Simulated Ischemia in Flow-Adapted Endothelial Cells Leads to Generation of Reactive Oxygen Species and Cell Signaling

Zhihua Wei, Karen Costa, Abu B. Al-Mehdi, Chandra Dodia, Vladimir Muzykantov, Aron B. Fisher

Abstract—We have previously shown that increased reactive oxygen species (ROS) generation occurs with ischemia in the oxygenated lung and have hypothesized that mechanotransduction is the initiating event. In the present study, we developed an in vitro model of oxygenated ischemia by interrupting medium flow to flow-adapted bovine pulmonary artery endothelial cells in an artificial capillary system. Cellular oxygenation during the "ischemic" period was maintained by perfusing medium over the abluminal surface of porous capillaries. Cells were assessed for ROS generation, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) binding activities, and DNA synthesis using dichlorofluorescein fluorescence by flow cytometry and spectrofluorometry, electrophoretic mobility shift assay of nuclear extracts with NF-κB–specific or AP-1–specific ^{32}P-labeled oligonucleotides, and ^{3}H-thymidine incorporation into DNA. Cells that were flow adapted for 2 to 7 days with 1 to 2 dyne/cm^2 shear stress exhibited a 1.6- to 1.9-fold increase in ROS generation during 1 hour of simulated ischemia compared with continuously perfused cells. This effect was abolished by diphenyleneiodonium chloride (DPI), indicating a role for a flavoprotein such as NADPH oxidase. The increase in ROS generation with ischemia was similar for cells from low and high passages. With ischemia, flow-adapted cells exhibited increases of 1.7-fold in nuclear NF-κB and 1.5-fold in nuclear AP-1; these changes were abolished by pretreatment with *N*-acetylcysteine or DPI. Ischemia for 24 hours resulted in a 1.8-fold increase of ^{3}H-thymidine incorporation into DNA and a significant increase of cells entering the cell cycle, as indicated by flow cytometry with propidium iodide. We conclude that flow-adapted endothelial cells generate ROS with ischemia that results in activation of NF-κB and AP-1 and an increase of DNA synthesis. This effect is not mediated by hypoxia, implicating a role for mechanotransduction in ischemia-mediated cell signaling. **(*Circ Res*. 1999;85:682-689.)**

Key Words: shear stress ■ artificial capillary ■ reactive oxygen species ■ signal transduction

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In organs with systemic circulation, the phenomenon of paradoxical generation of reactive oxygen species (ROS) with ischemia/reperfusion is attributed to the associated occurrence of anoxia/reoxygenation.[1] By contrast, an interruption of pulmonary blood flow in the air-ventilated lung does not lead to tissue anoxia because adequate oxygen is supplied from the alveolus. However, increased ROS generation occurs nevertheless.[2] Recently, we have proposed the mechanotransduction hypothesis as the mechanism for ROS generation in the lung during oxygenated ischemia.[3] Briefly, the hypothesis ascribes endothelial cell "sensing" of abrupt cessation of shear stress as the initiating factor for the biochemical events that lead to activation of endothelial NADPH oxidase and ROS generation.[4]

Normally, endothelial cells in vivo are subjected to pulsatile flow (shear stress). Shear stress affects endothelial cell structure and function, including cell orientation with flow direction, distribution of stress fibers, induction/suppression of genes, and production of vasoactive substances.[5–9] Most previous investigations of shear stress in endothelial cell biology have involved the de novo application of flow to cells maintained in static culture. These studies can be interpreted in terms of either increased shear stress or as reperfusion after adaptation to no-flow but may have little physiological relevance for normally flow-adapted endothelium in vivo. Other studies have used anoxia/reoxygenation in static endothelial cells to simulate ischemia/reperfusion,[10] although this approach obviously ignores any possible flow effects on the cellular response.

The goal of the present study was to develop an in vitro model of lung ischemia by interruption of medium flow while maintaining oxygenation to endothelial cells that have been adapted to shear stress in culture. We asked the

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following questions: Will the endothelial cells in vitro "sense" and respond with increased ROS generation to abrupt interruption of flow as demonstrated in vivo? What are the time course and the magnitude of shear stress required for adaptation of cultured cells to laminar flow as determined by their response to its removal? Does ROS generation in oxygenated ischemia represent a signal transduction event?

For the in vitro model, bovine pulmonary artery endothelial cells (BPAECs) were flow adapted in an artificial capillary system continuously perfused by pulsatile fluid flow.[11] Both structurally and functionally, this system may be more representative of a capillary bed in vivo than other models such as a parallel plate flow chamber or a cone viscosimeter.[12] Using this artificial capillary system for growing and flow adapting BPAECs, we show that abrupt cessation of shear stress leads to increased ROS generation, activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), and induction of DNA synthesis.

Materials and Methods

Cells and Culture Media

BPAECs (CCL-209) obtained from the American Type Culture Collection (ATCC, Manassas, Va) and primary BPAECs from Clonetics (Cambrex Co, San Diego, Calif) were propagated in MEM containing Earle's salts. Except where indicated, BPAECs used for experiments represented passages 17 to 25 of cells from ATCC. Early-passage cells from Clonetics were used after passages 3 to 4.

