adjusting the ratio of Na-HEPES to HEPES. All perfusates used for control and test perfusion contained BSA (10 mg/ml).

HX was purchased from MP Biomedicals (Solon, OH), and XO and SOD were from Sigma. H2O2 (30%; Fisher BioReagents) was diluted with albumin-Ringer solution to the desired final concentrations. All perfusates containing the test agent were freshly prepared before each cannulation. Data analysis and statistics. All values listed in the text are means ± SE, except where noted. Paired t-tests were used to analyze the mean values obtained before and after stimulation of the same vessel. Unpaired t-tests and one-way ANOVA were used to compare data between groups. A probability value of P < 0.05 was considered statistically significant. In summary figures, an asterisk (*) indicates a significant increase from the negative control and the dagger (†) indicates a significant decrease from the positive control.

RESULTS

Superoxide induced immediate and transient increases in microvessel Lp. To investigate the effect of superoxide on microvessel permeability HX (0.5 mM) and XO (10 μM) were used to generate superoxide in the perfusate. Figure 1 shows that RLU measured by CL increased from 103 ± 15 to 724 ± 69 (n = 3) when HX was added to the Ringer-albumin solution containing XO and remained at that level during 60 min of measurement. This magnitude of CL increase was comparable with that produced by fMLP-stimulated neutrophils (6–8-fold increase), which induced a transient increase in microvessel Lp (37, 38). The increased CL by HX/XO reaction was completely abolished by SOD (1,500 U/ml). In addition, H2O2 at either 100 or 500 μM did not cause any increases in CL, indicating that superoxide and not H2O2 contributed to HX/XO-produced CL. The effect of superoxide on microvessel Lp was examined in seven microvessels. The mean control Lp was 1.7 ± 0.2 × 10−7 cm²s⁻¹cmH2O⁻¹. Perfusion of HX/XO caused immediate increases in microvessel Lp. Figure 2A shows the time course of an individual experiment. The mean peak Lp, which occurred within 5 min of HX/XO perfusion, was 4.3 ± 0.6 times that of the control and fell close to control level after 15 min. Due to the potential binding of extracellular XO to
vascular endothelium (18), the effect of XO alone on microvessel \(L_p\) was examined in three vessels. No significant changes in \(L_p\) were observed. Figure 2 summarizes the results.

To distinguish the role of superoxide from that of \(H_2O_2\) in HX/XO-induced \(L_p\) increase, the effects of SOD on basal \(L_p\) and HX/XO-induced \(L_p\) increase were examined in four microvessels. The perfusion of SOD (1,500 U/ml) for 20 min did not change the basal \(L_p\) (\(L_p\)SOD/\(L_p\)control was 1.0 ± 0.1). When SOD plus HX/XO was applied to each vessel, the HX/XO-induced transient increase in \(L_p\) was completely abolished, which is consistent with the CL measurement. The mean \(L_p\)SOD+HX/XO was 1.1 ± 0.2 times the control value. An individual experiment is shown in Fig. 2B, and a summary is presented in Fig. 2C.

Superoxide induced immediate and transient increases in endothelial [\(Ca^{2+}\)], a pattern correlated to the \(L_p\) increases. HX/XO-induced changes in endothelial [\(Ca^{2+}\)], were measured with both the photometry system and \(Ca^{2+}\) imaging in a total of 13 microvessels. The photometer measurements were conducted in nine microvessels. The mean baseline endothelial [\(Ca^{2+}\)] was 90 ± 3 nM. Six of the vessels were perfused with HX/XO following control measurements. Endothelial [\(Ca^{2+}\)] transiently increased to a mean peak value of 397 ± 80 nM in 5 min and then declined to 154 ± 21 nM after 15 min. Figure 3A shows a representative time course of HX/XO-induced changes in endothelial [\(Ca^{2+}\)]. The effect of SOD on HX/XO-induced [\(Ca^{2+}\)] increases was examined in three microvessels. Perfusate of SOD did not change the baseline [\(Ca^{2+}\)], but the transient increase in endothelial [\(Ca^{2+}\)] was completely abolished when each vessel was perfused with HX/XO in the presence of SOD. When two of the vessels were re-exposed to HX/XO in the absence of SOD, the \(Ca^{2+}\) response was resumed. The time course of a single experiment is presented in Fig. 3B. Figure 3C summarizes the results.

