Mitochondrial requirement for endothelial responses to cyclic strain: implications for mechanotransduction

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Mitochondrial requirement for endothelial responses to cyclic strain: implications for mechanotransduction. Am J Physiol Lung Cell Mol Physiol 287: L486–L496, 2004. First published April 16, 2004; 10.1152/ajplung.00389.2003. —Mechanical strain triggers a variety of cellular responses, but the underlying mechanotransduction process has not been established. Endothelial cells (EC) respond to mechanical strain by upregulating adhesion molecule expression through a signaling process involving reactive oxygen species (ROS), but the site of their generation is unknown. Mitochondria anchor to the cytoskeleton and could function as mechanotransducers by releasing ROS during cytoskeletal strain. In human umbilical vein EC (HUVEC), ROS production increased 221 ± 17% during 6 h of cyclic strain vs. unstrained controls. Mitochondrial inhibitors diphenylene iodonium or rotenone abrogated this response, whereas inhibitors of nitric oxide (NO) synthase (l-nitroarginine), xanthine oxidase (allo- purinol), or NAD(P)H oxidase (apocynin) had no effect. The antioxidants ebselen and diethyldithiocarbamate inhibited the increase in ROS during cytoskeletal strain. In human umbilical vein EC (HUVEC), ROS production increased 221 ± 17% during 6 h of cyclic strain vs. unstrained controls. Mitochondrial inhibitors diphenylene iodonium or rotenone abrogated this response, whereas inhibitors of nitric oxide (NO) synthase (l-nitroarginine), xanthine oxidase (allo- purinol), or NAD(P)H oxidase (apocynin) had no effect. The antioxidants ebselen and diethyldithiocarbamate inhibited the increase in ROS, but the NO scavenger Hb had no effect. Thus strain induces ROS release from mitochondria. In other studies, HUVEC were rendered mitochondria deficient (ρ0 EC) to determine the requirement for electron transport in the response to strain. Strain-induced 2/7-dichlorofluorescein fluorescence was attenuated by >80% in ρ0 EC compared with HUVEC (43 ± 7 vs. 221 ± 17%). Treatment with cytochalasin D abrogated strain-induced ROS production, indicating a requirement for the actin cytoskeleton. Cyclic strain (6 h) increased VCAM-1 expression in wild-type but not ρ0 EC. Increases in NF-κB activation and VCAM-1 mRNA expression during strain were prevented by antioxidants. These findings demonstrate that mitochondria function as mechanotransducers in endothelium by increasing ROS signaling, which is required for strain-induced increase in VCAM-1 expression via NF-κB.

reactive oxygen species; mitochondria; vascular cell adhesion molecule-1; superoxide; hypertension; atherosclerosis; cytoskeleton; cyclic stretch

In the cardiovascular system, endothelial cells normally experience intermittent or continuous shear stress, and altered blood flow patterns have been implicated in the pathogenesis of cardiovascular diseases including atherosclerosis and atherosclerosis and atherosclerotic formation (24, 45). In the lung, endothelial cells experience shear stress as well as mechanical strain associated with cyclic lung inflation and deflation. Several mechanisms have been proposed to contribute to the mechanotransduction responses in vascular cells. These include flow-sensitive ion channels [Ca2+ (34) and K+ (40)], mechanosensitive kinases (50), and an integrin-cytoskeletal (tensility) model (31, 53, 54). Despite evidence to support the involvement of each of these systems, the mechanism responsible for initially triggering the mechanotransduction cascade leading to subsequent changes in gene expression in strained cells has not been identified. Moreover, although specific signaling cascades are activated in response to changes in flow (18, 22, 39), the relationship between these signaling systems and the upstream sensor(s) has not been established.

Studies by Medford and colleagues (33, 51) have implicated reactive oxygen species (ROS) as signal transduction agents in strained endothelium, and the activation of NF-κB and the subsequent increase in cell adhesion molecule (CAM) expression have been linked to the intracellular redox state (32). Chappell et al. (17) reported that the shear-induced increase in cell adhesion molecule expression in endothelial cells is attenuated in the presence of the antioxidant L-ascorbate (NAC). Likewise, Cheng et al. (19) reported that strain-induced increases in ICAM-1 expression are abolished when endothelial cells are pretreated with catalase or NAC. These studies implicate ROS as signaling elements in the upregulation of ICAM-1 and monocyte chemotactic protein-1 (57) in endothelial cells subjected to mechanical strain, but the source of these oxidants is not known.

Endothelial cells could conceivably generate ROS from an NAD(P)H oxidase system, from xanthine oxidase, or from other oxidase systems (Fig. 1A). A variety of endothelial cell subtypes express NAD(P)H oxidase, and this system has been implicated in the signaling activated during mechanical strain (4). Mice lacking the gp91phox subunit of NADPH oxidase generated fewer oxidants in vascular segments during PMA stimulation compared with segments from wild-type (WT) mice, suggesting that NAD(P)H oxidase systems participate in signaling (25). Several studies have concluded that NAD(P)H oxidase is involved, on the basis of the observation that strain-induced changes were inhibited by diphenylene iodonium (DPI) (28, 48, 56). However, the flavoprotein inhibitor DPI also blocks virtually all cellular oxidase systems, including mitochondrial complex I, nitric oxide (NO) synthase, and xanthine oxidase (36). Therefore, the inhibition by DPI is not
A

xanthine oxidase

mitochondria

Allopurinol

DPI

Rotenone

NAD(P)H oxidase

Ebselen

H₂O₂ glutathione

erosperoxidase

H₂O₂

superoxide

SOD

catalase

B

CoQ

Complex I

Complex II

CoQH₂

Fe-S

Cytochrome Oxidase

CoQ

O₂

Superoxide

SOD

H₂O₂

Signal Transduction

Antimycin A

Materials and Methods

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma Chemical except where otherwise noted. L-Nitroarginine (L-NA) was purchased from Calbiochem-Novabiochem, and 2′,7′-dichlorofluorescein (DCFH) diacetate was purchased from Molecular Probes. The Flexercell 3000 strain unit and BioFlex culture plates were purchased from Flexcell International.