Artificial Capillary System

BPAECs were cultured under flow using commercially available artificial capillary technology (CellMax Quad Artificial Capillary Cell Culture Systems; Cellco, Inc, Germantown, Md).[5,11] Briefly, each CellMax system comprised a central pump station capable of accommodating 4 flowpaths with cartridges. Each cartridge consisted of 230 semipermeable polypropylene hollow fibers (artificial "capillaries") mounted in a hard polycarbonate casing, with ports allowing perfusion via the luminal or the abluminal compartment. A schema of the system is shown in Figure 1.

Cell Culture in Artificial Capillary System

The inner lumen of the "capillary" fibers was coated with ProNectin F (Protein Polymers, San Diego, Calif). Cells from 5 confluent T 75-cm^2 flasks of BPAECs were seeded per cartridge. To prevent the unattached BPAECs from being flushed out of the fibers, the perfusing medium was routed to the abluminal side. After a 24-hour attachment period, the perfusion circuit was rerouted across the luminal side. Cells were cultured under pulsatile flow, generally for 2 days at 1 dyne/cm^2 shear rate. Shear rate was calculated from specifications supplied by the manufacturer of the cell culture modules (0.18 dyne/cm^2 per milliliter of perfusate flow).

Simulated Ischemia

Oxidant generation was assessed by loading cells for 30 minutes with 5 μmol/L 2′,7′-dichlorofluorescin (H_2DCF) diacetate (Kodak, Rochester, NY) and measuring its conversion to fluorescent dichlorofluorescein (DCF). Cells were then subjected to either 1 hour of continuous flow (control) or 1 hour of simulated ischemia. "Ischemia" was simulated by rerouting the flow from the luminal to the abluminal compartment. This protocol eliminated endothelial shear stress but allowed continued oxygenation. Analysis of medium samples obtained from the cartridge lumen using an oxygen electrode indicated that P_{O_2} during abluminal flow ("ischemia") was similar to control, indicating the adequacy of gaseous diffusion between the luminal and abluminal compartments. The perfusate for these experiments was Krebs-Ringer bicarbonate (KRB) solution, pH 7.4. In some experiments, 100 μmol/L diphenyliodonium chloride, 10 μmol/L diphenyleneiodonium chloride (DPI) (ICN Biochemicals), or 2.5 mmol/L *N*-acetylcysteine (NAC) (Sigma Chemical Co) was added to the perfusate medium as a potential inhibitor. Because similar values were obtained with the two iodonium compounds, results with the diphenyl and diphenylene forms were combined.

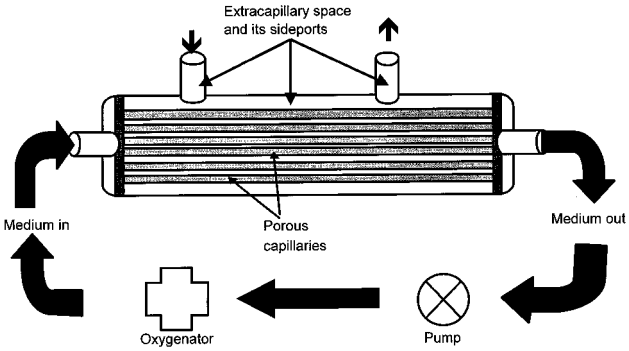

Figure 1. Schema of the artificial capillary system. A set of hollow, porous polypropylene fibers (capillaries) are conjoined in parallel at their ends forming a common inlet and an outlet port allowing simultaneous luminal perfusion of medium. The whole capillary bunch is encased in a sealed cylinder with two sideports for perfusing the extracapillary space (abluminal surface). A peristaltic pump exerts pulsatile flow to the medium that flows through a gas-permeable silicone tubing serving as an oxygenator. Endothelial cells are seeded and grown on the inner surface of the capillaries and subjected to flow adaptation by recirculating cell culture medium through the endports. For oxygenated "ischemia," luminal flow through the endports is interrupted and flow is switched to the sideports, which then serves as the source of oxygen from the abluminal side. A set of 4 cartridges, 4 pump heads, and 4 bottles with medium constitutes a unit of a CellMax Quad system. The whole system, excluding the electronic pump regulator, is housed in a cell culture incubator at 37°C with air/5% CO_2.

At the end of the 1-hour control (constant flow) or ischemic periods, cells were removed from the cartridges with trypsin. Cells were analyzed for DCF fluorescence by fluorescence microscopy, flow cytometry, and spectrofluorometry using standard methods. Nuclear extracts[13] were prepared from ischemic and control cells (no H_2DCF present) and were analyzed for NF-κB and AP-1 subunits by electrophoretic mobility shift assay (EMSA) using the appropriate ^{32}P-labeled oligonucleotides and by EMSA supershift and Western blot using commercially available antibodies (Santa Cruz Biotechnology).

