NADPH oxidase promotes NF-κB activation and proliferation in human airway smooth muscle

SUKHDEV S. BRAR,1 THOMAS P. KENNEDY,1 ANNE B. STURROCK,2 THOMAS P. HUECKSTEADT,2 MARK T. QUINN,3 THOMAS M. MURPHY,4 PASQUALE CHITANO,4 AND JOHN R. HOIDAL2

1Department of Internal Medicine, Cannon Research Center, Carolinas Medical Center, Charlotte 28232; 2Division of Pulmonary Diseases, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710; 3Division of Respiratory, Critical Care and Occupational (Pulmonary) Medicine, University of Utah, Salt Lake City, Utah 84132; and 4Department of Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717

Received 6 June 2001; accepted in final form 10 November 2001

EVIDENCE IS RAPIDLY ACCUMULATING that low-activity-reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases homologous to that in phagocytic cells generate reactive oxygen species (ROS) as signaling intermediates in both endothelium and vascular smooth muscle. We therefore explored the possibility of such an oxidase regulating growth of airway smooth muscle (AWSM). Proliferation of human AWSM cells in culture was inhibited by the antioxidants catalase and N-acetylcysteine, and by the flavoprotein inhibitor diphenylene iodonium (DPI). Membranes prepared from human AWSM cells generated superoxide anion (O2•−) measured by superoxide dismutase-inhibitable lucigenin chemiluminescence, with a distinct preference for NADPH instead of reduced nicotinamide adenine dinucleotide as substrate. Chemiluminescence was also inhibited by DPI, suggesting the presence of a flavoprotein containing oxidase generating O2•− as a signaling molecule for cell growth. Examination of human AWSM cells by reverse transcriptase-polymerase chain reaction consistently demonstrated transcripts with sequences identical to those reported for p22phox. Transfection with p22phox antisense oligonucleotides reduced human AWSM proliferation. Inhibition of NADPH oxidase activity with DPI prevented serum-induced activation of nuclear factor-κB (NF-κB), and overexpression of a superrepressor form of the NF-κB inhibitor IκBα significantly reduced human AWSM growth. These findings suggest that an NADPH oxidase containing p22phox regulates growth-factor responsive human AWSM proliferation, and that the oxidase signals in part through activation of the prototypical redox-regulated transcription factor NF-κB.

superoxoide anion; diphenylene iodonium; p22phox

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tic smooth muscle cells express p22phox (22, 23, 54), and p47phox is important for oxidase function (27, 45). However, gp91phox appears to be replaced in aortic smooth muscle cells by the unique homolog Mox-1 (54), reported by others as NADPH oxidase homolog-1 (NOH-1) (7), as the heme-containing membrane partner for p22phox. Yet another gp91phox homolog renox has been recently found in renal epithelial cells where it is postulated to serve as an oxygen sensor regulating renal endothrotein production (20, 48). A consensus terminology has been proposed for the growing family of NADPH oxidases: NOX1 for Mox-1/NOH-1; NOX2 for gp91phox; and NOX4 for renox (48).

Evidence is beginning to accumulate that a phagocyte-like NADPH oxidase is also present in airway smooth muscle (AWSM). Antioxidants and the flavoprotein inhibitor diphenylene iodonium (DPI) inhibit proliferation of rat and bovine AWSM in culture (13, 42). Fetal bovine serum (FBS) and platelet-derived growth factor (PDGF) stimulate generation of ROS by rat (13) and bovine (42) AWSM cells in vitro, and ROS release by cell cultures is inhibited by DPI (13). Treatment of bovine AWSM cells with PDGF or transfection with a constitutively active Rac1 (pEXV-Myc-V12Rac1) increases expression of the cell cycle regulator cyclin D1 in an antioxidant and DPI-inhibitable manner (42), and PDGF-induced expression of cyclin D1 is reduced by inhibition of the oxidase via overexpression of a dysfunctional NH2-terminal fragment of p67phox (42). Thus, a flavoprotein containing oxidase, presumably an NADPH oxidase, also appears to play a role in proliferative signaling of AWSM. However, the components of the oxidase and its functional activity in AWSM have not been determined. We therefore initiated studies to characterize the NADPH oxidase in cultured human AWSM and to better understand its contribution to signaling of growth and proliferation.

METHODS

Culture of human AWSM. Human AWSM cells were harvested and cultured using methods previously reported for rat and mouse AWSM (13). With approval of the Institutional Review Board, human lung was obtained from lobes resected during thoracotomy for lung cancer. Segmental or subsegmental bronchus was dissected free from vessels and lung parenchyma. Adventitia and bronchial epithelium were removed under a dissecting microscope, and the remaining parenchyma. Adventitia and bronchial epithelium were resectioned and cultured in Dulbecco’s modified medium of 5% CO2-95% air at 37°C in Hanks’ balanced salt solution (HBSS) containing 0.2% type IV collagenase and 0.05% type IV elastase. Enzyme digests were centrifuged at 500×g, and the pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin (250 ng/ml) in a humidified atmosphere of 5% CO2-95% air at 37°C. On reaching confluence, cells were passed with 0.25% trypsin-0.002% EDTA. Immunostaining was performed using a polyclonal antibody against α-smooth muscle actin (Sigma) and visualized using an avidin-biotin-immunoperoxidase technique. Smooth muscle cultures demonstrated the typical “hill and valley” appearance under phase-contrast microscopy and stained avidly for α-smooth muscle actin. Preliminary studies demonstrated that culture of cells in the presence of 10% FBS resulted in a linear growth phase up to 120 h. Cultures from passages 2–9 were used for experiments.

