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Original Research Communication

Depolarization-Associated Iron Release with Abrupt Reduction in Pulmonary Endothelial Shear Stress *In Situ*

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ABSTRACT

This study evaluated the roles of endothelial cell membrane potential and reactive oxygen species (ROS) in the increase of tissue free iron during lung ischemia. Oxygenated ischemia was produced in the isolated rat lung by discontinuing perfusion while ventilation with O_2 was maintained. We have shown previously that tissue oxygenation is maintained in this model of ischemia and that biochemical changes are the result of an abrupt reduction in endothelial shear stress. With 1 hr oxygenated ischemia, generation of ROS, evaluated by oxidation of dichlorodihydrofluorescein (H_2DCF) to fluorescent dichlorofluorescein, increased 8.0-fold, lung thiobarbituric acid reactive substances (TBARS) increased 3.4-fold, and lung protein carbonyl content increased 2.4-fold. Lung tissue free iron, measured in the lung homogenate with a fluorescent desferrioxamine derivative, increased 4.0-fold during ischemia. Pretreatment of lungs with thapsigargin abolished the increase in free iron with ischemia indicating that this effect is dependent on Ca^{2+} release from intracellular stores. Perfusion of lungs with high (25 m*M*) K^+ to depolarize the endothelium also led to a significant increase in tissue free iron. Pretreatment of lungs with 35 μM cromakalim, a K^+-channel agonist, significantly inhibited both ischemia-induced tissue oxidant injury and the increase in free iron with ischemia or with high K^+ perfusion. A similar increase in free iron was observed when lungs were ventilated with either O_2 or N_2 during the ischemic period or were pre-perfused with an inhibitor of ROS production (diphenyleneiodonium). These results indicate that ROS generation is not required for ischemia-mediated iron release. Thus, ROS generation and iron release with ischemia are independent although both are subsequent to endothelial cell membrane depolarization. Antiox. Redox Signal. 2, 335–345.

INTRODUCTION

LUNG ISCHEMIA refers to the reduction of pulmonary blood flow that occurs with pulmonary artery obstruction due to varying causes such as pulmonary embolism, vascular compression by a tumor, or *in situ* pulmonary artery thrombosis. In the absence of bronchial obstruction, ventilation to the ischemic segment should continue and lung tissue oxygenation is maintained. We have used the continuously ventilated isolated rat lung subjected to global ischemia to simulate this clinical paradigm (Fisher *et al.*, 1991). This experimental model shows a reproducible series of events that are initiated shortly after the onset of ischemia and include: endothelial cell membrane depolarization, assembly of endothelial NADPH oxidase; generation of reactive oxygen species (ROS), increased intracellular Ca^{2+}, re-

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lease of free iron from tissue stores, and lipid peroxidation and oxidation of tissue proteins (Al-Mehdi *et al.*, 1997a,b, 1998; Ayene *et al.*, 1992; Fisher *et al.*, 1991; Tozawa *et al.*, 1999; Zhao *et al.*, 1997a,b). An *in vitro* model has demonstrated the role of ischemia-induced ROS generation in cell signaling by the activation of endothelial NF-$\kappa\beta$ and AP-1 followed by cell proliferation (Wei *et al.*, 1999). These events in the lung occur during the ischemic period, provided that oxygen supply is maintained through ventilation, and do not require reperfusion. Modulation of these events by perfusate flow unrelated to changes in lung ATP has generated the hypothesis that the metabolic changes are initiated by the loss of shear stress (Al-Mehdi *et al.*, 1997a,b).

Our previous study showed that free iron in the lung was significantly elevated at 30 min of ischemia, the earliest time period evaluated (Zhao *et al.*, 1997a). That study did not evaluate possible mechanisms for the ischemic-mediated iron release. The present study was designed to explore the relationships between endothelial cell membrane depolarization, ROS generation, intracellular Ca^{2+}, and iron release during ischemia using the isolated perfused lung model. Tissue free iron was measured by a new method based on a fluorescent desferrioxamine derivative (FL-DFO), which decreases in fluorescence upon iron binding. ROS release was evaluated with the fluorescent trap, dichlorofluoroscein (DCF), and by oxidation of tissue components as indicated by generation of thiobarbituric acid reactive substances (TBARS) and protein carbonyls. The effect of Ca^{2+} was evaluated by perfusing lungs with Ca^{2+}-free medium or by pre-perfusion with thapsigargin to depelete intracellular Ca^{2+} stores. The role of membrane potential in the generation of free iron was evaluated with two separate approaches. First, a K^+-channel agonist was shown to prevent membrane depolarization associated with lung ischemia and its effect on release of free iron into the tissue was evaluated. Second, a model of perfusion with high K^+, which we have shown previously to cause membrane depolarization (Al-Mehdi *et al.*, 1997a), was used to simulate the effects of ischemia on endothelial cell membrane potential. The results of these experiments indicate that increase of free iron in the lung is associated with membrane depolarization and release of Ca^{2+} from intracellular stores and is independent of ROS generation.

MATERIALS AND METHODS

Reagents

Aprotinin, cromakalim, butylated hydroxytoluene, 2,4-dinitrophenyl-hydrazine (DNPH), leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), thapsigargin, and thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein (2′,7′-dichlorofluorescin, H_2DCF) diacetate, *N*-(fluorescein-5-thiocarbanoyl)-desferrioxamine (FL-DFO), and *bis*-oxonol were obtained from Molecular Probes (Eugene, OR). Desferrioxamine (desferal mesylate) was obtained from Novartis (Summit, NJ). Diphenyleneiodonium chloride (DPI) was obtained from Calbiochem (LaJolla, CA). Protein assay reagent concentrate and γ-globulin were from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were of analytical grade.