Cell culture. Primary culture human umbilical vein endothelial cells (HUVEC, Clonetics) were grown in endothelial growth media (EGM, Clonetics) and characterized by positive staining for von Willebrand factor, CD31, and factor VIII. Cells were grown to 80–100% confluence on collagen I-coated plates for stretch experiments (Bioflex, Flexcell International). We generated respiration-deficient HUVEC lacking mitochondrial DNA (p⁰ cells or p⁰ HUVEC) by incubating WT cells in ethidium bromide (25 ng/ml) for 2–3 wk in medium supplemented with pyruvate and uridine (14, 16). We then selected the p⁰ cells by exposing the cultures to mitochondrial inhibitors rotenone (1 μg/ml) and antimycin A (1 μg/ml), which are lethal to WT endothelial cells over the course of 7 days. The resulting mutant cells lacked mitochondrial DNA, which encodes critical subunits required for electron transport and ATP synthesis. We further confirmed respiration-deficient HUVEC by only using those p⁰ HUVEC that exhibited a >90% reduction in O₂ consumption as measured by respirometry (data not shown). We assessed cellular respiration by measuring the rate of decrease in O₂ tension of a stirred suspension of cells in a gas-tight chamber, as described previously (13). To standardize comparisons between WT and p⁰ HUVEC, results were normalized for total protein content as determined by the Bradford method. Cells between passages 2 and 6 were used for experiments.

Strain apparatus. Intermittent cyclic strain was applied to HUVEC as previously described (42). Briefly, confluent Bioflex plates were placed on a Flexercell 3000 base plate in the incubator, and membranes at the bottom of the plates were subjected to a vacuum of −20 kPa at a frequency of 15 cycles/min for up to 24 h. During each cycle, cells were strained for 3 s and relaxed for 1 s. This pattern was chosen because of its similarity to the frequency of lung vascular stretch during tidal ventilation and because it was feasible with the Flexercell apparatus. A vacuum of −20 kPa caused a deformation of the silastic membrane of ~25% with the loading posts installed. This level of stretch was well tolerated by the cells and is consistent with the level of strain potentially encountered in the ventilated lung. Confluent Bioflex plates not subjected to strain were placed in the incubator to serve as time-matched controls. HUVEC were preincubated with experimental treatment media (containing inhibitors, antioxidants, cytochalasin D, nucedazole, etc.) 2 h before strain treatments. The doses of pharmacological inhibitors were chosen after dose-response experiments demonstrated the lowest dose necessary to inhibit fluorescence in unstrained cells; these doses coincided with a 10-fold increase from the dose reported to inhibit 50% of enzyme activity for the targeted enzyme systems (apocynin for NADPH oxidase, allopurinol for xanthine oxidase, N-nitro-l-arginine for endothelial NO synthase, rotenone for mitochondrial complex I, and antimycin for mitochondrial complex III).

Measurement of ROS. ROS signaling was assessed with DCFH diacetate. ROS in the cells cause the oxidation of DCFH, yielding the fluorescent compound 2′,7′-dichlorofluorescein (DCF). DCFH was added to cells at a final concentration of 10 μM immediately before experimentation at time t = 0. To assess the intracellular levels of DCF after 6 h of strain, we removed the media and immediately lysed and centrifuged the cells to remove debris. DCF fluorescence was
measured (excitation = 488 nm, emission = 530 nm; Perkin-Elmer LS-5), and data were normalized to unstrained controls and expressed as percent change from unstrained controls. In preliminary studies, responses to strain were detected as early as 15 min into the experiment. Responses were larger in magnitude at 6 h, so this time point was selected for analysis. However, the pattern of responses was similar at the two time points, indicating that the results were not specifically dependent on the time of sampling.

Flow cytometry. Cells were harvested from the Bioflex plates with 0.1% collagenase and 0.25% EDTA and centrifuged, and the pellet was labeled with 1° antibody (anti-VCAM-1, 1:25; anti-E-selectin, 1:25; anti-ICAM-1, 1:10; R&D Systems) at 4°C for 20 min. The labeled cells were rinsed twice with PBS, centrifuged, and then labeled with 2° FITC antibody (IgG, 1:10; Caltag Laboratories) at 4°C for 20 min. Cells were then washed with PBS and fixed in 0.1% paraformaldehyde. Receptor expression was quantified with a fluorescence-activated cell sorter (FACS, Becton Dickinson). Forward and side scatter fluorescence data identified 10,000 viable cells in each experimental group for unlabeled cells, nonspecific labeled cells, VCAM-1-labeled cells, E-selectin-labeled cells, and ICAM-1-labeled cells. Data were accumulated from 10,000 cells at 530-nm emission. These data were expressed in the histogram format of fluorescent events vs. log fluorescence and were analyzed in comparison with the autofluorescence of unlabeled cells and the fluorescence of unstrained VCAM-1-, E-selectin-, and ICAM-1-labeled cells. Changes in fluorescence were expressed via the mean fluorescence intensity. All data were collected with Cell Quest on a Power Macintosh 7200.

