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An immediate endothelial cell signaling response to lung ischemia

CHUN SONG, ABU B. AL-MEHDI, AND ARON B. FISHER
Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

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Song, Chun, Abu B. Al-Mehdi, and Aron B. Fisher. An immediate endothelial cell signaling response to lung ischemia. *Am J Physiol Lung Cell Mol Physiol* 281: L993–L1000, 2001.—Abrupt cessation of lung perfusion induces a rapid endothelial response that is not associated with anoxia but reflects loss of normal shear stress. This response includes membrane depolarization, H_2O_2 generation, and increased intracellular Ca^{2+}. We evaluated these parameters immediately upon nonhypoxic ischemia using fluorescence videomicroscopy to image in situ endothelial cells in isolated, ventilated rat lungs. Lungs labeled with 4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl}1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS; a membrane potential probe), Amplex Red (an extracellular H_2O_2 probe), or fluo 3-AM (a Ca^{2+} indicator) were subjected to control perfusion followed by global ischemia. Endothelial di-8-ANEPPS fluorescence increased significantly within the first second of ischemia and stabilized at 15 s, indicating membrane depolarization by ~17 mV; depolarization was blocked by preperfusion with the K^+ channel agonist lemakalim. Increased H_2O_2, inhibitable by catalase, was detected in the vascular space at 1–2 s after the onset of ischemia. Increased intracellular Ca^{2+} was detected 10–15 s after the onset of ischemia; the initial increase was inhibited by preperfusion with thapsigargin. Thus the temporal sequence of the initial response of endothelial cells in situ to loss of shear stress (i.e., ischemia) is as follows: membrane depolarization, H_2O_2 release, and increased intracellular Ca^{2+}.

fluorescence microscopy; perfused lung; shear stress

ENDOTHELIAL CELLS LINING THE microvasculature are normally subjected to mechanical forces, including both mechanical strain resulting from intravascular pressure and shear stress associated with blood flow. Either of these forces can cause mechanical distortion of the cell that might be expected to induce a cellular response. Thus an increase of shear stress on endothelial cells in culture results in alignment of cells with their long axis in the direction of flow, induction of a spectrum of proteins, and a signaling response including the generation of reactive oxygen species (ROS) and nitric oxide (NO; see Refs. 8, 10, 11, 14, 19).

In contrast to these studies of increased shear, our studies have investigated the response of endothelial cells to the abrupt reduction of shear stress, i.e., ischemia, as might be caused in situ by obstruction of an afferent vessel. We used the isolated rat lung preparation, which is continuously ventilated, to avoid the changes in cellular oxygenation that accompany ischemia in other organs (5). We have shown that, within the initial several minutes after ischemia, lung endothelium responds with plasma membrane depolarization, generation of ROS and NO, and an increase in intracellular Ca^{2+} (3–5, 22). To simulate these conditions in vitro, bovine pulmonary artery endothelial cells were cultured in a parallel plate chamber (16) or in an artificial capillary system (24). Cells adapted to flow for 24–48 h demonstrated a response similar to that observed with the intact lung, although no response was shown for cells cultured under static conditions.

The goal of the present study was to determine the temporal relationships among the observed endothelial cell changes with ischemia to better understand their interrelationships. We used a microfluorographic technique to image ischemia-mediated endothelial cellular events in the intact rat lung using fluorescent dyes as reporter molecules. For monitoring membrane potential, a membrane-localizing fast-response probe, [4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl}1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS)], was selected. This hydrophobic dye is nonfluorescent in solution, becomes fluorescent upon binding to phospholipids in the plasma membrane, and increases in fluorescence upon membrane depolarization (12). For ROS detection, a cell-impermeable H_2O_2-sensitive probe (Amplex Red) was used to assess the release of H_2O_2 into the intravascular medium. For intracellular Ca^{2+} measurements, we used fluo 3-AM, a widely used Ca^{2+} indicator that is cell membrane permeable. We have previously described the temporal relationships for the change in intracellular Ca^{2+} and generation of NO in the intact lung in response to ischemia (3). The present studies describe the temporal relationships of changes in endothelial membrane potential, ROS generation, and intracellular Ca^{2+} during the first minute of ischemia.

Address for reprint requests and other correspondence: A. B. Fisher, Institute for Environmental Medicine, Univ. of Pennsylvania, Medical Center, One John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104 (E-mail: abf@mail.med.upenn.edu).

