Reactive oxygen species and cell signaling with lung ischemia.

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INTRODUCTION AND OVERVIEW OF ISCHEMIA-REPERFUSION

This presentation concerns reactive oxygen species (ROS) and cell signaling in lung ischemia and during reperfusion. A number of years ago, McCord, Granger and others described the now-classic paradigm for ischemia-reperfusion injury: anoxia during ischemia results in the breakdown of ATP to xanthines and a protease activation that converts xanthine dehydrogenase to an oxidase. ROS are generated when oxygen is supplied during reperfusion, resulting in tissue injury (1, 2). Our contribution was the demonstration that ROS generation is initiated during the ischemic period and represents a response to altered mechanotransduction rather than a primary response to metabolic events.

We began the studies described in this report approximately fifteen years ago to investigate ischemia-reperfusion injury in the lung. Unlike other tissues, the lung parenchyma does not rely on its circulation for cellular oxygen requirements. Thus, the lung adds rather than removes oxygen from the pulmonary arterial blood. Ischemia in the lung, unlike that in other organs, does not result in tissue anoxia. Our initial goal was to use the isolated lung model in order to separate the effects of ischemia-reperfusion from those resulting from anoxia-reoxygenation. We called this model oxygenated ischemia because we continued to ventilate the lungs with air (plus 5% CO_2 to maintain pH balance) during the ischemia period. The ventilation of the lungs with nitrogen resulted in a marked decrease in lung ATP content, compatible with anoxia (3, 4). By contrast, measurement of tissue ATP after one hour of oxygenated ischemia showed no change compared with continuously perfused lungs. This confirmed the adequacy of oxygenation during the ischemic period (3, 5). Therefore, this isolated lung preparation can be used to study ischemia without the confounding effects of tissue anoxia.

Oxidative Injury in Lung Ischemia

Our initial observation was that oxygenated ischemia in the lung resulted in evidence of oxidative injury similar to that observed with anoxic ischemia followed by reperfusion in other tissues. We found that thiobarbituric acid reactive substances (TBARS) and conjugated dienes, indices of tissue lipid peroxidation, and protein carbonyls, an index of protein oxidation, increased progressively in the lung between 15 and 60 minutes of oxygenated ischemia (3, 6). Since oxidant production by the lung was unrelated to either anoxia or reoxygenation, the ischemia-mediated injury could not be explained by the classic paradigm for ROS production with ischemia-reperfusion. We considered potential mechanisms other than reoxygenation as

possible causes for the ischemic effect. Manipulation of glucose delivery and tissue pH showed that changes in these parameters were not responsible for the ischemia-mediated events (3).

Shear Stress in Lung Ischemia

At this stage of perplexity, we realized that endothelial cells are normally subjected to mechanical forces associated with blood flow and that an alteration in these forces with ischemia (i.e. no-flow) represents a major physiological change. Although the effects of increased shear stress on endothelial cells *in vitro* had been recognized (7), possible alteration of endothelial function with loss of shear stress *in vivo* had not been considered. The exposure of endothelial cells cultured under the usual static conditions to subsequent laminar shear stress elicits an adaptation response. The response is characterized by changes in ion channel activity during the first minute after imposition of shear, activation of signaling molecules during the subsequent hour, changes in cell surface proteins, including adhesion molecules over the next several hours, and finally, changes in cell shape with cellular realignment in the direction of flow (7). From this background, we reasoned that cells normally accustomed to shear stress might respond similarly when the shear is removed. The remainder of this presentation deals with the response to ischemia from the perspective of altered shear stress.

Endothelial Response *In Vivo* with Ischemia

We have now developed a scheme describing the changes in endothelial cells that we observe in the first minute following abrupt cessation of flow in the pulmonary microvasculature (Figure 1). The initial event is the rapid inactivation of cell membrane K_{ATP} channels, resulting in endothelial cell membrane depolarization. It is followed by the activation of endothelial membrane-localized NADPH oxidase. The NADPH oxidase enzyme complex has been well studied in polymorphonuclear leukocytes (PMN) and now, we and others have shown that the components of this oxidase are present in endothelial cells (8, 9). The depolarization of the endothelial cell also leads to the opening of voltage-dependent calcium channels. This results in calcium influx, activation of eNOS, and NO generation (Figure 1). While we can only speculate on the adaptive nature of these effects, we propose that they represent a physiological attempt to increase blood flow and perhaps initiate a signaling response, which may serve to generate or repair blood vessels.