Primers and RT-PCR. The VCAM-1 primers for the PCR were (5’ → 3’) as follows: + strand, AGTGTGGCGCTCCTGTAATGG (nucleotides 1064–1083); − strand, CTGTGCTCTGTTGTCCTGCT (nucleotides 1744–1763). The 5’- and 3’-primers were chosen within exons 6 and 8, respectively, to avoid exon 5, which can be alternatively spliced. As previously described (20), linear phase of the exponential phase of the amplification was determined for each primer set to allow semiquantitative PCR analysis. The number of cycles was chosen in the linear phase of amplification and was set to 40 for VCAM-1. We verified the specificity of generated PCR products by noting the correct size on the basis of the known cDNA sequences, the placement of the internal primers, and the predicted size of fragments produced by restriction enzyme digests (data not shown).

Northern analysis. The human VCAM-1 cDNA probe was cloned by RT-PCR. Briefly, total RNA from HUVEC treated with 10 ng/ml TNF-α for 6 h was reverse transcribed, and the cDNA was amplified by PCR with a VCAM-1 primer set for 40 cycles. The 700-bp product was purified and used as the probe.

Strained and unstrained HUVEC were harvested after 6 h, and total RNA was isolated. We performed Northern analysis by loading 3–5 μg of total RNA onto a 2% agarose-6% formaldehyde gel, electrophoresis, and then transfer onto nylon membranes. After 4–6 h of prehybridization, membranes were hybridized with 32P-labeled VCAM-1 cDNA for 16 h at 42°C. After being washed, the blots were exposed to Kodak-X AR film at −70°C for 24 h. All blots were rehybridized with a 28S RNA probe (Ambion).

Electrophoretic mobility shift assay. We isolated nuclear protein extracts by suspending the cells in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 5 μg/ml aprotonin) for 15 min on ice. After adding 25 μl of 10% Nonidet P-40, we subsequently centrifuged the cells at 12,000 rpm for 30 s. The pellet was then resuspended in buffer B (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, and 5 μg/ml aprotonin) for 15 min at 4°C. Samples were prepared according to the instructions of the Gel Shift Assay System (Promega E-3300, catalog no. E3521) and were loaded onto 4% polyacrylamide gels and run in 0.5× Tris-borate-EDTA for 4 h at 120 V as described previously (9, 23). The NF-κB consensus sequence for the probe was 5’-AGTTGAGGGACTTTCCCAGG-3’.

Cytoskeleton experiments. Two hours before exposing cells to mechanical strain, we preincubated HUVEC in media containing cytochalasin D (0.5–10 μM). The lowest concentration that returned strain-induced DCF fluorescence to baseline levels was used for subsequent experiments (5.0 μM for HUVEC). Similar experiments were conducted with nocodazole (1.0–10 μg/ml).

Statistical analysis. The quantitative data acquired from the DCF fluorescence experiments and flow cytometry experiments were subjected to a two-way analysis of variance (ANOVA). After successful screening of the data for nonrandom tendencies, we confirmed a normal distribution of the data via graphical techniques (histogram of residuals, residuals vs. normality, and residuals vs. fit). When the ANOVA detected a statistically significant difference among groups or with strain, we conducted a t-test to determine whether a statistical difference existed between control unstrained cells and control strained cells. Further pairwise t-tests were then conducted to identify sources of variance in strained and unstrained groups. The groups of interest were those in which the difference in strained and unstrained cells was abolished. Significance was defined as P < 0.05, and all computations were conducted with Minitab software.

RESULTS

ROS signaling during strain. To assess the source(s) of oxidant production during cyclic strain, we incubated confluent HUVEC with DCFH, which becomes fluorescent (DCF) when oxidized. After 6 h (Fig. 2A), increases in DCF fluorescence were noted even in unstrained controls, indicating that some basal oxidant production occurs in quiescent cells. However, the extent of DCF oxidation was significantly increased in cells subjected to 6 h of strain (221 ± 17%), compared with unstrained controls (P < 0.05). This response was attenuated by the mitochondrial complex I inhibitor rotenone (2 μM) (68 ± 7%), but not by the NADPH oxidase inhibitor apocynin (300 μM) (191 ± 15%) or the xanthine oxidase inhibitor allopurinol (100 μM) (183 ± 27%). Antimycin A inhibits mitochondrial electron transport and increases mitochondrial ROS generation by prolonging the lifetime of ubisemiquinone in complex III (27). As expected, antimycin A (2 μM) increased DCFH oxidation in unstrained cells, but it did not amplify the increase in DCF signal elicited by strain. DPI is a flavoprotein inhibitor that blocks electron transport in a broad range of systems including mitochondrial complex I, NAD(P)H oxidases, NO synthase, and other systems (35). DPI (5 μM) virtually abolished the basal oxidation of DCFH in unstrained cells and significantly attenuated the strain-associated increase in DCF fluorescence compared with controls (75 ± 9%, P < 0.05). Endothelial cells release NO in response to shear stress (11), and NO can potentially contribute to the oxidation of DCFH (43). Therefore, we evaluated the participation of reactive nitrogen species (RNS) in the response to strain by inhibiting NO synthesis with l-NA. In both strained and unstrained cells, l-NA attenuated but did not abolish the DCF fluorescence signal compared with their respective controls. Thus both RNS and RNS participate in the response to strain, but ROS are primarily responsible for the DCF fluorescence changes.

