Membrane depolarization and NADPH oxidase activation in aortic endothelium during ischemia reflect altered mechanotransduction

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Matsuzaki, Ikuo, Shampa Chatterjee, Kris DeBolt, Yefim Manevich, Quinwei Zhang, and Aron B. Fisher. Membrane depolarization and NADPH oxidase activation in aortic endothelium during ischemia reflect altered mechanotransduction. Am J Physiol Heart Circ Physiol 288: H336–H343, 2005. First published August 26, 2004; doi:10.1152/ajpheart.00025.2004.—We previously showed that “ischemia” (abrupt cessation of flow) leads to rapid membrane depolarization and increased generation of reactive oxygen species (ROS) in lung microvascular endothelial cells. This response is not associated with anoxia but, rather, reflects loss of normal shear stress. This study evaluated whether a similar response occurs in aortic endothelium. Plasma membrane potential and production of ROS were determined by fluorescence microscopy and cytochrome c reduction in flow-adapted rat or mouse aorta or monolayer cultures of rat aortic endothelial cells. Within 30 s after flow cessation, endothelial cells that had been flow adapted showed plasma membrane depolarization that was inhibited by pretreatment with cromakalim, an ATP-sensitive K+ (KATP) channel agonist. Flow cessation also led to ROS generation, which was inhibited by cromakalim and the flavoprotein inhibitor diphenyleneiodonium chloride (DPI), a flavoprotein inhibitor. Aortic endothelium from mice with “knockout” of the KATP channel (KIR6.2) showed a markedly attenuated change in membrane potential and ROS generation with flow cessation. In aortic endothelium from mice with knockout of NADPH oxidase (gp91phox), membrane depolarization was similar to that seen in wild-type mice but ROS generation was absent. Thus rat and mouse aortic endothelial cells respond to abrupt flow cessation by KATP channel-mediated membrane depolarization followed by NADPH oxidase-mediated ROS generation, possibly representing a cell-signal response to altered mechanotransduction.

ATP-sensitive potassium channels; KIR6.2; gp91phox, fluorescence microscopy; flow adaptation

ENDOTHELIAL CELLS EXPOSED to blood flow are known to express mechanosensors that convert shear stress-related mechanical forces on the plasma membrane to specific cellular signals (5, 8). Increased shear stress can modulate endothelial cell function by initiating a wide range of responses (5, 9, 29), including activation of flow-sensitive ion channels (5–7), altered gene expression (14, 26), and cytoskeletal reorganization (10, 24). In contrast, the effects of decreased shear due to various causes, such as embolism, thrombosis, or shock, are relatively poorly understood.

We previously used imaging techniques with fluorescent indicators in an isolated rat lung model to detect pulmonary endothelial responses in situ to loss of shear stress. Flow cessation resulted in depolarization of the endothelial cell plasma membrane and generation of reactive oxygen species (ROS) in isolated perfused lungs (2–4, 28) and in flow-adapted pulmonary vascular endothelial cells in vitro (21). This response was shear dependent and independent of changes in intravascular pressure (4).

Vascular heterogeneity among vessel types from different organs has been well recognized. Thus it is not clear whether the responses observed in the pulmonary vasculature would be seen in a systemic vascular bed. The goal of this study was to evaluate the reaction of aortic endothelial cells to altered shear stress with use of in situ and cell culture systems. The present study shows that these cells respond to flow cessation with cell membrane depolarization followed by generation of ROS similar to that described for pulmonary endothelium. Using gene-targeted mice, we determined that an ATP-sensitive K+ (KATP) channel is responsible for the plasma membrane depolarization and that the plasma membrane NADPH oxidase is the ROS generator.

MATERIALS AND METHODS

Materials. Bis-(1,3-dibutylbarbituric acid)trimethine oxonol (bisoxonol), Amplex red, and 1,1’-dioctadecyl-3,3’,3’-tetramethylindodicarbocyanine perchlorate acetylated LDL were purchased from Molecular Probes (Eugene, OR). Cromakalim, a K+ channel agonist, diphenyleneiodonium chloride (DP), a flavoprotein inhibitor, bovine erythrocyte superoxide dismutase (SOD), and ferricytochrome c (cyto c) from horse heart were purchased from Sigma (St. Louis, MO). Catalase was obtained from Boehringer Mannheim (Indianapolis, IN). Rat aortic endothelial cells (RAEC) and complete RAEC culture medium were purchased from VEC Technologies (Rensselaer, NY); cells from passages 3–11 were used.

