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Original Research Communication

In Situ Imaging of Intracellular Calcium with Ischemia in Lung Subpleural Microvascular Endothelial Cells

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ABSTRACT

We propose that generation of reactive oxygen species (ROS) during ischemia is associated with an increase in intracellular calcium ($[Ca^{2+}]_i$) in pulmonary capillary endothelial cells. We used an isolated rat lung model and epifluorescence microscopy to evaluate $[Ca^{2+}]_i$ in subpleural microvascular endothelial cells *in situ* by ratio imaging of the fluorophores, Calcium Green and Fura Red (CG/FR). Lungs were ventilated continuously under control (continuously perfused) or global ischemia (no perfusion) and thus remained adequately oxygenated even with ischemia. Ischemia for 5 min led to increase in CG/FR, indicating increase in $[Ca^{2+}]_i$ in endothelial cells *in situ*; CG/FR remained elevated during a subsequent 10 min of ischemia. Ca^{2+}-free perfusion and gadolinium (100 μM) inhibited the increase in $[Ca^{2+}]_i$, while thapsigargin (250 nM) had no effect. These results indicate that increase in endothelial cell $[Ca^{2+}]_i$ with ischemia was due to influx from the extracellular medium. Perfusion with *N*-acetyl-L-cysteine (20 mM) or diphenyleneiodonium chloride (10 μM) prevented the ischemia-mediated $[Ca^{2+}]_i$ increase, suggesting a role for ROS in the Ca^{2+} changes with ischemia. Membrane depolarization by perfusion with high potassium (K^+) or glyburide also resulted in increased $[Ca^{2+}]_i$ whereas the K^+-channel agonist cromakalim, inhibited ischemia-mediated Ca^{2+} influx. We conclude that increased ROS generation with 'oxygenated' lung ischemia is associated with influx of Ca^{2+} and an increase in endothelial cell cytosolic calcium concentration. Antiox. Redox Signal. 1, 145–154, 1999.

INTRODUCTION

ISCHEMIA-REPERFUSION is now a well-recognized mediator of tissue injury associated with the generation of reactive oxygen species (ROS). The paradigm is that tissue injury results from oxidation of cellular components such as lipid and protein components of membranes and organelles. Recently, it has been postulated that ROS represent physiologic signaling agents in addition to their toxic potential and may be involved in the release of second messengers through activation of phospholipases and perhaps other enzymes. Thus, hydrogen peroxide (H_2O_2) causes release of inositol phosphates, at least in part due to activation of phospholipase C (PLC) with secondary effects on intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Shasby *et al.*, 1985, 1988; Muzykantov *et al.*, 1992). In addition, ROS-mediated damage

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to cellular membranes can increase their leakiness to Ca^{2+}, which then increases inside the cell as a manifestation of toxicity. Increased $[Ca^{2+}]_i$ in turn has signaling properties or may exert toxic manifestations due to its effect on enzymes and other biomolecules.

An increase in $[Ca^{2+}]_i$ with ischemia and reperfusion has been suggested for several different organs, based largely on circumstantial evidence. Calcium channel blockers prevented injury in rat intestine and edema in rabbit lungs with ischemia and reperfusion (Kimura *et al.*, 1998; Karck and Haverich 1992). Cardiac ischemia resulted in a rapid decline in left ventricular function and gradual rise in $[Ca^{2+}]_i$ (Hampton *et al.*, 1998). Brain ischemia led to Ca^{2+} influx via T-type channels in rat white-matter astrocytes (Fern, 1998). Ischemia in these studies was accompanied by anoxia with ROS generation during the reperfusion period.

In the air-ventilated lung, an interruption of pulmonary blood flow does not lead to tissue anoxia (Fisher *et al.*, 1991; Minamiya *et al.*, 1998). This model represents the condition associated with pulmonary embolism, for example. But an increase in ROS generation occurs nonetheless with its onset during the ischemic period (Al-Mehdi *et al.*, 1997a; Zhao *et al.*, 1997). The initiating event appears to be endothelial cell membrane depolarization. We have postulated that these cellular manifestations of oxygenated ischemia are due to mechanotransduction of decreased shear stress sensed by the endothelial cells. It is not known whether endothelial cell $[Ca^{2+}]_i$ also changes during oxygenated ischemia in parallel with the production of ROS.