To decipher the nature of the reactive molecule(s) produced during cyclic strain, we measured DCF fluorescence in HUVEC in the presence of various intracellular antioxidant compounds (Fig. 2B). Strain-induced DCF oxidation was diminished by the glutathione peroxidase mimetic ebselen (50 μM) and by NAC (1 mM), a thiol reductive agent (P < 0.05).
Diethyldithiocarbamate (DDC, 1 mM), an inhibitor of Cu/Zn superoxide dismutase also attenuated the strain-induced increases in DCF fluorescence, as did 4,4′-disothiooceanostilbene-2,2′-disulfonic acid (DIDS, 100 μM), an anion channel blocker that also acts on the inner mitochondrial membrane anion channel (7). To clarify the relative roles of NO and H₂O₂ in this response, we added free hemoglobin (Hb, 5 μM) to the media in an attempt to scavenge NO. This produced a modest attenuation of the DCF response to strain, suggesting that NO is a minor contributor to the strain-induced oxidant signal in these cells.

To clarify the requirement for mitochondrial electron transport in the response to strain, ρ⁰ cells were subjected to strain in the presence of DCFH. The ρ⁰ cells lack critical subunits of mitochondrial complexes I, III, IV, and V that are required for electron transport (15). Compared with their WT counterparts, the DCF signal was attenuated in the ρ⁰ cells (43 ± 7 vs. 221 ± 17%; P < 0.05, Fig. 2C). The small residual increase in DCFH oxidation during strain in ρ⁰ cells was not decreased by rotenone but was attenuated by l-NA or DPI (data not shown). Consistent with their lack of an electron transport system, ρ⁰ cells failed to increase DCFH oxidation in response to antimycin A. Antioxidants that had attenuated the DCF fluorescence response in WT cells failed to diminish the small residual strain-induced increase in signal in ρ⁰ cells (Fig. 2D). However, addition of Hb did attenuate the small DCF fluorescence response to strain, suggesting that the residual increase in DCF oxidation may be mediated by NO.

**Activation of NF-κB by mitochondrial ROS.** Exogenously applied oxidants have been shown to induce activation of NF-κB (6). We sought to determine whether endogenous oxidant signals generated during strain were biologically significant in terms of their ability to activate NF-κB by a similar mechanism. Electrophoretic mobility shift assays (EMSA) for DNA binding of NF-κB were conducted on nuclear extracts from HUVEC assayed after 15 min of cyclic strain. Compared with unstrained cells, DNA binding by NF-κB was increased in HUVEC (Fig. 3A). A similar increase was observed in unstrained cells treated with antimycin A to augment mitochondrial ROS generation. Strain-induced increases in DNA binding of NF-κB were attenuated by rotenone and by DPI. Analysis with antibodies to p65 and p50 confirmed that the active NF-κB in HUVEC treated by cyclic strain or lipopolysaccharide (LPS) consisted of the p65/p50 heterodimer (Fig. 3A and data not shown). To further implicate ROS in this activation, we treated strained cells with antioxidants and then subjected them to 15 min of cyclic strain. At concentrations that had decreased DCFH oxidation in the cell, antioxidants (ebselen, NAC, DIDS, and DDC) also inhibited the strain-induced activation of NF-κB (Fig. 3B). By contrast, neither apocynin (an inhibitor of neutrophil NADPH oxidase) nor allopurinol (an inhibitor of xanthine oxidase) attenuated the NF-κB response to strain.

The effects of cyclic strain on NF-κB activation were also evaluated in ρ⁰ cells (Fig. 3C). Unlike WT cells, ρ⁰ cells failed to activate DNA binding of NF-κB in response to strain. More prolonged straining regimens lasting 30, 60, or 120 min also failed to increase in NF-κB activity in the ρ⁰ cells, indicating that the response was not merely delayed. These cells also failed to respond to antimycin A. However, the ρ⁰ cells were still capable of activating the p65/p50 heterodimer in response to LPS, which can activate NF-κB by an ROS-independent
pathway (5). To further confirm that \( \rho^0 \) cells retained the capacity to respond to an ROS signal, we treated these cells with \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \)) exogenously for 15 min. This exposure resulted in activation of NF-\( \kappa \)-B (Fig. 2C), indicating that \( \rho^0 \) cells were capable of responding to an oxidant signal despite an absence of functional mitochondria. Thus the absence of mitochondrial electron transport prevented the generation of an ROS signal in response to strain, but it did not prevent the cells from responding to an externally applied oxidant stimulus.

**Regulation of VCAM-1 mRNA by mitochondrial ROS.** The VCAM-1 promoter region contains two NF-\( \kappa \)-B sites (30), making it a potential target for activation in response to strain-induced ROS and NF-\( \kappa \)-B signaling. To test for possible activation of this gene in response to cyclic strain, we assessed WT HUVEC for changes in VCAM-1 mRNA over several hours (Fig. 4A). Basal expression of VCAM-1 mRNA was low in unstrained endothelium, but strain-induced increases were observed within 3 h. This response peaked near 6 h and remained high until the strain was removed (data not shown). Subsequent experiments therefore were conducted for a period of 6 h.

To determine whether mitochondrial ROS released in response to strain were required for this response, we carried out studies with rotenone and antimycin A (Fig. 4B). Augmentation of mitochondrial ROS generation by antimycin A increased VCAM-1 mRNA in unstrained cells, indicating that mitochondrial ROS have the ability to trigger this response. When strained cells were treated with rotenone or DPI, the strain-induced increase in VCAM-1 was decreased. To clarify the involvement of potential sources of the ROS signal, we treated HUVEC with allopurinol, apocynin, and L-NA (Fig. 4C). None of these curtailed the strain-induced increase in VCAM-1 mRNA expression. DIDS and DDC (data not shown) attenuated the mRNA increase observed during strain. These results suggest that changes in mRNA levels are the result of superoxide generated by the mitochondria in the response to mechanical strain.