Measurement of cultured AWSM proliferation. Proliferation of cultured AWSM was quantitated using a previously reported colorimetric method based on metabolic reduction of the soluble yellow tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble purple formazan by the action of mitochondrial succinyl dehydrogenase (13). This assay empirically distinguishes between dead and living cells. For proliferation studies, cells were seeded into 24-well uncoated plastic plates (Costar) at 15,000–50,000 cells/well and cultured with DMEM and mitogens. After 24–96 h, medium was removed, cells were washed twice with 1 ml of sterile Dulbecco’s modified PBS without Ca2+ or Mg2+ (DBPS), the medium was replaced with 1 ml/well fresh DMEM containing 100 μg/ml MTT and 0.5% FBS, and plates were incubated an additional 1 h. MTT-containing medium was removed, 0.5 ml of dimethyl sulfoxide was added to each well, and the absorbance of the solubilized purple formazan dye was measured at 540 nm. A total of four to six wells were studied at each treatment condition. Preliminary studies were performed to optimize the concentration of MTT and incubation time, and to confirm that the absorbance of the MTT formazan reduction product correlated with direct counts of stained cells within the range employed. For antioxidant interventions and in studies with a final cell density of 40,000 cells/well, cells were fixed in ice-cold buffered formalin (5% in DBPS), permeabilized by two 30-min treatments of ice-cold methanol, stained with Wright’s-modified Geimsa, and counterstained with eosin. Direct cell counts were performed on five random fields well viewed at a magnification of ×100 using a 1-mm2 ocular grid.

Cell culture treatments. Cell proliferation was studied in cultures stimulated with 10% FBS. To explore the role of oxidants in signaling mitogenesis, we tested the effects on cell proliferation of supplementing media with the O2 scavenger superoxide dismutase (SOD; 300–3,000 U/ml), the H2O2 scavenger catalase (300–3,000 U/ml), the sulfhydryl donor and hydroxyl radical (·OH) scavenger N-acetylcysteine (20 mmol/l), the xanthine oxidase inhibitor allopurinol (1 mmol/l), the nitric oxide synthase inhibitor Nω-nitro-arginine (100 μmol/l), and the flavoprotein inhibitor diphenyl-eneiodonium (DPI) (5–100 μmol/l). To probe for expression of NADPH oxidase components, monolayers of human AWSM cells were grown on 25-mm dishes to near confluence in 10% FBS, and RNA was harvested for analysis by the reverse transcriptase-polymerase chain reaction at this point or after an additional 48 h of growth arrest in serum-free DMEM. To study the effect of flavoprotein inhibition on signal transduction pathways, confluent human AWSM cells in 25-mm dishes were growth arrested for 48 h in serum-free DMEM and stimulated with 10% PBS and DMEM, in the presence and absence of 50 μmol/l DPI. After 0–6 h, cells were lysed and immunoassayed for the dually phosphorylated active forms of the mitogen-protein kinase/extracellular signal-regulated kinases ERK1 and ERK2 and p38 stress-activated kinase, and for total and phosphorylated inhibitory factor κB (IκB), as detailed below. To study the effect of flavoprotein inhibition on activation of the redox-regulated transcription factor nuclear factor (NF)-κB, confluent human AWSM cells in 100-mm dishes were growth arrested for 48 h in serum-free DMEM and stimulated with 10% PBS and DMEM, in the presence or absence of 50 μmol/l DPI at the time of stimulation. In some experiments, growth-arrested monolayers were also preincubated with DPI 30 min before...
stimulation. After 30, 60, or 90 min, total cell lysate or nuclear protein was isolated for immunoblot assay of phosphorylated IkBa or electrophoretic mobility shift assay of NF-κB DNA binding, as described below.

Measurement of ROS generation. Generation of ROS by intact monolayers of FBS-stimulated airway smooth muscle cells was studied by reduction of ferrocyanochrome c, employing a modification allowing absorbance reading with an automatic enzyme immunoassay reader, as previously reported (13). Human AWSM cells grown on 24-well plates in the presence of 10% FBS to near confluence were washed with DPBS and incubated in serum-free medium at 37°C with 160 μmol/l ferrocyanochrome c in a total volume of 400-μl HBSS (bicarbonate containing, phenol red free), with and without copper-zinc SOD (300 U/ml). The absorbance of each well was measured at 550 nm initially and again after 120 min of incubation at 37°C using an ELX800UV automated microplate reader (Biotek Instruments, Highland Park, VT). Monolayers were then washed with DPBS and cell protein was measured using the bicinchoninic protein assay (Pierce). O2 generation normalized to cell protein, was computed from the Beer-Lambert relationship as the quotient of SOD-inhibitable increase in absorbance over time divided by the difference between the molar extinction coefficients for ferrocyanochrome c and ferrocyanochrome c (ΔEM = 2.1 × 10^4 M^-1·cm^-1) and a measured light path length of 1 mm.

Generation of ROS by cell membranes was measured by SOD-inhibitable lucigenin chemiluminescence. The method of Pagano et al. (40), with centrifugation speeds modified according to the work of Mohazzab and Wolin (38), was used to prepare membranes for measurement of ROS. Human AWSM cells from six near-confluent T-75 flasks were harvested with trypsin-EDTA, washed once with ice-cold DPBS, and centrifuged for 5 min at 675 g. The pellet was resuspended in 500 μl of ice-cold Tris-sucrose buffer (pH 7.1) composed of (in mmol/l) 10 Trizma base, 340 sucrose, 1 phenylmethylsulfonyl fluoride (PMSF), 1 EDTA, and 10 μg/ml protease inhibitor cocktail (Sigma) and sonicated by four 15-s bursts. The cell sonicate was centrifuged at 1,475 g and 4°C for 15 min with the use of a Beckman Optima TL ultracentrifuge to remove nuclei and unbroken cells. The supernatant was then centrifuged at 29,000 g and 4°C for 15 min. The pellet was discarded and the supernatant was further centrifuged at 100,000 g and 4°C for 75 min. The pellet was resuspended in 100 μl Tris-sucrose buffer and stored at −80°C. Supernatant from the last centrifugation was also saved as representative of lactate dehydrogenase containing-soluble elements of cytoplasm (38). Generation of ROS was measured by SOD-inhibitable lucigenin chemiluminescence, as recently reported (33, 51) in 500 μl of 50 mmol/l phosphate buffer (pH 7.0), containing 1 mmol/l EGTA, 150 mmol/l sucrose, 5 μmol/l lucigenin, 15 μg membrane protein, 50 μg cytosolic protein, and 100 μmol/l NADH or NADPH as substrate. Chemiluminescence (in arbitrary light units) was measured at 60-s intervals for 5 min using a Turner model 20/20D luminoimeter (Turner Designs, Sunnyvale, CA). SOD (300 U) and DPI (50 μmol/l) were added to determine dependence of light generation on O2 generation and flavoprotein-containing enzymatic activity, respectively. The signal was expressed as the sum of all measurements after subtraction of the buffer blank (51).