To evaluate DNA synthesis, BPAECs were cultured in 1% FBS under flow for 2 days, incubated with 0.5 μCi/mL of methyl-^{3}H-thymidine (ICN Biochemicals) plus or minus 10 μmol/L DPI or 2.5 mmol/L NAC for 30 minutes, and subjected to 24 hours of continuous flow (control) or oxygenated "ischemia." A similar protocol was used for flow cytometric analysis of cell cycle with fixed cells stained by propidium iodide.

Statistics

Data are presented as mean±SE. Statistical analysis was carried out by ANOVA using SigmaStat (Jandel, San Rafael, Calif).

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

BPAECs under control conditions demonstrated oxidation of H_2DCF to DCF during the 1-hour incubation after

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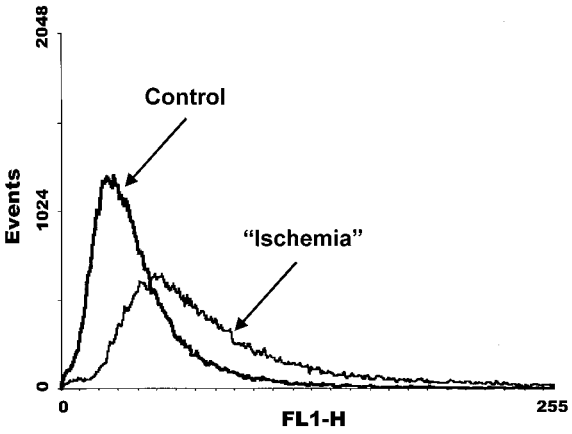

Figure 2. DCF fluorescence distribution of BPAECs by flow cytometry. BPAECs were cultured under flow for 2 days at 1 dyne/cm^2 of shear stress. After 1 hour of control or "ischemia" conditions, the cells were harvested, washed with KRB, and fixed in 4% paraformaldehyde. DCF fluorescence distribution of 10^4 cells was detected by flow cytometry. Ischemia shifts DCF fluorescence distribution to the right compared with control, indicating an increase of ROS production. FL1-H indicates fluorescence intensity; events indicate cell counts.

loading. This basal rate of DCF oxidation is indicated by fluorescence distribution on flow cytometry (Figure 2). Oxidation of the fluorophore indicates a baseline rate of ROS generation by the cultured cells, although oxidation due to the interaction of H_2DCF with other electron transfer pathways, such as the mitochondrial electron transport chain, is possible. The fluorescence distribution of BPAECs subjected to 1 hour of "ischemia" was significantly increased on flow cytometry compared with control, indicating increased H_2DCF oxidation. The mean fluorescence ratio of "ischemic" to control cells is 1.7 (Table 1). Fluorescence microscopic images of cells that had been subjected to ischemia and then detached from the flow chamber by trypsinization corroborate the increased fluorescence measured by flow cytometry (Figure 3).

TABLE 1. Flow Cytometric Analysis of ROS Generation by BPAECs With Simulated "Ischemia"

	DCF Fluorescence	
	Arithmetric Mean±SE	Geometric Mean±SE
Control	50.3±0.9	40.4±0.3
"Ischemia"	86.3±8.3*	68.5±7.6*
Ratio ("ischemia"/control)	1.7	1.7

Cells from 3 separate experiments were analyzed as described in Figure 2.
*$P<0.05$ vs control.

DCF fluorescence with "ischemia" also was significantly increased in sonicates of endothelial cells (Figure 4). The ratio of DCF fluorescence in cell sonicates of ischemic versus control cells was 1.6, similar to the results with flow cytometry of intact cells. The increase in ROS generation with "ischemia" in intact and sonicated cells was abolished by the presence of the flavoprotein inhibitor DPI (Figures 3 and 4).

Our studies showed that a period of flow adaptation was necessary to elicit a response to simulated ischemia. The time course for adaptation of cultured BPAECs to flow was studied in cells subjected to 1 dyne/cm^2 shear stress during adaptation (Figure 5). There were no differences in basal ROS production of control cells adapted to flow for periods ranging from 0 to 7 days. Nonadapted cells that