To illustrate the variations in \(Ca^{2+}\) responses to HX/XO at individual cell levels in each vessel, \(Ca^{2+}\) imaging was conducted in four microvessels and the changes in [\(Ca^{2+}\)] were analyzed from 45 endothelial cells (ROIs) ranging from 8 to 15 ROIs per vessel. The mean baseline [\(Ca^{2+}\)] of all ROIs in the four vessels was 56 ± 7 nM, and the mean peak response to HX/XO was 405 ± 138 nM, which occurred at 2.3 ± 0.5 min after the start of HX/XO perfusion. The peak [\(Ca^{2+}\)] of individual endothelial cells in each vessel varied. The mean relative variability of [\(Ca^{2+}\)] at individual endothelial levels of four vessels significantly increased from 11 ± 2% under...
control conditions to 32 ± 7% at peak [Ca\textsuperscript{2+}] \textsubscript{i} after exposure to HX/XO. The rate of the change in [Ca\textsuperscript{2+}] \textsubscript{i} (time to peak) varied between 1 to 6 min at individual cell levels, and the mean relative variability of four vessels was 23 ± 5%. Figure 4 shows representative images from one of the vessels, and Fig.

Fig. 3. A: representative time course of HX/XO-induced increases in endothelial intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}] \textsubscript{i}). The transient increase in endothelial [Ca\textsuperscript{2+}] \textsubscript{i}, closely correlated with the time course of HX/XO-induced \textit{Lp} increases. B: individual experiment demonstrating that SOD had no effect on basal [Ca\textsuperscript{2+}] \textsubscript{i}, and preperfusing vessel with SOD completely abolished HX/XO-induced increases in [Ca\textsuperscript{2+}] \textsubscript{i}. The Ca\textsuperscript{2+} response to HX/XO was recovered in the same vessel after washout of SOD from the vessel lumen. C: summary of HX/XO-induced peak [Ca\textsuperscript{2+}] \textsubscript{i} increases in the absence (n = 5) and presence (n = 3) of SOD. *Significant increase from negative control (P < 0.05); †significant decrease from positive control (P < 0.05). EC, endothelial cell.

Fig. 4. Heterogeneous peak Ca\textsuperscript{2+} responses in HX/XO-perfused microvessels. A: Ca\textsuperscript{2+} images from 1 representative vessel under control conditions and after exposure to HX/XO. The scale bar at left indicates the color-coded [Ca\textsuperscript{2+}] \textsubscript{i}. B: time courses of HX/XO-induced changes in endothelial [Ca\textsuperscript{2+}] \textsubscript{i}, measured in 11 regions of interest (ROIs) as marked in the control image, which illustrated the variations of the peak [Ca\textsuperscript{2+}] \textsubscript{i}, and the time to reach the peak value at individual cell level in 1 vessel. C and D: histograms of 45 ROIs from 4 vessels showing the heterogeneity of the peak responses of [Ca\textsuperscript{2+}] \textsubscript{i}, and the rate of the changes in [Ca\textsuperscript{2+}] \textsubscript{i} (time to peak) within each vessel and between vessels. Different filled patterns are used to indicate different vessels.
4B plots the time courses of [Ca^{2+}], changes in 11 individual endothelial cells (ROIs) identified in that vessel segment. The histograms presented in Fig. 4, C and D, demonstrate the variations of peak [Ca^{2+}], and the time to reach the peak response at individual cell levels within each vessel as well as between vessels.