Reverse transcriptase-polymerase chain reaction detection of NADPH oxidase components. To probe for presence of components of the neutrophil NADPH oxidase and NOX1, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as recently described (13). Cell monolayers were washed twice with DPBS and lysed with 4 mol/l guanidine thiocyanate, 25 mmol/l sodium citrate, and 0.5% sarkosyl. After being scraped, lysates were sheared with four passes through a pipette. RNA was extracted by the phenol-chloroform method (16) and quantitated spectrophotometrically at 260 and 280 nm. RNA (2 μg) was reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) in a reaction mixture containing 1 mmol/l dATP, dCTP, dGTP, and dTTP; 40 units of RNase inhibitor; 25 μmol/l random hexamers; 5 mmol/l MgCl2; 500 mmol/l KCl; and 100 mmol/l Tris-HCl (pH 8.3), in a total volume of 50 μl. The resultant cDNA was PCR amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p22^phox, gp91^phox, p47^phox, p67^phox, and NOX1 using human gene-specific sense and antisense primers based on sequences published in GenBank: GAPDH: 5'-ACCCACATGGAGAAGCTGG, 3’-CTCAGTGT-AGCCCGAAGTCG; p22: 5'-ATGGAGGGCGTGCGCAAGACGACATG, 3’-GATGTTGGCCTGCATGCTGGGCGG; gp91^phox, 5'-TCAATAATCTGATCTTATATCG, 3’-TGTCACACACTGTTATATATGC; NOX1: 5’-CTCGGTGTTTACGACTCTATGTC, 3’-GAACTCTAGAGCCGTAACCA; p47^phox, 5’-ACCCACAGCAATGCTGGGT, 3’-AGTACGTCTGACGTCAT, p67^phox, 5’-CGAGGGCAACACTGATAGA, 3’-CATGTGAACACTGACGTTCA. PCR was carried out on a Perkin-Elmer DNA thermal cycler 480. Amplification was carried out for 32 cycles for GADPH and p22^phox and 36 cycles for all other primers at 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by an extension step at 72°C for 10 min. PCR-amplified DNA was separated on 1.2% agarose gel, stained with ethidium bromide, and visualized and photographed under ultraviolet light. PCR products from defined bands were purified with QIAquick gel extraction kits (Qiagen, Chatsworth, CA) and sequenced automatically by an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the same respective primers for sequencing as for PCR. Results of RT-PCR from human AWSM cells were always compared with positive controls (human PMNs for p22^phox, gp91^phox, p47^phox, and p67^phox, and Caco colon carcinoma cells for NOX1).

Immunoblotting for proteins. To study activation of signal transduction cascades, cells were lysed and proteins were isolated and quantitated by immunoblotting as previously detailed (13) using antibodies against IkBa and phosphospecific antibodies (New England Biolabs, Beverly, MA) against the dual phosphorylated active forms of ERK1 and ERK2 (phosphorylated at Thr202/Tyr204 and p38 stress-activated kinase (phosphorylated at Thr180/Tyr182), and against phosphorylated IkBa (phosphorylated at Ser12), and p47^phox, and peroxi
dase-labeled donkey polyclonal anti-rabbit IgG. Cells were placed on ice, washed twice with cold DPBS, scraped into 0.5-ml boiling buffer [10% vol/vol glycerol and 2% wt/vol sodium dodecyl sulfate (SDS) in 83 mM Tris, pH 6.8] and sheared by four passages through a pipette. Aliquots were removed for protein determination, using the bicinchoninic protein assay (Pierce). After 10% β-mercaptoethanol and 0.05% bromphenol blue were added, lysates were boiled for 5 min and stored at −80°C until immunoblotting was performed. Proteins in defrosted samples were separated by SDS-PAGE on 12% polyacrylamide gels (15 μg protein/lane) and electrotransferred to 0.45 μm Hybond-enhanced chemiluminescence nitrocellulose membranes (Amersham Life Sciences) using the wet transblot method in transfer buffer (0.025 mol/l Tris, 0.192 mol/l glycine, 2.6 mol/l SDS, and 20% vol/vol methanol; pH 8.8) at 100 V for 1 h. Blots were blocked overnight at 4°C with blocking buffer (PBS with 0.1% Tween 20) containing 5% fat-free milk powder (Carnation, Glendale, CA). After being rinsed five times for 5 min each in PBS containing 0.1% Tween 20, blots were incubated for 1 h
at room temperature with primary antiserum diluted 1:2,000. After being rinsed again as above, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody diluted 1:2,000 in blocking buffer. Immunoblots were rinsed again as above and detected using an enhanced chemiluminescence method (ECL Western blotting detection system; Amersham Life Science, Buckinghamshire, UK) and autoradiography.

The NF-κB inhibitor IκBα and phosphorylated IκBα were assayed with several modifications of the above procedure. Cells were lysed in boiling buffer to which 50 mmol/l dithiothreitol (DTT) had been added as a reducing agent. Immunoblot assay of IκBα proceeded as above, but samples for measurement of phosphorylated IκBα were blocked for 2 h at room temperature and incubated overnight at 4°C with primary phospho-specific antibodies diluted 1:1,000 in PBS with 0.1% Tween and 5% BSA.

To measure protein expression of p22phox in the 100,000 g plasma membrane fraction, prepared as detailed earlier, immunoblot assays were performed as detailed above using previously described rabbit polyclonal (E31799) or mouse monoclonal (44.1) antibodies (14) at dilutions of 1:1,000.