Lung perfusion

The isolated perfused rat lung model used for this study has been described previously (Fisher *et al.*, 1980, 1991). Briefly, Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 180–200 grams were anesthetized with intraperitoneal pentobarbital sodium (30 mg/kg). Ventilation was maintained at 60 cycles/min, 2 ml tidal volume, and 2 cm H_2O end-expiratory pressure. The chest was opened and the pulmonary circulation was cleared of blood by gravity flow of perfusate with 25 cm H_2O pressure through a cannula inserted in the main pulmonary artery. The perfusate medium, Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 m*M* glucose and 3% (wt/vol) fatty acid-free bovine serum albumin (BSA) or 5% (wt/vol) clinical grade dextran was preincubated with the same gas mixture subsequently used for lung ventilation. In some experiments, Ca^{2+}-free perfusion was done with perfusate without calcium

and supplemented with 1 m*M* EGTA. The cleared lungs were freed of cardiac and other nonpulmonary tissues and were suspended in a water-jacketed perfusion chamber maintained at 37°C. Perfusion was maintained using a peristaltic pump at a constant flow rate of 10 ml/min with a recirculating volume of 40 ml. Global ischemia was produced by discontinuing perfusion for 1 hr while ventilation continued. In some experiments, ischemic lungs were reperfused at 10 ml/min for 1 hr following the 1 hr of ischemia. For high K^+ perfusion, the concentration of KCl in Krebs-Ringer bicarbonate buffer was increased to 24 m*M* K^+ with a corresponding reduction in NaCl for isosmolality. Lungs were ventilated with 95% O_2:5% CO_2, except for some experiments when 95% N_2:5% CO_2 was used as the ventilating gas during ischemia (all gases were supplied by BOC Group, Inc., Murray Hill, NJ). Perfusate PO_2 measured at 30 min and 60 min of perfusion was approximately 650 mmHg for the oxygenated (control) perfusion and less than 15 mmHg during N_2 ventilation. In some experiments, 35 μM cromakalim (a K^+-channel agonist) or 1 mg/ml desferrioxamine (an Fe chelator) was added to the perfusate 30 min before ischemia or high K^+ perfusion. Other lungs were preperfused for 30 min with 10 μM DPI, which has been shown previously to inhibit ROS generation in this model of lung ischemia (Zhao *et al.*, 1997b, Al-Mehdi *et al.*, 1998), or with 2 μM thapsigargin to deplete intracellular Ca^{2+} stores (Tozawa *et al.*, 1999). At the end of the perfusion experiment, the lungs were rapidly frozen by clamping with aluminum tongs pre-cooled in liquid N_2. Separate perfused lung preparations were required to measure: (1) ROS generation; (2) free iron release; and (3) lipid peroxidation/protein oxidation.

Measurement of ROS generation

Generation of ROS in lung tissue was monitored with dichlorodihydro-fluorescein (H_2DCF) fluorescence as previously described (Al-Mehdi *et al.*, 1994). After internalization of the diacetate compound, it is converted by intracellular esterases to nonfluorescent H_2DCF, which serves as a substrate for intracellular ROS to generate highly fluorescent DCF. Briefly, a 5 m*M* solution of H_2DCF diacetate was prepared in absolute ethanol, stored under N_2 at −20°C in darkness and added to the lung perfusate to make a final concentration of 5 μM. Lungs were perfused with the fluorophore for 30 min before initiation of ischemia. The frozen lungs suspended in saline were homogenized under N_2, and fluorescence of the homogenate was determined using a spectrofluorometer (Hitachi Perkin-Elmer, model MPF-2A) with 488 nm excitation and 530 nm emission.

Determination of lipid peroxidation

For assay of TBARS, a portion of the frozen lung tissue was homogenized under N_2 in 10 volumes of ice-cold 0.9% NaCl containing 0.2% butylated hydroxytoluene (Fisher *et al.*, 1991). An aliquot of the homogenate was extracted with TCA and reacted with TBA at 95°C for 15 min. TBARS were calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$ at 535 nm (Buege and Aust, 1978).

Determination of protein oxidation

Protein oxidation was determined by reaction with DNPH as described previously (Ayene *et al.*, 1992). A portion of the frozen lung was homogenized under N_2 in 10 m*M* HEPES buffer, pH 7.4, containing 137 m*M* NaCl, 4.6 m*M* KCl, 1.1 m*M* KH_2PO_4, 0.6 m*M* $MgSO_4$, protease inhibitors [leupeptin, 0.5 μg/ml; pepstatin, 0.7 μg/ml; aprotinin, 0.5 μg/ml; phenylmethylsulfonyl fluoride (PMSF), 40 μg/ml], and 1.1 m*M* EDTA. The homogenate was centrifuged at 100,000 × *g* for 5 min. The soluble protein in the supernatant was incubated with 10 m*M* DNPH. The DNPH derivative of the oxidized protein was separated from the unbound DNPH by Sephadex G-25 column chromatography and measured spectrophotometrically. Protein carbonyl content was calculated by using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ at 360 nm (Ayene *et al.*, 1992).

Measurement of free iron

Free iron in lung tissue was measured with FL-DFO fluorescence. Binding of low levels of either Fe^{2+} or Fe^{3+} strongly quenches the 515-nm fluorescence of FL-DFO. Briefly, a 0.24 m*M*

solution of FL-DFO was prepared in 0.9% NaCl and added to the lung perfusate to make a final concentration of 2.4 μM. Lungs were perfused with FL-DFO for 30 min and then with FL-DFO-free perfusate for 5 min to remove free FL-DFO in vasculature. Lungs were then subjected to ischemia with or without reperfusion or to high K^+ perfusion. Rapidly frozen lungs at the end of perfusion were suspended in saline and homogenized under N_2. Fluorescence of the homogenate was determined using a spectrofluorometer (Hitachi Perkin-Elmer, model MPF-2A) with 493 nm excitation and 515 nm emission. The amount of iron in each sample was determined from a standard curve using $FeSO_4$ reacted with FL-DFO.

