Ca\(^{2+}\)- and Phosphatidylinositol 3-Kinase-dependent Nitric Oxide Generation in Lung Endothelial Cells in Situ with Ischemia*

Endothelial cells generate nitric oxide (NO) in response to agonist stimulation or increased shear stress. In this study, we evaluated the effects of abrupt cessation of shear stress on pulmonary endothelial NO generation and its relationship to changes in intracellular Ca\(^{2+}\). In situ endothelial generation of NO and changes in intracellular Ca\(^{2+}\) are essential for the response of endothelial cells to abrupt flow cessation-induced NO generation in endothelial cells of subpleural microvessels in situ occurred between 30 and 90 s after onset of ischemia and was preceded by an increase in intracellular Ca\(^{2+}\) due to both influx of extracellular Ca\(^{2+}\) and release from intracellular stores. Flow cessation-induced NO generation in endothelial cells in situ was Ca\(^{2+}\)-, calmodulin-, and PI3-kinase-dependent. The similarity of endothelial cell response (increased NO generation) to either increased flow or cessation of flow suggests that cells respond to an imposed alteration from a state of adaptation. This response to flow cessation may constitute a compensatory vasodilatory mechanism and may play a role in signaling for cell proliferation and vascular remodeling.

Nitric oxide (NO) is a potent regulator of vascular tone in systemic and pulmonary vessels and plays an important role in cellular signaling and respiration (1–4). This mediator is generated by endothelial cells in response to agonist stimulation and also as a response to increased shear stress (5). Although the effect of increase in flow on endothelial cell NO generation has been characterized, the effect of abrupt cessation of shear stress (i.e., acute ischemia as in pulmonary embolism or donor lung ischemia for transplantation) on pulmonary endothelial NO generation in situ is unknown.

Endothelial cells in situ are constantly exposed to shear stress associated with blood flow and thus become flow-adapted. These cells respond with an increase in cytosolic Ca\(^{2+}\) and generation of reactive oxygen species when flow is stopped but oxygenation is maintained (6, 7). A similar response with reactive oxygen species generation subsequent to the abrupt cessation of flow has been demonstrated with endothelial cells adapted to flow in vitro (8). We hypothesized that the basis for this early response is mechanotransduction related to removal of endothelial cell shear stress (9). Increased intracellular Ca\(^{2+}\) is known to activate endothelial nitric-oxide synthase (eNOS) resulting in increased NO generation and has been demonstrated in cells exposed to increased shear stress (10, 11). In these nonadapted endothelial cells, shear-stress-induced NOS activation was biphasic, with an initial Ca\(^{2+}\)-dependent phase and a second, sustained phase that was Ca\(^{2+}\)-independent and phosphorylation-dependent (10–12). In this study, we evaluated whether the increased intracellular Ca\(^{2+}\) associated with the early response to flow cessation leads to increased NO generation. This paradigm constitutes a model for the response to acute ischemia. We have used the term oxygenated ischemia to refer to the abrupt cessation of flow in pulmonary circulation where oxygenation remains adequate during ischemia because of the lung alveolar air, and an analogous effect should apply to the initial several minutes of ischemia in a systemic vessel prior to critical oxygen depletion. Our studies show that in air-ventilated, isolated, intact rat lungs, endothelial cells in situ respond to removal of flow with increased generation of NO.

EXPERIMENTAL PROCEDURES

We have used an established fluorescence microscopic technique for visualizations of subpleural endothelium in situ in isolated, ventilated, and perfused rat lungs to monitor endothelial cell Ca\(^{2+}\) and NO (6, 7, 9). NO generation was monitored by labeling the pulmonary endothelium with 4,5-diaminofluorescein diacetate (DAF-2 DA, Calbiochem) that is de-esterified intracellularly to DAF-2 NO and its higher oxides, such as NO\(_3\) or nitrous anhydride (N\(_2\)O\(_3\)), provide the third nitrogen to form a triazoo ring from the two amino groups of the nonfluorescent DAF-2 and convert it to diaminotriazolofluorescein (DAF-2T) that is detected at 490 nm excitation and 530 nm emission (13). Changes in intracellular Ca\(^{2+}\) levels were monitored with Fluo-3 acetoxymethyl ester.