Electrophoretic mobility shift assays. Nuclear protein was isolated as described by Ogata et al. (39). Human AWSM cells on 100-mm dishes were scraped in 800 μl of buffer A composed of (in mmol/l) 10 HEPES (pH 7.9), 10 KCl, 0.1 EDTA, 0.1 EGTA, 1.0 DTT, and 1.0 PMSF (plus protease inhibitor cocktail, 10 μg/ml) at 4°C and kept on ice for 15 min, followed by the addition of 50 μl of 10% Nonidet P-40, and cells were homogenized by vortexing for 10 s. The homogenate was centrifuged at 3,000 g at 4°C for 10 min to prepare nuclei, and the pelleted nuclei were resuspended in 25 μl of ice-cold buffer B composed of (in mmol/l) 20 HEPES (pH 7.9), 400 NaCl, 1.0 EDTA, 1.0 EGTA, 1.0 DTT, 1.0 PMSF, and protease inhibitor cocktail (10 μg/ml). Nuclear proteins were extracted by incubation on ice for 30 min, and the supernatant containing nuclear protein was collected after centrifugation at 8,000 g at 4°C for 10 min. Protein concentration was determined on an aliquot by the Pierce method and the remainder of nuclear protein was frozen at −80°C until use.

Electrophoretic mobility shift assay were performed using the consensus binding oligonucleotides, 5′-GGTTGAGGG-GACTTTCACGAGGCGAGT-3′ and 3′-TCAACTCTCCGAAAGGCTCG-5′, for the p50 component of NF-κB (Promega, Madison, WI), end-labeled by phosphorylation with [32P]ATP and T4 polynucleotide kinase. DNA-protein binding reactions were performed with 5 μg of nuclear protein (as determined by the Pierce method) and 50–100,000 cpm of [32P]-end-labeled double-stranded DNA probe in binding buffer composed of (in mmol/l) 10 HEPES (pH 7.9), 25 KCl, 1.0 DTT, 1.0 EDTA, and 100 μg/ml poly(dI-dC) and 5% glycerol. All components of the binding reaction, with the exception of the labeled probe, were combined and incubated on ice for 30 min. After the labeled probe was added, the binding reaction was incubated for an additional 20 min at room temperature.

Competition experiments were performed with 10× unlabeled oligonucleotides for NF-κB. Supershift assays were performed by the addition of 2.0 μg of supershift-specific antibodies for p65, p50, p52, Rel B, or c-Rel components of NF-κB and incubated on ice for 30 min before the probe was added. Samples were electrophoresed on a 5% nondenaturing polyacrylamide gel in Tris-glycine-EDTA (120 mmol/l glycine and 1 mmol/l EDTA in 25 mmol/l Tris, pH 8.5) buffer. Gels were dried and analyzed by autoradiography at −80°C using an image-intensifier screen. Densitometry was performed using Kodak Digital Science 1D image analysis software (Eastman Kodak, Rochester, NY).

Transfection protocols for IκBα gene transfer. To repress activation of NF-κB, cells were transduced with adenoviral (Ad serotype 5; Ad5) vectors that were E1a/E1b-deleted and expressed a superrepressor of NF-κB (Ad-IκBαSR, 2 × 1011 plaque-forming U/ml) under the regulation of the cytomegalovirus (CMV) immediate-early promoter region (8) or expressed the CMV immediate-early promoter region alone (AdCMV-3, 2.05 × 1011 plaque-forming U/ml, control vector). These Ad vectors were constructed in the Vector Core Laboratory at the Gene Therapy Center of the University of North Carolina School of Medicine and were generous gifts, respectively, from Dr. Albert S. Baldwin of the Lineberger Cancer Center and Dr. Andrew Ghio of the Environmental Protection Agency Human Health Effects Center (Chapel Hill, NC). Transduction was performed using previously published protocols (8). Human AWSM cells were seeded onto 24-well plates at a density of 20,000 cells/well and grown for 6 h in DMEM with 10% FBS. Media was removed and replaced with 200 μl complete medium containing ~2.5 × 105 colony-forming units of Ad-IκBαSR or AdCMV-3. After overnight incubation, the vector containing medium was removed, and cells were washed once with warm DPBS and reincubated with fresh complete media. After an additional 24 h, cells were washed twice with ice-cold DPBS, fixed twice for 30 min in ice-cold 5% buffered formalin in DPBS, permeabilized twice with ice-cold methanol for 30 min, stained with Wright's-modified Giemsa, and counterstained with eosin before direct counting of cells using an ocular grid, as outlined earlier.

Transfection protocol for p22phox antisense treatment of human AWSM. To transfect antisense oligonucleotides for p22phox, human AWSM cells were cultured in six-well plates at a density of 20,000 cells/well and grown in DMEM containing 10% FBS. After 24 h, wells were washed once with DPBS and 800 μl of DMEM (serum and antibiotic-free) was added to each well. Previously reported (34) p22phox sense (5′-GGTTCTCACCAGGCGAGTAC-3′) or antisense (5′-GATGTTGCCATCAGGAGGCAAC-3′) oligonucleotides (2 μg) were mixed with 5 μl Lipofectace Reagent (Life Technologies) and 200 μl serum- and antibiotic-free DMEM at room temperature for 15 min. This mixture was then added to each well and cells were incubated at 37°C. After 6 h, the transfection mixture was gently removed and replaced with 2.5 ml of DMEM containing 10% FBS. Cells were incubated an additional 48 h before photographs were taken and growth was quantitated with the MTT assay.

Statistical analysis. Data are expressed as means ± SE for a minimum number of four observations, unless indicated. Differences between two groups were compared using Student’s t-test. Two-tailed tests of significance were employed. Differences between multiple groups were compared using one-way ANOVA. The post hoc test used was the Newman-Keuls multiple comparison test. Significance was assumed at P < 0.05.