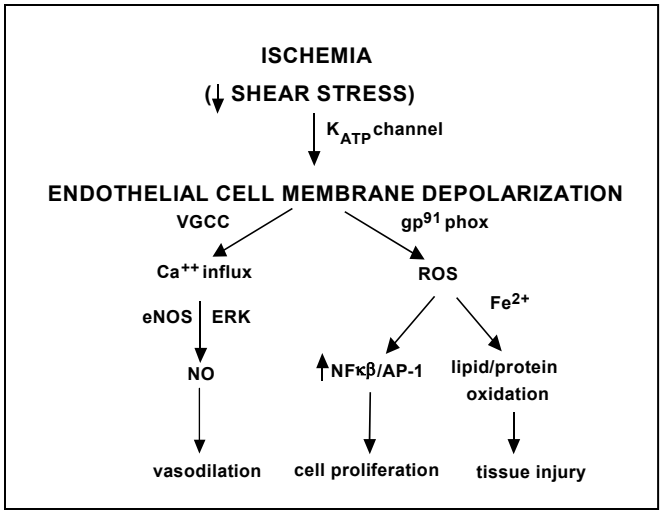

Fig. 1. Schematic demonstrating the response of the pulmonary vascular endothelium to oxygenated ischemia. See text for details**.**

Now, I will describe some of the experiments in greater detail. Studies with the isolated perfused lung utilized pleural surface fluorescence measurement as well as a subpleural imaging system in order to visualize these initial events with flow cessation. For the measurement of surface fluorescence, a light guide was placed against the pleural surface for fluorophore excitation and capture of fluorescence emission (10). For imaging, the lung was placed on the stage of an inverted epifluorescence microscope equipped with a

Metamorph imaging system or a confocal microscope, enabling us to visualize the endothelium of subpleural microvessels in the range of 20 to 50 μm in size (5). Imaging studies were carried out using a variety of fluorophores that were infused into the intact lung through the pulmonary circulation. Endothelial localization of fluorophores was confirmed by their co-localization with diI-acetylated LDL, an endothelial cell-specific marker (8, 11), and by a localization that was distinctly different from that observed with an epithelial cell marker instilled into the airway (5). Endothelial cell membrane potential was imaged with bis-oxonol or the more rapidly responding di-8-ANEPPS. We used a variety of probes to detect ROS: dichlorofluorescein (DCF), administered as the diacetate, is an intracellular detector not specific for ROS and might also detect reactive nitrogen species; hydroethidine, also intracellular, is oxidized by superoxide anion ; and amplex red, which is confined to the extracellular space, and in the presence of added peroxidase, detects H_2O_2. Fluo 3 was used to image intracellular calcium, and diaminofluorescein 2-T (DAF-2T) was used as a detector of NO.

Ischemia resulted in endothelial cell membrane depolarization, as indicated by increased bis-oxonol fluorescence (5, 12). This change was reversible with reperfusion and the cycle of depolarization-repolarization could be repeated for several cycles of ischemia and reperfusion (5). Ischemia also resulted in ROS generation (8, 10, 12) and increased intracellular Ca^{2+} (11). Our next goal was to couple video imaging to the microscopy system in order to determine timing for the observed changes with ischemia. Somewhat surprisingly, the increase in fluorescence compatible with membrane depolarization was extremely rapid and occurred within the initial one to two seconds after flow was suddenly stopped (13). The rapid increase within the first second raised the concern that this effect might represent a mechanical artifact, but a video scan at 18 frames per second demonstrated a gradual change in signal that rapidly reached a plateau value. Further evidence against an artifactual result was obtained by pre-treatment of lungs with lemakalim, a K_{ATP} channel agonist, which prevented the increase in fluorescence with ischemia (12, 13). Thus, lung ischemia resulted in rapid cell membrane depolarization that could be prevented with a K_{ATP} channel opener. Using a relatively crude calibration curve, we estimated that the membrane potential change with ischemia was about twenty millivolts (13).

Amplex red was used in video imaging studies as a probe for ROS (13). With ischemia, increased fluorescence was noted in approximately two to four seconds, progressively increasing during the subsequent several minutes of observation. ROS production was calcium-independent but was blocked with catalase, as expected. Fluo 3 was used as an indicator of intracellular calcium (13). An increased fluorescence in endothelial cells was observed at 10 to 15 seconds after the onset of ischemia. Based on results with thapsigargin, increased intracellular Ca^{2+} was due to an initial release from intracellular stores followed by influx from the extracellular space (13).

We also evaluated endothelial generation of NO using DAF-2T as the fluorescent indicator. This probe showed an increased fluorescence at 45 to 60 seconds after onset on ischemia (14). NO generation was blocked by the use of calcium-free perfusion medium or by a calmodulin inhibitor. Ca^{2+}/calmodulin-dependence is compatible with eNOS as the enzyme responsible for NO generation (14).

In summary, the use of fluorescent probes indicated a sequence of events associated with lung ischemia characterized by rapid membrane depolarization occurring in the initial one to two

seconds, ROS generation at two to four seconds, Ca^{2+} increase at 10 to 15 seconds, and NO generation at 45 to 60 seconds (Figure 2).

