Requirement for Reactive Oxygen Species in Serum-induced and Platelet-derived Growth Factor-induced Growth of Airway Smooth Muscle*

(Received for publication, January 7, 1999, and in revised form, March 12, 1999)

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Reactive oxygen species have been recently identified as important mediators of mitogenic signaling in a number of cell types. We therefore explored their role in mediating mitogenesis of airway smooth muscle. The antioxidants catalase, N-acetylcysteine, and probucol significantly reduced proliferation in primary cultures of rat tracheal smooth muscle stimulated with fetal bovine serum or platelet-derived growth factor, without affecting cell viability or inducing apoptosis. N-Acetylcysteine also significantly reduced serum-stimulated elevation of c-Fos but did not prevent the normal mitogen-induced increase in c-fos mRNA. Fractionation of ribosomes by sucrose density centrifugation and subsequent dot-blot Northern analysis revealed that antioxidants reduced incorporation of c-fos mRNA into the heaviest polyribosomes, suggesting redox regulation of c-fos mRNA translation. Serum treatment of monolayers produced a small but reproducibly significant rise in superoxide dismutase-inhibitable reduction of ferricytochrome c by myocyte monolayers. Serum-induced ferricytochrome c reduction, cellular proliferation, and c-Fos elevation were decreased by the flavoprotein-dependent enzyme inhibitor diphenyleneiodonium. Growth responses to fetal bovine serum and superoxide dismutase-inhibitable reduction of ferricytochrome c were not different between cultured tracheal myocytes from wild-type versus gp91 phagocyte oxidase null mice. These results suggest that mitogen stimulation of airway smooth muscle induces signal transduction of cell proliferation that is in part dependent on generation of partially reduced oxygen species, generated by an NADH or NADPH oxidoreductase that is different from the oxidase in phagocytic cells.

Reactive oxygen species have long been recognized as important mediators of inflammation, injury, and cell death. Now, evidence is accumulating that small amounts of reactive oxygen species generated in select cell compartments can trigger signal transduction leading to gene expression (1–6). Oxidant signaling might be especially important in the asthmatic airway, where smooth muscle proliferation in chronic severe asthma contributes to development of fixed airways obstruction. H2O2 treatment of tracheal myocytes successively stimulates protein kinase C, Raf-1, and mitogen-activated protein kinase/extracellular signal-regulated kinase 1 pathways, leading to activation of extracellular signal-regulated kinases, serine/threonine kinases of the mitogen-activated protein kinase superfamily important in transduction of mitogenic signals to the nucleus (7, 8). This has important implications for the pathogenesis of remodeling in the asthmatic airway, where myocytes are exposed to reactive oxygen species from activated eosinophils, neutrophils, monocytes, and macrophages. However, airway smooth muscle is also constantly stimulated by mitogens, including platelet-derived growth factor, endothelin, histamine, tumor necrosis factor-α, and even serum as a consequence of submucosal edema and increased submucosal vascular permeability in asthma (10–13). Recently, growth factor treatment of fat cells (14, 15), renal mesangial cells (2, 5, 9), endothelium (3), and fibroblasts (4, 6) has been shown to stimulate production of superoxide anion (O2-) and H2O2, which are important for mitogenic signaling. One proposed source of these species is a cytochrome b558* and flavoprotein-dependent membrane NADH or NADPH oxidoreductase similar to that found in phagocytes (2, 4, 9). However, the exact structural nature of this oxidase and its relation to growth factor-stimulated proliferative responses are not completely defined. We therefore investigated whether mitogen-stimulated tracheal myocytes also generate reactive oxygen species that are important for signaling cellular proliferation.