To further clarify the role of mitochondrial ROS generation in the increase in VCAM-1 mRNA, we conducted experiments in \( \rho^0 \) HUVEC. Unlike the WT HUVEC, 6 h of strain produced no significant increase in the mRNA message in \( \rho^0 \) HUVEC (Fig. 4D). Similarly, the \( \rho^0 \) HUVEC failed to increase mRNA expression of VCAM-1 in response to antimycin A treatment. However, they did retain the ability to augment VCAM-1 mRNA message in response to LPS, indicating that transcriptional activation by non-ROS pathways was intact.

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**Fig. 3.** Activation of NF-\( \kappa \)-B during mechanical strain in HUVEC. **A**: EMSA demonstrate an increase in activation in the presence of 15 min of cyclic strain that is abolished in the presence of rotenone or DPI. A similar increase was observed when unstrained cells were treated with antimycin for 15 min. LPS (100 \( \mu \text{M} \)) treatment was used as a positive control, and antibodies (Ab) were used to demonstrate \( \text{p}65/\text{p}50 \) as the active heterodimer in HUVEC. **B**: effects of antioxidants on strain-induced NF-\( \kappa \)-B activation. Antioxidants that decrease cellular levels of ROS diminished NF-\( \kappa \)-B activation, but apocynin and allopurinol had no effect. Supershift in the presence of \( \text{p}65 \) Ab in the last lane demonstrated that strain-induced activation involved \( \text{p}65/\text{p}50 \) heterodimer. **C**: effects of strain on NF-\( \kappa \)-B activation in \( \rho^0 \) HUVEC. Although the strain-induced increase in expression was not observed in \( \rho^0 \) HUVEC, these cells did increase NF-\( \kappa \)-B activation in response to exogenous administration of \( \text{H}_2\text{O}_2 \). Note that the LPS-induced response in \( \rho^0 \) HUVEC was preserved. Concentrations of inhibitors were as follows: antimycin A (2 \( \mu \text{M} \)), rotenone (2 \( \mu \text{M} \)), DPI (5 \( \mu \text{M} \)), allopurinol (100 \( \mu \text{M} \)), apocynin (30 \( \mu \text{M} \)), chelerythrine (50 \( \mu \text{M} \)), NAC (1 mM), DIDS (100 \( \mu \text{M} \)), and DDC (1 mM).
ings indicate that \( \rho^0 \) HUVEC fail to increase VCAM-1 mRNA in response to strain due to the lack of a mitochondrial electron transport system rather than to a general signaling defect.

**CAM surface expression during strain.** To further test the physiological significance of strain-induced mitochondrial ROS signaling, we measured CAM cell surface expression in response to mechanical strain. In strained cells, VCAM-1 cell surface expression increased by 5.5-fold over 6 h (Fig. 5A). This increase was attenuated by rotenone and by DPI, but not by allopurinol or apocynin. Unstrained HUVEC, cell surface expression of VCAM-1 was also increased by antimycin A and by LPS treatment. The VCAM-1 response to strain was attenuated by the antioxidants ebselen, NAC, and DDC and the anion channel inhibitor DIDS when used at the same concentrations that attenuated oxidant signaling (Fig. 5B). VCAM-1 cell surface expression in unstrained cells was moderately increased when NOS activity was inhibited with L-NA (unstrained + L-NA, 26 ± 4). However, in the presence of L-NA, strained HUVEC did not have a higher VCAM-1 expression than in those subjected to strain alone (strained + L-NA, 61 ± 11; \( P > 0.2 \)). Other cell adhesion molecules were also examined, including E-selectin, which increased cell surface expression 3.1-fold in response to cyclic strain (unstrained, 15 ± 4; strained, 46 ± 7), and ICAM-1, where cell surface expression was 2.2-fold higher (unstrained, 38 ± 7; strained, 84 ± 13) (data not shown). Again, the increases in CAM expression in response to cyclic strain were attenuated by rotenone or ebselen but were unresponsive/mildly increased in response to L-NA (data not shown). These results indicate that strain-induced mitochondrial ROS are capable of stimulating CAM expression at the cell surface in HUVEC.

To further explore the effects of mitochondrial ROS on CAM expression, we conducted flow cytometry on \( \rho^0 \) HUVEC (Fig. 5C). The increase in VCAM-1 cell surface expression during strain was attenuated in \( \rho^0 \) HUVEC compared with WT cells. The mild increase that was observed was not blocked by the inhibitors rotenone or DPI or by the antioxidants ebselen, NAC, DDC, or DIDS. Antimycin A failed to produce a detectable increase in expression in the \( \rho^0 \) HUVEC. Despite lower baseline expression (\( \rho^0, 6 ± 3; \) unstrained WT, 11 ± 6), LPS still elicited increases in VCAM-1 expression in both unstrained and strained \( \rho^0 \) cells. Similar results were obtained with \( \rho^0 \) cells for E-selectin and ICAM-1 (data not shown).