Fluorescence imaging

Additional experiments were carried out to visualize endothelial membrane potential and free iron in intact isolated perfused lungs. Imaging was carried out using an inverted microscope, a computer controlled CCD camera (MicroMAX, Princeton Instruments, Inc., Trenton, NJ), and graphics control software (Metamorph Imaging System, Universal Imaging Corp., West Chester, PA) as previously described (Al-Mehdi *et al.*, 1997b). Fluorescence images during control and ischemic periods were obtained for *bis*-oxonol, a plasma membrane potential-sensitive dye, and FL-DFO, the iron chelation dye. The *bis*-oxonol signal increases with depolarization while the FL-DFO signal decreases as free iron increases.

Protein assays

The protein content of the lung homogenate was measured by the Coomassie blue method, using bovine γ-globulin as standard (Zhao *et al.*, 1997a).

Statistical analysis

Data are summarized as means ± SE. Data were analyzed statistically by one-way analysis of variance for multiple comparisons followed by Bonferroni's test using SigmaStat software (Jandel Scientific, San Rafael, CA). The level of statistical significance was taken as $p < 0.05$.

RESULTS

ROS generation during lung ischemia and effects of cromakalim and desferrioxamine

Similar to our previous results (Fisher *et al.*, 1991; Ayene *et al.*, 1992; Al-Mehdi *et al.*, 1994, 1997c; Zhao *et al.*, 1997a,b), lung oxygenated ischemia resulted in increased ROS generation and lipid and protein oxidation as detected by lung DCF fluorescence, TBARS, and protein carbonyls, respectively (Fig. 1). The fold increase compared with control for these parameters was 8.0 for DCF, 3.4 for TBARS, and 2.4 for protein carbonyls. Addition of a potassium channel agonist, cromakalim (35 μM), to the perfusate prior to ischemia inhibited the subsequent increase of lung DCF fluorescence by 64%, the increase of TBARS by 61%, and the increase of protein carbonyls by 65% (Fig. 1). Similar effects on these indices were observed by addition of an iron chelator, desferrioxamine (1 mg/ml), to the perfusate prior to ischemia (Fig. 1).

Lung free iron content during ischemia

A low level of tissue free iron (0.08 nmol/mg protein) was present in rat lungs that had been cleared of blood. This basal level did not change during 2 hr of normal (control) perfu-

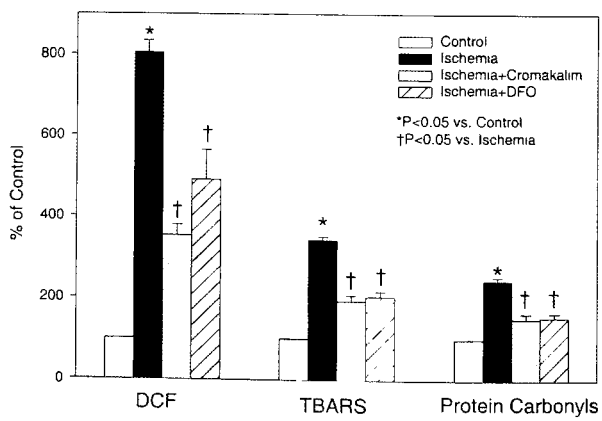


FIG. 1. Effect of cromakalim and desferrioxamine (DFO) on lung tissue ROS generation, lipid peroxidation, and protein oxidation with ischemia. Control lungs were continuously ventilated with O_2 and perfused for 2 hr. Ischemia is no perfusion with continued ventilation. *$p < 0.05$ compared with control; † $p < 0.05$ compared with ischemia group. Data are mean ± SE for four to six lungs for each condition.

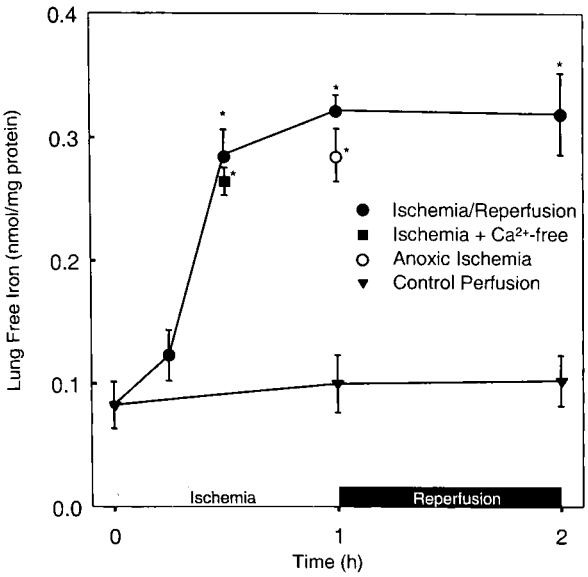


FIG. 2. Changes in lung tissue free iron during oxygenated ischemia/reperfusion or anoxic ischemia. Ischemia/reperfusion, Ca^{2+}-free perfusion, and control perfusion were done with oxygen ventilation. Anoxic ischemia was ischemia with N_2 ventilation. $^*p < 0.05$ versus control perfusion. Data are mean ± SE for three to four lungs for each condition.

sion (Fig. 2). The free iron level of lung tissue was increased 46% at 15 min and then increased about 3.5- to 4-fold above control between 30 and 60 min of ischemia (Fig. 2). There was no significant change in tissue free iron during 1 hr reperfusion (Fig. 2). The change at 1 hr of ischemia was similar whether lungs were ventilated with O_2 or N_2 during the ischemic period (Fig. 2). There was no difference in iron release with 30 min ischemia ($p > 0.05$; $n = 3$) when lungs were perfused with zero Ca^{2+} or with the standard 2.5 m*M* Ca^{2+} in the perfusion medium (Fig. 2).