The goal of the present study was to assess the status of $[Ca^{2+}]_i$ in lung endothelial cells *in situ* with early ischemia and determine its potential relationship with the other observed events such as membrane depolarization and ROS generation. We used an *in situ* lung endothelial $[Ca^{2+}]_i$ imaging technique in perfused rat lung similar to the one described by Ying *et al.* (1996). In the previous study, fura-2 was used as a calcium indicator for measuring agonist-stimulated $[Ca^{2+}]_i$ change while the present study used Calcium Green (CG) and Fura Red (FR). This approach, using the isolated perfused lung, enabled us to measure *in situ* Ca^{2+} by ratiometric fluorescence imaging and thereby to monitor changes of $[Ca^{2+}]_i$ as a consequence of ischemia in an intact tissue. We found that early ischemia leads to increase in $[Ca^{2+}]_i$ and that this increase is associated with ROS generation.

MATERIALS AND METHODS

Materials

FR acetoxymethyl esters, CG-1 acetoxymethyl esters (CG), and BODIPY acetylated low-density lipoproteins (LDL) were obtained from Molecular Probes (Eugene, OR). Ionomycin, gadolinium (III) chloride (Gd^{3+}), *N*-acetyl-L-cysteine (NAC), thapsigargin, glybenclamide (glyburide), and cromakalim were purchased from Sigma Chemical Co. (St. Louis, MO). Diphenyleneiodonium chloride (DPI) was obtained from Calbiochem (La Jolla, CA)

Isolated lung perfusion

The isolated perfused rat lung model used for this study has been described previously (Al-Mehdi *et al.*, 1998). Briefly, Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–220 grams were anesthetized with intraperitoneal pentobarbital (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 cm H_2O end-expiratory pressure. The chest was opened 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' Ringer bicarbonate solution (KRB; NaCl 118.45, KCl 4.74, $MgSO_4$ 7 H_2O 1.17, $CaCl_2$ 2 H_2O 1.27, KH_2PO_4 1.18, and $NaHCO_3$ 24.87 in mmol/liter) with added 5% dextran and 10 m*M* glucose. Perfusion was maintained using a peristaltic pump at a constant flow rate of 7 ml/min with a recirculating volume of 40 ml. Lungs were ventilated with 95% air/5% CO_2 (all gasses were supplied by BOC Group, Inc., 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.

Intravital subpleural microvascular endothelial cell microscopy

Intravital microscopy was performed as described previously for use with other fluoroprobes (Al-Mehdi *et al.*, 1998a). A similar model has been used by others to monitor changes of intracellular Ca^{2+} concentration in lung endothelial cells (Ying *et al.*, 1996). Briefly, the chamber with an isolated rat lung was placed on the stage of an epifluorescence microscope fitted with a 100× objective (Nikon Diaphot TMD) and equipped with an optical filter changer (Lambda 10-2, Shutter Instrument Co., Novato, CA).

A local anesthetic (0.05 mg of xylazine) was injected subepicardially into the posterior wall of the right atrium to abolish lung movement artifact due to contraction of remaining cardiac muscle. Excitation of the lung surface was accomplished with a mercury lamp fiberoptic light source, a fluoroisothiocyanate (FITC) filter set for CG (HQ41001 with 480/40 excitation filter, 505 LP dichroic mirror, and 535/40 emission filter; Chroma Technology Corp., Brattleboro, VT) and a TRITC filter set for FR (HQ41002b with 545/30 excitation filter, 565 LP dichroic mirror, and 610/75 emission filter; Chroma Technology Corp., Brattleboro, VT). The objective was positioned so that the microvascular diameter was maximum at the focal plane. Images from the microscope were acquired during 3 sec exposure time with a computer-controlled cooled CCD camera (MicroMAX, Princeton Instruments, Inc.), using graphics control software (Metamorph Imaging System, Universal Imaging Corp., West Chester, PA).