RESULTS

Antioxidants and flavoprotein inhibitors suppress human AWSM cell proliferation. Analogous to what we observed in cultured rat AWSM cells (13), antioxidants also reduced proliferation of cultured human AWSM (Fig. 1, A and B). Whereas catalase produced substantial inhibition of growth, SOD inhibited proliferation only to a slight, albeit statistically significant, degree. These results suggest that H2O2, and not O2, is the proximate ROS important for proliferative signaling in
Fig. 1. Antioxidants and flavoprotein inhibitors reduce growth of human airway smooth muscle (AWSM) cells. A: human AWSM cells stimulated with 10% fetal bovine serum (FBS) were seeded at a density of 50,000 cells/well in 24-well plates and grown in the presence of the antioxidants catalase (Cat; 3,000 U/ml), N-acetylcysteine (NAC; 20 mmol/l), or superoxide dismutase (SOD; 3,000 U/ml). After 48 h, cells were fixed in ice-cold buffered formalin (5% in Dulbecco’s modified phosphate-buffered saline (DPBS)), permeabilized with two 30-min treatments of ice-cold methanol, stained with Wright’s modified Geimsa, and counterstained with eosin. Direct cell counts were performed on 5 random fields at a magnification of ×100 using a 1-mm² ocular grid. Each observation represents counts taken in at least 6 culture wells. As previously reported for rat AWSM (13), the H₂O₂ scavenger catalase and the sulfhydryl donor N-acetylcysteine substantially inhibited growth of human AWSM. The O₂ scavenger SOD inhibited growth to a slight but statistically significant degree. B: Geimsa and eosin stains of human AWSM cells from experiments in A. a: Control cells grown with 10% FBS and no antioxidants; b: catalase-treated cells; c: SOD-treated cells; d: cells treated with N-acetylcysteine. Images are taken at ×200. C: growth was also inhibited when human AWSM cells were grown for 48 h in the presence of the flavoprotein inhibitor diphenylene iodonium (DPI), suggesting that proliferation is dependent in part on generation of reactive oxygen species (ROS) from a flavoprotein-dependent enzymatic source. Proliferation was quantitated by assessing the cell number-dependent reduction of the soluble yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan, measured as the absorbance at 540 nm (A₅₄₀) (13). *P < 0.05 vs. FBS alone; +P < 0.001 vs. FBS alone.
this cell type. Proliferation of human AWSM was also reduced in a dose-dependent manner by the flavoprotein inhibitor DPI (Fig. 1C), with growth suppression reaching a plateau at 50 μmol/l. Human AWSM growth was not reduced by allopurinol or Nω-nitro-L-arginine (data not shown), indicating that a flavoprotein-dependent oxidase other than xanthine oxidase or nitric oxide synthase mediates an important but exclusive pathway for proliferative signaling.

Human AWSM cell membranes produce ROS. Confluent human AWSM cell monolayers incubated for 3 h in HBSS progressively reduced ferricytochrome c in an SOD-inhibitable manner, generating 4.2 ± 0.4 pmol of O2/μg of cell protein over 3 h (n = 12 each with and without SOD). To determine whether O2 production was from a plasma membrane or cytosolic source, ROS generation of cell fractions was studied with the use of lucigenin chemiluminescence. Lucigenin chemiluminescence was stimulated by the membrane, but not cytosolic fraction, of proliferating human AWSM cells (Fig. 2). The preferred substrate was NADPH rather than NADH. Addition of cytosol to membranes failed to increase, and SOD or DPI (at 50 μmol/l) substantially decreased chemiluminescence. These findings suggest a membrane electron transport system utilizing NADPH as a donor of reducing equivalents for generation of O2.

Proliferating human AWSM cells express the p22phox component of the NADPH oxidase. To begin to determine expression of membrane components of the putative NADPH oxidase, we performed RT-PCR on RNA extracted from human AWSM cells under two conditions: cells grown to near confluence in the presence of 10% FBS; and cells grown to near confluence, then growth arrested in serum-free media for 48 h. Proliferating nearly confluent human AWSM cells strongly expressed the α-subunit of cytochrome b558, p22phox (Fig. 3A, lanes 1–3), but expression was more variable in growth-arrested, serum-starved cells (lanes 4–6). The 252-base pair PCR product obtained is identical to bases 221–372 of the reported human mRNA sequence (26), except for exhibiting the C242-to-T transition in p22phox recently reported to be associated with accelerated coronary disease (15). Protein product for p22phox was also clearly detectable by immunoassay in membranes from humans AWSM cells (Fig. 3B). The cytochrome and flavin-bearing β-subunit of the cytochrome, gp91phox, and the p67phox cytosolic component were also detected but expression was variable and required 36 cycles of amplification for detection (data not shown). No evidence was found for the NOX1 homolog of gp91phox or the p47phox cytosolic component of the leukocyte NADPH oxidase (data also not shown), but clear expression for both components was seen in controls (human PMN for p47phox and Caco colon carcinoma cells for NOX1).

p22phox is necessary for human AWSM proliferation. Transfection of sense oligonucleotides for p22phox had little effect on growth of human AWSM (Fig. 4B (sense) vs. 4A (control), and Fig. 4D). However, antisense oligonucleotides for p22phox reduced expression of p22phox...
protein (Fig. 4E), and significantly impaired AWSM proliferation (Fig. 4C antisense) vs. 4B (sense), and Fig. 4D). This suggests that the p22phox is important and necessary for signaling-induced proliferation.