The increase in free iron with lung ischemia by biochemical assay was corroborated by *in situ* imaging of endothelium in the pulmonary subpleural region. These images demonstrated 76 ± 5% ($p < 0.05$; $n = 3$) decrease in FL-DFO fluorescence at 30 min of ischemia compared with control, as shown in Fig. 3. The decreased fluorescence reflects increased cellular free iron and indicates that the endothelium is the predominant site of lung iron release with ischemia. Ischemia mediated decrease in endothelial cell FL-DFO in the presence of DPI (Fig. 3) was 89 ± 8% versus control ($p < 0.05$; $n = 3$), comparable to results in the absence of the inhibitor. Thus, ROS generation via the NADPH oxidase or other flavoprotein-associated pathway had no effect on endothelial cell iron release with ischemia. *In situ* imaging confirmed that absence of Ca^{2+} in the perfusate had no effect on endothelial free iron release with ischemia (Fig. 4). However, depletion of intracellular Ca^{2+} stores with thapsigargin completely abolished the ischemia-mediated increase in endothelial free iron (Fig. 4).

The measured iron level with ischemia in this study was comparable to our previously published data using a different assay, except that in our previous study, the iron level continued to increase during the reperfusion period (Zhao *et al.*, 1997a). We focussed on two possible methodological explanations for the difference between the two results; first, that fluorescence quenching of FL-DFO in lung tissue reached a maximum (saturation) during ischemia in the present study or, second, that iron was artifactually released from lung homogenate *in vitro* (in the test tube) in the previously used assay. The latter effect could have occurred during the standard *in vitro* incubation at 37°C for 1 hr that was used to develop the color. To test the possibility that the fluorescence quenching of FL-DFO in lung tissue was already maximal, we added 0.5, 1.0, or 1.5 nmol Fe^{2+}/mg protein to the homogenate of ischemic lungs. Fluorescence of FL-DFO was linearly quenched by the added iron (not shown), indicating that quenching of FL-DFO fluorescence was not saturated by the iron released during ischemia. To test the possibility that iron was released from lung homogenate during the assay previously used, free iron was measured in lung homogenates with incubation for 1 hr either at 37°C or 4°C. Incubation at the two different temperatures gave similar results for control lungs but disparate results for ischemic/reperfused rat lungs. Under control conditions, free iron after 1 hr of incubation was 90 ± 30 pmol/mg protein (mean ± range) for homogenate incubated at 4°C and 120 ± 40 for incubation at 37°C. For ischemic/reperfused lungs, free iron was 320 ± 40 pmol/mg protein for homogenate incubated at 4°C and 530 ± 40 with incubation at 37°C. These data indicate that iron was released dur-

FIG. 3. Effect of ischemia on FL-DFO fluorescence in subpleural microvascular endothelial cells *in situ* in the intact, isolated lung. Images are in pseudocolor with black representing lowest and white the highest fluorescence, as shown by the intensity bar at the lower right. The brightest structures are the vascular areas whereas the dark areas are the alveolar space. Ischemia led to decrease in FL-DFO fluorescence, indicating increase in endothelial cell free iron **(upper panels)**. Pretreatment of lungs for 30 min with 10 μM DPI did not inhibit the decrease in FL-DFO fluorescence with ischemia **(lower panels)**. The figures are representative of three experiments for each condition (see text in Results).

ing the 1 hr incubation of ischemic/reperfused lungs *in vitro* at 37°C and could account for an artifactually elevated free iron level during reperfusion in the previous studies.

Role of endothelial membrane potential in release of free iron

To test the role of endothelial depolarization with ischemia in triggering iron release, we utilized the K^+-channel agonist, cromakalim, to inhibit depolarization. Similar to our previous results (Al-Mehdi *et al.*, 1997b), ischemia was accompanied by depolarization, as indicated by increased fluorescence of the dye, *bis*-oxonol (Fig. 5). The addition of cromakalim prevented the increase in *bis*-oxonol fluorescence with ischemia (Fig. 5) indicating maintenance of endothelial membrane potential in the presence of this agonist, and also significantly inhibited the increase in lung free iron (Fig. 6). This latter result is compatible with the ability of cromakalim to prevent oxidative lung injury with ischemia as shown in Fig. 1.

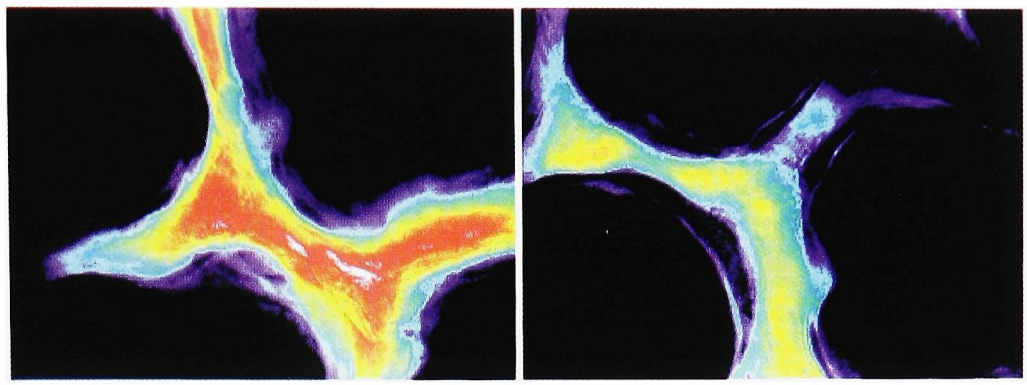
The role of membrane potential in iron release was evaluated further with a high K^+ (24 mM) perfusion model that, like lung ischemia, results in membrane depolarization and ROS generation. High K^+ perfusion led to increase tissue free iron which was blocked by the presence of cromakalim (Fig. 6).