After an equilibration period of 30 min with the isolated lung to allow uptake of CG (968 n*M*) and FR (570 n*M*) fluorophores, the intravascular dye was removed by perfusion with dye-free medium for 5 min to reduce background fluorescence. Ventilation was stopped briefly (<15 sec) to permit collection of fluorescence images. Five to seven exposures were taken from randomly selected areas of each lung and then ventilation was resumed. For quantification, an area of interest containing one or more endothelial cells was outlined. CG and FR intensities were measured in the area of interest and their ratio of intensities was calculated (CG/FR). We normalized the fluorescence values in different areas by using "% change of ratio fluorescence intensity (% of baseline)." Values for five to seven areas of interest were averaged to obtain a mean value for each lung. In some lungs, localization of the fluorophores was determined by comparison of BODIPY-acetylated LDL, an endothelial cell marker, and FR images after 30 min of coperfusion.

As a control group, after the equilibration period, images were taken every 5 min for 15 min during continuous perfusion. For the ischemia group, after taking control images, the peristaltic pump was stopped to create global ischemia. Some lungs were pretreated with cromakalim (30 μM), DPI (10 μM), NAC (20 m*M*), thapsigargin (250 n*M*), or gadolinium (100 μM) by perfusate administration during the dye equilibration period. Additional lungs were perfused with calcium-free KRB containing 1 m*M* EGTA prior to ischemia. The effect of depolarization in the absence of ischemia was studied by switching to high K^+-KRB (24 m*M* K^+ replacing equimolar Na^+) perfusion or by perfusion with glyburide (10 μM), a K^+ channel inhibitor. In other experiments, ionomycin (1 μM) was used as a positive control for calcium influx.

Statistical analysis

Results are expressed as mean ± SE for each condition unless otherwise stated. Significance of parametric differences among groups was evaluated with one-way analysis of variance (ANOVA) and Bonferroni's test for multiple comparison using SigmaStat (Jandel Scientific, San Rafael, CA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Lungs were perfused with BODIPY-acetylated LDL and FR for 30 min and evaluated for fluorescence. There was essentially complete colocalization of fluorescence from FR and BODIPY-acetylated LDL, an endothelial cell-specific marker (Fig. 1). This is clearly indicated in the overlay fluorescence of both images, as

shown by the yellow color with no significant red or green. Thus, FR appears to be localized to the endothelium in this model. Fluorescence of the calcium fluorophores in vascular endothelial cells was unchanged after 15 min of control perfusion (Fig. 2). With 15 min of ischemia, there was a significant increase in CG fluorescence, a moderate decrease in FR fluorescence, and a marked increase in the CG/FR image (Fig. 2). This increased ratio indicates an increase in $[Ca^{2+}]_i$. To evaluate the time course of fluorescence change, average CG/FR in endothelial cells from five to seven randomly selected areas for each condition was measured in three to six different lungs under each condition at 5-min intervals during 15 min of control perfusion or ischemia. $[Ca^{2+}]_i$ was increased significantly at 5 min of ischemia, the earliest time point studied, and did not change significantly during the next 10 min (Fig. 3).

The increase in $[Ca^{2+}]_i$ during ischemia was prevented with Ca^{2+}-free perfusion (Fig. 4). Addition of thapsigargin to the perfusate during perfusion with Ca^{2+}-free medium led to increase $[Ca^{2+}]_i$ within 10 min followed by its return to baseline at 30 min (data not shown), compatible with release of Ca^{2+} from thapsigargin-sensitive stores in lung endothelial cells and its efflux from the cells. Thapsigargin is an inhibitor of Ca^{2+} adenosine triphosphatase (ATPase) of the endoplasmic reticulum and has been widely used to deplete intracellular Ca^{2+} stores (Doan *et al.*, 1994; Oike *et al.*, 1994; Chetham *et al.*, 1999). The ischemic increase in the $[Ca^{2+}]_i$ was not affected by thapsigargin pretreatment, either in the presence or absence of external Ca^{2+}. Ischemic increase in $[Ca^{2+}]_i$ was blocked by Gd^{3+}, a cation channel blocker (Fig. 4). These results imply that the increase in $[Ca^{2+}]_i$ during ischemia was due to influx from the extracellular medium.

A possible relationship between the change in $[Ca^{2+}]_i$ and increased ROS generation with ischemia was evaluated by pretreatment of lungs with NAC (a sulfhydryl-based antioxidant) or DPI (a flavoprotein inhibitor, commonly used as an inhibitor of membrane bound NADPH oxidase). DPI has been shown previously to markedly inhibit ROS generation in lung ischemia (Al-Mehdi *et al.*, 1998), whereas NAC is considered an intracellular ROS scavenger. Both NAC and DPI markedly inhibited the increase in $[Ca^{2+}]_i$ with ischemia (Fig. 5).