EXPERIMENTAL PROCEDURES

Materials—Male adult Sprague-Dawley rats were purchased from Charles River (Raleigh, NC). Transgenic mice lacking the gene for the gp91 component of phagocyte oxidase (gp91phox-/-) were a gift from Dr. Mary Dinauer (Indiana University, Indianapolis, IN) (16). Protease inhibitors and guanidine thiocyanate were obtained from Roche Molecular Biochemicals. Diphenyleneiodonium chloride was from Aldrich. Dulbecco’s modified Eagle’s medium (DMEM),1 Hanks’ balanced salt solution (HBSS), HEPEP, antibiotic-antimycotic (10,000 units of penicillin, 10,000 µg of streptomycin, and 25 µg of amphotericin B/ml) and

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ balanced salt solution, FBS, fetal bovine serum; PDGF, platelet-derived growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; DPBS, Dulbecco’s PBS; Me3SO, dimethyl sulfoxide; NAC, N-acetylcysteine; DPI, diphenyleneiodonium; SOD, superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; PCR, polymerase chain reaction.
cells were lysed and c-fos mRNA was measured by RT-PCR, Northern blotting, and ribonuclease protection assay, as described below. To study whether levels of c-Fos or Fos-B were affected, confluent rat myocyte monolayers on 6-well plates were growth-arrested for 48 h in 0.5% FBS and DMEM. Cells were then pretreated with catalase (3000 units/ml), superoxide dismutase (SOD) (100 units/ml), catalase (3000 units/ml) and catalase and SOD and DMEM and stimulated with 10% FBS and DMEM for 15, 30, or 60 min, in the presence of additional scavengers or DPI added at the time of stimulation. Cells were then lysed, and c-Fos and Fos-B were assayed by immunoblotting as described below. To determine whether antioxidants influenced the rate of c-Fos elimination, confluent rat myocytes grown to confluence in 24-well plates were treated for 48 h in 0.5% FBS and DMEM and stimulated with 10% FBS and DMEM. After 60 min, stimulated cells were treated with 20 mM NAC or DPsB vehicle, and cycloheximide (25 μg/ml) was added to media to block further transcription. Cells were then harvested, 1.2, and 4 h later for immuno- blot assay of c-Fos. Finally, to study potential regulation of c-Fos production at the ribosomal level, rat tracheal myocytes were grown to confluence in T-25 flasks, growth-arrested in DMEM for 72 h, preincubated with 3000 units/ml catalase or 20 μM NAC for 2 h, and stimulated for 30 min with 10% FBS in the presence or absence of antioxidants. Cells were then harvested, polysomes were fractionated by sucrose gradient centrifugation, and purified mRNA from each fraction was subjected to dot-blot Northern assay for c-fos as described below.

**Measurement of Superoxide Anion (O2−) Generation—**
By FBS stimulated airway smooth muscle cells was measured by the technique of SOD-inhibitable reduction of ferricytochrome c (21), employing a modification allowing absorbance reading with an automatic enzyme immunoassay reader (22). Confluent cells grown on 24-well plates were growth-arrested for 72 h in serum-free DMEM, washed with DPBS, and incubated at 37 °C with 160 μM ferricytochrome c in total volume of 400 μl of HBSS (phenol red-free) with and without 10% FBS or copper-zinc SOD (300 units/ml). The absorbance of each well, blanked to replicate wells incubated with ferricytochrome c and SOD without FBS, was measured at 550 nm initially and again after 60 min of serum stimulation using an ELx800UV automated microplate reader (Biotek Instruments, Highland Park, VT). Monolayers were then washed with DPBS, and cell protein was measured using the BCA protein assay (Pierce). OD4, normalizing, to cell protein, was computed from the Beer-Lambert relationship (23) as the quotient of SOD-inhibitable increase absorbance over time divided by the difference between the molar extinction coefficients for ferricytochrome c and ferrocytochrome c (EM = 2.1 × 10^4 M^−1 cm^−1) (21) and a measured light path length of 1 mm. In some experiments, DPI (10 μM) was added to stimulated cultures at the time of stimulation to determine the magnitude of ferricytochrome c reduction that might be attributable to flavoprotein containing NADH or NADPH oxidoreductases.