DISCUSSION

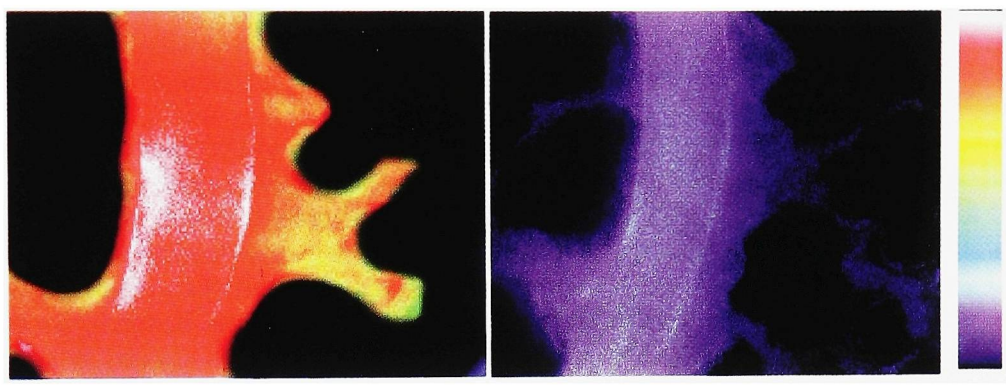
A role for free iron in tissue injury associated with a variety of oxidative insults is now widely accepted (Halliwell and Gutteridge, 1990). Iron may mediate these toxic effects through several possible pathways. For example, Fe^{2+} in the presence of $O_2^{-\cdot}$ and hydrogen peroxide (H_2O_2) can generate ·OH, a potent oxidant. Oxidation reactions may also generate the perferryl radical, another potent oxidant. Fe^{2+} also has been ascribed a role in site-specific protein oxidation through its binding to specific amino acids where it can mediate reaction with H_2O_2 (Stadtman and Oliver, 1991). To minimize these and other potentially damaging reactions, cells normally sequester iron within intracellular "transit" iron pools and within ferritin molecules, the major intracellular storage site.

Delocalization of iron from intracellular stores has been documented for several models of ischemia and reperfusion. These models include cardiac arrest and resuscitation, the isolated rat heart, isolated rabbit lungs, and ischemia of the intestine and hindlimb (Holt *et al.*, 1986; Paller and Hedlund, 1988; Robinson and Hedlund, 1989; Bysani *et al.*, 1990; Boucher *et al.*, 1992; Chiao *et al.*, 1994; Oubidar *et al.*, 1994). In one study, increased tissue iron was shown during a 2-hr period of cardiac ischemia, indicating that reperfusion is not necessary for iron release in the heart (Holt *et al.*, 1986). Previously, we have come to a similar conclusion, confirmed in the present study, based on studies with the isolated rat lung (Zhao *et al.*, 1997a). The present study showed progressive increase of tissue free iron during global ischemia, with a plateau at 30–60 min and no change during a subsequent 1-hr reperfusion. Elevation of tissue free iron with is-

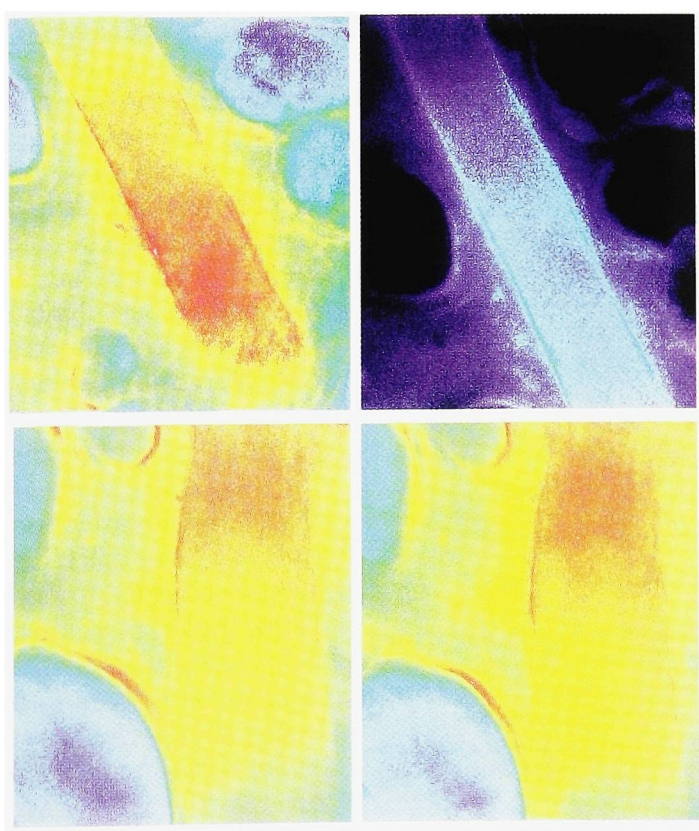
FIG. 4. Effect of Ca^{2+}-free medium and thapsigargin on subpleural microvascular endothelial cell free iron with ischemia. Images were obtained as in Fig. 3. The upper left and lower left represent control experiments with Ca^{2+}-free perfusion and pre-perfusion with 2 μM thapsigargin, respectively. There was no effect of Ca^{2+}-free medium on the ischemic-mediated decrease of FL-DFO fluorescence (upper right panel) whereas thapsigargin markedly inhibited the normal decrease in fluorescence with ischemia (lower right panel). This result indicates that depletion of intracellular Ca^{2+} stores abolishes the ischemia-mediated iron release. The result is representative of two independent experiments.