The ischemic increase in $[Ca^{2+}]_i$ also was inhibited with cromakalim, a K^+-channel agonist (Table 1). This agent was used based on our previous results, which indicated that cromakalim protects against ischemia-mediated lipid peroxidation (Fisher and Al-Mehdi, 1996). In addition, we have postulated K^+-channel inactivation as the mechanism for membrane depolarization, an initial manifestation of lung ischemia (Al-Mehdi *et al.*, 1996, 1997b, 1998a). To study the effect of membrane potential on endothelial $[Ca^{2+}]_i$, lungs were perfused continuously with high-potassium KRB or glyburide, a K^+-channel blocker. These treatments have been shown to result in endothelial cell membrane depolarization in our isolated perfused lung model (Al-Medhi *et al.*, 1996, 1997b). Both treatments led to increase endothelial $[Ca^{2+}]_i$ in the absence of ischemia (Table 1). Ionomycin, a calcium ionophore, was used as a positive control in these experiments and led to the expected increase in $[Ca^{2+}]_i$ (Table 1).

DISCUSSION

For these experiments, we used ratiometric imaging with two visible wavelength calcium indicators: FR and CG-1. With an increase in $[Ca^{2+}]_i$, CG fluorescence is significantly increased whereas FR fluorescence is moderately decreased (Haugland, 1996; Wächtler *et al.*, 1996). A ratiometric image using these two probes simultaneously will eliminate fluorescence measurement artifacts due to differential dye loading and compartmentalization. The study of $[Ca^{2+}]_i$ with ratio imaging has been previously reported. The simultaneous use of fluo-3 and FR was used to measure $[Ca^{2+}]_i$ by confocal microscopy or flow cytometry in cardiac myocytes or lymphocytes (Lipp and Niggli, 1993; Novak and Rabinovitch, 1994). The distribution of the ratio images in these cell models was uniform (Lipp and Niggli, 1993). CG indicators are almost identical to those of fluo-3, but they are more fluorescent in resting cells, less phototoxic, and can be used at lower concentration (Eberhard and Erne, 1991; Haugland, 1996). Wächtler *et al.* (1996) used CG-1

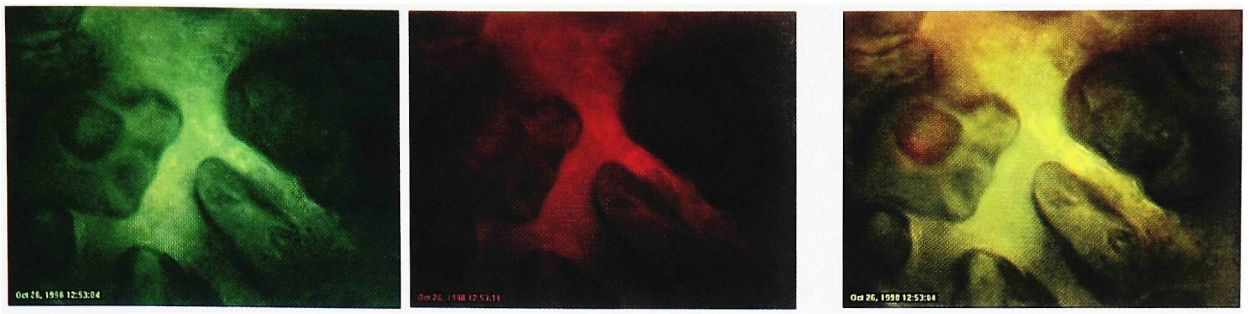

FIG. 1. Colocalization of calcium fluorescence and endothelial cells based on pseudocolor images of a calcium-dependent fluorophore (Fura Red) and endothelial cell-specific marker (BODIPY-acetylated LDL). Isolated rat lungs were placed on a microscope stage for *in situ* imaging of subpleural endothelial cells as described in Materials and Methods. Lungs were perfused with BODIPY-acetylated LDL and FR for 30 min. **Left panel:** Endothelial cells labeling with BODIPY-acetylated LDL. **Middle panel:** FR fluorescence. **Right panel:** Overlay fluorescence of both images; yellow indicates the colocalization of the two fluorophores. The horizontal dimension of each panel is 87 μm.