Sprague-Dawley male rats weighing 180–220 g were obtained from Charles River Breeding Laboratories (Kingston, NY). KATP channel (KIR6.2)-knockout mice (22) were obtained originally from Dr. S. Seino (Dept. of Pharmacology, Chiba University) and bred in our institutional Animal Care Facilities. NADPH oxidase (gp91phox) knockout mice and wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). KIR6.2- and gp91phox-knockout mice had been backcrossed to the C57BL/6 background. All animal study protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Vessel preparation and laminar flow chamber. A parallel plate chamber was used to study flow effects in aortic tissue. Animals (rats or mice) were anesthetized with pentobarbital sodium (50 mg/kg ip), tracheotomized, and placed on a ventilator. After the chest was opened, the thoracic aorta was immediately removed and carefully trimmed to remove excess adventitial tissue. The aorta was cut longitudinally into 5-mm-long sections (~20–30 mg each) and then immediately fixed to a glass slide with instant glue on one corner, with the endothelial cell layer facing up (Fig. 1A). The edges of the tissue were sealed with adhesive tape to keep the preparation flat. The slide with affixed tissue was placed in the tissue flow chamber (Fig. 1B).

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The total ischemic time from death of the animal to the start of reflow adaptation was <5 min.

The flow chamber consisted of a steel plate (7.5 × 3.5 × 0.4 cm) with a central hollow slot. The glass slide containing aortic tissue was placed on the top, and the bottom was sealed with a coverslip (2.2 × 2.2 cm) to create a rectangular flow channel (1.5 × 1.5 × 0.02 cm). The flow chamber was installed between two reservoirs containing culture medium; after passage through the flow chamber, medium collected in the first reservoir and was recirculated to the second reservoir by means of a peristaltic pump (Fig. 1B). This double-reservoir system resulted in laminar flow to the aortic preparation. The volume of recirculating perfusate was ~10 ml, and the volume of the chamber was ~200 µl. The perfusate was Krebs-Ringer bicarbonate solution (in mmol/l: 118 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 MgSO₄·7H₂O, 1.3 CaCl₂·2H₂O, 1.2 KH₂PO₄, and 24.9 NaHCO₃) with 10 mM glucose + 25 mM HEPES, pH 7.4, equilibrated with 5% CO₂ in air. For experiments with high K⁺, NaCl was replaced equivalently with KCl. Aortic tissue in the flow chamber was subjected to shear stress, generally at 10 dyn/cm², for 1 h (reflow adaptation) before experiments. The shear stress (τ) to which the cells were exposed was calculated as follows: τ = (6µ/h²)bQ, where µ is the dynamic viscosity, b is the flow chamber height, h is the flow chamber height, and Q is the flow rate. Ischemia was produced by abrupt cessation of perfusate flow. During the ischemic period, the upper plate was loosened, so that the static perfusate was exposed in part to air to maintain oxygenation. In some experiments, a graded alteration of shear stress was produced by decreasing, rather than stopping, the perfusate flow.

Cell culture. RAEC in complete medium (pH 7.4) were plated on a glass slide (44 × 20 mm) that had been coated with 0.2% gelatin. Cells were allowed to grow for ≥24 h until they became fully confluent. Cells were then cultured in a laminar flow chamber under static conditions or continuous laminar flow as described above for aortic preparations. The chamber used for flow adaptation of RAEC in monolayer culture was a commercially available apparatus (Confocal Imaging chamber RC-30, Warner Instruments, Hamden, CT). For adaptation to flow, a slide with adherent cells was perfused in the flow chamber with growth medium supplemented with 25 mM HEPES (pH 7.4) at 37°C generally for 24 h at an estimated shear stress at the cell surface of 5 dyn/cm². A similarly prepared slide was maintained under static culture conditions for the same duration (i.e., static cells). Microscopic evaluation showed that cells exposed to laminar flow reoriented with the long axis in the direction of flow (not shown) as described previously for endothelial cell flow adaptation (9, 10). After flow adaptation, the growth medium was substituted with a standard Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose, 25 mM HEPES, and 3% dextran (Sigma), pH 7.4, and the cells were incubated as described above.