Control Ischemia



Control + DPI Ischemia + DPI



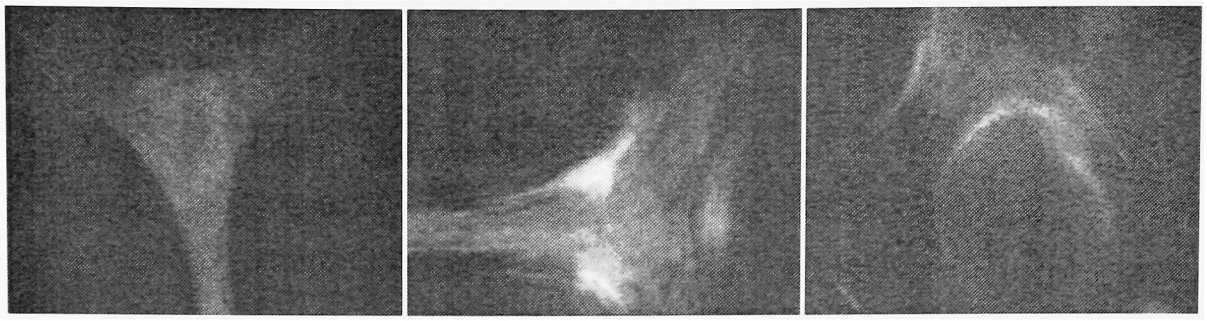


FIG. 5. Effect of cromakalim on *bis*-oxonol fluorescence in subpleural microvascular endothelial cells *in situ* in the intact, isolated lung. Ischemic increase in endothelial *bis*-oxonol fluorescence indicates membrane depolarization. Cromakalim pretreatment inhibited the increase in *bis*-oxonol fluorescence during ischemia. Data are representative of four experiments.

chemia could mediate oxidative injury during either the ischemic or reperfusion phase in large part depending on the simultaneous generation of ROS. In systemic organs, ROS are generated and oxidative injury occurs when oxygen is resupplied during reperfusion. By contrast, the adequate oxygenation of ventilated lung during ischemia permits the generation of ROS and iron-mediated tissue injury during the ischemic phase.

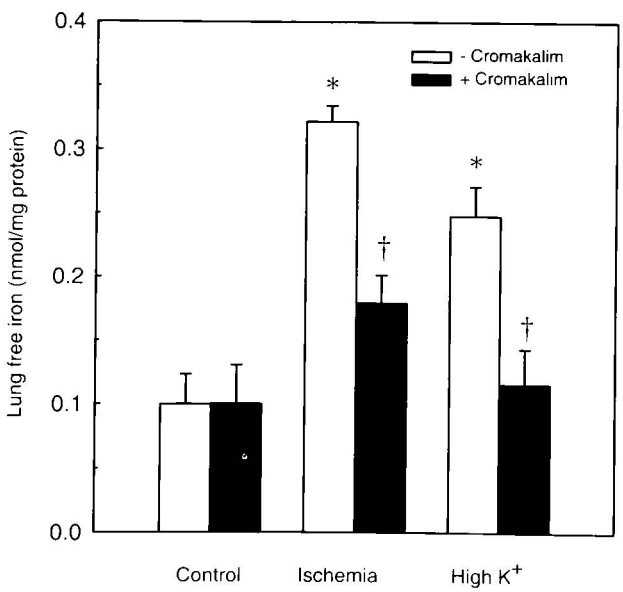


FIG. 6. Effect of cromakalim on lung free iron during lung ischemia and high K^+ perfusion. Both ischemia and high K^+ led to increased free iron levels in lung tissue that was prevented with cromakalim pretreatment. $*p < 0.05$ compared with control; $\dagger p < 0.05$ compared with ischemia group. Data are mean ± SE for four lungs for each condition.

A role for free iron in oxidative injury with ischemia and reperfusion is indicated by the results with the iron chelator desferrioxamine. A protective effect of desferrioxamine or other iron chelators has been observed in ischemia/reperfusion models utilizing the heart, intestine, kidney, or skeletal muscle as well as the lungs (Fuller *et al.*, 1987; Haynes *et al.*, 1990; Lesnefsky *et al.*, 1990; Lutz *et al.*, 1990; Conte *et al.*, 1991; Ayene *et al.*, 1993; Fantini and Yoshioka, 1993; Zhao *et al.*, 1997a). In the present study, desferrioxamine significantly inhibited lipid peroxidation, as indicated by TBARS, and protein oxidation, as indicated by protein carbonyl formation, during lung ischemia. Desferrioxamine also inhibited oxidation of the fluorophor DCF, suggesting a role for iron-mediated oxidants in this reaction.

Our previous studies have indicated that lung ischemia results in a relatively rapid (within minutes) depolarization of the endothelial cell membrane that is ATP independent (Al-Mehdi *et al.*, 1997b). We have speculated that depolarization is due to a mechanical sensing by the endothelial cell membrane, or, more specifically, to the loss of shear stress. Evidence linking membrane depolarization to shear stress was obtained by studies relating perfusate flow rate to membrane potential in the isolated perfused rat lung (Al-Mehdi *et al.*, 1997b). This relationship may be mediated through shear stress-activated K^+ channels,

which are thought to be responsible for maintaining endothelial cell membrane potential during flow (Olesen *et al.*, 1988). In the present study, we confirmed that lungs made ischemic demonstrate increased *bis*-oxonol fluorescence, compatible with endothelial membrane depolarization as previously described (Al-Mehdi *et al.*, 1997b). On the basis of the hypothesis that a K^+-channel agonist could stabilize membrane potential during ischemia, lungs were perfused with cromakalim and evaluated for its effect on ischemia-mediated membrane depolarization as indicated by *bis*-oxonol fluorescence. Pretreatment of lungs with cromakalim partially prevented both ischemia-mediated depolarization and also the increase in lung free iron, suggesting an association between membrane potential and release of free iron from its intracellular stores. In further experiments, we perfused lungs with high K^+ to depolarize the endothelium and simulate the effects of lung ischemia (Al-Mehdi *et al.*, 1997a). Perfusion with high K^+ (in the absence of ischemia) led to increased lung tissue free iron, which was partially inhibited by the presence of cromakalim. Thus, our results show that tissue free iron is increased with cell membrane depolarization induced by lung ischemia or perfusion with high K^+ and this effect is partially prevented by the presence of an agonist that stabilizes the endothelial cell membrane potential. Because cromakalim did not completely block iron release with ischemia, it is possible that a second mechanism unrelated to membrane potential plays an additional role in these events.