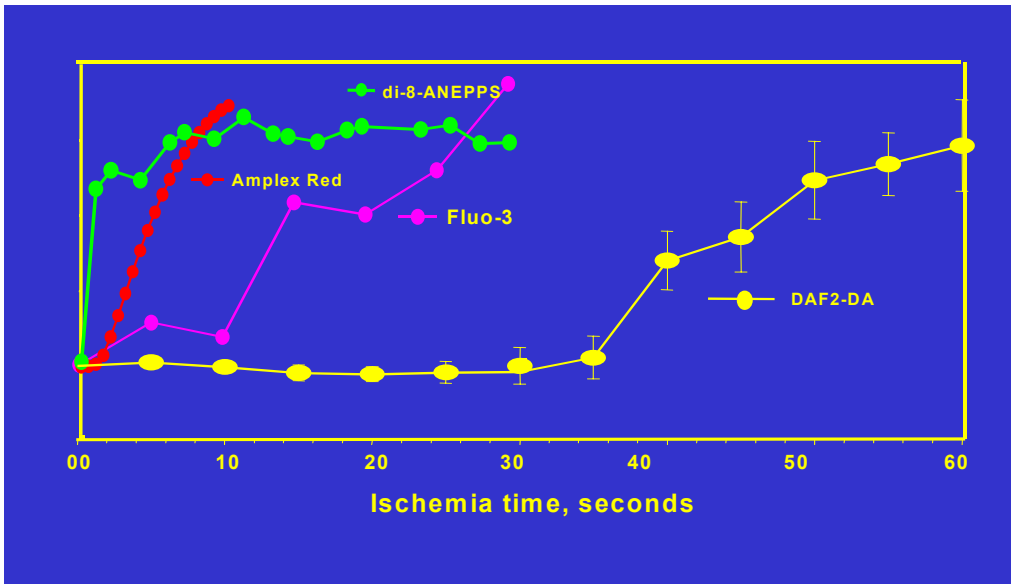

Fig 2. Temporal sequence of events in pulmonary microvascular endothelial cells, as indicated by fluorescent probes following the acute onset of lung ischemia. Di-8-ANEPPS, cell membrane potential; Amplex red, ROS production; Fluo-3, intracellular Ca^{2+}; DAF-2T, NO. Data from (13, 14).

Ischemia vs. Anoxia-Reoxygenation

We compared mechanisms for ROS generation during ischemia versus anoxia/reoxygenation (4). Ventilating the lungs with nitrogen for one hour, followed by one hour ventilation with oxygen produced anoxia/reoxygenation. DCF was used as an index of ROS generation in the absence or presence of inhibitors. Allopurinol was used as an inhibitor of xanthine oxidase. Diphenylene iodonium (DPI), an inhibitor of flavoproteins, has been used to inhibit NADPH oxidase activity in phagocytes, though it is not specific for that enzyme. DPI inhibits $gp91^{phox}$, the flavoprotein component of NADPH oxidase that transfers the electron to O_2 to generate superoxide anion. ROS production was observed with both ischemia and anoxia/reoxygenation, the former giving a somewhat greater level of DCF oxidation (4). However, the effects of the inhibitors were diametrically opposed (4). Allopurinol had no effect on ROS production during ischemia but as predicted, blocked ROS production with anoxia/reoxygenation. On the other hand, DPI blocked ROS production with ischemia but had no effect with anoxia/reoxygenation. These results suggest that NADPH oxidase is the enzyme responsible for ROS generation in lung endothelium with ischemia while xanthine oxidase is the ROS generator with anoxia/reoxygeneration.

NADPH Oxidase and ROS Generation

To study further the role of NADPH oxidase in lung ischemia, we utilized mice with "knockout" of $gp91^{phox}$ (kindly supplied by Mary Dinauer, University of Indiana). These mice were generated as a model of chronic granulomatous disease. Increased DCF fluorescence with ischemia in the wild-type mouse lung was similar to that described for the rat. However, there

was no increase in DCF fluorescence in lungs from $gp91^{phox}$ knockout mice, providing additional evidence that the source of ROS with ischemia is the NADPH oxidase (8).

In Vitro Models of Endothelial Ischemia

We extended our studies to *in vitro* models to further investigate the mechanisms for the response of pulmonary vascular endothelial cells to ischemia. Yefim Manevich designed a parallel plate laminar flow chamber with the dimensions of a standard curvette holder. It could be put into a fluorometer or spectrophotometer for direct and continuous read-out (15). Endothelial cells were grown under laminar flow conditions on an optically clear plastic slide. The initial studies utilized bovine pulmonary artery endothelial cells, but subsequently, rat pulmonary microvascular endothelial cells were evaluated when such preparation became available.