**Measurement of Cytotoxicity and Apoptosis—**To assess for cytotoxicity, DPI (5–50 μM), catalase (3000 units/ml) or NAC (20 mM) was added to wells of airway smooth muscle cells grown to confluence on 24 well plates and growth-arrested for 72 h (0.5% FBS). After 24 h, lactate dehydrogenase activity in microcentrifuged supernatant was measured using a commercially available assay (DG-1340K from Sigma). Cell death was assessed by trypan blue dye exclusion.

To confirm that antioxidants were suppressing growth mechanisms rather than inducing apoptosis, cells grown to confluence on 35-mm Petri dishes or on glass slides were treated with 3000 units/ml catalase or 20 mM NAC for 24 h. Apoptosis was studied by visually assessing endonuclease dependent DNA fragmentation on ethidium bromide-stained agarose gels, as previously reported (18). Briefly, confluent cultures treated with drug for 24 h were washed twice with DPBS, scraped on ice into 1.5-ml microcentrifuge tubes and centrifuged at 250 × g for 5 min at 4 °C. The cell pellet was gently resuspended in 30 μl of DPBS and lysed by addition of 30 μl of lys buffer (50 mM EDTA, 1.6% (w/v) sodium lauryl sarcosinate, and 5 mM protease K in 200 mM Tris-HCl, pH 8.0). Lysate was incubated at 50 °C for 24 h. RNase (0.2 mg/ml) was added, and the lysate was incubated for another 2 h at 37 °C. DNA was extracted by the phenol-chloroform method (24), and bands were separated along with a DNA ladder standard on a 1% agarose gel at 60 V for 1 h, intercalated with ethidium bromide, and visualized under ultraviolet light. Photographs of stained gels were stud-
quantitated as described previously (18). Cells were placed on ice, washed twice with cold DPBS, scraped into 0.5 ml of boiling buffer (10% (v/v) glycerol and 2% (v/v) SDS in 83 mM Tris, pH 6.8) and sheared by four passages through a pipette. Aliquots were removed for protein determination, as described previously. After 10% β-mercaptoethanol and the blue color, the aliquots were added to 5 ml of cold 200 mM Tris (pH 7.5), 0.1 M NaCl, and stored at −80 °C until immunoblotting was performed. Proteins in defrosted samples were separated by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels (15 μg of protein/lane) and electrophoresed at 0.45 μm Hybrid ECL nitrocellulose membranes (Amersham Pharmacia Biotech) using the wet trans blot method in transfer buffer (90 mM Tris, 0.192 M glycine, 20% methanol; pH 8.8) at 100 volts 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 rinsing five times for 5 min each in PBS containing 0.1% Tween 20, blots were incubated for 1 h at room temperature with 2.0 μg/ml of c-Fos polyclonal antibody. After rinsing again as above, blots were incubated for 1 h at room temperature with anti-rabbit IgG/ hors eradish peroxidase antibody diluted 1:2000 in blocking buffer as the secondary antibody for c-Fos. Immunoblots were rinsed again as above and detected via an enhanced chemiluminescence method (ECL Western blotting detection system, Amersham Pharmacia Biotech). Autoradiographic film (X-OMAT AR, Eastman Kodak) was exposed to immunoblots for 10, 30, or 60 s to obtain optimal exposures of bands that were quantitated by laser densitometry using Kodak 1D imaging analysis software (Kodak). Fos B was assayed similarly using polyclonal goat anti-Fos B primary and anti-goat IgG/ horseradish peroxidase secondary antibodies. Reverse Transcription-Polymerase Chain Reaction—Semi-quantitative RT-PCR was performed by modification of procedures previously described (18, 20). Cell monolayers were washed twice with DPBS and lysed with 4% guanidine thiocyanate, 25 mM sodium citrate, and 0.5% Sarkosyl. After scraping, lysates were sheared with four passes through a pipette. RNA was extracted using the phenol-chloroform method (24) and quantitated spectrophotometrically at 260 and 280 nm. RNA (2 μg) was reverse transcribed using 200 units of M-MLV reverse transcriptase (Promega) in a reaction mixture containing 1 mM dATP, 1 mM dCTP, dGTP, and dTTP; 40 units of RNase inhibitor; 25 μM random hexamers; 5 mM MgCl2; 500 mM KCl; and 100 mM Tris-HCl (pH 8.3), in a total volume of 20 μl. The reaction was performed at 42 °C for 60 min followed by heat inactivation for 5 min at 95 °C. The resultant cDNA was PCR amplified for 30 and 35 cycles for GAPDH and c-fos, respectively, using rat gene-specific sense and antisense primers (18) based on sequences published in GenBankTM: GAPDH, 5′ ACCACCATGG-9′; c-fos, 5′ ACTGGATAAGCCGGCGGAG 3′ and 5′ GTTGAGGTAGATGCCTGTC 3′ (331 bp product); c-fos, 5′ ACTGGATAGGCCGGCAG 3′ and 5′ GCTCGGAGTGATGGCTGC 3′ (331 bp product), respectively. The specificity of primers for rat c-fos was confirmed by Southern blotting performed using a sequence internal to the chosen primers. PCR was carried out on a Perkin-Elmer DNA thermal cycler 480. Amplification for GAPDH was carried out by 30 cycles at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 1 min, followed by an extension step at 72 °C for 1 min. PCR conditions for c-fos were 35 cycles at 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 1 min followed by an extension step at 72 °C for 10 min. PCR-amplified DNA was separated on a 1.6% agarose gel, stained with ethidium bromide, and visualized and photographed under ultraviolet light. The resulting Polariod negative was quantitated by laser densitometry. The intensity of the GAPDH cDNA bands (a housekeeping gene unaffected by stimulation with FBS) for each sample was then used to normalize for loading differences in c-fos band intensities.