**Cytoskeletal/mitochondrial association in mechanotransduction.** To explore the involvement of the cytoskeleton in the strain-induced mitochondrial ROS signaling, WT HUVEC were treated with increasing concentrations of cytochalasin D to disrupt the actin cytoskeleton beginning 2 h before straining. Although cytochalasin elicited changes in cell shape, total RNA harvested from cells treated with cytochalasin D (5 \( \mu M \)) did not increase until after 12 h of treatment (data not shown), indicating that the cells did not detach from the substrate during the experiment. After 6 h of strain, DCF fluorescence was measured (Fig. 6A). In the presence of cytochalasin D (5 \( \mu M \)), strain-induced increases in DCF fluorescence were virtually abolished. However, these cells retained the ability to augment mitochondrial ROS generation in response to antimycin A, indicating that cytochalasin D did not inhibit mitochondria. To determine the role of microtubules in the oxidant signaling response, we preincubated cells with nocardazole (1.0–10 \( \mu g/ml \)) and measured the DCF response. Nocardazole failed to abolish the strain-induced increases in DCF fluorescence. To rule out the possibility that nocardazole produces a nonspecific increase in ROS production, we incubated cells with rotenone in the presence of nocardazole. Mitochondrial inhibition with rotenone abolished the strain-induced increase in DCF oxidation, implicating mitochondria as the oxidant source (Fig. 6B). Cytochalasin D treatment attenuated the strain-induced mRNA expression of VCAM-1 in a concentration-dependent manner (Fig. 6C). However, at 5 \( \mu M \) cytochalasin D did not block the ability of the cells to upregulate VCAM-1 mRNA message in response to antimycin A, again suggesting the absence of a nonspecific inhibition. To determine whether cytochalasin D was interfering with the downstream effector pathways involved in gene expression, we exposed HUVEC to strain for 15 min after 2 h of treatment.
with cytochalasin D. Nuclear extracts analyzed by EMSA showed that the level of p65/p50 binding to DNA was attenuated by cytochalasin D at 2.5 or 5.0 μM, but not at lesser concentrations (Fig. 6D). In HUVEC treated with cytochalasin D, strain-induced NF-κB activation could still be elicited by antimycin A, indicating that cytochalasin D did not interfere with the mitochondrial ability to generate the required ROS signal or interfere with the downstream signaling involved in NF-κB activation (data not shown). Strained HUVEC treated with cytochalasin D and L-NA showed a significant attenuation of DCF fluorescence (cytochalasin D, 96 ± 8%; cytochalasin D + L-NA, 66 ± 16%; P < 0.05). Thus disruption of the actin cytoskeleton interfered with strain-induced mitochondrial ROS production but did not significantly affect strain-induced NOS activity.

To evaluate the role of the tubulin cytoskeleton in strain-induced ROS signaling, we treated confluent cells with varying concentrations of nocodazole to block microtubule polymerization. In HUVEC monolayers pretreated with nocodazole (10 μg/ml), the oxidation of DCFH in response to strain or antimycin A was not significantly altered, but the signal was attenuated by rotenone (Fig. 6B).

**DISCUSSION**

These studies sought to identify the significance of mitochondria in the transduction of mechanical strain into biological responses in cultured vascular endothelial cells. Using fluorescent redox-sensitive dyes, we found evidence of a significant increase in mitochondrial ROS signaling during cyclic strain. Chemically dissimilar antioxidants abolished the responses to strain, and p0 HUVEC lacking a functional electron transport system selectively lost the ability to respond to mechanical strain but not the ability to respond to LPS. The site of ROS generation appears to be mitochondrial complex III, on the basis of the observation that inhibitors that block electron flux into that site (rotenone, DPI) attenuated the strain-induced increases in the ROS signal, along with subsequent NF-κB activation, mRNA expression of VCAM-1, and cell surface expression of adhesion molecules. Previous studies have shown that mitochondrial inhibitors that block electron transfer into complex III attenuate superoxide generation by preventing the generation of ubisemiquinone, whereas inhibitors that block electron flux distal to that site tend to increase ROS generation by prolonging the lifetime of ubisemiquinone (27).

Collectively, these findings suggest that mitochondrial inhibition downstream from complex III by antimycin A increased the ROS signal and mimicked the response to strain. Consistent with those findings, we observed that mitochondrial inhibition downstream from complex III by antimycin A increased the ROS signal and mimicked the response to strain. Collectively, these findings support a model in which the mitochondria release ROS from complex III as an early signal in response to strain, which is required for the subsequent activation of gene expression.

Previous investigators have implicated ROS in the signal transduction process activated by mechanical strain. However, our findings conflict with other groups who have suggested that an endothelial NAD(P)H oxidase functions as the source of the strain-induced ROS signal (29). Their conclusions were based on the pharmacological inhibition of ROS generation by DPI, a flavoprotein inhibitor that blocks electron transfer in the NAD(P)H oxidase but also in a wide variety of other oxidase systems including mitochondrial complex I. In our study, apocynin, a more specific inhibitor of the neutrophil NADPH oxidase (26), failed to abrogate the response to strain, suggesting that the NAD(P)H oxidase is not importantly involved.
Superoxide is normally generated in mitochondria through univalent electron transfer to \( \text{O}_2 \) primarily at the ubisemiquinone site in complex III (12). Superoxide is rapidly dismutated to \( \text{H}_2\text{O}_2 \) by Mn-superoxide dismutase in the mitochondria and by Cu/Zn superoxide dismutase in the cytosol. The resulting \( \text{H}_2\text{O}_2 \) is capable of initiating a physiologically relevant signal transduction process. The present study examined NF-\( \kappa \)B activation and subsequent transcriptional activation of genes including VCAM-1 as a marker of the activation of a downstream response. Whether or not superoxide anion generated in the mitochondria can escape to the cytosol is controversial (47). Our studies with DIDS, which inhibits the mitochondrial inner membrane anion channel (7), showed a significant inhibition of the strain-induced ROS, NF-\( \kappa \)B, and mRNA responses. It is therefore possible that the ROS signaling pathway requires an anion channel for superoxide to reach the cytosol, where it acts as a signaling agent.