NADPH oxidase influences growth-signaling transduction cascades. ROS from growth factor-responsive NADPH oxidase might affect AWSM proliferation by acting on a number of signal transduction cascades. The flavoprotein inhibitor DPI prevents activation of cyclin D1 expression in bovine AWSM cells by a constitutively active phosphatidylinositol 3-kinase (PI3K) (43), suggesting that the PI3K pathway is upstream from the putative Rac1-activated oxidase. Also, whereas activation of ERK1 and ERK2 is unaffected by ROS scavengers or flavoprotein inhibition, cyclin D1 expression induced in bovine AWSM cells by PDGF or a constitutively active Rac1 is nonetheless prevented by antioxidants or DPI (42). Thus the putative NADPH oxidase must affect proliferation by influencing other signal transduction pathways. Using antibodies specific for their respective phosphorylated active forms or phosphorylated products, we studied the effect of flavoprotein inhibition on activity of the IκB kinase, the ERK1/ERK2 mitogen-activated kinases, and the p38 stress-activated kinase. Serum promptly activated the
IκB kinase, resulting in phosphorylation of IκBα (Fig. 5A), and stimulated the ERK1/ERK2 kinase (Fig. 5B) and p38 stress-activated kinase cascades (Fig. 5C). Treatment of cells with DPI (50 μmol/l) did not reduce phosphorylation of ERK1/ERK2 (Fig. 6A) or p38 mitogen-activated protein (MAP) kinase (Fig. 6B). However, DPI did reduce phosphorylation of IκBα, shown in immunoassays using a phospho-specific antibody (Fig. 6C), suggesting that phosphorylation of IκBα in human AWSM cells may be in part redox regulated by the putative NADPH oxidase. Phosphorylation of IκBα is the initiating event in activation of NF-κB, the prototypical redox-regulated transcription factor (5, 6, 46, 47). We therefore performed electrophoretic mobility shift assays to determine whether serum stimulation activates NF-κB. Serum stimulation for 90 min induced a dramatic increase in DNA binding activity for NF-κB (Fig. 7A, lane 2). Supershift experiments with antibodies specific for potential NF-κB components identified the top band (arrowhead) as the relevant p50 (lane 3) and p65 (lane 5) dimer. Consistent with its reduction of IκBα phosphorylation, DPI treatment also significantly and substantially inhibited serum stimulation of DNA binding activity for NF-κB in nuclear protein of human AWSM cells (Fig. 7, B and C). These results imply that the putative NADPH oxidase influences AWSM proliferation in part through redox regulation of NF-κB transcriptional activity.

**Transcription factor NF-κB is important for human AWSM proliferation.** The transcription factor NF-κB plays a well-recognized role in inflammation but has recently (25, 30) also been found important in regulating G0/G1 to S-phase transition in cell proliferation through increasing expression of cyclin D1. In vascular smooth muscle, NF-κB is essential for proliferation. When nuclear activation of NF-κB is interrupted by microinjection of IκBα or NF-κB consensus oligonucleotides, growth is disrupted (11). However, the importance of NF-κB for AWSM proliferation has not been established. We therefore studied the effect on human AWSM proliferation of retarding NF-κB activation by transduction of cells with an adenovirus linked super-repressor form of IκBα (Ad-IκBαSR). Transduction of cells with the adenoviral vector linked to the CMV promoter (Ad-CMV) as a control had no inhibitory effect on proliferation. However, transduction with Ad-IκBαSR resulted in prominent IκBαSR expression (Fig. 8C), which substantially impaired growth of human AWSM cells (Fig. 8, A and B). Thus interruption of NF-κB activation by DPI might explain in part the reduction in human AWSM proliferation by this flavoprotein inhibitor.

**DISCUSSION**

Signal transducing oxidases have now been reported in a number of cell types from the blood vessel wall, including vascular endothelium (22, 29), vascular smooth muscle (7, 15, 23, 27, 38, 45, 54, 56), and blood vessel adventitial fibroblasts (40, 57). In endothelium (22) and adventitial fibroblasts (41), the membrane oxidase components appear identical to the respective α- and β-subunits, p22phox and gp91phox, present in leukocytes. In vascular smooth muscle cells, gp91phox appears to be replaced by a homolog NOX1 (22, 41, 54). Functionally, these oxidases have been implicated in ANG II-induced cellular hypertrophy (23, 56), mitogenic signaling (1, 23, 28, 55), ANG-dependent hypertension (23, 62), impaired endothelial-dependent vascular relaxation (37), and progression of atherosclerosis (15, 23, 60). In the airway mucosa, a p22phox and gp91phox-containing NADPH oxidase regulating K+ channel activity has been reported by one group to serve as an O2 sensor in pulmonary neuroepithelial bodies (19). In airway smooth muscle, ANG II induces hypertrophy in cell cultures (36), and a constitutively active form of Rac1 stimulates cyclin D1 expression in a DPI-inhibitable manner (42), suggesting that a functionally important NADPH oxidase analogous to that in vascular smooth muscle exists. However, its components and relationship to growth signaling cascades have not been explored.

In this report, we demonstrate evidence for a growth-regulatory oxidase activity in human AWSM membranes. Serum-induced proliferation of human AWSM
cells in culture was strongly inhibited by the antioxidants catalase and N-acetylcysteine (Fig. 1A), indicating the importance of redox signaling in growth factor-dependent cell cycling. Human AWSM membranes (Fig. 2) supported generation of lucigenin chemiluminescence in a SOD-inhibitable manner, indicating that the initial product of the growth regulating oxidase activity is likely O$_2^-$. However, because proliferation of human AWSM cells was strongly inhibited by catalase but only slightly by SOD (Fig. 1A), it is likely that H$_2$O$_2$ formed from dismutation of O$_2^-$ is the important proximate signaling species. DPI inhibited cell proliferation (Fig. 1C) and lucigenin chemiluminescence by isolated membranes (Fig. 2), suggesting that the putative growth regulatory oxidase is a flavoprotein-dependent enzyme. Neither the xanthine oxidase inhibitor allopurinol nor the nitric oxide synthase inhibitor $N^\omega$-nitro-L-arginine had an inhibitory effect on growth. Therefore, neither of these flavoprotein-containing enzymes is a likely source of growth regulating ROS in human AWSM cells. Similar to what has been reported for vascular smooth muscle cells (33, 51), SOD-inhibitable lucigenin chemiluminescence stimulated by human AWSM cell membranes was approximately five-fold higher with NADPH rather than NADH as the electron donating substrate (Fig. 2). Thus the putative NADPH growth regulating oxidase of AWSM cells shares some of the functional properties reported for its vascular smooth muscle cell counterpart, but with important differences.