The present results confirm our previous findings of increased ROS generation and oxidant injury during oxygenated lung ischemia (Fisher *et al.*, 1991; Ayene *et al.*, 1992; Al-Mehdi *et al.*, 1997c, 1998; Zhao *et al.*, 1997a,b). Ischemic lungs showed increased DCF fluorescence as well as increased generation of TBARS and protein carbonyls. Like its effects on membrane potential and iron release, ROS generation with ischemia was partially prevented by the presence of cromakalim. Based on previous studies with this class of compounds, this latter effect of cromakalim is not due to ROS scavenging and likely was a result of its effects on K^+ channels (Pieper and Gross, 1992). Because ROS generation and iron release both occur early in response to membrane depolarization, iron release could be a secondary response to increased ROS. However, this possibility is excluded because ventilation of the lung with N_2 during ischemia prevents the generation of ROS (Ayene *et al.*, 1992; Al-Mehdi *et al.*, 1997c) but did not affect the liberation of free iron (Zhao *et al.*, 1997a and present study). This result was corroborated by perfusion of DPI, which blocks ROS generation with ischemia (Zhao *et al.*, 1997b; Al-Mehdi *et al.*, 1998) but had no effect on iron release in the present study. Another possible mechanism is that membrane depolarization leads to Ca^{2+} influx or Ca^{2+} release from intracellular stores, which then results in liberation of iron from its intracellular binding sites. Although Ca^{2+}-free medium had no effect on iron release with ischemia, thapsigargin markedly inhibited this effect, raising the possibility that membrane depolarization-induced release of Ca^{2+} from internal stores triggers the release of iron. Other possible mechanisms are that cell membrane depolarization leads to instability of mitochondrial Fe-containing enzymes or to the generation of NO, which triggers release of iron from intracellular storage sites. Whether one of these or some other mechanism is responsible for linkage between cell membrane depolarization and the increased cellular free iron with lung ischemia will require additional study.

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ABBREVIATIONS

BSA, bovine serum albumin; DCF, dichlorofluoroscein; DNPH, dinitrophenyl hydrazine; DPI, diphenyleneiodonium chloride; FL-DFO, fluoroscein desferrioxamine; H_2DCF, dichlor-

odihydrofluorescein; H_2O_2, hydrogen peroxide; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

REFERENCES

AL-MEHDI, A.B., SHUMAN, H., and FISHER, A.B. (1994). Fluorescence microtopography of oxidative stress in lung ischemia-reperfusion. Lab. Invest. **70,** 579–587.

AL-MEHDI, A.B., SHUMAN, H., and FISHER, A.B. (1997a). Oxidant generation with K^+-induced depolarization in the isolated perfused lung. Free Rad. Biol. Med. **23,** 47–56.

AL-MEHDI, A.B., ZHAO, G., and FISHER, A.B. (1997b). ATP-independent membrane depolarization with ischemia in the oxygen-ventilated isolated rat lung. Am. J. Respir. Cell Mol. Biol. **18,** 653–661.

AL-MEHDI, A., SHUMAN, H., and FISHER, A.B. (1997c). Intracellular generation of reactive oxygen species during non-hypoxic lung ischemia. Am. J. Physiol. (Lung Cell. Mol. Physiol. 16). **272,** L294–L300.

AL-MEHDI, A.B., ZHAO, G., DODIA, C., TOZAWA, K., COSTA, K., MUZYKANTOV, V., ROSS, C., BLECHA, F., DINAUER, M., and FISHER, A.B. (1998). Endothelial NADPH oxidase as the source of oxidants with lung ischemia or high K^+ Circ. Res. **83,** 730–737.

AYENE, I.S., DODIA, C., and FISHER, A.B. (1992). Role of oxygen in oxidation of lipid and protein during ischemia/reperfusion in isolated perfused rat lung. Arch. Biochem. Biophys. **296,** 183–189.

AYENE, S.I., AL-MEHDI, A.B., and FISHER, A.B. (1993). Inhibition of lung tissue oxidation during ischemia/reperfusion by 2-mercaptopropionylglycine. Arch. Biochem. Biophys. **303,** 307–312.

BOUCHER, F.S., PUCHEU, S., CONDRAY, C., FAVIER, A., and DELERIS, J. (1992). Evidence of cytosolic iron release during post-ischemic reperfusion of isolated rat heart-influence on spin-trapping experiments with DMPO. FEBS Lett. **302,** 261–264.

BUEGE, J.A., and AUST, S.D. (1978). Microsomal lipid peroxidation. Methods Enzymol. **52,** 302–310.

BYSANI, G.K., KENNEDY, T.P., KY, N., RAO, N.V., BLAZE, C.A., and HOIDAL, J.R. (1990). Role of cytochrome P-450 in reperfusion injury of the rabbit lung. J. Clin. Invest. **86,** 1434–1441.

CHIAO, J.J., KIRSCHNER, R.E., and FANTIM, G.A. (1994). Iron delocalization occurs during ischemia and persists on reoxygenation of skeletal muscle. J. Lab. Clin. Med. **124,** 432–438.

CONTE, J.V., JR., KATZ, N.M., FOEGH, M.L., WALLACE, R.B., and RAMWELL, P.W. (1991). Iron chelation therapy and lung transplantation. Effects of deferoxamine on lung preservation in canine single lung transplantation. J. Thorac. Cardiovasc. Surg. **101,** 1024–1029.

FANTINI, G.A., and YOSHIOKA, T. (1993). Deferoxamine prevents lipid peroxidation and attenuates reoxygenation injury in postischemic skeletal muscle. Am. J. Physiol. **264,** H1953–H1959.

FISHER, A.B., DODIA, C., and LINASK, J. (1980). Perfusate composition and edema formation in isolated rat lungs. Exp. Lung Res. **1,** 13–22.

FISHER, A.B., DODIA, C., TAN, Z., AYENE, I., and ECKENHOFF, R.G. (1991). Oxygen-dependent lipid peroxidation during lung ischemia. J. Clin. Invest. **88,** 674–679.

FULLER, B.J., LUNEC, J., HEALING, G., SIMPKIN, S., and GREEN, C.J. (1987). Reduction of susceptibility to lipid peroxidation by desferrioxamine in rabbit kidneys subjected to 24–h cold ischemia and reperfusion. Transplantation **43,** 604–606.