Microscopy. An inverted epifluorescence microscope (Diaphot TMD, Nikon) equipped with an optical filter changer (Lambda 10-2, Sutter Instrument, Novato, CA), a digital camera (model ORCA-100, Hamamatsu), and MetaMorph imaging software (Universal Imaging, West Chester, PA) was used for imaging as previously described (28). The flow chamber with intact aorta or the cultured cell monolayer was mounted on the stage of the microscope. Tissue or cells in the flow chamber were preperfused with the membrane potential-sensitive fluorophore bis-oxonol (200 nM) with or without inhibitors for 30 min or with the H₂O₂-sensitive fluorophore Amplex red (2.5 µM) + horseradish peroxidase (0.01 U/ml) for 10 min before flow cessation. Excitation for fluorescence imaging was accomplished with a mercury lamp fiber-optic light source and appropriate filter set: for bis-oxonol, HQ-41001 with 480 ± 20 nm excitation, 505LP dichroic, and 535 ± 25 nm emission; for Amplex red or 1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate acetylated LDL, HQ-41002b with 545 ± 15 nm excitation, 570 LP dichroic, and 610 ± 37.5 nm emission (Chroma Technology, Brattleboro, VT). For quantitation, areas of interest were randomly selected, and the fluorescence intensity of each was measured. Fluorescence intensity was normalized as a percent change in intensity level from baseline.

Biochemical measurements. O₂⁻ production by aortas during graded decrease of flow was measured by reduction of cyto c. Cyto c (100 µM) was added to the perfusate during the 60-min period of reflow adaptation at 10 dyn/cm² shear stress; catalase (50 µM) was added to prevent reoxidation of cyto c by H₂O₂. The flow was then abruptly reduced to give a shear stress of 0 (“ischemia”), 0.25, 0.5, or 1 dyn/cm². A different aorta preparation was used for each reduced shear experiment. The perfusate was collected during the first min of reduced flow or aspirated from the chamber for the zero-flow condition, and its absorbance and that of a sample of recirculated perfusate were measured at 550 nm. O₂⁻ production during the 60-min reflow-adaptation period was calculated from the change in absorbance at the start and end of reflow adaptation. The absorbance reading of the 0 and 0.25 dyn/cm² samples exceeded 10 times the threshold sensitivity of the spectrophotometer. O₂⁻ production was calculated from the...
An increase in ROS generation indicated by increased Amplex red fluorescence was observed after ~2–3 min of flow cessation in reflow-adapted aortas and increased linearly over the next 10 min (Fig. 3A). Pretreatment with catalase (1,000 U/ml) to scavenge H$_2$O$_2$ markedly suppressed the change in Amplex red fluorescence (Fig. 3A). ROS generation with flow cessation was not seen in aortic tissue during continuous flow (Fig. 3A). Preincubation with 10 $\mu$M DPI or 30 $\mu$M cromakalim significantly suppressed the changes in Amplex red fluorescence, indicating inhibition of ROS generation (Fig. 3B). The use of Amplex red to detect cellular generation of ROS primarily reflects H$_2$O$_2$ and is supported by the inhibiting effect of catalase. Although the Amplex red probe and catalase are extracellular, H$_2$O$_2$ could arise from intra- or extracellular sources, because H$_2$O$_2$ readily crosses cell membranes.

RESULTS

The relation between magnitude of shear stress during 1 h of reflow adaptation of the aorta and the subsequent ischemic response was studied. Aortas were flow adapted with shear stress varying from 0 to 15 dyn/cm$^2$, and ROS generation with ischemia was measured with Amplex red as a fluorophore. Aortas flow adapted to 1 dyn/cm$^2$ showed a significant increase in Amplex red fluorescence with ischemia and a greater response when reflow adaptation was 2 dyn/cm$^2$ (Fig. 2). There was no significant difference in ischemic response with shear stress that varied from 2 to 15 dyn/cm$^2$ during reflow adaptation (Fig. 2). Further studies of ischemic response were carried out using 10 dyn/cm$^2$ shear stress during reflow adaptation.