The vascular smooth muscle oxidase contains a distinct membrane p22$^{phox}$ component that is critical for mediation of oxidase activity and ANG II-induced hypertrophy (55). Human AWSM cells also demonstrate clear expression of p22$^{phox}$ mRNA that, when sequenced, is identical to the sequence reported for the leukocyte analog (Fig. 3A). Furthermore, immunoassays show conspicuous evidence of p22$^{phox}$ protein in human AWSM cell membranes (Fig. 3B). When antisense oligonucleotides for p22$^{phox}$ are transfected into AWSM cells (Fig. 4), growth is significantly impaired. Thus the p22$^{phox}$ NADPH oxidase subunit is expressed in human AWSM cells and is critically important for their proliferation. The structure of the remaining components of the oxidase is less certain. It has been suggested that vascular smooth muscle cells express the gp91$^{phox}$ homolog NOX1 as the partner for p22$^{phox}$ in forming the membrane components of cytochrome b$_{558}$ (7, 22, 54). We detected no mRNA for NOX1 and only weak expression of gp91$^{phox}$. Thus, it is not presently clear whether the partner for p22$^{phox}$ in AWSM is gp91$^{phox}$, expressed at an extremely low level, or a unique tissue-specific protein, such as the recently reported thyroid THOX1 and THOX2 oxidases (17, 18) or NOX4, recently reported in renal tubular epithelium (20, 48) and bone osteoclasts (61). Vascular smooth muscle cells express the p47$^{phox}$ cytosolic component of the leukocyte oxidase (27, 45). Also, overexpression of a dysfunctional NH$_2$-terminal fragment of p67$^{phox}$ disrupts growth factor-stimulated cyclin D1 promoter ac-
tivity in bovine AWSM cells (42), suggesting that this cytosolic component is present in bovine airway smooth muscle. We detected PCR product for p67phox in human AWSM cells only after 36 cycles of amplification, and were unable to demonstrate any product for p47phox. Thus the two cytosolic components of the leukocyte oxidase are either absent or expressed at low levels. Either circumstance might account for the low rate of ROS production in human AWSM cells compared with the oxidase of leukocytes (32).

In vascular smooth muscle, the putative NADPH oxidase appears to signal through certain isoforms of MAP kinases (1) and p38 stress-activated protein kinase (56). In AWSM cells, the situation appears different. The AWSM oxidase seems to be part of the PI3K cascade activated by PDGF in signaling cell proliferation (43). Catalase and DPI block stimulation of the cyclin D1 promoter by expression of either a constitutively active Rac1 (V12Rac1) or a PI3K catalytic subunit (p110PI 3-KCAAX). However, whereas the PI3K inhibitor wortmannin blocks cyclin D1 promoter activation by PDGF, it does not prevent stimulation of the promoter by V12Rac1. This would suggest that the putative NADPH oxidase is downstream from and possibly activated by phosphorylation of one or more of its components by the PI3K, similar to the situation in human polymorphonuclear neutrophils, where receptor-stimulated Rac2 activation is downstream from and mediated by PI3K (2). Also, stimulation of the NADPH oxidase with V12Rac1 does not activate the MAP kinases ERK1 and ERK2, and neither antioxidants nor DPI inhibit ERK1 and ERK2 activation by PDGF (42). In our studies, we confirmed that serum growth factor-induced activation of ERK1 and ERK2 is not inhibited by DPI, and also found that DPI does not prevent serum activation of the p38 stress-activated MAP kinase. Flavoprotein inhibition with DPI did, however, prevent serum-induced phosphorylation of IkB (Fig. 6C), the first step toward initiating cytosol to nuclear transport of the transcription factor NF-κB. DPI also inhibited serum-induced activation of p65/p50-containing NF-κB DNA binding activity (Fig. 7, B and C). Therefore, in human AWSM, redox regulation of NF-κB appears to be a major pathway mediating the influence of NADPH oxidase activity on cell proliferation.

In unstimulated normal cells NF-κB resides in the cytoplasm as a dimeric protein complex bound to an inhibitor protein, designated IκBα (49). Agonist stimulation activates NF-κB DNA binding activity in human AWSM cells. Human AWSM cells were grown to confluence, growth arrested for 48 h and stimulated with 10% FBS for 90 min. Nuclear protein was harvested and electrophoretic mobility shift assays were performed using a 32P-labeled consensus oligonucleotides specific for the p50 component of nuclear factor (NF)-κB. A: compared with unstimulated cells (lane 1), FBS-stimulated cells demonstrated prominent nuclear DNA binding activity for NF-κB (lane 2). Supershift experiments demonstrated that the relevant band (lane 2, arrowhead) contained p65 (lane 5) and p50 (lane 3), but not p52 (lane 4), Rel-B (lane 6), or c-Rel (lane 7). Competition experiments show specificity: lane 8, human AWSM nuclear protein incubated with 32P-labeled NF-κB consensus oligonucleotides; lane 9, addition of ×10 unlabeled NF-κB consensus oligonucleotides; lane 10, addition of ×10 unlabeled consensus oligonucleotides specific for the cAMP response element. B: replicate (n = 4) cultures of human AWSM were grown to confluence, growth arrested in serum-free media for 48 h and stimulated with 10% FBS for 90 min, with and without DPI (50 μmol/l) 15 min before serum exposure. DPI prevented serum stimulation of NF-κB DNA binding activity. C: densitometry of the p65/p50 containing bands from electrophoretic mobility shift assay shown in B. * P < 0.001 vs. FBS alone.