Northern Blotting—GAPDH and c-fos antisense probes were generated from rat pTRI-GAPDH and mouse p-TRI-c-fos/exon 4 linearized plasmids (Ambion, Austin, TX) using T7 RNA polymerase, producing fragments of 383 and 299 bases, respectively. The probe generated for c-fos had 95% homology with the sequence for rat c-fos. Probes were labeled for 1 h at 37 °C with 32P-UTP (Amersham Pharmacia Biotech) at a final concentration of 12.5 μl, purified on SELECT-DRF Spin Chromatography columns (5 Prime → 3 Prime, Inc., Boulder, CO) and quantitated on a Beckman LS 6000IC scintillation counter. Northern blots were performed with a kit from Ambion according to the manufacturer’s instructions. Briefly, RNA (15 μg) was electrophoresed on a 1.2% agarose gel and transferred overnight to a nylon membrane. After UV cross-linkage, membranes were prehybridized at 65 °C for 2 h and hybridized at 65 °C for 14 h with 106 cpm/ml of 32P-labeled c-fos cRNA. Membranes were washed twice for 5 min at room temperature with low stringency wash solution (Ambion), washed twice for 15 min at 65 °C with high stringency solution (Ambion), and exposed overnight to Kodak XAR-5 film in an imaging intensifying cassette at 80 °C. Bands were quantitated by laser densitometry. Equivalent loading of total RNA was verified by stripping c-fos probe from the membrane by boiling in 0.1% SDS in diethyl pyrocarbonate-treated water and hybridizing with 106 cpm/ml of 32P-labeled cRNA probe for G3PDH generated similarly from rat pTRI-GAPDH (Ambion). The unprotected RNA was digested at 37 °C for 30 min with RNase T1 in RNase digestion buffer. The reaction was stopped by addition of 300 μl of RNase inactivation/precipitation mixture. RNA samples were resuspended in 15 μl of formaldehyde loading buffer, heat denatured for 4 min at 95 °C and electrophoresed on 5% acrylamide/8 M urea denaturing gels. Gels were transferred to filter paper, imaged by autoradiography as described above, and quantitated by laser densitometry.