The functional consequences of strain-induced mitochondrial ROS generation were assessed in HUVEC in terms of their involvement in the activation of NF-\( \kappa \)B. Previous studies have shown that exogenous oxidants can activate NF-\( \kappa \)B and trigger subsequent transcription (6). However, our results show that strain can activate a similar response by triggering ROS production from mitochondria. We found that DNA binding of

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**Fig. 6.** Effects of cytoskeletal disruption on strain-induced changes in HUVEC. A: effects of cytochalasin D (cyto D) on strain-induced DCF fluorescence (\( n = 6 \)) over 6 h. B: effects of nocodazole (Noco, in \( \mu \)g/ml) on strain-induced DCF fluorescence (\( n = 6 \)) over 6 h. C: effect of cytochalasin D on strain-induced VCAM-1 expression over 6 h. D: effects of cytochalasin D on strain-induced NF-\( \kappa \)B activation as measured by EMSA (15 min). Concentrations of inhibitors were as follows: antimycin A (AntiA, 2 \( \mu \)M) and rotenone (Rot, 2 \( \mu \)M). *\( P < 0.05 \) comparison between strained and unstrained cells within the same intervention group; †\( P < 0.05 \) comparison between strained control cells and strained group with intervention.
NF-κB p50/p65 heterodimer was increased by cyclic strain and that this increase was abrogated by inhibitors of the proximal region of the mitochondrial electron transport chain or by antioxidants. By contrast, no inhibition was seen with inhibitors of the NAD(P)H oxidase system or of xanthine oxidase. The activation by strain was mimicked by antimycin A treatment in unstrained cells, indicating that increases in mitochondrial ROS are sufficient to activate this response. Finally, the responses to strain or antimycin A were abolished in ρ0 HUVEC that lack a mitochondrial electron transport chain and are therefore incapable of generating ROS from that system. The absence of a response in these cells could not be attributed to nonspecific effects, since these cells could still activate NF-κB in response to LPS or exogenous H2O2. Collectively these findings indicate that ROS generated from the mitochondrial electron transport chain are capable of activating NF-κB in cells subjected to cyclic strain.

The data obtained from ρ0 HUVEC add additional evidence that the mitochondrial ROS production during cyclic strain is important in mediating activation of NF-κB and upregulation of VCAM-1 expression. In these cells, antimycin A did not increase ROS generation and subsequent NF-κB and VCAM-1 expression; however, these cells did retain the functional ability to activate these signaling pathways in response to LPS, which utilizes proinflammatory signaling pathways that are capable of activating NF-κB independently of mitochondrial ROS (16). These mitochondria-deficient cells complement the findings of the pharmacological inhibitors of ROS production. Moreover, the observation that these cells retained the ability to respond to LPS stimulation indicates that these cells are not metabolically incapable of activating the signaling pathways studied in these experiments. Although the ρ0 cells serve as a useful adjunct to the pharmacological agents used in these studies, a targeted genetic disruption of a critical component of the mitochondrial electron transport chain would be a more specific approach to attempt in subsequent studies.

The triggering of transcription in response to this NF-κB activation was evaluated in terms of the mRNA message for several cell adhesion molecules. The VCAM-1 promoter contains two NF-κB sites located at −63 and −77 bp relative to the transcriptional start site (30), which are critical for the increased expression in inflammatory states. The increase in mRNA expression of VCAM-1 during cyclic strain was prevented by inhibitors of the proximal region of the electron transport chain and by antioxidants. Moreover, ρ0 HUVEC failed to increase VCAM-1 mRNA levels in response to mechanical strain. However, ρ0 HUVEC were still capable of increasing VCAM-1 expression in response to LPS, thus demonstrating the specificity of the mitochondrial role in the response to mechanical strain. Collectively these results are consistent with the NF-κB activation data, and they support our conclusion that increases in mitochondrial ROS signaling are responsible for the strain-induced increase in expression of VCAM-1 mRNA.

Although our findings clearly implicate NF-κB in the response to strain, we cannot exclude the possibility that other redox-sensitive factors such as activator protein-1 or GATA binding proteins may have contributed to this response (1, 41). Our findings nevertheless demonstrate that strain-induced increases in CAM expression do not require the shear stress response elements such as those found in ICAM-1 or PDGF-B (44, 49).

The increases in VCAM-1 mRNA message were also associated with increases in cell surface expression of adhesion molecules. Using flow cytometry, we found that expression of all these adhesion molecules increased in response to strain, with the greatest effects seen in VCAM-1 and E-selectin. The high constitutive expression of ICAM-1 made it difficult to evaluate changes in its mRNA and cell surface expression in response to strain. However, basal expression of VCAM-1 was lower, and previous studies have demonstrated that NO and ROS play significant roles in the regulation of its expression (32, 37). The present study extended those results by showing that strain-induced mitochondrial ROS production was capable of activating this downstream response. Consistent with the NF-κB and VCAM-1 mRNA results, the cell surface expression of VCAM-1 was abrogated by inhibitors of the proximal region of the electron transport chain and by antioxidants. Moreover, ρ0 cells failed to increase cell surface expression in response to strain but were still capable of increasing expression in response to LPS. The intact response to LPS in the ρ0 cells further demonstrates that mitochondrial ATP production is not required for the activation of gene expression in HUVEC. Consistent with this interpretation is the observation that the pharmacological inhibitors of electron transport, rotenone and antimycin A, had diametrically opposite effects on NF-κB activation and mRNA expression, yet these compounds both inhibit mitochondrial electron transport.