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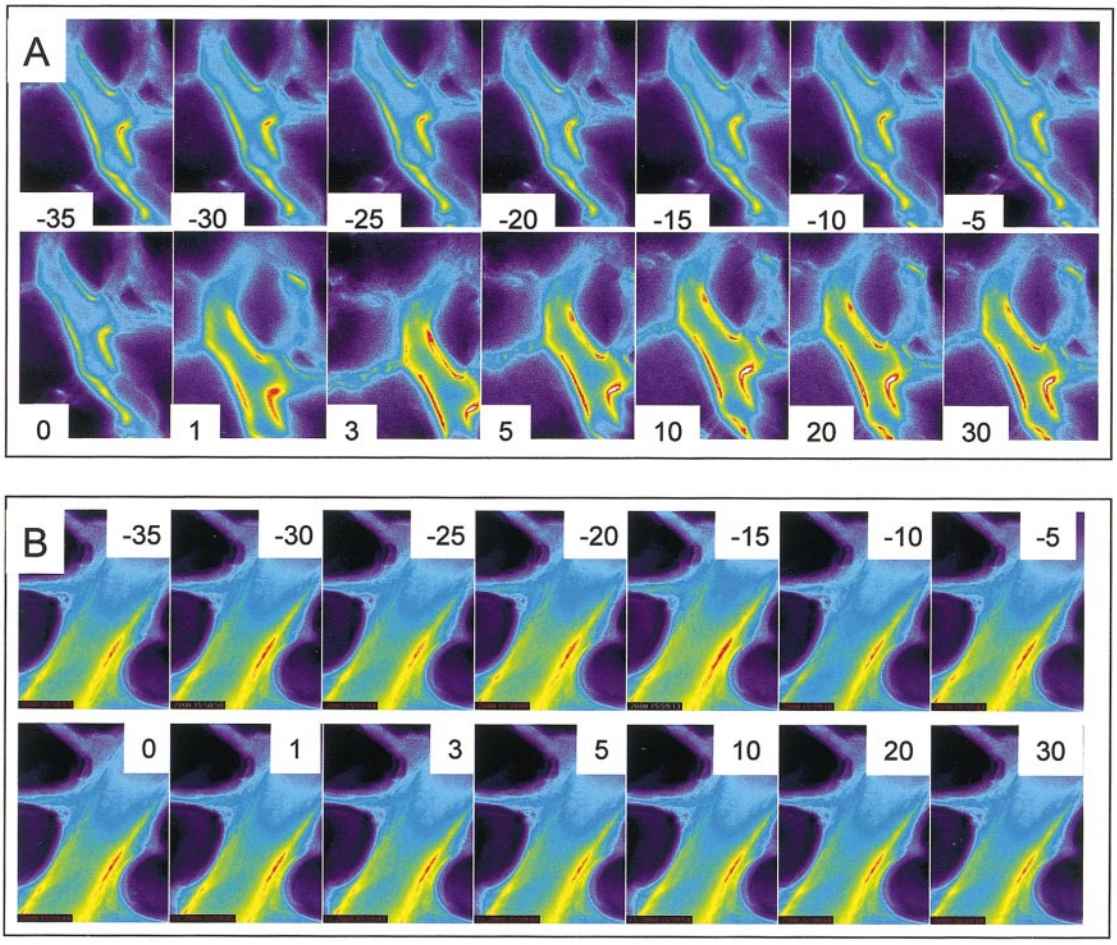
MATERIALS AND METHODS

Materials. Di-8-ANEPPS, Amplex Red, and fluo 3-AM were obtained from Molecular Probes (Eugene, OR). Lemakalim, a K^+ channel agonist, was a gift from SmithKline Beecham Pharmaceuticals. Thapsigargin was purchased from Calbiochem (La Jolla, CA). Catalase was obtained from Boehringer Mannheim (Indianapolis, IN).

Isolated lung perfusion. The isolated perfused rat lung model used for this study has been described previously (4). Briefly, Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 180–220 g were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). A tracheostomy was performed, and ventilation was maintained by positive-pressure ventilation at 60 cycles/min, 2 ml of tidal volume, and 2 cmH_2O end-expiratory pressure. The chest was incised, and the pulmonary circulation was cleared through a cannula inserted in the main pulmonary artery, exiting from a cannula inserted in the left atrium. The perfusate was Krebs-