**H_{2}O_{2} induced delayed but progressive increases in microvessel L_{p}**. The effects of H_{2}O_{2} at different concentrations and perfusion times on microvessel permeability were studied in 34 microvessels. The mean control L_{p} was 1.6 ± 0.1 × 10^{-7} cm·s^{-1}·cmH_{2}O^{-1}. The immediate effect of H_{2}O_{2} on microvessel L_{p} was examined in nine microvessels. In these experiments, L_{p} was measured right after microvessels were perfused with H_{2}O_{2} at a concentration of 100 μM (n = 4) or 500 μM (n = 5). No significant changes in L_{p} were observed up to 15 min of the H_{2}O_{2} perfusion at either concentration. Figure 5, A and B, shows individual representative experiments. The mean ratios of L_{pH_{2}O_{2}} relative to control L_{p} at both concentrations were 1.1 ± 0.1.

The effects of longer periods of H_{2}O_{2} perfusion on microvessel L_{p} were also investigated. Perfusion of H_{2}O_{2} at 100 μM for 30 min resulted in a slightly increased L_{p} but not significantly different from that of the control. The mean ratio of H_{2}O_{2} L_{p}/control L_{p} was 1.6 ± 0.3 (n = 4). A significant L_{p} increase was observed after 30 min perfusion of H_{2}O_{2} at 500 μM, which was 3.6 ± 0.6 times that of the control (n = 9). After 1 h perfusion of H_{2}O_{2} at 100 or 500 μM, the L_{p} increased to 6.6 ± 0.9 (n = 7) and 11.3 ± 3.6 (n = 5) times that of the control.
control, respectively. Figure 5, C–F, shows the individual representative experiments. Figure 6A shows the summary.

The reversibility of the increased $L_p$ induced by H$_2$O$_2$ was also studied. In the same seven vessels that were exposed to H$_2$O$_2$ at 100 $\mu$M for 60 min, perfusion of albumin-Ringer perfusate for 15 min partially recovered $L_p$ from 6.6 ± 0.9 to 3.6 ± 0.7 times the control value. However, the same maneuver did not reverse the increased $L_p$ in microvessels that were exposed to H$_2$O$_2$ at 500 $\mu$M for 60 min ($n$ = 4).

H$_2$O$_2$-induced increases in endothelial [Ca$^{2+}$], correlated to the time course of $L_p$ changes. H$_2$O$_2$-induced changes in endothelial [Ca$^{2+}$], were examined with both a photometry system and Ca$^{2+}$ imaging in a total of 18 microvessels. The photometer measurements were conducted in 12 microvessels with a mean baseline [Ca$^{2+}$], at 101 ± 6 nM. After baseline measurements, six of the vessels were perfused with 100 $\mu$M H$_2$O$_2$ and the other six vessels were perfused with 500 $\mu$M of H$_2$O$_2$. The [Ca$^{2+}$], was continuously measured for 70 min after the start of H$_2$O$_2$ perfusion with 1-min intervals for the first 15 min and 2-min intervals for the remaining of the perfusion period. In contrast with the immediate transient increases in endothelial [Ca$^{2+}$], in HX/XO perfused vessels, endothelial [Ca$^{2+}$], did not show an immediate increase after the start of H$_2$O$_2$ perfusion at either concentration but progressively increased with time, a pattern correlated with the time course of $L_p$. Figure 6B demonstrates the pooled time courses of 100 and 500 $\mu$M H$_2$O$_2$-induced increases in endothelial [Ca$^{2+}$], from two groups of vessels. Endothelial [Ca$^{2+}$], increased to 224 ± 28, 440 ± 102, and 559 ± 106 nM after the perfusion of 100 $\mu$M H$_2$O$_2$ for 30, 60, and 70 min, respectively. In vessels perfused with 500 $\mu$M H$_2$O$_2$, endothelial [Ca$^{2+}$], was 388 ± 80 nM at 30 min and reached a plateau at 954 ± 231 nM after 50 min of perfusion. Procedure control was conducted in two microvessels by the perfusion of albumin-Ringer solution for 70 min, which showed no significant changes in endothelial [Ca$^{2+}$].