When a cell is subjected to mechanical distortion, a signaling sequence is initiated that leads to the functional response to strain. Early signaling events such as NF-κB activation and the appearance of mRNA message for target genes should occur before events such as VCAM-1 expression that require protein synthesis and transport to the cell surface. To the extent that downstream functional responses are triggered by ROS from mitochondria, the ROS signal should be the earliest to appear. In this study, DCF fluorescence was measured at 6 h, yet other responses were sampled before that time. However, this does not indicate that the oxidant signal temporally followed the downstream responses. The DCF fluorescence reflects the accumulation of oxidized probe in the cell over time, which is a function of its rate of oxidation and the duration of accumulation. It is reasonable to assume that the rate of oxidant production begins to increase as soon as the stretch begins, even though some time may be required to accumulate sufficient oxidized probe to detect. In preliminary studies, we detected increases in DCFH oxidation as early as 15 min into strain, indicating that the initiating signal was present at an early time point. With continued strain, DCF fluorescence continued to increase, resulting in a larger value at 6 h, so this was the time point selected for analysis.

Other studies were carried out to examine the involvement of the actin cytoskeleton in transmitting mechanical forces from the plasma membrane to the mitochondria. Cytochalasin D pretreatment caused disruption of the actin cytoskeleton and attenuated the strain-induced mitochondrial ROS signaling, NF-κB activation, and VCAM-1 transcription in HUVEC, thus demonstrating a requirement for the actin cytoskeleton in these responses. Importantly, cytochalasin D did not block the ability of antimycin A to stimulate mitochondrial ROS generation, indicating that the mitochondria retained the ability to respond.
By contrast, the tubulin cytoskeleton appeared to be minimally involved in the responses to strain in confluent HUVEC based on the failure of nocodazole-induced microtubule disassembly to interfere with the response to strain. Although the spatial distribution of mitochondria in dividing cells is regulated by the tubulin cytoskeleton (58), the actin cytoskeleton appears to play a more important role in strain transmission to the mitochondria in confluent endothelial cells. Further studies are required to determine whether the tubulin cytoskeleton participates in mechanotransduction in other cell types or at different stages of the cell cycle.

Our proposed model of mechanotransduction is complementary to the “tensegrity” model proposed by Ingber (31), in that mechanical forces are transmitted from integrins at the cell surface throughout the cell via the cytoskeleton. Some studies have identified a possible role of the integrins themselves in the mechanotransduction process (53). Meyer et al. (38) showed that cAMP signaling in response to the twisting of magnetic beads anchored to the RGD-ligand binding site on β1-integrins occurs even when the actin cytoskeleton is disrupted with cytochalasin D or when the microtubule system is disassembled with nocodazole. Their findings indicate that certain signaling processes capable of activating transcription do not require normal cytoskeletal organization. Our studies suggest that an intact actin cytoskeleton is required for the induction of other transcriptional systems during cyclic strain. However, the underlying biophysical process by which force transmitted through the cytoskeleton might lead to an increase in mitochondrial ROS signaling is not known. Although cytoskeletal disruption did not impair the ability of the mitochondria to increase ROS signaling, it is still possible that the connection between integrins and the mitochondria involves a signaling system activated at the integrin receptors such as G proteins (38). Future studies are needed to fully clarify the relationships among different signaling pathways activated by mechanical strain in cells, and the role of the cytoskeleton in these pathways.

The lung parenchyma and its blood vessels normally experience cyclic strain during tidal ventilation. In patients with acute hypoxic respiratory failure treated with mechanical ventilation involving high tidal volumes and positive end-expiratory pressure, the magnitude of that strain may be increased. These patients exhibit higher mortality, they require a more prolonged period of ventilatory support, and they exhibit increased circulating levels of cytokines (3). Hence, excessive lung stretch may initiate an inflammatory cascade that contributes to the preexisting lung injury and triggers the release of inflammatory mediators that could potentially affect other organ systems. Our study identifies a possible molecular mechanism for that condition, whereby excessive stretch elicits an increase in oxidant release by mitochondria, leading to the activation of NF-κB and the transcriptional upregulation of inflammatory genes. The specific cells contributing to that response in vivo have not been identified but could potentially include endothelial cells, epithelial cells, and interstitial cells in the lung. We used HUVEC in our study because 1) they are primary cells capable of recapitulating in vitro the responses of vascular cells in vivo, 2) they are human cells for which appropriate reagents were available, and 3) many previous mechanotransduction studies have focused on the endothelium. Future studies are therefore necessary to determine whether the initial findings of this study are representative of responses in various types of cells in the lung and its blood vessels. Similarly, the magnitude and frequency of stretch in this study were limited by the capabilities of the Flexercell apparatus but were nevertheless chosen to be comparable in some respects to the conditions experienced by lung cells. The ability to reproduce the stress and strain conditions of lung endothelial cells in an in vitro system is technically limited, so caution must be used in extrapolating the conditions used in our study, or similar studies, to the intact lung. Despite these limitations, the mechanism suggested by our findings could conceivably apply to many different cell types and to a variety of different mechanical perturbations. Again, future studies are required to determine whether the signaling cascade identified in this study is applicable to cells in the intact lung.

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