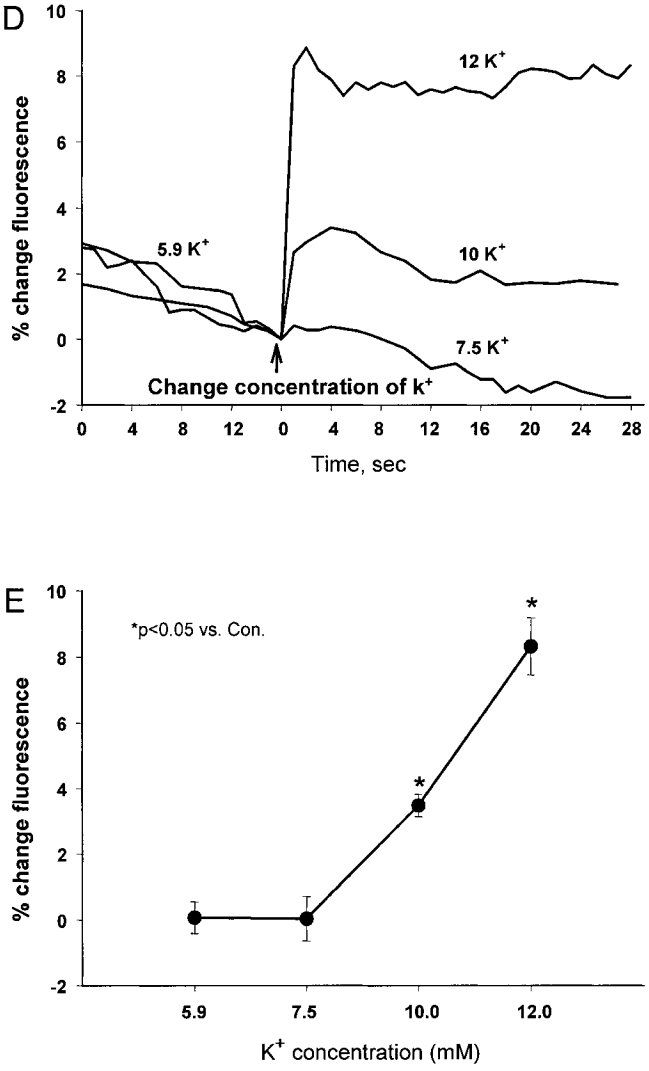


Fig. 1.—*Continued*

Ringer bicarbonate solution (KRB; in mmol/l: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.3 $CaCl_2 \cdot 2H_2O$, 1.2 KH_2PO_4, and 24.9 $NaHCO_3$) with added 5% dextran and 10 mM glucose. In experiments with high K^+, NaCl was replaced equivalently with KCl to maintain isosmolality. Perfusion was maintained using a peristaltic pump at a constant flow rate of 8 ml/min with a recirculating volume of 40 ml. Lungs were ventilated with 95% air-5% CO_2 (all gases were supplied by BOC Group, Murray Hill, NJ). The cleared lungs were removed from the chest and were placed horizontally on a 48 × 60-mm coverglass window in a specially designed chamber. The use of rats for this study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Intravital subpleural microvascular endothelial cell microscopy. Intravital microscopy was performed as described previously (4). Briefly, the chamber with an isolated rat lung was placed on the stage of an epifluorescence microscope fitted with a ×60 objective (Nikon Diaphot TMD) and equipped with an optical filter changer (Lambda 10–2; Sutter Instrument, Novato, CA). A local anesthetic (0.05 mg of xylazine) was injected subepicardially in the posterior wall of the right atrium to abolish lung movement artifacts resulting from contraction of remaining cardiac muscle. Excitation of the lung surface was accomplished with a mercury lamp fiber- optic light source and the appropriate filter set as follows: for di-8-ANEPPS, 440 ± 15 nm excitation, 505 LP dichroic, 530 ± 20 emission; for Amplex Red, HQ-41002b with 545 ± 15 excitation, 565 LP dichroic, and 610 ± 37.5 emission; and for fluo 3, HQ-41001 with 480 ± 20 excitation, 505 LP dichroic mirror, and 535/40 emission (Chroma Technology, Brattleboro, VT). An inverted Nikon TMD epifluorescence microscope, a Hamamatsu ORCA-100 digital camera, and MetaMorph imaging software (Universal Imaging, West Chester, PA) were used for high-resolution digital imaging. To image membrane potential or intracellular Ca^{2+}, lungs were preperfused for 30 min with di-8-ANEPPS (5 μM) or fluo 3 (1.1 μM), and the intravascular dye was then removed by perfusion with dye-free medium for 5 min to reduce background fluorescence. To image H_2O_2 generation, lungs were preperfused with Amplex Red (25 μM) plus horseradish peroxidase (25 μg/ml), which was allowed to remain in the lung during the ischemic period. Some lungs were pretreated by perfusate administration during the dye equilibration period with 30 μmol/l lemakalim, a K^+ channel agonist, or with 1 μmol/l thapsigargin to deplete intracellular Ca^{2+} stores. Other lungs were perfused during the equilibration period with Ca^{2+}-free KRB containing 1 mM EGTA.

Fluorescent images of di-8-ANEPPS- or fluo 3-stained vascular endothelial cells and intravascular Amplex Red were taken from the same area either as a stream (18 frames/s) for up to 10 s or every 30 s for up to 30 min. Ventilation was suspended during the period of data acquisition. Although movement of tissue resulting from flow cessation did occasionally shift the field of observation out of view, this could be minimized by maintaining an appropriate alveolar static inflation pressure during the observation period. Images

Fig. 1. 4-{2-[6-(Dioctylamino)-2-naphthalenyl]ethenyl}1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) fluorescence in subpleural endothelial cells in situ in the intact rat lung. Lungs were perfused with di-8-ANEPPS for 30 min of equilibration, and then images were acquired, usually at 1-s intervals, before and after ischemia (global cessation of flow). Images are in pseudocolor with the intensity scale shown in *C*. Nos. in the images indicate time in seconds for the control period (negative numbers) or ischemic period (positive numbers) for the same lung, with start of ischemia at 0. *A*: endothelial response to ischemia. Lungs were perfused under control conditions and then subjected to abrupt ischemia. *B*: effect of a K^+ channel activator. Lemakalim (30 μM) was added to the perfusate during the 30-min equilibration period. *C*: quantitation of time course of membrane potential with ischemia. Fluorescence intensity of 3 lungs (each representing the averaged value for 4–7 endothelial cells) for control and lemakalim-treated lungs was plotted as means ± SE. In *inset*, images of di-8-ANEPPS-stained vascular endothelial cells were taken as a stream (18 frames/s) for up to 5 s to increase resolution during the 1st s. *D*: calibration of di-8-ANEPPS fluorescence change by perfusion of isolated lungs with elevated K^+ to induce membrane depolarization. Cells were loaded with di-8-ANEPPS and perfused with Krebs-Ringer bicarbonate (KRB; 5.9 mM K^+) to obtain a baseline. The perfusate was abruptly changed to modified KRB with 7.5, 10, or 12 mM K^+. Each curve represents the mean response of 3 endothelial cells from 1 lung. *E*: mean ± SE plot from 3 different lungs for each K^+ concentration to calibrate di-8-ANEPPS fluorescence change. Con, control.

were acquired during a control period of continuous perfusion that lasted at least 10 min and then after the peristaltic pump was stopped to create ischemia.