A}
Table 1. $O_2^-$ generation by reflow-adapted rat aorta as detected by cytochrome c reduction under various shear stress conditions

<table>
<thead>
<tr>
<th>Shear Stress, dyn/cm²</th>
<th>$O_2^-$ Generation, nmol·min⁻¹·mg wet wt⁻¹</th>
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<tbody>
<tr>
<td>10</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>13±2</td>
</tr>
<tr>
<td>0.25</td>
<td>140±1</td>
</tr>
<tr>
<td>0</td>
<td>148±7</td>
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Values are means ± SE (n = 10) for 10 dyn/cm² and mean ± range (n = 2) for other conditions. Aortas were reflow-adapted to 10 dyn/cm² shear stress for 1 h. $O_2^-$ production by cytochrome c reduction was measured during reflow-adaptation period at 10 dyn/cm² and then during the subsequent 1 min of reduced flow to give the indicated shear stress. Each step change in flow utilized separate aortas for measurement.

ROS generation in reflow-adapted aortas also was evaluated by reduction of cyto c added to the perfusate. This method indicates extracellular generation of $O_2^-$, because cyto c remains in the extracellular space and $O_2^-$ crosses cell membranes relatively slowly. $O_2^-$ generation was minimal under flow conditions, but production increased markedly with ischemia (Table 1). The graded decrease in flow showed a threshold effect with no change in $O_2^-$ production until shear stress was reduced by ~95% from the reflow-adaptation value (Table 1). Reduction of cyto c with ischemia was completely abolished by addition of SOD (100 μM) to the perfusate (not shown).

Increased ROS generation with flow cessation also was seen in RAEC that had been flow adapted at 5 dyn/cm² shear stress in cell culture (Fig. 4). The increase in Amplex red fluorescence with flow cessation was completely blocked by the presence of catalase (100 U/ml). Control RAEC that had been cultured under static conditions and exposed to flow for only 10 min to load the dye showed no significant change in Amplex red fluorescence with flow cessation (Fig. 4).

Bis-oxonol was used as a fluorescent indicator for changes in endothelial cell membrane potential. RAEC in situ that had been reflow adapted showed a rapid initial increase in bis-oxonol fluorescence with flow cessation followed by a slower rate of increase to an apparent plateau value at ~2–3 min (Fig. 5). The increase in fluorescence indicates plasma membrane depolarization. The change in bis-oxonol fluorescence with flow cessation was markedly attenuated by pretreatment of aortas with cromakalim, a K<sub>ATP</sub> channel agonist (Fig. 5). To assess tissue oxygenation during ischemia, the perfusate in contact with the aorta was aspirated 10 min after flow cessation. Analysis of the sample by phosphorescence assay showed $P_{O_2} > 45$ mmHg, indicating adequate oxygenation during the ischemic period.

The membrane depolarization response of endothelium to ischemia was also studied with RAEC in monolayer culture. Cells were cultured under static conditions or were flow adapted at 5 dyn/cm² for 24 h before study. Cells that were flow adapted showed an increase in bis-oxonol fluorescence after flow cessation (Fig. 6), indicating depolarization similar to that in the intact aorta. There was no significant change in bis-oxonol fluorescence with cessation of flow in static (control) RAEC that were subjected to flow for only 30 min (to load the dye) or in continuously perfused flow-adapted cells (Fig. 6B). ATP content of RAEC was measured to determine whether decreased ATP could be responsible for the membrane depolarization. ATP content was 13.7 ± 2.1 nmol/mg protein in control cells adapted to 5 dyn/cm² and 15.4 ± 3.2 nmol/mg protein in flow-adapted cells 10 min after cessation of flow (mean ± SE; n = 3); this difference was not statistically significant. The lack of change in ATP content indicates that cellular oxygenation was adequate during the stop-flow period.

To confirm that a change in cell membrane potential of aortic endothelium could result in ROS production, aortas were perfused with an isotonic solution containing 24 mM K⁺, instead of the usual 5.9 mM K⁺, to depolarize the endothelium during continuous flow. ROS production by rat aortic tissue during continuous flow was detected 5 and 10 min after the...
solution was switched from normal to high K⁺ and was markedly inhibited by the presence of DPI (Fig. 7).

To further investigate the link between endothelial cell membrane depolarization and ROS generation after flow cessation, we evaluated aortas from mice with knockout of Kir6.2, the pore-forming unit of the K<sub>ATP</sub> channel in endothelial cells (22). We showed previously in pulmonary vascular endothelial cells in culture that induction of this channel occurs during...
flow adaptation and is required for membrane depolarization with cessation of flow (7). Membrane potential change in aortas from KIR6.2-knockout mice was significantly diminished compared with that from wild-type mice (Fig. 8A). ROS generation with cessation of flow also was significantly smaller in the KIR6.2-knockout than in the wild-type mice (Fig. 8B).