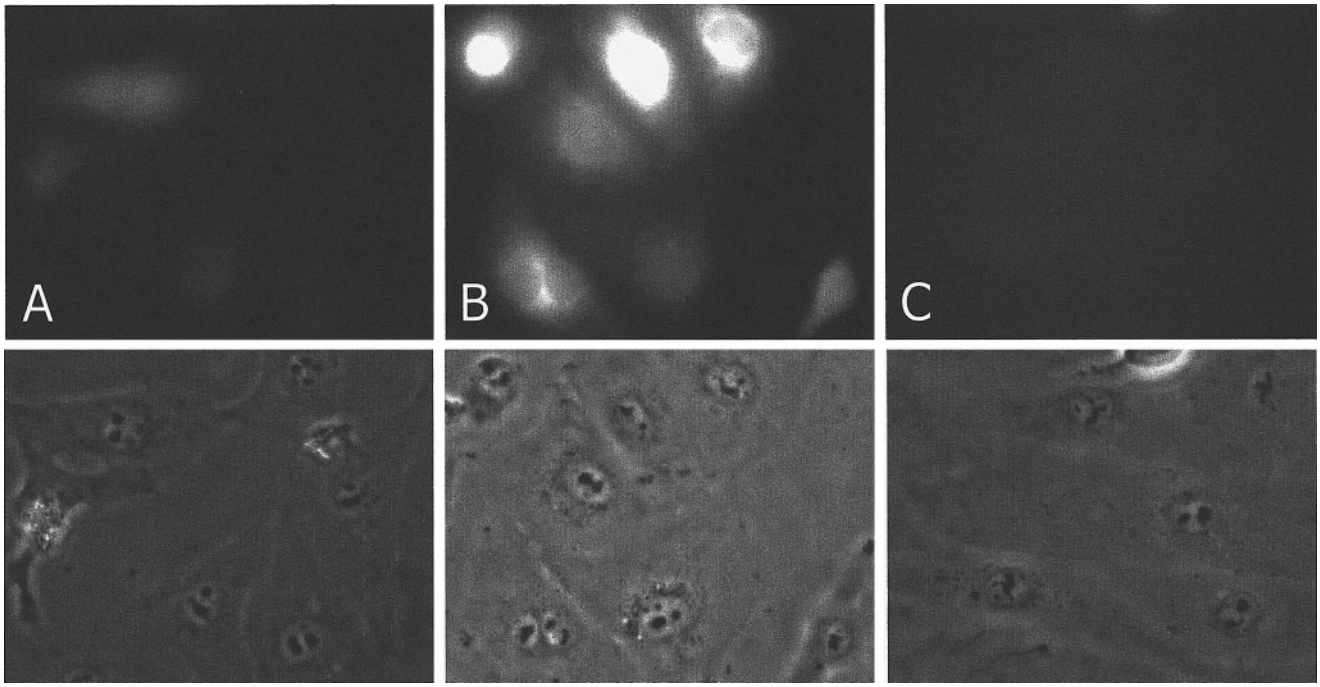

Figure 3. Fluoromicrography of BPAECs studied under control and "ischemia" conditions. Cells were trypsinized from the culture cartridges and examined immediately using an inverted fluorescence microscope. Top panels, Fluorescence images. Bottom panels, Phase-contrast images. A, Control (continuous perfusion) cells. B, Cells subjected to 1 hour of "ischemia." C, Cells subjected to "ischemia" in the presence of the inhibitor DPI.

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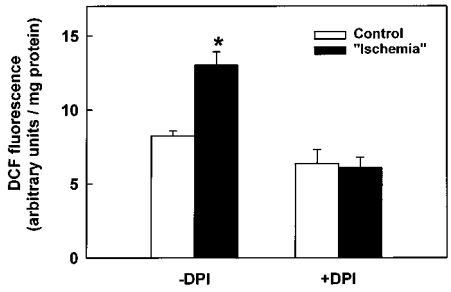

Figure 4. ROS generation with "ischemia" measured by DCF fluorescence in cell sonicates. BPAECs were cultured for 2 days at 1 dyne/cm^2 flow. Cells were loaded with DCF during a 30-minute preincubation and were subjected to either 1 hour of continuous flow (control) or 1 hour of simulated "ischemia." The cells were harvested, sonicated, and DCF fluorescence was measured at 530 nm (excitation 490 nm). DPI (10 μmol/L) when present was added to the perfusate during the preincubation period. *$P<0.05$ vs control; n=9 for −DPI; n=7 for +DPI.

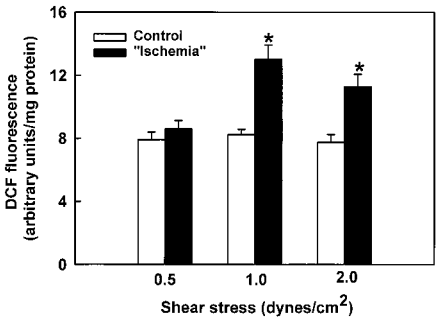

Figure 6. Effects of magnitude of shear stress during adaptation on subsequent ROS generation with ischemia. BPAECs were cultured for 2 days under flow-induced shear stress of 0.5 to 2 dyne/cm^2 and subjected to 1 hour of control perfusion or 1 hour of "ischemia." The cells were harvested, washed, sonicated, and DCF fluorescence was measured. ROS production with "ischemia" was significantly increased compared with control in cells adapted to shear stress of 1 and 2 dyne/cm^2 but was unchanged with adaptation to 0.5 dyne/cm^2. *$P<0.05$ vs corresponding control; n=3 to 9.

were perfused for a brief period or cells that were adapted to flow for 12 hours showed no increase in ROS production with simulated "ischemia." After 1 day of flow adaptation, cells exhibited a significant increase in ROS production when subjected to 1 hour of "ischemia." The magnitude of the "ischemic" response was significantly greater in cells adapted to flow for 2 days, whereas cells adapted for 7 days showed no significant further effect.