Statistical analysis. For quantitation, randomly selected endothelial cells were outlined, and the fluorescence intensity of each was measured over time. Mean fluorescence intensities of four to seven endothelial cells were averaged to calculate the response for each lung. For each condition, fluorescence intensity of three to four lungs was calculated and is expressed as means ± SE. Comparison among conditions was made using ANOVA with Bonferroni's test using SigmaPlot 2000 (SPSS). Differences were considered to be significant at $P < 0.05$.

RESULTS

Endothelial cells in situ in subpleural microvessels of rat lungs demonstrated a stable level of di-8-ANEPPS fluorescence during a 30-s period of control perfusion (Fig. 1). With cessation of shear stress (i.e., ischemia), there was an increase in di-8-ANEPPS fluorescence that was maximal within the first second, indicating rapid membrane depolarization (Fig. l*A*). The fluorescence level increased slightly over the next 14 s and then stabilized. The increase in fluorescence represented nearly 7% of the baseline value and was quite reproducible (Fig. 1*C*). When images were recorded in a single endothelial cell on a more expanded time scale, a gradual depolarization with ischemia was observed during the first second (Fig. 1*C*, *inset*). Pretreatment with a K^+ channel activator (lemakalim) completely blocked the increase in di-8-ANEPPS fluorescence with ischemia (Fig. 1, *B* and *C*). The slight but progressive decrease in fluorescence observed during ischemia in the presence of lemakalim presumably was the result of photobleaching. There was a slight decrease in fluorescence during the equilibration period in the presence of lemakalim (data not shown), compatible with endothelial hyperpolarization by the K^+ channel agonist.

Endothelial depolarization by perfusion with increased K^+-containing medium was used to provide an approximate calibration for the di-8-ANEPPS fluorescence signal. Lungs were loaded with the di-8-ANEPPS dye, perfused under control conditions with KRB (5.9 mM K^+) to obtain baseline fluorescence, and then abruptly changed to high-K^+ KRB containing either 7.5 mM K^+, 10 mM K^+, or 12 mM K^+. Two lungs were studied under each condition, and the result for each lung represents the mean of fluorescence images from three endothelial cells (Fig. 1, *D* and *E*). By extrapolating the results with high K^+, a 6.7% increase in di-8-ANEPPS fluorescence observed at 15 s of ischemia would require perfusion with a 11.6 mM KCl solution. Calculation using the Nernst relationship and assuming an initial endothelial cell membrane potential of −70 mV indicates that the decrease in cell membrane potential with ischemia was 17 mV.

Amplex Red fluorescence in the lumen of subpleural microvessels was stable during control perfusion but exhibited a progressive increase in fluorescence during ischemia, indicating H_2O_2 release in the medium (Fig. 2*A*). The initial change was observed between 1 and 2 s after flow cessation and reached an apparent plateau at ~8 s (Fig. 2*D*). However, we determined that this plateau was artifactual as a result of photobleaching because of continuous exposure during image acquisition at 18 frames/s. When images were acquired every 30 s, Amplex Red fluorescence continuously increased during 30 min of ischemia, indicating sustained H_2O_2 generation (Fig. 2*E*). The increase in Amplex Red fluorescence with ischemia was completely blocked by catalase pretreatment (Fig. 2, *B* and *D*), but removal of extracellular Ca^{2+} (plus EGTA) had no effect (Fig. 2, *C* and *D*).

Endothelial cell intracellular Ca^{2+} as indicated by fluo 3 fluorescence remained stable during 30 s of control perfusion (Fig. 3). Ischemia led to increased intracellular Ca^{2+} in endothelial cells (Fig. 3*A*) that was observed between 10 and 15 s after cessation of flow (Fig. 3*D*). There was an apparent plateau in intracellular Ca^{2+} concentration at 15–20 s followed by a subsequent further increase. The increase in intracellular Ca^{2+} was partially prevented by replacement of the usual perfusate with Ca^{2+}-free medium containing EGTA (Fig. 3*B*). The initial increase in intracellular Ca^{2+} was abolished by pretreatment with thapsigargin, an inhibitor of Ca^{2+}-ATPase of endoplasmic reticulum, although Ca^{2+} subsequently increased to a value similar to that observed with Ca^{2+}-free medium (Fig. 3, *C* and *D*). These results suggest that the ischemia-mediated increase in endothelial cell Ca^{2+} is the result of an initial Ca^{2+} release from intracellular stores followed by influx of Ca^{2+} from the extracellular medium.