HALLIWELL, B., and GUTTERIDGE, J.M.C. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. **186,** 1–85.

HAYNES, J., JR., SEIBERT, A., BASS, J.B., and TAYLOR, A.E. (1990). U74500A inhibition of oxidant-mediated lung injury. Am. J. Physiol. **259,** H144–H148.

HOLT, S., GUNDERSON, M., JOYCE, K., NAYINI, N.R., EYSTER, G.F., GARITANO, A.M., ZONIA, C., KRAUSE, G.S., AUST, S.D., and WHITE, B.C. (1986). Myocardial tissue iron delocalization and evidence for lipid peroxidation after two hours of ischemia. Ann. Emerg. Med. **15,** 1155–1159.

LESNEFSKY, E.J., REPINE, J.E., and HORWITZ, L.D. (1990). Deferoxamine pretreatment reduces canine infarct size and oxidative injury. J. Pharmacol. Esp. Ther. **253,** 1103–1109.

LUTZ, J., AUGUSTIN, A., and FRIEDRICH, E. (1990). Severity of oxygen free radical effects after ischemia and reperfusion in intestinal tissue and the influence of different drugs. Adv. Exp. Med. Biol. **277,** 683–690.

OLESEN, S.P., CLAPMAN, D.E., and DAVIES, P.F. (1988). Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. Nature **331,** 168–170.

OUBIDAR, M., BOQUILLON, M., MARIE, C., SCHREIBER, L., and BRALET, J. (1994). Ischemia-induced brain iron delocalization: effect of iron chelators. Free Rad. Biol. Med. **16,** 861–867.

PALLER, M.S., and HEDLUND, B.E. (1988). Role of iron in postischemic renal injury in the rat. Kidney Int. **34,** 474–480.

PIEPER, G.M., and GROSS, G.J. (1992). EMD 52692 (bimakalim), a new potassium channel opener, attenuates luminol-enhanced chemiluminescence and superoxide anion radical formation by zymosan-activated polymorphonuclear leukocytes. Immunopharmacology **23,** 191–197.

ROBINSON, E., and HEDLUND, B. (1989). Role of iron in ischemia and reperfusion. Circ. Shock **27,** 367–3624.

STADTMAN, E.R., and OLIVER, C.N. (1991). Metal-catalyzed oxidation of proteins. Physiological consequences. J. Biol. Chem. **266,** 2005–2008.

TOZAWA, K., AL-MEHDI, A.B., MUZYKANTOV, V.,

and FISHER, A.B. (1999). *In situ* imaging of intracellular calcium with ischemia in lung subpleural microvascular endothelial cells. Antiox. Redox Signal. **1,** 145–154.

WEI, Z., COSTA, K., AL-MEHDI, A.B., DODIA, C., MUZYKANTOV, V., and FISHER, A.B. (1999). Simulated ischemia in flow adapted endothelial cells leads to generation of reactive oxygen species and cell signaling. Circ. Res. **85,** 682–689.

ZHAO, G., AYENE I.S., and FISHER, A.B. (1997a). Role of iron in ischemia-reperfusion oxidative injury of rat lungs. Am. J. Respir. Cell Mol. Biol. **16,** 293–299.

ZHAO, G., AL-MEHDI, A.B., and FISHER, A.B. (1997b). Different pathways for oxidant generation with anoxia/ reoxygenation versus ischemia/reperfusion in rat lungs. Am. J. Physiol. (Lung Cell. Mol. Physiol.) **273,** L1112–L1117.

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2. Uta Kerkweg, Katja Pamp, Judith Fieker, Frank Petrat, Robert C. Hider, Herbert de Groot. 2009. Release of redox-active iron by muscle crush trauma. *Shock* 1. [CrossRef]
3. Cheng-Tan Li, Wei-Ping Zhang, Yun-Bi Lu, San-Hua Fang, Yu-Mei Yuan, Ling-Ling Qi, Li-Hui Zhang, Xiao-Jia Huang, Lei Zhang, Zhong Chen, Er-Qing Wei. 2009. Oxygen-glucose deprivation activates 5-lipoxygenase mediated by oxidative stress through the p38 mitogen-activated protein kinase pathway in PC12 cells. *Journal of Neuroscience Research* **87**:4, 991-1001. [CrossRef]
4. Shampa Chatterjee, Kenneth E. Chapman, Aron B. Fisher. 2008. Lung Ischemia: A Model for Endothelial Mechanotransduction. *Cell Biochemistry and Biophysics* **52**:3, 125-138. [CrossRef]
5. Qunwei Zhang , Shampa Chatterjee , Zhihua Wei , Wei Dong Liu , Aron B. Fisher . 2008. Rac and PI3 Kinase Mediate Endothelial Cell–Reactive Oxygen Species Generation During Normoxic Lung IschemiaRac and PI3 Kinase Mediate Endothelial Cell–Reactive Oxygen Species Generation During Normoxic Lung Ischemia. *Antioxidants & Redox Signaling* **10**:4, 679-690. [Abstract] [PDF] [PDF Plus]
6. Tatyana Milovanova , Yefim Manevich , Alex Haddad , Shampa Chatterjee , Jonni S. Moore , Aron B. Fisher . 2004. Endothelial Cell Proliferation Associated with Abrupt Reduction in Shear Stress Is Dependent on Reactive Oxygen SpeciesEndothelial Cell Proliferation Associated with Abrupt Reduction in Shear Stress Is Dependent on Reactive Oxygen Species. *Antioxidants & Redox Signaling* **6**:2, 245-258. [Abstract] [PDF] [PDF Plus]
7. Aron B. Fisher, Abu B. Al-Mehdi, Yefim Manevich. 2002. Shear stress and endothelial cell activation. *Critical Care Medicine* **30**:Supplement, S192-S197. [CrossRef]