Ca$^{2+}$ imaging was conducted in six microvessels. Three of the vessels were exposed to 100 $\mu$M H$_2$O$_2$ and the other three vessels were exposed to 500 $\mu$M H$_2$O$_2$. Changes in [Ca$^{2+}$], were analyzed from a total of 84 endothelial cells (ROIs) ranging from 10 to 18 ROI per vessel, depending on the number of available cells located in the same focal plane of the vessel wall. The mean baseline [Ca$^{2+}$], and H$_2$O$_2$-induced time-dependent changes in [Ca$^{2+}$], from all of the ROIs in six vessels were summarized in Fig. 7A. The mean magnitudes of H$_2$O$_2$-induced increases in endothelial [Ca$^{2+}$], measured at individual cell levels were slightly higher than the photometry measurements, but not statistically significant. Heterogeneous responses were observed in all vessels. The mean relative variability of six vessels was 15 ± 2% under control conditions and significantly increased to 35 ± 2% and 33 ± 2% after perfusion of H$_2$O$_2$ for 45 and 60 min at 100 and 500 $\mu$M, respectively. Figure 7, B and D, illustrates Ca$^{2+}$ images from two representative microvessels that were exposed to 100 and 500 $\mu$M H$_2$O$_2$. The histograms (Fig. 7, C and E) illustrate the variations of individual endothelial Ca$^{2+}$ responses at different time points from the vessel presented in Fig. 7, B and D.

Ca$^{2+}$ influx contributed to HX/XO-induced increases in endothelial [Ca$^{2+}$], and microvessel $L_p$. To examine the main source contributing to HX/XO-induced increases in endothelial [Ca$^{2+}$], LaCl$_3$ (50 $\mu$M), an inhibitor of the capacitive cationic channels in endothelial cells, was applied to the perfusate for 20 min before each vessel was exposed to HX/XO. Experiments were conducted in five microvessels. The mean control endothelial [Ca$^{2+}$], was 83 ± 3 nM. The perfusion of LaCl$_3$ for 20 min did not significantly change [Ca$^{2+}$], and the mean was 76 ± 2 nM. When HX/XO was added to the perfusate in the presence of LaCl$_3$, [Ca$^{2+}$], was briefly increased to 145 ± 7 nM within 5 min and quickly returned to baseline value. This small transient increase represented the Ca$^{2+}$ release from internal stores. Each microvessel was then perfused with albumin-Ringer for 30 min to remove LaCl$_3$. When HX/XO was applied the second time, a transient increase in [Ca$^{2+}$], resumed with a mean peak of [Ca$^{2+}$], at 313 ± 69 nM, a magnitude similar to the HX/XO-induced Ca$^{2+}$ response in the absence of LaCl$_3$. Figure 8A shows an individual experiment, and Fig. 8B summarizes the results.

To examine whether HX/XO-induced increases in $L_p$ are Ca$^{2+}$ influx dependent, the effect of LaCl$_3$ on HX/XO-induced increases in $L_p$ was examined in five microvessels. The mean control $L_p$ of five microvessels was 1.5 ± 0.3 × 10$^{-7}$ cm$^2$·s$^{-1}$·cmH$_2$O$^{-1}$. Perfusing microvessels with LaCl$_3$ (50 $\mu$M) for 20 min did not significantly change $L_p$ (0.9 ± 0.1 times of the control). When each vessel was perfused with
HX/XO in the presence of LaCl₃, the transient increases in \( L_p \) were completely abolished. The mean ratio of \( L_p \)HX/XO relative to \( L_p \)control was 0.8 ± 0.1. Figure 8, C and D, shows a single experiment and the results of five microvessels.