To summarize the temporal changes observed for pulmonary microvascular endothelial cells in situ after the onset of ischemia, membrane depolarization occurred within the first second, an increase in intravascular H_2O_2 was detected between 1 and 2 s, and an increase in intracellular Ca^{2+} occurred between 10 and 15 s (Fig. 4).

DISCUSSION

Although numerous in vitro studies with endothelial cells have demonstrated a cellular response to increased shear stress (8, 10, 11, 14, 19), there have been relatively few studies of the effects of loss of shear stress, i.e., ischemia. Our previous studies with the isolated rat lung have indicated a relatively rapid response by the endothelium characterized by cell membrane depolarization (5), generation of ROS via a membrane-localized NADPH oxidase (4), increased intracellular Ca^{2+} (3, 22), activation of NO synthase with NO generation (3), and release of intracellular iron from its stores (6). In these studies, ventilation of the lung was continued during the ischemic period so that changes in tissue oxygenation were not responsible for the observed cellular responses and tissue ATP content was unchanged (5). We have reproduced these effects with pulmonary artery endothelial cells in vitro using both artificial capillary (24) and parallel plate (16) cell culture systems. These in vitro studies indicated that a period of flow adaptation (24–48 h) was necessary to elicit a subsequent response to ischemia. Obviously,

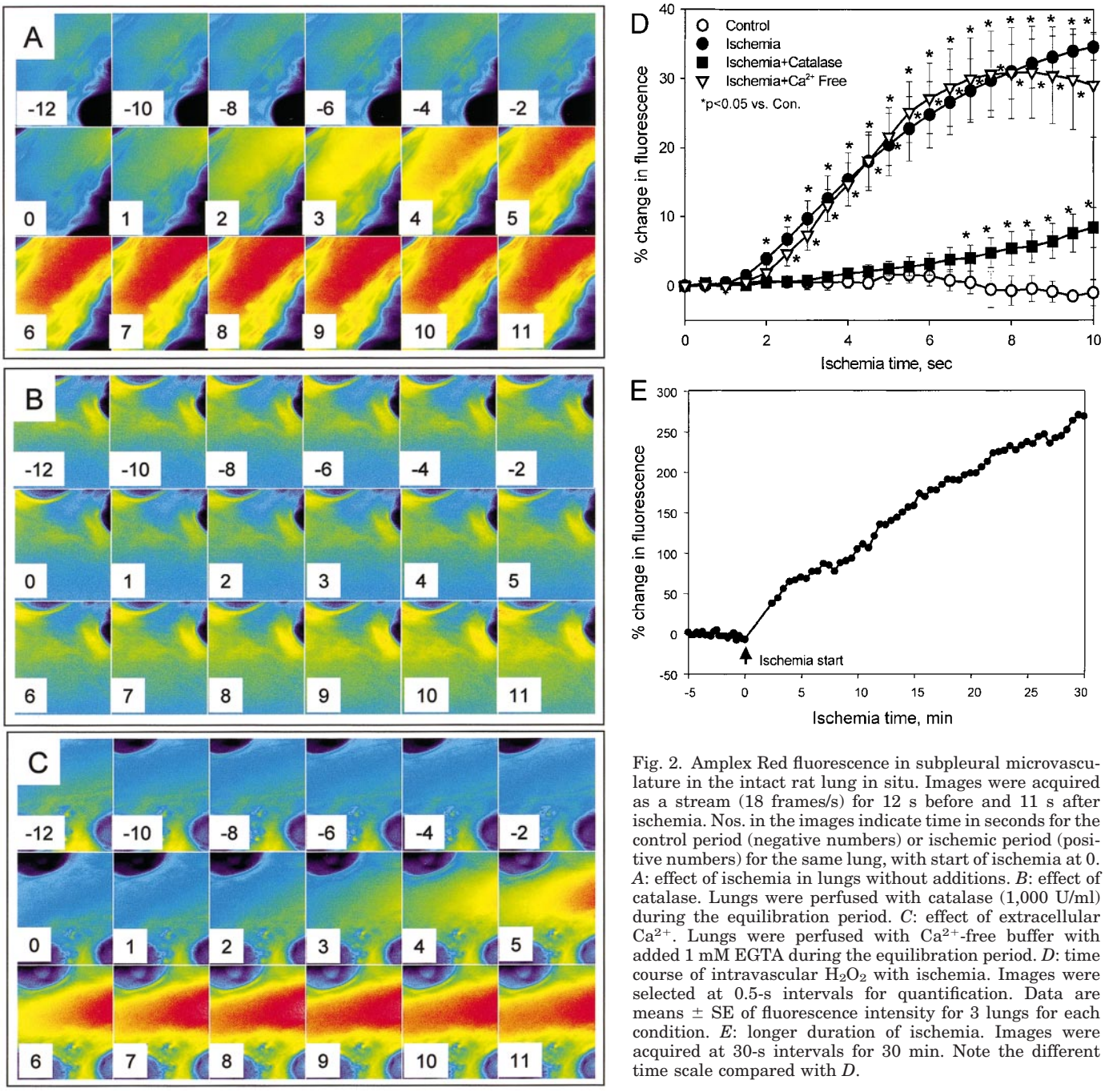


Fig. 2. Amplex Red fluorescence in subpleural microvasculature in the intact rat lung in situ. Images were acquired as a stream (18 frames/s) for 12 s before and 11 s after ischemia. Nos. in the images indicate time in seconds for the control period (negative numbers) or ischemic period (positive numbers) for the same lung, with start of ischemia at 0. *A*: effect of ischemia in lungs without additions. *B*: effect of catalase. Lungs were perfused with catalase (1,000 U/ml) during the equilibration period. *C*: effect of extracellular Ca^{2+}. Lungs were perfused with Ca^{2+}-free buffer with added 1 mM EGTA during the equilibration period. *D*: time course of intravascular H_2O_2 with ischemia. Images were selected at 0.5-s intervals for quantification. Data are means ± SE of fluorescence intensity for 3 lungs for each condition. *E*: longer duration of ischemia. Images were acquired at 30-s intervals for 30 min. Note the different time scale compared with *D*.

endothelial cells in situ would be expected to be in a flow-adapted state.

To determine the temporal relationships among these responses to ischemia, we used high-speed videomicroscopy to image the temporal changes occurring in individual endothelial cells. We have shown previously, using similar dyes, that these fluorescent reporter molecules colocalize in cells that have accumulated fluorescent acetylated low-density lipoprotein and are clearly distinct from a fluorescent epithelial marker; thus, this imaging method reflects endothelial cells (4, 5, 22). In a previous investigation, we reported results for imaging endothelial NO production and intracellular Ca^{2+} in response to ischemia (3). In the present study, we imaged the changes in membrane potential, H_2O_2 generation, and intracellular Ca^{2+} concentration that occur during the first minute after loss of shear stress.

Our previous studies demonstrating ischemia-related endothelial depolarization used bis-oxonol, a slow-response membrane potential probe (5). Observation of fast kinetics in the present study was possible by using a fast-response probe (di-8-ANEPPS) and a near-video-rate digital-imaging technique. Dyes of the aminonaphthylethenylpridinium class have been widely used for the detection of submillisecond membrane potential changes in a variety of tissue, cell, and