To study the relationship between magnitude of shear stress during adaptation and subsequent "ischemic" response, cells were cultured under flow for 2 days with shear stress varying from 0.5 to 2 dyne/cm^2. There was no significant increase in ROS production with "ischemia" in cells that had been adapted to a shear stress of 0.5 dyne/cm^2 (Figure 6). DCF fluorescence with "ischemia" increased similarly in cells adapted to shear stress of 1 or 2 dyne/cm^2.

To determine whether the passage number affected the response to ischemia, early passages of BPAECs (passages 3 and 4) adapted to flow for 2 days were compared with the standard high passages of BPAECs. The ratio of DCF fluorescence in "ischemia" to control was similar between early and high passages of flow-adapted BPAECs (Table 2).

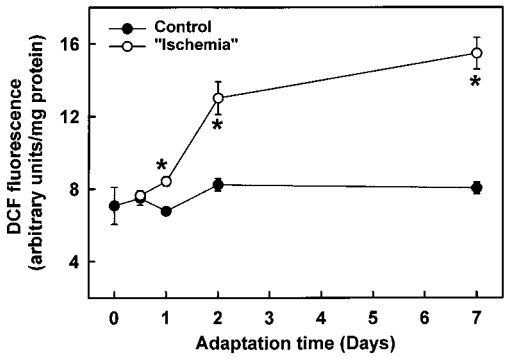

Figure 5. Effect of flow adaptation time on ROS generation as detected by DCF fluorescence in sonicates of BPAECs. BPAECs were adapted to 1 dyne/cm^2 flow for periods varying from 0 to 7 days. After 1 hour of control perfusion or 1 hour of "ischemia," the cells were collected, sonicated, and DCF fluorescence was measured. DCF fluorescence indicating ROS generation is significantly increased with oxygenated "ischemia" after 1 day of flow adaptation and reaches a plateau at 2 days of adaptation. *$P<0.05$ vs corresponding control; n=3 to 9 for each time point.

Flow-adapted BPAECs were evaluated for possible activation of NF-κB with "ischemia" (Figure 7). Specific detection of NF-κB components in cell nuclear extracts by EMSA indicates the presence of the p65/p65 and p50/p65 dimeric bands (Figure 7A, lane 2). The bands were specifically supershifted with anti-p65 and anti-p50 antibodies (Figure 7A, lanes 3 and 4). The darkest bands near the bottom of the gels were unidentified but could represent the monomeric p52 component of NF-κB. Simulated "ischemia" in BPAECs resulted in NF-κB activation that was inhibited by the antioxidant NAC and the flavoprotein inhibitor DPI (Figure 7B and 7C). Figure 7B is a representative EMSA, and Figure 7C represents densitometry of p65/p65 and p50/p65 bands on autoradiograms similar to those shown in Figure 7B. One hour of simulated "ischemia" led to a 1.7-fold increase in the NF-κB signal compared with control perfusion. The addition of 2.5 mmol/L NAC or 10 μmol/L DPI completely blocked the ischemic increase in NF-κB.

Cells also were analyzed for possible activation of AP-1 with simulated "ischemia." The components of the AP-1

TABLE 2. Effect of Passage Number on Response to Simulated "Ischemia"

	DCF Fluorescence in Cell Sonicates, Arbitrary Units per Milligram of Protein	
	Early Passages (n=3)	High Passages (n=9)
Control	6.79±0.23	8.24±0.34
"Ischemia"	10.14±0.20	13.02±0.90
"Ischemia"/control	1.50±0.04	1.59±0.11

DCF fluorescence in BPAECs from passages 3 to 4 (early) or 17 to 25 (high) was studied after 2 days of flow adaptation (luminal flow) following the 24-hour seeding period. Data are mean±SE. The ratio of "ischemia" to control is not significantly different between early and high passages of BPAECs.

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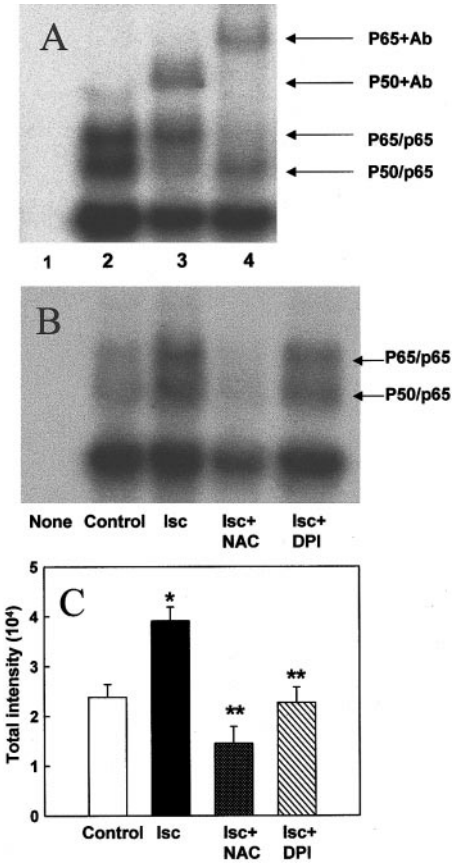