Ca²⁺ influx-dependent increases in endothelial [Ca²⁺] and microvessel \( L_p \) in H₂O₂-perfused microvessels. The effect of LaCl₃ (50 μM) on H₂O₂-induced increases in endothelial [Ca²⁺] was examined in nine microvessels. Mean control

**Fig. 7. Heterogeneous Ca²⁺ responses in H₂O₂-perfused microvessels.** A: summary of Ca²⁺ imaging data demonstrating H₂O₂-induced time-dependent changes in [Ca²⁺], in vessels exposed to 100 μM (n = 3) and 500 μM (n = 3) H₂O₂. Each point is the mean [Ca²⁺], calculated from all ROIs of 3 vessels. B and D: Ca²⁺ images from the representative vessels under control conditions and after exposure to 100 or 500 μM H₂O₂. The color changes are correlated to the changes in [Ca²⁺], as indicated in the scale bar shown at left. This demonstrates delayed but progressive increases in [Ca²⁺]. C and E: histograms of [Ca²⁺], changes from 16 and 13 ROIs selected from the images in B and D, respectively. Each histogram illustrates the variations of [Ca²⁺] at each time point after exposure to 100 or 500 μM H₂O₂ in that vessel. Different filled patterns indicate the same group of ROIs in the vessel at different H₂O₂ exposure time.
endothelial \([\text{Ca}^{2+}]\), was 86 ± 3 nM. Perfusion of LaCl₃ for 20 min did not significantly change the endothelial \([\text{Ca}^{2+}]\); the mean was 87 ± 4 nM. Each vessel was then perfused with either 100 μM \((n = 5)\) or 500 μM \((n = 4)\) of H₂O₂ for 60 min in the presence of LaCl₃. LaCl₃ abolished 100 μM H₂O₂-induced \([\text{Ca}^{2+}]\) increases (mean was 116 ± 5 nM at the end of 1 h perfusion; \(P > 0.05\)). It also significantly attenuated 500 μM H₂O₂-induced \([\text{Ca}^{2+}]\) increases from 945 nM (in the absence of LaCl₃) to 265 ± 35 nM. Figure 9, A and B, shows an individual experiment and the summarized results.

The effect of LaCl₃ on H₂O₂-induced \(L_p\) increases was examined in another nine microvessels. The mean control \(L_p\) was 1.7 ± 0.3 × 10⁻⁷ cm·s⁻¹·cmH₂O⁻¹. The perfusion of LaCl₃ for 20 min did not significantly change \(L_p\), and the mean ratio of \(L_p\) relative to \(L_p\)control was 0.9 ± 0.04. Each vessel was then perfused with either 100 μM \((n = 5)\) or 500 μM \((n = 4)\) of H₂O₂ for 60 min in the presence of LaCl₃. No significant increases in \(L_p\) were observed at the end of 1 h H₂O₂ perfusion at either concentration. The mean ratio of H₂O₂, \(L_p\)LaCl₃ relative to \(L_p\)control was 1.1 ± 0.1 and 1.1 ± 0.6, respectively. Figure 9C shows an individual representative experiment, and Fig. 9D summarizes the results.

**DISCUSSION**

Our study was the first to show that superoxide and H₂O₂ have differential effects on endothelial \([\text{Ca}^{2+}]\) and microvessel permeability in individually perfused microvessels. Superoxide anions generated by HX/XO induced immediate and transient increases in endothelial \([\text{Ca}^{2+}]\) and microvessel permeability, whereas H₂O₂-induced increases in endothelial \([\text{Ca}^{2+}]\) and microvessel permeability were delayed and progressive, demonstrating a concentration and time dependence. The results indicated that these two oxygen species and their direct derivatives activate endothelium, or evoke cell damage, via different cellular mechanisms. Their distinct actions may explain the transient versus prolonged permeability increases induced by ROS released from activated blood cells under different experimental conditions (37, 38). Heterogeneous \([\text{Ca}^{2+}]\) responses at individual endothelial cell levels were illustrated by \([\text{Ca}^{2+}]\) imaging in both H₂O₂ and HX/XO perfused vessels, a pattern consistent with observations in inflammatory mediator-stimulated microvessels (24, 27). The results that inhibition of \([\text{Ca}^{2+}]\) influx by LaCl₃ prevented HX/XO and H₂O₂-induced \(L_p\) increases indicate a critical role of \([\text{Ca}^{2+}]\) influx in both superoxide and H₂O₂-induced permeability increases.