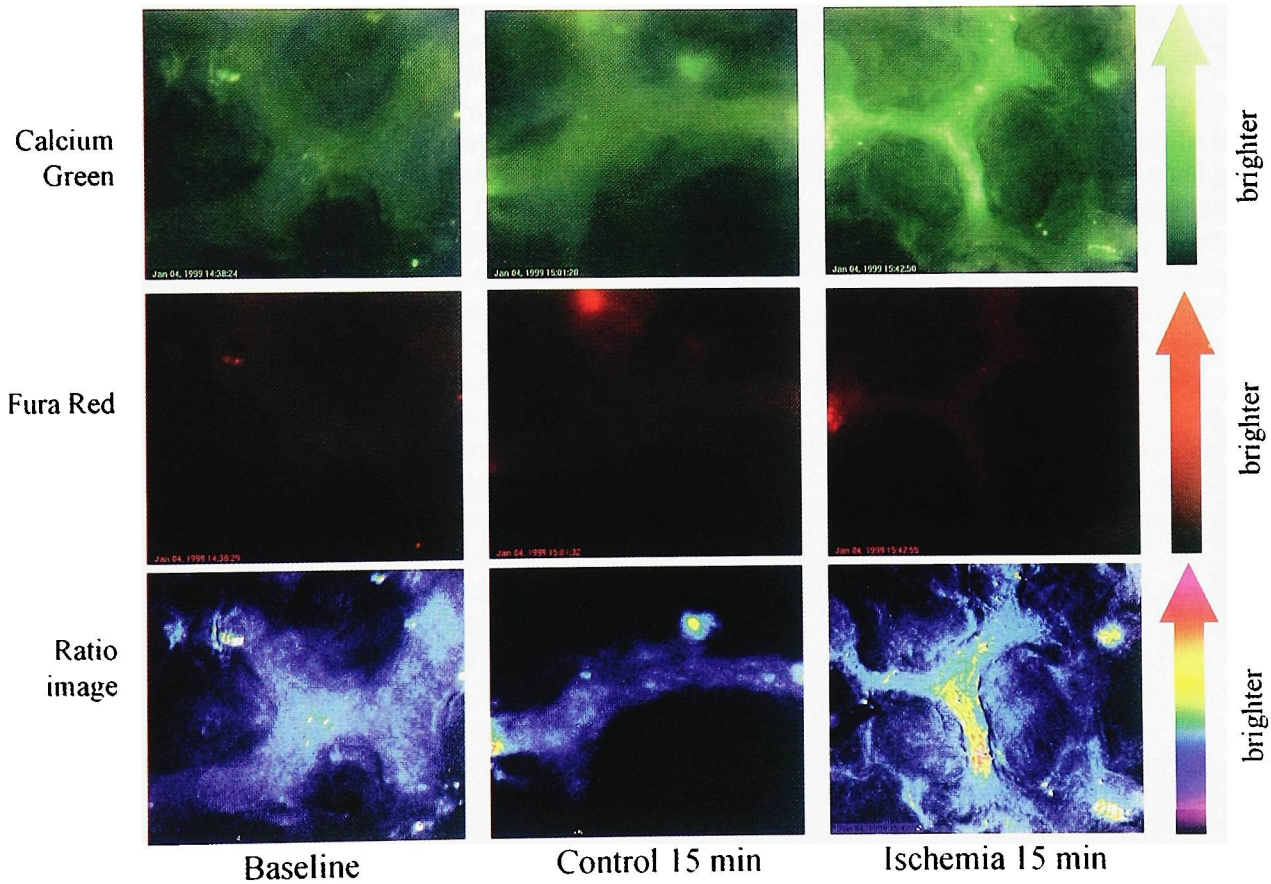

FIG. 2. *In situ* fluorescence images with CG-1 (top), FR (middle), and their ratio (bottom). Isolated rat lungs were placed on a microscope stage for *in situ* imaging of subpleural endothelial cells as described in Materials and Methods. Lungs were perfused with CG-1 and FR for 30 min for equilibration. Then baseline images were taken. Fluorescence is shown in pseudocolor with the scales on the right indicating intensity level. CG-1 and FR fluorescence did not change during continuous perfusion (control). With ischemia for 15 min, there was increased CG-1 fluorescence, slight decreases or no change in FR fluorescence, and increased fluorescence in the ratiometric image.