Figure 7. Oxidant-mediated NF-κB activation with simulated "ischemia" in BPAECs. A, Specific detection of NF-κB components by EMSA. Nuclear extracts (4 μg of protein) from flow-adapted cells after 1 hour of "ischemia" were incubated with 50-fold unlabeled NF-κB–specific oligonucleotide before adding ^{32}P-labeled probe (lane 1), with 50-fold unlabeled nonspecific oligonucleotide (lane 2), with 0.4 μg of anti-p50 antibody (lane 3), or with 0.4 μg of anti-p65 antibody (lane 4). B, Representative EMSA. Individual lanes are negative control with no nuclear extract (None); nuclear extracts from flow-adapted cells containing 4 μg of protein per lane—after 1 hour of control perfusion (Control); after 1 hour of simulated "ischemia" (Isc); after 1 hour of "ischemia" plus 2.5 mmol/L NAC (Isc+NAC); and after 1 hour of "ischemia" plus 10 μmol/L DPI (Isc+DPI). C, Densitometry of p65/p65 and p50/p65 bands on autoradiograms similar to those shown in panel B using an optical scanner and image-processing software (SigmaScan Pro, SPSS Inc). Abbreviations are as in panel B. *$P<0.05$ vs Control; **$P<0.05$ vs Isc. n=7 for Control and Isc; n=3 for Isc+NAC and Isc+DPI.

complex were analyzed by both antibody supershift assay and immunoblot analysis (Figure 8). Western blot analysis showed an increase in both c-*jun* and c-*fos* subunits of AP-1 with "ischemia" (Figure 8A), suggesting that the specific AP-1 activated with "ischemia" is a c-*jun*/c-*fos* heterodimer. The presence of c-*jun* in the nuclear extracts of ischemic cells also was demonstrated by EMSA, which showed one band that was supershifted by an antibody specific for c-*jun* (Figure 8B). For unexplained reasons, there was no supershift by anti–c-*fos* (data not shown). AP-1 activation with "ischemia" in flow-adapted BPAECs was confirmed by EMSA (Figure 8C and 8D). Figure 8C is a representative EMSA, and Figure 8D represents quantification of AP-1 band intensity on autoradiograms from 5 to 6 separate experiments. One hour of "ischemia" led to a 1.5-fold increase in the AP-1 band intensity compared with control perfusion. The addition of 2.5 mmol/L NAC or 10 μmol/L DPI completely blocked the ischemic increase in AP-1 binding activity (Figure 8A, 8C, and 8D).

Cells were evaluated for the effect of ischemia on cell proliferation. BPAECs were cultured under flow in 1% serum for 2 days for adaptation. ^{3}H-thymidine incorporation into DNA was measured during an additional 24 hours of control perfusion or 24 hours of "ischemia." "Ischemia" resulted in a 1.8-fold increase in radiolabel incorporation expressed either in terms of cellular protein (Figure 9A) or cellular DNA[14] (data not shown). This effect was inhibited by the presence of NAC or DPI. To evaluate whether the increased ^{3}H-thymidine incorporation represented de novo synthesis or DNA repair, cells were analyzed by flow cytometry for their distribution in the cell cycle. Flow cytometric analysis of cell cycle with propidium iodide indicated that ischemia led to a decrease in the number of cells in the G0G1 phase and a concomitant increase in the number of cells in the S phase (Figure 9B and 9C). This indicates that the increase in ^{3}H-thymidine incorporation with "ischemia" is due to de novo DNA synthesis as opposed to DNA stabilization and is compatible with cellular proliferation.

Discussion

The isolated rat lung model used in our laboratory has provided extensive evidence that production of ROS occurs during the ischemic period in the ventilated lung.[2,15] Continued ventilation provides oxygenation in this model and maintains tissue energy (ATP) status.[3,15] We have postulated that removal of the mechanical stimulus as a result of cessation of perfusate flow (ischemia) initiates oxidant generation by endothelial cells[3] through the NADPH oxidase pathway.[4]

We tested this hypothesis by developing an in vitro model of ischemia using BPAECs grown in an artificial capillary system. To measure ROS, we used H_2DCF diacetate, a nonfluorescent probe that is converted by oxidants such as H_2O_2 to the fluorescent product DCF. This probe has been widely used as a sensitive detector of oxidants in a variety of cells and organs.[16–19] To simulate oxygenated ischemia as experienced by endothelial cells in the air-ventilated lung, we interrupted the perfusate flow over the luminal surface of the cells while providing oxygen from the medium flow over the abluminal sides of the porous capillaries. With interruption of luminal flow for 1 hour, BPAECs that had been flow adapted for 2 to 7 days showed a significant increase (1.6- to 1.9-fold) in conversion of H_2DCF to DCF, indicating an increased generation of oxidants compared with continuously perfused cells. Like the intact lung, H_2DCF oxidation with "ischemia" was blocked by the presence of DPI, a flavoprotein inhibitor that has been shown to inhibit endothelial NADPH oxidase.[4] The increased DCF fluorescence with "ischemia" was confirmed in early endothelial passage cells indicating that it was not a phenomenon related to passage number. Therefore, the BPAECs that lined the artificial capillaries simulated the behavior of lung endothelial cells in situ with respect to oxidant generation in "ischemia."