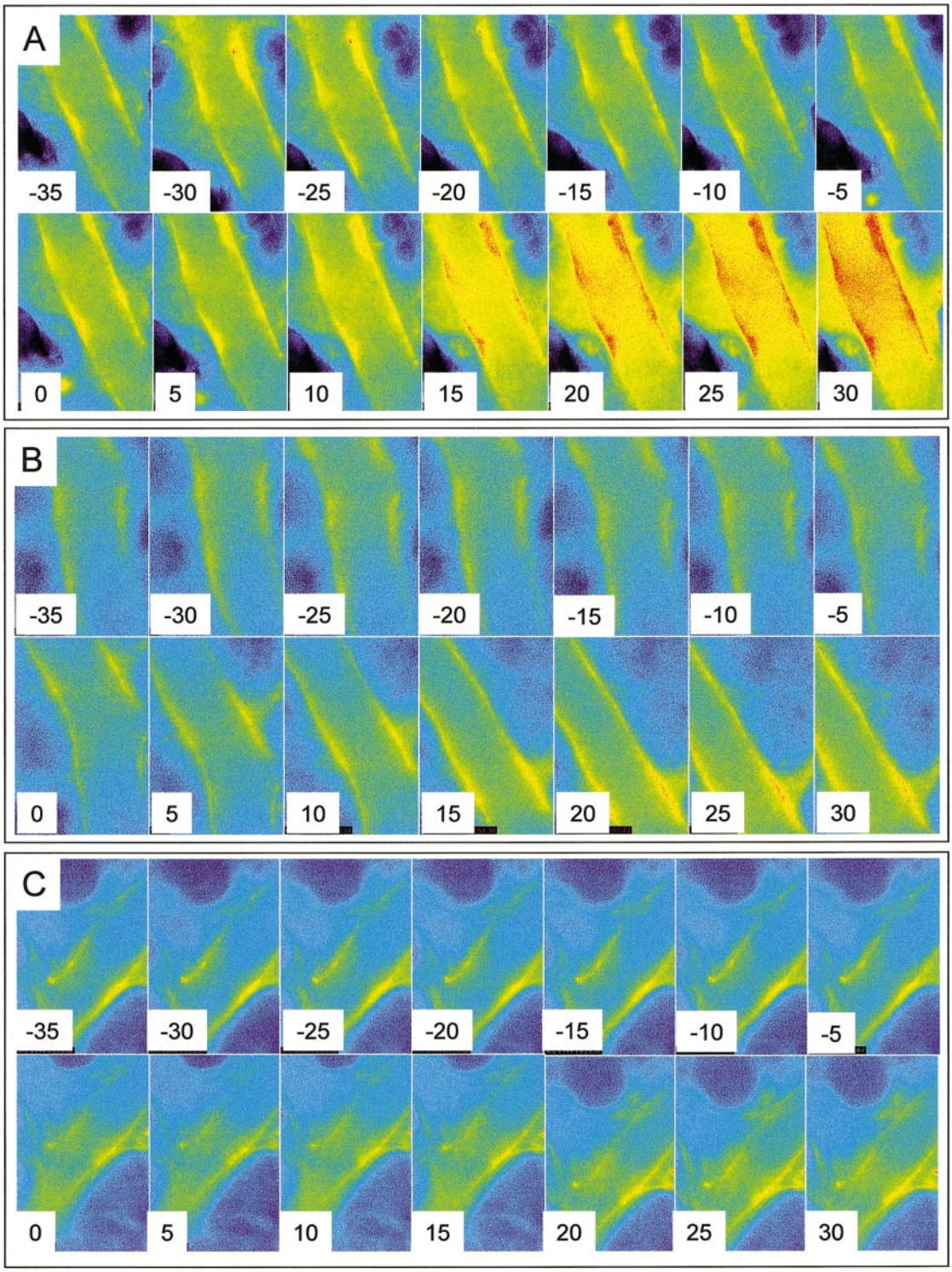


Fig. 3. Effect of ischemia on fluo 3 fluorescence in lung endothelial cells in situ. Fluo 3 was preperfused for 30 min before the onset of ischemia. Nos. in the images indicate time in seconds for the control period (negative numbers) or ischemic period (positive numbers) for the same lung, with start of ischemia at 0. *A*: fluo 3 fluorescence with ischemia under control conditions. *B*: effect of Ca^{2+}-free perfusion on changes in fluo 3 fluorescence with ischemia. Lungs were perfused with Ca^{2+}-free medium containing 1 mM EGTA. *C*: effect of depletion of intracellular Ca^{2+} stores on changes in fluo 3 fluorescence with ischemia. Thapsigargin (TG; 1 μM) was administered in the perfusate for 30 min during the equilibration period. *D*: time course for changes in intracellular Ca^{2+} with ischemia. Each data point represents the mean ± SE for 3 lungs for each condition.

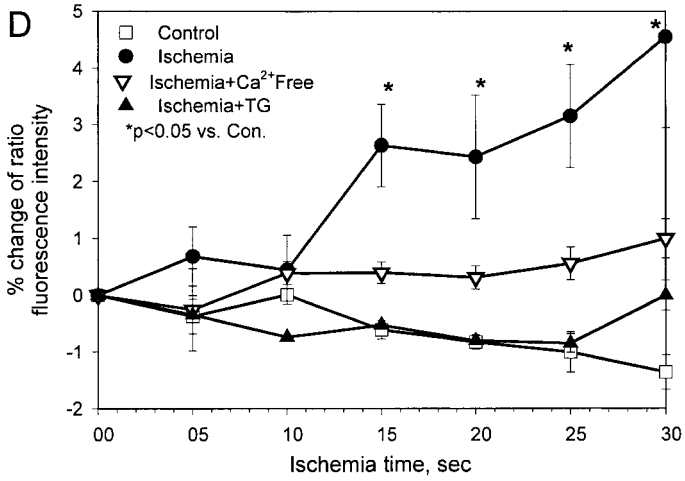


Fig. 3—*Continued*

model membrane systems (12). Di-8-ANEPPS, an especially hydrophobic member of this class, is retained in the outer leaflet of the plasma membrane and thus reflects plasma membrane potential (12). In our observations with pulmonary endothelium, the dye had an apparent plasma membrane localization with no microscopic evidence for subcellular organellar distribution. Endothelial cells in situ exhibited increased di-8-ANEPPS fluorescence within the first second of ischemia, compatible with rapid plasma membrane depolarization. The gradual depolarization observed with a high-resolution time scale and the complete block of depolarization with lemakalim indicate that the response is not related to mechanical artifacts associated with abrupt stoppage of flow. The rapid depolarization (within a second) provides a strong indication that the phenomenon is an ion channel-mediated event. The plasma membrane electrical potential for endothelial cells in culture and perhaps in situ is maintained principally by inwardly rectifying K^+ channels (1). Evidence for a role for K^+ channels in ischemia-mediated depolarization is its inhibition with the K^+ channel activators cromakalim (5) or lemakalim (present study).