In recent years, reported evidence has suggested that ROS are no longer merely injurious by-products of metabolism but are essential participants in cell signaling to regulate cellular functions (3, 8, 17, 35). Our previous studies demonstrated that...
ROS released from activated neutrophils, either in suspension (37) or attached to microvessel walls (38), caused a transient increase in endothelial [Ca\textsuperscript{2+}], and microvessel permeability, whereas the addition of SOD inhibited the response (37). Superoxide anions are the initial product generated through NADPH oxidase-mediated respiratory burst upon neutrophil activation by fMLP (2, 5, 37). Our previous CL measurements confirmed such superoxide production with fMLP-stimulated neutrophils (37). However, the released superoxide anion may spontaneously be catalyzed into H\textsubscript{2}O\textsubscript{2} and other oxidants radicals. It is important to understand the role of each individual species in the permeability increase. Our present study used the enzymatic reaction of HX/XO in the perfusate to simulate the ROS released from activated blood cells. The magnitude of superoxide generated by HX/XO was made comparable with that produced by fMLP-stimulated neutrophils (2 \times 10^6/ml) (37) by measuring CL. Vessels perfused with HX/XO produced immediate and transient increases in endothelial [Ca\textsuperscript{2+}], and microvessel Lp, a pattern similar to that observed in vessels perfused with fMLP-stimulated neutrophils in suspension. The enzymatic reaction of HX/XO would be expected to generate both superoxide and H\textsubscript{2}O\textsubscript{2} (10). We then used SOD, a scavenger of superoxide, and a direct application of H\textsubscript{2}O\textsubscript{2} to further define the individual roles of superoxide and H\textsubscript{2}O\textsubscript{2} in HX/XO-induced permeability increases. SOD completely abolished the enzymatic reaction of CL, as well as HX/XO-induced increases in endothelial [Ca\textsuperscript{2+}], and microvessel Lp, which indicated that in the absence of superoxide, the amount of H\textsubscript{2}O\textsubscript{2} produced by HX/XO reaction plus that catalyzed by SOD from HX/XO-generated superoxide does not have the same effect on Lp and [Ca\textsuperscript{2+}] when superoxide was present. Additionally, the direct perfusion of H\textsubscript{2}O\textsubscript{2} at 100 or 500 \muM showing no immediate increase in either permeability or endothelial [Ca\textsuperscript{2+}], supports that superoxide generated by HX/XO or released by fMLP-stimulated neutrophils, and not the H\textsubscript{2}O\textsubscript{2} produced by HX/XO reaction or spontaneously catalyzed from superoxide, is responsible for the immediate and transient increases in microvessel permeability.

Early studies by Del Maestro (6, 7) demonstrated that superfusion of XO in the hamster check pouch transiently increased the number of leakage sites and leukocyte interactions with endothelium. Based on their observations that pre-superfusion of either SOD or catalase resulted in decreased macromolecular extravasation, it was concluded that superoxide and H\textsubscript{2}O\textsubscript{2} presence is necessary for the transiently increased leakage. Due to the potential contamination of catalase with SOD (11), we distinguished the role of superoxide from...
that of H₂O₂ in microvessel permeability by using SOD and directly applied H₂O₂. Unlike studies using the whole vascular bed, our studies focused on the direct interaction of reactive species with endothelial cells, which were conducted in the absence of the effect of ROS-stimulated leukocyte on leakage formations. Furthermore, our results showed that perfusing vessels with XO alone did not have effect on microvessel L̄p, unlike the results from whole vascular bed (6, 7). It appears that in the absence of blood, endothelial cells in the vessel walls do not have sufficient substrate to interact with XO to produce superoxide.