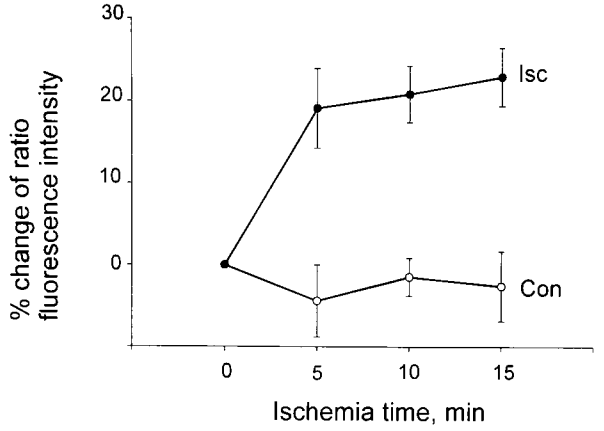

FIG. 3. Time course of $[Ca^{2+}]_i$ with ischemia in isolated rat lungs. After loading with CG-1 and FR as described in Fig. 2, control images of the fluorescence ratio were taken, and then the peristaltic pump was stopped to create ischemia. Images were taken at 5-min intervals. Normalized mean fluorescence intensity was calculated for each lung as described in Materials and Methods. Ischemia significantly increased the fluorescence ratio indicating increased $[Ca^{2+}]_i$ in endothelial cells of perfused rat lungs ($p < 0.05$). (○) control (Con); (●) ischemia (Isc). Data are mean ± SE for $n = 3$ for control, $n = 6$ for ischemia.

and FR simultaneously for study of the rat isolated spinal root. Other studies have used a single fluorophore such as fura-2 for measuring Ca^{2+} in cultured cells or organs (McDonough and Button, 1989; Williams and Fay, 1990; Ying *et al.*, 1996). Compared with fura-2, which requires ultraviolet excitation, visible light-excitable Ca^{2+} indicators such as CG offer several advantages including reduced interference from sample autofluorescence, less cellular photodamage, and higher absorbance.

Our previous studies of lung endothelial cells *in situ* using the isolated rat lung have established membrane depolarization and increased ROS generation as early events with ischemia (Al-Mehdi *et al.*, 1996, 1997b, 1998a; Zhao *et al.*, 1997). Because calcium plays an important role in cell signaling processes, a logical next step was to determine the effect of ischemia on lung endothelial cell calcium. The experimental design allowed us to study the calcium response of intact vascular endothelial cells *in situ*. Ischemia, *i.e.*, abrupt cessation of flow without anoxia, led to increase in $[Ca^{2+}]_i$ within the initial 5 min.

The increase in $[Ca^{2+}]_i$ with ischemia could be due to influx from the extracellular medium, release from intracellular stores, or both. Normal endothelial cell cytosolic calcium concentration is about 100 n*M* (Allbritton *et al.*, 1992; Moore *et al.*, 1998). This cytosolic calcium concentration is much lower than in extracellular

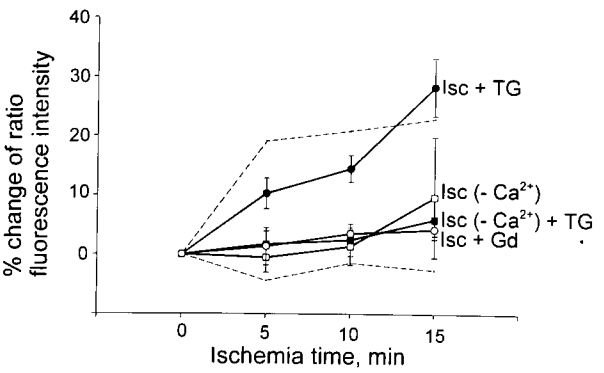

FIG. 4. Evaluation of source of increase in $[Ca^{2+}]_i$ with ischemia. Isolated rat lungs were perfused for 30 min with 250 n*M* thapsigargin (TG) or 100 μM gadolinium (Gd) in normal KRB or with calcium-free ($-Ca^{2+}$) KRB. Fluorophores (CG-1 and FR) were coperfused. After control images were taken, the peristaltic pump was stopped to create ischemia. Values were calculated as in Fig. 3. Dashed lines show results with ischemia **(top)** and control perfusion **(bottom)**, reproduced from Fig. 3. Data are mean ± SE for $n = 3$ for each condition.