Sucrose Gradient Fractionation of Polyribosomes—Cells were trypsinized, harvested by centrifugation, and washed twice with ice-cold DPBS containing 50 μg/ml cycloheximide. Cells were then lysed by incubation for 10 min in 1 ml of ice-cold lysis buffer (0.25 mM sucrose, 250 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 100 μg/ml cycloheximide, 0.5% (v/v) Triton X-100, and 0.2 mg/ml heparin (as a ribonuclease inhibitor) in 20 mM HEPES, pH 7.5). The lysate was then microcentrifuged for 10 min at 4 °C and 14,000 rpm. Linear 15–50% (w/w) sucrose gradients (5.5 ml) were prepared using a low speed gradient maker (Amersham Pharmacia Biotech) and pumped into centrifuge tubes (Beckman Ultra-Clear 14 × 80 mm). The non-sucrose component of the gradient solution was the same as the lysis buffer, except that the concentration of KCl and heparin were 500 mM and 0.5 mg/ml, respectively. Aliquots (800 μl) of microcentrifugated supernatant, representing material from approximately 4 × 106 cells, were layered onto the gradient and centrifuged at 40,000 rpm for 110 min at 4 °C in a Beckman Ultracentrifuge using a 40T rotor. The bottom of the tube was punctured with a needle and tubing connected to a peristaltic pump, and gradient fractions of 600 μl were collected into 1.5-ml microcentrifuge tubes containing 3 μl of 20% (w/v) SDS and 50 μl of 4 mM sodium acetate, pH 5.0. RNA in each fraction was extracted by the phenol chloroform protocol (24) and dot-blotted onto nylon membrane as described previously (27). Membranes were then subjected to Northern blotting for c-fos as described above.

Statistical Analysis—Data are expressed as mean values ± S.E. The minimum number of replicates for all measurements was four, unless otherwise indicated. Differences between multiple groups were compared using one-way or two-way analysis of variance. The post hoc test used was the Newman-Keuls multiple comparison test. Two-tailed tests of significance were employed. When data was not found by the Shapiro-Wilk test to be normally distributed, the Wilcoxon rank sum test was employed. Significance was assumed at p < 0.05.

Results—FBS and PDGF promoted rat airway smooth muscle cell growth in a dose-dependent manner, as previously reported (18). PDGF was only about half as potent as FBS. SOD slightly enhanced cell growth in cultures stimulated with PDGF, but catalase at 300 and 3000 units/ml and NAC at concentrations of 10 mM and greater significantly inhibited airway smooth muscle proliferation in response to FBS or PDGF (Fig. 1). Antioxidant treatment of confluent rat tracheal myocyte monolayers did not induce LDL release, cause uptake of trypan blue or produce DNA fragmentation, as studied by ethidium bromide stained agarose gels or by 3′-OH fluorescent end-labeling of DNA fragments (data not shown). This suggests that reactive oxygen species and cellular redox status are important for mitogen-stimulated signal transduction in these cells. Treatment with NAC also caused a significant decrease in FBS-stimulated production of the early response gene product c-fos (Fig. 2). Fos-B was expressed at identical low levels in both growth-arrested and serum-stimulated cultures and was not influenced by antioxidants (data not shown). Antioxidants did not prevent serum stimulation of c-fos mRNA (Fig. 3), which we studied by three different methodologies. Semiquantitative PCR, Northern blotting, and ribonuclease protection assays all consistently confirmed that antioxidants did not block the normal early response increase of myocyte c-fos.

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mRNA from mitogenic stimulation. This suggests that reactive oxygen species and alterations in redox status effected in response to mitogens may also regulate early response genes at a posttranscriptional level. To probe whether antioxidants might affect rates of proteolytic destruction, we stimulated c-Fos expression for 60 min with 10% FBS and then inhibited further translation of mRNA by addition of cycloheximide (25 μg/ml) to cultures. Fig. 4A shows that the rate of elimination of c-Fos was not changed substantially by treatment of cells with NAC, raising the possibility that antioxidants might regulate levels of protein by influencing the degree of message translation. This was confirmed by Northern dot-blot analysis of c-fos mRNA in polysomes size fractionated by linear sucrose gradient centrifugation. Fig. 4B demonstrates that compared with stimulation with FBS alone (rows A and B), pretreatment with 3000 units/ml catalase (rows C and D) or 20 mM NAC (rows E and F) eliminates detection of c-fos mRNA in the heaviest polysomal fractions that actively translate mRNA into protein.