Materials—DAF-2 DA and N\(_7\)-nitro-L-arginine methyl ester (l-NAME) were obtained from Calbiochem (La Jolla, CA); Fluo-3/AM and DiI-acetylated LDL were from Molecular Probes (Eugene, OR).

Isolated Lung Perfusion and Intact Organ Endothelial Cell Microscopy—We used an established intact organ microscopy technique to image microvascular endothelial cells in situ in isolated, ventilated, and perfused rat lungs in real time using an epifluorescence microscope (6, 7, 9). Briefly, Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 150–200 g were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). A tracheostomy was performed, and artificial ventilation with 95% air + 5% CO\(_2\) (BOC Group, Inc., Murray Hill, NJ) was started through a cannula. The abdomen was opened and the animal was exsanguinated by transection of major abdominal vessels. A cannula was inserted into the main pulmonary artery via a puncture in the right ventricle, and another was inserted into the left atrium. The lung was cleared of blood by gravity perfusion via the pulmonary artery with an artificial medium (Krebs-Ringer bicarbonate buffer KRB: NaCl, 118.45; KCl, 4.74; MgSO\(_4\)_7H\(_2\)O, MgSO\(_4\)_7H\(_2\)O, 39807
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1.7; CaCl2/2H2O, 1.27; KH2PO4, 1.18; and NaHCO3, 24.87 in mmol/liter with 5% dextran and 10 mM glucose at pH 7.4). The flow-through perfusate left the lung via the left atrial cannula. Once the lung became visibly cleared of blood (appeared white in color), the heart-lung preparation was dissected en bloc and was placed on a 48 × 60 × 0.16 mm coverglass window in a specially designed Plexiglas chamber with ports for the tracheal, pulmonary, and left atrial cannulae. The cardiovascular ports were connected to a peristaltic pump that recirculated 40 ml of perfusate at a constant flow rate of 8 ml/min through the pulmonary vascular bed. The chamber 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 Co., Novato, CA). A local anesthetic (0.05 mg of xylazine, Sigma) 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 fiber optic light source, a fluorescein isothiocyanate filter set for DAF-2T or Fluo-3 (HQ41001 with 480/40 excitation filter, 505 LP dichroic mirror, and 535/40 emission filter; a tetramethylrhodamine isothiocyanate filter set for Dil-acetylated LDL, Chroma Technology Corp., Brattleboro, VT). The integrity of the preparation was continuously monitored by online measurements of intratracheal and pulmonary artery perfusion pressures. Endothelial cells in the subpleural vasculature were positively identified by labeling with Dil-acetylated LDL added to the perfusate. We have used a Nikon Diaphot TMD epifluorescence microscope, a Hamamatsu ORCA-100 digital camera, and MetaMorph imaging software (Universal Imaging) for imaging. After an equilibration period of 45 min with the isolated lung to allow uptake of DAF-2 DA (5 μmol/liter), intravascular dye was removed by perfusion with dye-free medium for 5 min to reduce background fluorescence. Images of DAF-2T- or Fluo-3-stained vascular endothelial cells were taken from the same area as a stream (18 frames per s) or every 3 s for up to 10 min during which ventilation was stopped. As a control, after the equilibration period, images were taken during continuous perfusion. Then the peristaltic pump was stopped to create ischemia. Some lungs were pretreated with t-NAMe (1 mmol/liter) by administration of perfusate during the dye equilibration period or with 1 μM thapsigargin. Additional lungs were perfused with calcium-free KreB containing 1 mmol/liter EGTA prior to ischemia.