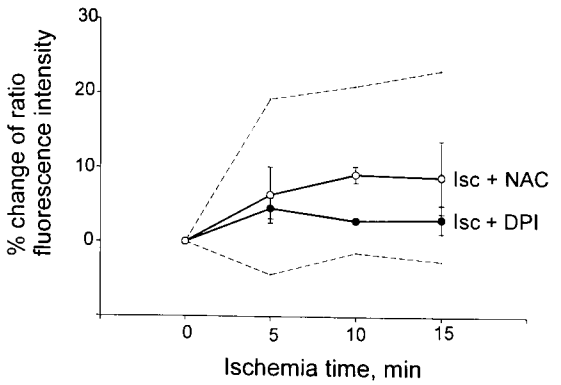

FIG. 5. Effect of ROS inhibitors during ischemia. Lungs were perfused with NAC or DPI plus CG-1 and FR for 30 min. After control images were taken, the peristaltic pump was stopped to create ischemia. (○) Ischemia with 20 m*M* NAC (Isc + NAC); (●) ischemia with 10 μM DPI (Isc + DPI). Values were calculated as in Fig. 3. Dashed lines show results with ischemia **(top)** and control perfusion **(bottom)**, reproduced from Fig. 3. Data are mean ± SE for $n = 3$ for NAC, $n = 4$ for DPI.

TABLE 1. EFFECT OF MEMBRANE DEPOLARIZATION ON INTRACELLULAR CALCIUM

Experimental group	*Percent change of ratio fluorescence intensity*
Control	−2.7 ± 3.5
Ischemia (15 min)	23.0 ± 3.5[a]
Ischemia (15 min) + Cromakalim, 30 μM	10.3 ± 4.4
Glyburide, 10 μM (no ischemia)	29.6 ± 3.6[a]
High (24 mM) K^+ (no ischemia)	24.8 ± 0.5[a]
Ionomycin, 1 μM (no ischemia)	30.9 ± 9.0[a]

Results are mean ± SE for $n = 3$ to 6.
[a] $p < 0.05$.

medium (in this experiment, Ca^{2+} concentration of perfusate was 2.5 mM) or in intracellular stores (high μM to low mM Ca^{2+} in smooth endoplasmic reticulum) (Moore *et al.*, 1998). To determine the source of increase in endothelial Ca^{2+} with ischemia, we modulated the extracellular source by calcium-free perfusion or Gd^{3+} treatment to block Ca^{2+} entry. Both treatments prevented the increase in fluorescence ratio with ischemia. To determine the role of intracellular stores, we depleted the stores by pretreatment with thapsigargin and then conducted ischemic experiments in the presence or absence of extracellular Ca^{2+}. Thapsigargin pretreatment had no effect on the subsequent ischemic increase in $[Ca^{2+}]_i$. These experiments suggest that intracellular release was not a mechanism of Ca^{2+} increase and that the source of Ca^{2+} was influx from outside the cell.

The relationships among cell membrane depolarization, ROS generation and the influx of Ca^{2+} were investigated by experiments using membrane potential modulators and ROS inhibitors. We have previously shown that ischemia results in membrane depolarization (Al-Mehdi *et al.*, 1996, 1997b, 1998a). The present study shows an increase in $[Ca^{2+}]_i$ with high K^+ or glyburide (depolarizing agents) in the perfusion medium (no ischemia). Partial prevention of increase in Ca^{2+} during ischemia was observed with cromakalim, a K^+-channel opener that hyperpolarizes endothelial cells membrane and thus attenuates ischemia-induced depolarization. These results indicate that depolarization of the endothelial cell membrane results in increased intracellular Ca^{2+}. Thus, Ca^{2+} influx could possibly be mediated by voltage-dependent Ca^{2+} channels that are activated with ischemia-induced membrane depolarization. Although previous observations indicated that endothelial cells do not possess voltage-dependent Ca^{2+} channels (Nilius *et al.*, 1997) and endothelial cell $[Ca^{2+}]_i$ was shown to decrease with depolarization and increase with hyperpolarization (He and Curry, 1991), those studies used statically cultured cells. It is possible that endothelial cells *in situ* (*i.e.*, acclimatized to flow) possess voltage-dependent calcium channels that are lost with static culture. Indeed, voltage-dependent calcium channels have been shown in freshly isolated endothelial cells (Bossu *et al.*, 1989, 1992). If that is the case, then increase in $[Ca^{2+}]_i$ with ischemia could result from a depolarization activated Ca^{2+} channel.