Amplex Red was used to image the generation of ROS. This probe, which does not cross cell membranes, reacts with H_2O_2 in the presence of horseradish peroxidase to form the fluorescent product resorufin (12). The probe can detect 50 nM H_2O_2 (12). We have found that Amplex Red does not form resorufin in the presence of other reactive oxygen and nitrogen species such as superoxide anion, NO, peroxynitrite, and hydroxyl radical (data not shown). Complete inhibition of the ischemia-induced increase in Amplex Red fluorescence with catalase confirms specificity of the reaction for H_2O_2 under these conditions. Because the dye is not taken up by endothelial cells and fluorescence is retained in the perfusate, it serves as a marker of H_2O_2 released extracellularly. Although H_2O_2 is freely diffusible and could have originated from intracellular sites of generation, the rapid time course of its appearance (1–2 s) is suggestive of its formation from extracellularly released superoxide. Superoxide released on the endothelial cell outer surface can be converted spontaneously to H_2O_2 and more rapidly by the presence of membrane-bound extracellular superoxide dismutase. We propose that the superoxide-producing enzyme is a membrane-bound endothelial NADPH oxidase analogous to the oxidase of phagocytic cells. Endothelial cells possess the components of this superoxide-generating enzyme (4, 13), and we have shown its involvement in generation of ROS during ischemia by the loss of response in $gp91^{phox}$ knockout mice (4). The temporal response for H_2O_2 generation by the enzyme in the endothelial cells in situ appeared to be significantly faster than the response of phagocytes, where a longer lag period is observed between application of stimulus and enzyme activation (15). This lag period for phagocytes has been attributed to the requirement for translocation of cytosolic components to the membrane and assembly of the enzyme complex during activation (9). It is possible that the NADPH oxidase in endothelial cells is constitutively assembled but is minimally active under resting conditions; however, with ischemia, the activity may be enhanced as a result of some other regulatory mechanisms. The Ca^{2+} independence of the ischemia-mediated increase of Amplex Red fluorescence in the present study indicates that influx of Ca^{2+} is not required for activation of endothelial cell NADPH oxidase.