On the basis of our previous studies with lungs (3), we postulated that a cell membrane NADPH oxidase is responsible for ROS production with flow cessation. We used mice with knockout of gp91phox (the integral membrane flavoprotein component of NADPH oxidase) to evaluate the role of the enzyme complex in the aortic endothelium. Membrane depolarization following flow cessation was similar for wild-type and gp91phox-knockout aortic endothelium (Fig. 8A), but ROS generation was markedly diminished in the NADPH-deficient aortas (Fig. 8B).

**DISCUSSION**

We reported previously that cessation of flow (ischemia) results in endothelial cell membrane depolarization and subsequent generation of ROS in isolated lung in situ (3, 4, 28) and flow-adapted pulmonary vascular endothelial cells in vitro (21). We have termed this model “oxygenated” or “normoxic” ischemia, because the studies are carried out with continued oxygenation from ambient sources, in contrast to most ischemia models, where anoxia results. Anoxia would, of course, abolish ROS production because of the absence of O2 and could independently influence membrane potential through change in ATP. This lung model has enabled us to establish an endothelial cell response paradigm related to altered shear stress. Previously published evidence for a similar response in organs other than the lung is the finding of ROS generation during the early phase of ischemia when O2 is available. ROS generation in ischemia has been indicated by electron paramagnetic resonance studies of the heart (13) and by the salicylate hydroxylation method for the gut (30), although the emphasis in those studies was on the more robust ROS production with reperfusion. To directly study the response of a systemic vascular bed, we have developed a method using isolated perfused rat or mouse aorta that allows imaging of the endothelial cell layer. Although it is unlikely that aortic endothelium would be normally subjected to the large decrease in shear necessary to elicit the ROS response, these cells can serve as a convenient model for other systemic endothelia. We have used the isolated aorta preparation and cultured aortic endothelial cells that have been flow adapted in vitro to investigate a shear stress-associated pathway for ROS generation with flow cessation.

Membrane depolarization was observed with cessation of flow in aortic endothelial cells in situ. A similar response was observed in flow-adapted RAEC in vitro but did not occur in cells that had been maintained in culture under static conditions. On the basis of our previous studies with pulmonary microvasculature, we propose that membrane depolarization with ischemia results from inactivation of cell membrane KATP channels (7). Endothelial cells from rat aorta have demonstrated the presence of membrane currents associated with...
K_{ATP} channels (16). In the present experiments, a K_{ATP} channel agonist, cromakalim, suppressed the bis-oxonol response to flow cessation, providing evidence that depolarization is associated with K_{ATP} channel inactivation. To further evaluate whether a K_{ATP} channel is the element responsible for membrane depolarization, we studied aortas isolated from mice with knockout of K_{irb}6.2 (22). Compared with aortas from wild-type mice, endothelial cell membrane potential change after ischemia in the K_{irb}6.2-knockout mice was greatly diminished. Thus results obtained with the K_{ATP} channel agonist and the K_{ATP} channel-knockout mice indicate that this channel is involved in the cell membrane depolarization response to cessation of flow. Our recent studies showed that rat pulmonary microvascular endothelial cells in culture demonstrate only low levels of K_{ATP} channel expression, and channel expression and inwardly rectified membrane current increase significantly with flow adaptation (7). Increased channel expression may be responsible in part for the augmented response to ischemia of aortic endothelial cells after flow adaptation. Endothelial cells in situ would be expected to be in a flow-adapted state.

We used Amplex red as an indicator of ROS generation. This probe reacts with H₂O₂ in the presence of horseradish peroxidase to form the fluorescent product resorufin. The studies using cyto c reduction indicate that O₂⁻ is generated extracellularly during ischemia. Therefore, extracellular H₂O₂ detected by Amplex red likely arises from dismutation of extracellular O₂⁻, either spontaneously or catalyzed by extracellular SOD (25). ROS generation with flow cessation was observed in flow-adapted endothelial cells but not in cells that had been cultured under static conditions. Recently published studies indicate that endothelial cells possess a plasma membrane NADPH oxidase-like enzyme system (3, 12). Studies with the flavoprotein inhibitor DPI are compatible with membrane NADPH oxidase (3, 12). We previously observed in flow-adapted endothelial cells but not in cells that had been cultured under static conditions. 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