An alternative mechanism is that changes in $[Ca^{2+}]_i$ are dependent on ROS generation. The observed increase in endothelial $[Ca^{2+}]_i$ with ischemia was markedly inhibited by NAC or DPI, inhibitors of ROS generation, suggesting a direct effect of ROS. ROS have been shown to lead to increase in $[Ca^{2+}]_i$ in endothelial cells in culture (Shasby *et al.*, 1985, 1988; Franceschi *et al.*, 1990; Doan *et al.*, 1994; Dreher and Jundo, 1995; Siflinger-Birnboim *et al.*, 1996; Volk *et al.*, 1997; Hu *et al.*, 1998) and other cells (Roychoudhury *et al.*, 1996; Hoyal *et al.*, 1998). Hydrogen peroxide apparently activates a Ca^{2+}-influx pathway in endothelial cells (Doan *et al.*, 1994; Siflinger-Birnboim *et al.*, 1996), although the mechanism remains to be established. Several possibilities have been postulated as the

mechanism for calcium influx with ROS challenge including: (1) lysis of the membrane with formation of pores that allow Ca^{2+} entry, (2) production of cation ionophores through plasma membrane lipid peroxidation or cross-linking of proteins, (3) direct activation of calcium channels by ROS, and (4) agonist-stimulated Ca^{2+} influx through activation of G proteins (Franceschi *et al.*, 1990; Doan *et al.*, 1994). The present studies with inhibitors do not differentiate between these mechanisms but do suggest that ROS are the primary mediator of the observed increased endothelial $[Ca^{2+})_i$ with lung ischemia as opposed to membrane potential-mediated activation of voltage-dependent Ca^{2+} channels. Although these studies indicate primarily an effect of ROS on cell membrane Ca^{2+} permeability, other studies with cultured endothelial cells have indicated that ROS also can activate release of Ca^{2+} from intracellular stores (Shasby *et al.*, 1988).

Although the effect of ischemia on lung endothelial $[Ca^{2+}]_i$ has not been previously reported, others have shown that subjecting statically cultured endothelial cells to an abrupt onset of flow also leads to increase in $[Ca^{2+}]_i$ (James *et al.*, 1995; Murase *et al.*, 1998). The similarity of endothelial cell calcium response to increased flow or no-flow (*i.e.*, ischemia) may reflect an imposed alteration from a state of adaptation (Davies, 1995). Thus, either increased or decreased flow imposed on the adapted state could induce an increase in $[Ca^{2+}]_i$ as a signaling mechanism in endothelial cells.

In summary, we postulate the following sequence of events in lung endothelium during early ischemia. Membrane depolarization occurs rapidly and is associated with an increase in ROS generation. ROS generation and possibly activation of voltage-dependent Ca^{2+} channels lead to a change in cell membrane Ca^{2+} permeability and increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$, in turn, can activate enzymatic sources of ROS to amplify the initial response to ischemia. Thus, both ROS and Ca^{2+}, in addition to their role as mediators of cellular injury, appear to be signaling molecules that can shape the response of the endothelial cells to an acute decrease in mechanical shear stress as a manifestation of ischemia.

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ABBREVIATIONS

ANOVA, analysis of variance; ATPase, adenosine triphosphatase; $[Ca^{2+}]_i$, intracellular calcium; CG, calcium green; DPI, diphenyleneiodonium chloride; FITC, fluoroisothiocyanate; FR, Fura Red; H_2O_2, hydrogen peroxide; KRB, Krebs Ringer bicarbonate; LDL, low-density lipoproteins; NAC, *N*-acetyl-L-cysteine; PLC, phospholipase C; ROS, reactive oxygen species.

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