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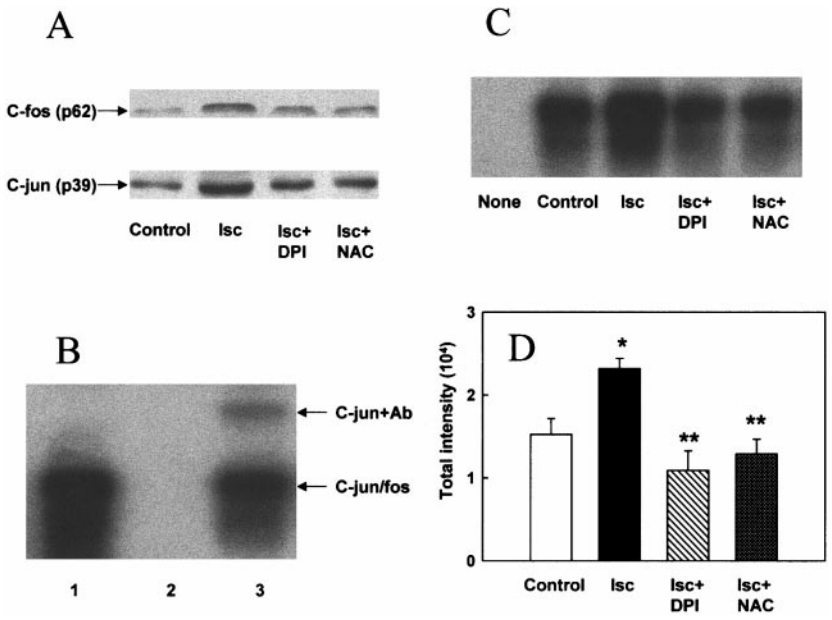

Figure 8. ROS-mediated AP-1 activation with "ischemia" in BPAECs. A, AP-1 analysis of control and "ischemic" cells by Western blot. Immunoblots were performed with nuclear extracts (20 μg) from control, "ischemia" (Isc), Isc+10 μmol/L DPI, and Isc+2.5 mmol/L NAC. Bands for c-*jun* and c-*fos* are indicated. B, Specificity of AP-1 analysis by EMSA. Nuclear extracts (2 μg of protein) from flow-adapted cells after 1 hour of "ischemia" were incubated with 50-fold unlabeled nonspecific oligonucleotide (lane 1), with 50-fold unlabeled specific oligonucleotide (lane 2), or with 0.4 μg of anti–c-*jun* antibody (lane 3) before adding ^{32}P-labeled probe. Note the supershift of radiolabel in the presence of c-*jun* antibody. C, Representative EMSA of control and "ischemic" cells. Lanes are negative control with no nuclear extract (None); nuclear extracts from flow-adapted cells containing 2 μg of protein per lane after 1 hour of continuous flow (Control); after 1 hour of simulated "ischemia" (Isc); after 1 hour of Isc+10 μmol/L DPI; and after 1 hour of Isc+2.5 mmol/L NAC. D, Densitometry of AP-1 band on autoradiograms similar to those shown in panel A. Abbreviations as in panel C. *$P<0.05$ vs Control; **$P<0.05$ vs Isc. n=6 for Control, Isc, and Isc+NAC; n=5 for Isc+DPI.

One of the major findings from the present studies is that endothelial cells require a period of flow adaptation to acquire their response to no-flow with ROS generation. Previous reports indicate that the period of adaptation of endothelial cells to shear stress ranges from minutes to days and depends on the type of cellular response.[20] Our data show that 12 to 24 hours of adaptation to flow was necessary for development of a significant ischemic response in these endothelial cells and that 1 to 2 days were required for maximal effect. The mechanism for priming this response to altered flow presumably requires increased expression of cellular mechanoresponsive elements.

The mechanism for activation of ROS generation by simulated "ischemia" is not yet clear. Previously we have shown that lung endothelial cells in situ exhibit plasma membrane depolarization during "ischemia," and this could serve as a link between mechanosensing and NADPH oxidase activation.[3] A similar mechanism may operate for this in vitro model of lung ischemia, but this possibility was not tested because the cell culture system is not readily amenable to the study of membrane potential of individual cells. The signals linking cell membrane depolarization and activation of oxidant generation are also unknown, but one possibility may be changes in cellular ionic homeostasis. Ziegelstein et al[21] recently showed increased pH_i in rat aortic endothelial cells in response to abrupt reduction in fluid shear stress, but the mechanisms for this or other potential signals for activation of ROS generation remain to be investigated.