When rat airway smooth muscle monolayers were incubated with ferricytochrome c, there was a small but reproducibly significant increase in absorbance over 60 min after stimulation by FBS; and this increase could be inhibited by addition of SOD to the medium (Fig. 5A). Calculating superoxide (O$_2^-$) generation as the SOD inhibitable difference in A$_{550}$, airway smooth muscle cells generated 4 ± 2 and 21 ± 3 pmol of O$_2^-$/min/μg of cell protein before and after stimulation with 10% FBS, respectively. DPI reduced FBS-stimulated ferricytochrome c reduction by over 60% (Fig. 5B), suggesting that much of the measurable O$_2^-$ originates from a flavoprotein-dependent process. We therefore studied the effect of DPI on rat airway smooth muscle proliferation. The same concentration of DPI that decreased serum-induced ferricytochrome c reduction (10 μM) also reduced FBS-stimulated cell proliferation (Fig. 6A) but was not cytotoxic, as measured by LDH activity in supernatant or trypan blue dye exclusion (data not shown). DPI treatment of rat airway myocytes also reduced expression of c-Fos (Fig. 6B). Nitro-l-arginine did not block serum-induced proliferation (Fig. 7), and allopurinol decreased proliferation to only a minor, albeit statistically significant, degree (Fig. 7) compared with other antioxidant strategies (Fig. 1). This mitigates against either nitric oxide synthase or xanthine oxidase as major flavoprotein-containing enzymatic sources of reactive oxygen species involved in airway myocyte growth. Taken together, these results imply that the origin of reactive oxygen species that are important for mitogen-induced signaling in airways smooth muscle may be a membrane-localized flavoprotein-dependent NADH or NAPDH oxidoreductase. The best studied NADPH oxidoreductase is that responsible for the respiratory burst in neutrophils, where it exists as a combination of two membrane proteins, p22 and the flavoprotein bearing gp91, which together form a complete cytochrome, b558. To determine whether an analogous enzyme is responsible for O$_2^-$ generation by tracheal myocytes, we compared the growth responses of cultured tracheal smooth muscle cells from wild-type and gp91$^{phox}$ null mice to 0.5, 2.5, 5.0, and 10% FBS. Fig. 8 shows that there was no significant difference in serum growth responses of airway smooth muscle from wild-type versus gp91$^{phox}$ knockout mice. Myocytes from wild-type and gp91$^{phox}$ knockout mice also generated equivalent amounts of O$_2^-$ in response to stimulation with 10% FBS (19 ± 4 and 19 ±...
cells against invading organisms, reactive oxygen species are now understood to be major mediators of human disease (28). Initially, the involvement of reactive oxygen species in illness was conceptualized as the chemistry of “scorched earth,” in which critical cell proteins and lipids were indiscriminately oxidized and rendered metabolically inactive for their roles in normal cell function (28). More recently, it has been realized that oxidants can operate as signaling molecules, controlling even gene expression (1). In the airway, treatment of tracheal myocytes with exogenous H$_2$O$_2$ has been shown to activate extracellular extracellular signal-regulated kinases (7) via successive activation of protein kinase C, Raf-1, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (8). This provides a scheme by which oxidant stimulation could directly effect transduction of mitogenic signals to the nucleus. We have extended these observations by showing that antioxidant treatment of tracheal myocytes dramatically reduces cell proliferation in response to mitogenic stimulation with serum or PDGF (Figs. 1 and 9). Antioxidants also inhibited expression of c-Fos (Fig. 2), the product of an early response gene subsequently up-regulated by mitogens in response to cooperative protein kinase C and Ras/Raf stimulation of members of the mitogen-activated protein kinase superfamily of serine-threonine kinases. Finally, serum stimulation of airway smooth muscle cells promoted a small but reproducibly significant increase in O$_2^*$ (Fig. 5) release by myocytes into culture medium. Taken together, these results provide evidence that reactive oxygen species are generated directly in response to mitogenic stimulation of airway smooth muscle and that these reactive molecules may be physiologically important in signaling subsequent events leading to proliferation.