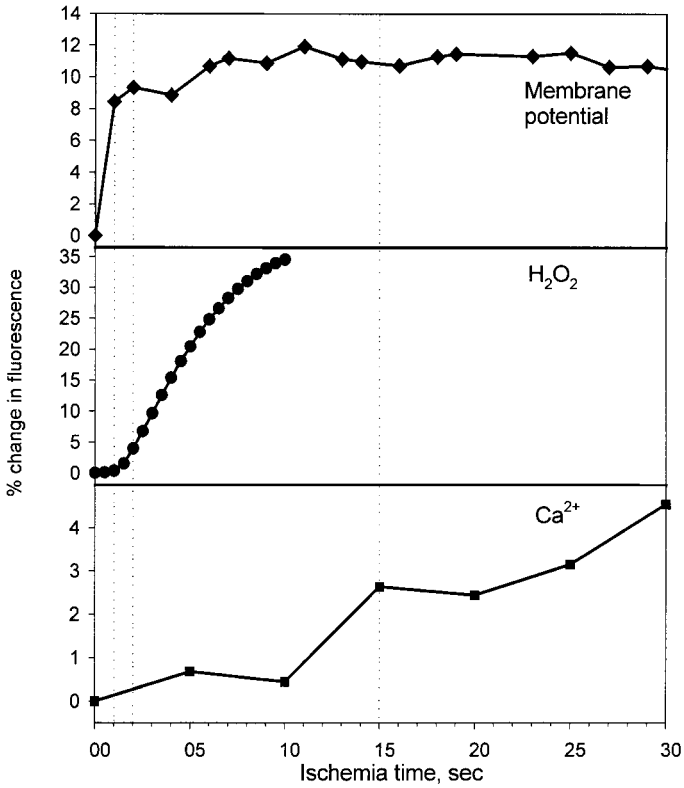


Fig. 4. Temporal relationships among changes in membrane potential, reactive oxygen species, and Ca^{2+} with ischemia. Changes in average pixel intensity from separate experiments are plotted vs. ischemia time. Each data point represents the mean ± SE of 4–7 endothelial cells for each of 4 lungs with di-8-ANEPPS, Amplex Red, or fluo 3.

Fluo 3 was used as an intracellular probe that markedly increases in fluorescence emission upon binding free Ca^{2+} (12). An increase in fluo 3 fluorescence indicated an increase in intracellular Ca^{2+} that occurred after 10–15 s of ischemia. This initial increase of intracellular Ca^{2+} was inhibited by preperfusion with thapsigargin. At a later stage (>25 s), the increase in Ca^{2+} was less sensitive to thapsigargin but was inhibited by elimination of Ca^{2+} from the perfusate. This suggests a two-phase process, with an initial Ca^{2+} elevation resulting from release from intracellular stores and a subsequent influx of extracellular Ca^{2+}. The Ca^{2+} influx could be through Ca^{2+} release-activated channels (17). Alternatively, depolarization could lead to opening of slowly responding voltage-dependent Ca^{2+} channels (e.g., T-type channels). Previous reports have indicated that voltage-dependent Ca^{2+} channels are not present in cultured endothelial cells (18), although a voltage-dependent Ca^{2+} current compatible with a T-type channel has been shown for freshly isolated bovine adrenal capillary endothelial cells (7) and recently for rat lung microvascular endothelial cells (21). We have proposed that pulmonary endothelial cells in situ constantly exposed to shear stress possess these channels although they may be lost during static cell culture (22). The estimated change in endothelial cell membrane potential of −17 mV with ischemia would be sufficient to reach the activation potential for T-type Ca^{2+} channels (17).

The temporal relationships thus established by combining this and our previous study using the isolated rat lung (3) indicate the following sequence of events occurring in endothelial cells during the first minute of ischemia: membrane depolarization, H_2O_2 generation, increase in intracellular Ca^{2+}, and NO generation. This sequence is similar to that observed with bovine pulmonary artery endothelial cells adapted to flow in vitro in a parallel plate chamber (16). These initial events with ischemia are followed by activation of nuclear factor-κB and activator protein-1 and endothelial cell proliferation (24). The fast and orderly occurrence of the initial responses indicates that shear stress and its sensing by appropriate sensors play an important role in homeostasis of the endothelial cell.

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