Generation of ROS with "ischemia" was associated with activation of NF-κB and AP-1 and an increase of DNA synthesis indicating cellular proliferation. These effects were blocked by the antioxidant NAC and the flavoprotein inhibitor DPI, indicating that ROS can mediate signal transduction in this in vitro model.[22–24] NF-κB and AP-1, transcription factors for a variety of genes including those for cytokines and growth factors such as vascular endothelial growth factor, have been shown previously to be oxidant sensitive.[25–27] NF-κB and AP-1 were shown to interact functionally with the shear stress response element in the platelet-derived growth factor α and β genes and to induce a mitogenic response.[28,29] Recently, it has been shown that ROS can mediate Ras-induced cell cycle progression and mammalian cell proliferation.[29,30] Therefore, the increased DNA synthesis with ischemia may be associated with ROS-mediated activation of one or more of these transcription factors.

The present report demonstrates increased ROS production and activation of NF-κB and AP-1 in flow-adapted endothelial cells in response to abrupt removal of shear stress. A similar response has been noted with endothelial cells cultured under static conditions and subsequently exposed abruptly to shear stress.[31,32] Rac1 has been shown to play a key role in the initiation of shear-induced ROS generation.[33] The similarity of response in switching from flow to no-flow and vice versa may reflect the response to an alteration from the adapted state.[20] Thus, either increased flow with statically adapted cells or decreased flow with flow-adapted cells could induce ROS generation and activation of transcription factors or other mediators. This phenomenon has been described by Davies et al,[34] who noted that pinocytosis increased with initiation of flow in statically adapted cells or with cessation of flow in flow-adapted cells. Thus, the adapted state appears to determine the subsequent response of endothelial cells to altered mechanical forces.

In summary, we have developed an in vitro model of oxygenated "ischemia." Flow-adapted endothelial cells respond to cessation of flow with increased ROS production, resulting in activation of NF-κB and AP-1 and cellular proliferation. This model may be useful for studying the mechanisms for lung response to "ischemia" and the role of ROS as second messengers in the endothelial cell.

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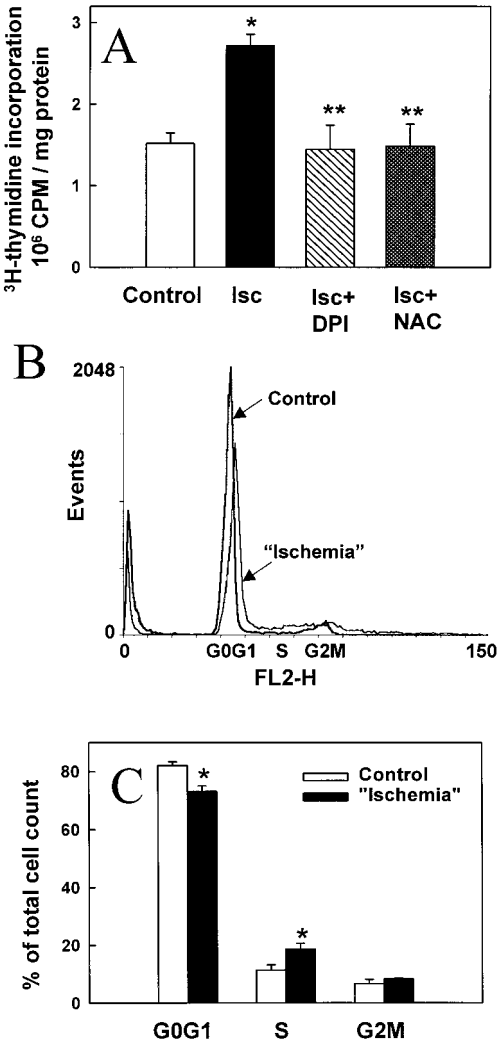

Figure 9. ROS-mediated increase of DNA synthesis with "ischemia." BPAECs were cultured in 1% FBS for 2 days at 1 dyne/cm^2 flow. A, ^{3}H-thymidine incorporation. After preincubation for 30 minutes with 0.5 μCi/mL of ^{3}H-thymidine with or without inhibitors (10 μmol/L DPI or 2.5 mmol/L NAC), cells were subjected to 24 hours of "ischemia" or 24 hours of control perfusion. The cells were harvested and lysed, and radioactivity indicating ^{3}H-thymidine incorporation into DNA was counted. ^{3}H-thymidine incorporation is expressed as 10^6 cpm per mg of protein. *$P<0.05$ vs Control; **$P<0.05$ vs Isc. n=12 for Control and Isc; n=3 for Isc+NAC and Isc+DPI. B and C, Flow cytometric analysis of cell cycle using propidium iodide. Cells were subjected to 24 hours of control perfusion (Control) or "ischemia" as in panel A. The cells were harvested and fixed in ethanol, treated with RNase, and stained with propidium iodide as described in Materials and Methods. The cell cycle distribution of 10^4 cells for each condition was analyzed with FACScan flow cytometer. B, Fluorescence cell scan representative of 4 experiments. The peaks for G0G1 and G2M phases of the cell cycle and the interval between them representing the S phase are marked on the x-axis. FL2-H indicates fluorescence intensity; events, cell counts. C, Calculated results for n=4 experiments similar to those in panel B. "Ischemia" results in a decrease in the percentage of cells in the G0G1 phase and an increase in the percentage of cells in the S phase. *$P<0.05$ vs Control.

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