RESULTS AND DISCUSSION

Endothelial cells in situ in subpleural microvessels of rat lungs exhibited progressive increase in DAF-2T fluorescence during ischemia indicating increased NO generation with abrupt cessation of shear stress compared with control perfusion (Fig. 1a). The fluorescent endothelial cells in subpleural precapillary arterioles could be readily identified as elongated, flow-aligned structures with tapered ends pointing against the flow direction and most of the fluorescence in the thicker part of the cells in the perinuclear area (Fig. 1a). Specific endothelial cellular identification was confirmed by colabeling with Dil-acetylated LDL that showed cellular colocalization with DAF-2T fluorescence (data not shown). Quantification of fluorescence intensity in the same endothelial cells over time and averaged for a few cells shows that the increase in DAF-2T fluorescence with ischemia was completely blocked by t-NAMe (Fig. 2a), indicating specificity of the signal for NO. Endothelium possesses at least two isoforms of NOS: endothelial (eNOS) and inducible (iNOS) (14). eNOS requires increased levels of Ca2+ and calmodulin binding for its dissociation from caveolin and activation whereas activation of iNOS is Ca2+-independent (15). Ca2+-free medium containing 1 mM EGTA led a to marked decrease in DAF-2T fluorescence with ischemia (Fig. 2a), suggesting that eNOS is responsible for endothelial NO generation under these conditions. The calmodulin inhibitor, calmidazolium chloride, and the PI3-kinase inhibitor, wortmannin, markedly inhibited the ischemic increase in DAF-2T fluorescence (Fig. 2b). These results indicate a role for calmodulin binding and the protein kinase B/Akt-mediated phosphorylation for the activation of NOS in oxygenated ischemia. Dependence of NOS activation on both Ca2+ and PI3-kinase in pulmonary endothelial cells in situ with flow cessation contrasts with the dependence on PI3-kinase alone for sustained NOS activation associated with imposition of shear stress to cells in static culture (16). There was no evidence for a Ca2+-independent component as described previously for increased flow (10, 11, 17, 18).

To establish a temporal relationship between the NO and Ca2+ changes, we used Fluor-3 to monitor changes in endothelial cell Ca2+. Ischemia led to increased Ca2+ in endothelial cells in situ that was partially prevented by perfusion with either Ca2+-free, 1 mM EGTA-containing medium or by pretreatment with thapsigargin, an inhibitor of Ca2+-ATPase of endoplasmic reticulum (Fig. 1b and Fig. 2, c and d). These results indicated that ischemic increase in endothelial cell Ca2+ is due to both influx and intracellular release.

Comparison of the time course for the NO and Ca2+ responses to ischemia revealed that the onset of Ca2+ increase occurs between 10 and 20 s whereas the onset of NO increase is between 30 and 90 s for individual endothelial cells (Fig. 3a).

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Fig. 2. a, effect of NOS inhibition on DAF-2T fluorescence with ischemia. L-NAME pretreatment (Ischemia + L-NAME) completely blocked the increase in DAF-2T fluorescence with ischemia (Ischemia). Average pixel intensity of 4–7 endothelial cell areas as outlined using MetaMorph Imaging software was followed over time for each lung and expressed as percent change from initial fluorescence intensity. Each data point represents mean ± S.E. for 3–4 lungs. b, effect of inhibitors of calmodulin (calmidazolium chloride) and PI3-kinase (wortmannin) on DAF-2T fluorescence with ischemia. c, effect of ischemia and Ca^{2+}-free perfusion on Fluo-3 fluorescence. Ca^{2+}-free perfusion also included 1 mM EGTA. d, effect of depletion of intracellular Ca^{2+} stores on ischemic increase in Ca^{2+}. Thapsigargin was administered in the perfusate 30 min before ischemia. For each lung, results for 4–7 endothelial cells were averaged; each data point represents the mean ± S.E. for 3–4 lungs for each condition.

Fig. 3. a, temporal relationship between Ca^{2+} and NO changes with ischemia in pulmonary endothelial cells in situ. Changes in average pixel intensity from separate experiments are plotted as ischemia time of the same cell. Each data point represents mean ± S.E. for 4–7 endothelial cells for each of 4 lungs with DAF-2T or Fluo-3. b, schematic representation of the mechanotransduction hypothesis for response of endothelial cells in situ to ischemia. One or more primary flow sensors may be involved in the ischemic response. Voltage-dependent Ca^{2+} channels (VDCC) have been demonstrated in freshly isolated endothelial cells (19); Ca^{2+} increase during ischemia subsequent to cell membrane depolarization suggests their presence in endothelial cells in situ. The mechanism relating mechanotransduction (summarized as Ca^{2+}-Releasing Factors) to Ca^{2+} release from intracellular stores with ischemia is not understood: a direct effect may involve force transmission via cytoskeleton, and an indirect effect may include Ca^{2+}-induced Ca^{2+} release or other secondary pathways.

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