Fig. 7. Serum stimulation increases and DPI decreases NF-κB DNA binding activity in human AWSM cells. Human AWSM cells were grown to confluence, growth arrested for 48 h and stimulated with 10% FBS for 90 min. Nuclear protein was harvested and electrophoretic mobility shift assays were performed using a 32P-labeled consensus oligonucleotides specific for the p50 component of nuclear factor (NF)-κB. A: compared with unstimulated cells (lane 1), FBS-stimulated cells demonstrated prominent nuclear DNA binding activity for NF-κB (lane 2). Supershift experiments demonstrated that the relevant band (lane 2, arrowhead) contained p65 (lane 5) and p50 (lane 3), but not p52 (lane 4), Rel-B (lane 6), or c-Rel (lane 7). Competition experiments show specificity: lane 8, human AWSM nuclear protein incubated with 32P-labeled NF-κB consensus oligonucleotides; lane 9, addition of ×10 unlabeled NF-κB consensus oligonucleotides; lane 10, addition of ×10 unlabeled consensus oligonucleotides specific for the cAMP response element. B: replicate (n = 4) cultures of human AWSM were grown to confluence, growth arrested in serum-free media for 48 h and stimulated with 10% FBS for 90 min, with and without DPI (50 μmol/l) 15 min before serum exposure. DPI prevented serum stimulation of NF-κB DNA binding activity. C: densitometry of the p65/p50 containing bands from electrophoretic mobility shift assay shown in B. * P < 0.001 vs. FBS alone.
Ser32 and Ser36 near the NH2-terminus of IκBα targeting the inhibitor for ubiquitination and proteolytic degradation by the 26S proteasome (44). The removal of IκBα unmasks the nuclear localization signal (10), allowing the NF-κB complex to translocate to the nucleus, where it binds to its respective nucleotide sequence and transcriptionally regulates expression or repression of target genes. The initial upstream response to agonist activation of IκB kinase and subsequent degradation of IκBα in normal cells has been proposed to be production of O2•−, followed by generation of H2O2 (46). The compelling argument for this sequence of events is the observation that many of the known inducers of NF-κB also induce generation of ROS (5, 6, 46, 47). In human aortic smooth muscle cells, PDGF stimulates generation of O2•− and increased gel mobility shift assay NF-κB DNA binding activity that is blocked by the O2•− scavengers SOD or Tiron, the flavoprotein inhibitor DPI, the protein kinase C inhibitor Ro 31-8220, and the PI3K inhibitor wortmannin (35). O2•− also triggers activation of NF-κB in human umbilical vein endothelial cells through a pathway involving phosphorylation and activation of the IκB kinase by O2•−-stimulated protein kinase C (39). These events are consistent with growth factor stimulation of a PI3K and NADPH oxidase-dependent pathway, in which O2•− stimulates protein kinase C through thiol modification of the zinc finger motif in the protein.
Activation of NF-κB by ROS generated from a putative NADPH oxidase would be expected to facilitate human AWSM cellular proliferation by promoting cell cycle G0/G1 to S phase transition through enhanced expression of cyclin D1 (25, 30). NF-κB activation might also enhance resistance of vulnerable proliferating cells to apoptosis through increasing expression of antipapoptotic Bcl-2 family proteins (9, 57, 59). In vascular smooth muscle, NF-κB is essential for proliferation. When nuclear activation of NF-κB is interrupted by microinjection of IкBα or NF-κB consensus oligonucleotides, growth is disrupted (11). NF-κB appears to be equally important for proliferation of human AWSM. When activation of NF-κB is disrupted through expression of the superrepressor form of IкBα (Fig. 8C), proliferation of human AWSM is substantially impaired (Fig. 8, A and B). Conversely, activation of NF-κB might therefore be expected to promote cellular AWSM growth. This has significant implications for the pathogenesis of airway wall remodeling in the inflamed, asthmatic airway, where in mucosal biopsies NF-κB has been shown to be universally activated (24).

Given the important contribution of the putative NADPH oxidase to regulation of airway smooth muscle proliferation (13, 42, 43), much additional work is needed to fully characterize the components of this oxidase and better understand its regulation. It is unclear whether the regulatory cytosolic component p47phox is actually missing or simply expressed at such low levels as to be undetectable by our PCR methods. It has been postulated that the growth regulatory NADPH oxidase in AWSM and other mesenchymal tissues is the more primitive form of a signaling enzyme system that has subsequently evolved into a host defense function in leukocytes (4). Consistent with this hypothesis is the demonstration that purified cytochrome b558 alone can be activated by an anionic amphiphile to catalyze O2•− production at a low steady rate when provided an environment containing oxygen, reduced flavins, and Rac1-GTP•γS, and at a much higher rate simply with addition of p67phox (32). Thus not all the specialized leukocyte cytosolic regulatory components are necessary for basic enzymatic oxidase function at the low rate of O2•− generation observed in human AWSM (4). Also, we have previously reported that genetic deletion of gp91phox does not impair the growth response of murine AWSM cells to serum (13). Thus, AWSM either expresses a unique gp91phox homolog, or gp91phox is conceivably replaced in null mice by upregulated expression of a gp91phox homolog such the renal tubular epithelial oxidase NOX4 (renox) (17, 48), which is expressed 2.5-fold higher in osteoclasts from gp91phox null mice than in wild-type controls (61).

Understanding which oxidase components are necessary, their molecular structure, and the nature of their interactions present compelling tasks for delineating the potentially important role of an AWSM NADPH signaling oxidase in the remodeling of the asthmatic airway wall (12). Airway wall remodeling, caused in large part by hypertrophy and hyperplasia of AWSM within the medial layer, leads to the phenomenon of fixed airways obstruction characteristic of severe asthma (12). Strategies that disrupt the function of a growth regulatory oxidase might retard development of this incapacitating condition. Furthermore, if there is analogy between the condition in the airway and in vascular smooth muscle, polymorphisms in oxidase components predisposing subjects to coronary disease, such as the C424G->T transition of p22phox (15), might also pose a risk for development of airways remodeling in severe asthma.

We thank Drs. Mark Reames and Edward Lipford for assistance in procuring the human airway specimens from which AWSM was cultured.

This work was funded by grants from the Charlotte-Mecklenberg Health Care Foundation and by National Institutes of Health Grants HL-50153-07 (Dr. Hoidal), RO1-AR-42426, and HL-66767 (Dr. Quinn).

REFERENCES


14. Burritt JB, Quinn MT, Jutila MA, Bond CW, and Jesaitis AJ. Topological mapping of neutrophil cytochrome B epitopes...
NADPH OXIDASE ACTIVATES NF-κB IN SMOOTH MUSCLE


26. Homo sapiens cytochrome b-245: alpha polypeptide (C.Y.B.A.), mRNA. GenBank accession number XM008040; beta polypeptide (CYBA), mRNA, GenBank accession number NM000397; homolog sapiens p47phox, similar to neutrophil cytosolic factor 2 (65 kd, chronic granulomatous disease, autosomal 2), clone MGC:2275, mRNA, GenBank accession number BC001666.

27. Hsieh-hsiang Segal BH, Pagnan PJ, Rey FE, Paigen B, Deleou Nardis J, Hoyt RF, Holland SM, and Finekl T. Expression of phagocyte NADPH oxidase beta polypeptide (CYBA), mRNA. GenBank accession number (C.Y.B.A.), mRNA.