Another important ROS is hydroxyl radical, which can be a direct derivative of superoxide or H₂O₂, but contributes to permeability increases in both cases. Hydroxyl radicals derived from superoxide anions have been reported as a key factor in increased vascular permeability produced by ischemia and systemic infusion or local superfusion of HX/XO (7, 28, 29). The distinct patterns of HX/XO- and H₂O₂-induced increases in endothelial [Ca²⁺], and microvessel permeability indicate that, if hydroxyl radical plays a role in permeability, the magnitude and time course of superoxide-generated hydroxyl radicals must be quite different from that derived from H₂O₂.

The results from Ca²⁺ channel blocker LaCl₃ on HX/XO-induced endothelial [Ca²⁺], indicated that HX/XO-induced transient increases in [Ca²⁺]i, are involved in an initial Ca²⁺ release from the intracellular stores and a subsequent extracellular Ca²⁺ influx. Pretreating the vessels with LaCl₃ did not affect HX/XO-induced Ca²⁺ release from internal stores but blocked the Ca²⁺ influx and HX/XO-induced L̄p increase. This indicated that Ca²⁺ influx is responsible for the permeability increase. Theses results are consistent with our previous observations that in agonist-stimulated microvessels, the Ca²⁺ release from internal stores only contributes about 30% of the initial peak of [Ca²⁺]i, which is not sufficient to increase the microvessel L̄p, and the magnitude of Ca²⁺ influx determines the magnitude of the peak L̄p increase (13, 16). The heterogeneity of peak Ca²⁺ responses between endothelial cells may also account for the variations in leakage site formation as reported in many in vivo studies. Our results indicated that even though superoxide anion is a more potent ROS, it acts like an agonist stimulus. The transient pattern of HX/XO-induced increases in endothelial [Ca²⁺], and microvessel L̄p is similar to that induced by inflammatory mediators such as bradykinin and platelet activating factor (16, 19). The transient nature indicated that superoxide and superoxide-derived hydroxyl radical, in the concentration range that we applied, acts as an agonist that initiates Ca²⁺-dependent increases in microvessel permeability rather than causing damages of proteins or the cell membrane.

H₂O₂ is a relatively stable reactive oxygen metabolite. Because the application of SOD demonstrated protection on oxidant stress-induced tissue damages, H₂O₂ has been considered a less injurious oxygen species. In addition, H₂O₂ is a recognized potent dilator of resistance vessels and may play an important role in the regulation of blood flow under certain pathological conditions (23, 30). Our results indicated that the long-term effect of H₂O₂ on endothelial cells and microvessel permeability, even at subcytotoxic concentrations, should not be overlooked. The physiological concentrations of H₂O₂ responsible for normal intracellular cell signaling were reported less than 1 µM, usually 7- to 10-fold below the extracellular concentration, whereas local pathological concentrations of H₂O₂ can be significantly higher (34). The H₂O₂ generated from activated leukocytes at a density of 2 × 10⁹ cells/mL can reach 300 µM after 37 min (22). Currently, the direct effect of H₂O₂ on endothelial cells is mainly studied in cultured endothelial cells with H₂O₂ concentration ranging from 100 µM to 10 mM (3, 8, 17, 31, 32). Most reported H₂O₂-induced increases in [Ca²⁺], in cultured endothelial cells were rapid and transient with peaks occurring within seconds to minutes of H₂O₂ exposure (8, 17, 32). The H₂O₂-triggered Ca²⁺ entry appears via a pathway that is sensitive to Ni²⁺, La³⁺ (8), or through transient receptor potential melastatin 2 channels (17, 25). H₂O₂-induced permeability increases measured as decreases in endothelial monolayer resistance usually occur rapidly and last about a few hours. Only one of the in vitro studies reported a permeability increase after 70 min of exposure (26). The H₂O₂-induced decreases in endothelial monolayer resistance have been reported as either independent (32) or partially dependent on Ca²⁺ influx (17).