When cells were subjected to laminar flow for 24 hours, they reoriented in the direction of the flow, a well-known phenomenon, indicating flow adaptation (15). These flow-adapted cells were then studied with simulated ischemia by abruptly stopping the flow. With bis-oxonol as a probe of membrane potential, cessation of the flow resulted in a rapid increase in fluorescence compatible with membrane depolarization. This was followed by rapid repolarization when the flow was started again, reproducing the changes noted in perfused lungs (15). There was no change in bis-oxonol fluorescence with flow cessation in cells that had been subjected to flow for only thirty minutes before the experiment and therefore, were not flow-adapted. Thus, membrane depolarization in response to ischemia required flow-adapted cells which presumably is the state of the endothelium in the normal lung .

We next evaluated the generation of ROS in a spectrophotometer using cytochrome c added to the medium as a trap for extracellular superoxide anion (15). We postulated by analogy with PMN that the membrane-bound NADPH oxidase of endothelium would generate superoxide extracellularly. Reduction of cytochrome c and its inhibition by superoxide dismutase indicated the generation of superoxide anion with ischemia. The generation of superoxide reached a plateau after several minutes, likely because of the decreasing O_2 content of the medium. The time to plateau could be extended four to fivefold by gassing the medium with O_2 instead of air, though the initial rate of cytochrome c reduction was similar for the two conditions. Superoxide production was blocked by the addition of DPI to inhibit the NADPH oxidase. Cells that were not flow-adapted showed no change in superoxide production with simulated ischemia.

NO production by the cells was evaluated using DAF-2T as the fluorophore (15). Flow-adapted cells show NO production that was blocked by L-NAME, an inhibitor of NO synthase, and by the addition of EGTA to the medium, indicating calcium-dependence. Thus, simulated ischemia in this *in vitro* system reproduced the effects we observed with the perfused lung.

Cell Signaling with Ischemia

The above *in vitro* model permitted only short-term studies since O_2 availability became limiting and eventuated in anoxic cells. Therefore, we developed an *in vitro* model that permitted longer-term studies by using a commercially available artificial capillary system (16). This system consists of multiple porous capillaries that we pre-coat with fibronectin . During the cell attachment period, medium is perfused via abluminal ports in order to provide oxygen and substrate. Following attachment, we switch to luminal perfusion for flow adaptation. To impose ischemia, the perfusate is switched back to the abluminal ports so that the cells can be provided

with oxygen but do not experience shear stress. This protocol simulates our lung model of oxygenated ischemia. As with the isolated lung and parallel plate *in vitro* system, cells that were flow-adapted and then subjected to simulated ischemia showed DPI-inhibitable ROS generation, as indicated by DCF fluorescence (16) and NO generation, as detected with DAF-2T (17).

We used this *in vitro* model to investigate signaling mechanisms that might be initiated in response to ischemia. Simulated ischemia for ten minutes resulted in significant increase of phophorylated extracellular signal-regulated kinase (ERK) by immunoblot with no change in total ERK, indicating the activation of this kinase (17). Nuclear factor-kappa B (NF-kB) activation was demonstrated by electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts from cells following one hour of ischemia (shorter time points were not studied) (16). There appeared to be activation of both the P65 homodimer and P50/P65 heterodimer. An increase also was seen by assay for activator protein-1 (AP-1) (16). Treatment with DPI to block ROS generation prevented the increases in ERK, NF-kB, and AP-1, as did ROS scavengers (catalase or N-acetyl cysteine) (16, 17).

These experiments indicate that the generation of superoxide with ischemia results in a signaling cascade, presumably with downstream effects. We evaluated cell proliferation as one of the possible responses to the ROS-induced signals. Ischemia resulted in a significant increase in ^{3}H-thymidine incorporation into DNA after 24 hours ischemia, indicating increased DNA synthesis or repair (16). The use of a fluorescence-activated cell sorter (FACS) showed a decrease in the percentage of cells in the G_0G_1 phase, and an increased percentage of cells in the S phase with ischemia, suggesting that ischemia did, in fact, activate cell division (16).

SUMMARY AND CONCLUSIONS

In summary, our studies, utilizing the intact lung and several *in vitro* models, have shown a characteristic response of flow-adapted endothelial cells to ischemia. We believe that this effect represents a response to decreased shear stress since it is unrelated to cellular oxygenation. The response is characterized by endothelial cell depolarization, followed by activation of the membrane-bound NADPH oxidase with generation of ROS, cell signaling, activation of transcription factors, and increased cell division. We postulate that the physiologic role of this response is an attempt to restore blood flow through vasodilation and the repair or genesis of blood vessels.

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