Our present study first demonstrated that, in intact microvessels, H₂O₂ at pathophysiological relevant concentrations (17) showed no immediate effect on either endothelial [Ca²⁺]i or microvessel permeability. Its effect was instead accumulative, demonstrating a concentration and time dependence. A significant increase in L̄p was observed after 30 min of H₂O₂ perfusion at 500 µM and after 1 h of H₂O₂ perfusion at 100 µM. Our measurements of H₂O₂-induced increases in endothelial [Ca²⁺], demonstrated a time course correlated to that of the L̄p changes. In contrast with superoxide-induced transient increases in endothelial [Ca²⁺], and microvessel permeability, the H₂O₂-induced increases in [Ca²⁺]i and microvessel permeability can only be partially reversed after the perfusion of H₂O₂ at 100 µM for 1 h and are not reversible after 1 h of H₂O₂ perfusion at 500 µM. This indicates that long-term exposure to H₂O₂ caused irreversible damage in the vascular wall. The H₂O₂-induced delayed but progressive permeability increase may parallel the late-phase sustained permeability increases observed in vessels with platelet/leukocyte aggregate adhesion (13). The two distinct time courses of permeability increases elicited by different species of reactive oxygen may explain the transient and prolonged permeability increases observed during inflammation. The initial release of superoxide during leukocyte activation may first trigger an immediate activation of endothelial cells, resulting in transient endothelial gap formations and the subsequent activation of platelets and platelet/leukocyte aggregate adhesion. The activated leukocytes and platelets may further cause the augmented release of superoxide. Due to the transient and highly reactive nature of superoxide, the endothelial cells may experience continuous exposure to superoxide-converted H₂O₂ and its secondary derivatives. The long-term exposure to H₂O₂, even at a low dosage, may cause accumulative lipid oxidation and membrane damage, resulting in overloaded Ca²⁺ and prolonged irreversible permeability increases. The measurements of H₂O₂-induced accumulative increases in endothelial [Ca²⁺], appear to support this hypothesis. The time course of H₂O₂-induced increases in endothelial [Ca²⁺], is similar to that of the L̄p increases. Our results also showed that blocking Ca²⁺ entry with a Ca²⁺ channel blocker, LaCl₃, completely abolished the 100 µM H₂O₂-induced increases in endothelial [Ca²⁺], and prevented...
the $L_p$ increase. In 500 $\mu$M H$_2$O$_2$ perfused vessels, LaCl$_3$ prevented the $L_v$ increase and significantly reduced the maximum mean [Ca$^{2+}$], to 265 ± 35 nM after 1 h perfusion of H$_2$O$_2$. This small magnitude increase in [Ca$^{2+}$], can be attributed to Ca$^{2+}$ leak via different pathways but does not appear sufficient to cause permeability increase. Theses results indicated that the H$_2$O$_2$-induced increases in microvessel permeability depend on Ca$^{2+}$ influx via LaCl$_3$-sensitive pathways.

Our study provided the first evidence that superoxide and H$_2$O$_2$ have differential actions on endothelial [Ca$^{2+}$], and microvessel permeability and that increased Ca$^{2+}$ influx contributes to both ROS-induced permeability increases in intact microvessels. The distinct time courses of superoxide and H$_2$O$_2$-induced increases in endothelial [Ca$^{2+}$], and permeability indicate that two ROS may trigger signaling pathways or evoke cellular damage via different cellular mechanisms. Currently, in vitro studies indicate that endothelial gap formation, the disruption of adhesion proteins, and the changes in cytoskeleton arrangements are the mechanisms for ROS-induced endothelial barrier dysfunction, which are similar to those reported in inflammatory mediator-induced permeability increases (1, 9, 12, 17, 20, 21, 36). Whether different cellular mechanisms are responsible for such distinct patterns of permeability increases in intact microvessels remains to be determined. The results obtained from this study suggest that during acute inflammation superoxide released from activated neutrophils is responsible for immediate increases in microvessel permeability and that the subsequent accumulation of H$_2$O$_2$ contributes to the late-phased, sustained permeability increases. Both acute and delayed permeability increases are dependent of endothelial Ca$^{2+}$ influx. Blocking Ca$^{2+}$ influx can be a strategy in the prevention of ROS-induced permeability increases and vascular dysfunction under different pathological conditions.

GRANTS

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REFERENCES