At present, the enzymatic source and chemical identity of the reactive oxygen to species proximate in signaling are not clear. Previous studies have suggested evidence for both O$_2^*$ (2, 20) and H$_2$O$_2$ (3, 4, 6, 14, 15) generation in response to mitogenic stimulation of mesangial cell, adipocytes, fibroblasts and aortic vascular smooth muscle cells. Increased ferricytochrome c reduction (Fig. 5B), serum-induced cell proliferation (Fig. 6A), and c-Fos expression (Fig. 6B) were prevented by pretreatment of cells with DPI. This suggests that mitogen-induced reactive oxygen species in airway smooth muscle cells are from a flavoprotein-dependent source, such as an NADH or NADPH oxidoreductase, as previously reported for other cell types (2, 3, 6). The enhancement of PDGF-stimulated cell proliferation by SOD supports this supposition. Whether NADH or NADPH is an important electron donor for generation of reactive oxidant species important for signaling may be H$_2$O$_2$. The enhancement of PDGF-stimulated cell proliferation by SOD supports this supposition. Whether NADH or NADPH is an important electron donor for generation of reactive oxygen species by airway smooth muscle, whether the oxidase is localized to the cell membrane or cell interior, and whether H$_2$O$_2$ is a primary enzymatic product or is in part formed from dismutation of O$_2^*$ spontaneously or by extracellular SOD, are issues that are currently being investigated.

Knowledge about the structure and function of NADH/ NADPH oxidoreductase comes mostly from studies in neutrophils, where it is responsible for the respiratory burst essential to the microbicidal activity of these cells. The neutrophil oxidase is a multisubunit complex that generates O$_2^*$ in one-electron reduction of O$_2$ using electrons supplied by NADPH (29). The oxidase is expressed at high levels in phagocytes, where O$_2^*$ is the precursor to H$_2$O$_2$ and other reactive oxidants that are used to kill bacteria and fungi. Structurally, the oxidase consists of two membrane proteins, gp91 and p22, that together form a unique cytochrome with a redox midpoint potential of

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**DISCUSSION**

Once regarded as merely waste byproducts of aerobic metabolism or molecules of defense produced by host inflammatory proteins to confer protection against invading organisms, reactive oxygen species are now understood to be major mediators of human disease (28). Initially, the involvement of reactive oxygen species in illness was conceptualized as the chemistry of “scorched earth,” in which critical cell proteins and lipids were indiscriminately oxidized and rendered metabolically inactive for their roles in normal cell function (28). More recently, it has been realized that oxidants can operate as signaling molecules, controlling even gene expression (1). In the airway, treatment of tracheal myocytes with exogenous H$_2$O$_2$ has been shown to activate extracellular extracellular signal-regulated kinases (7) via successive activation of protein kinase C, Raf-1, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (8). This provides a scheme by which oxidant stimulation could directly effect transduction of mitogenic signals to the nucleus. We have extended these observations by showing that antioxidant treatment of tracheal myocytes dramatically reduces cell proliferation in response to mitogenic stimulation with serum or PDGF (Figs. 1 and 9). Antioxidants also inhibited expression of c-Fos (Fig. 2), the product of an early response gene subsequently up-regulated by mitogens in response to cooperative protein kinase C and Ras/Raf stimulation of members of the mitogen-activated protein kinase superfamily of serine-threonine kinases. Finally, serum stimulation of airway smooth muscle cells promoted a small but reproducibly significant increase in O$_2^*$ (Fig. 5) release by myocytes into culture medium. Taken together, these results provide evidence that reactive oxygen species are generated directly in response to mitogenic stimulation of airway smooth muscle and that these reactive molecules may be physiologically important in signaling subsequent events leading to proliferation.

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−245 mV and a reduced minus oxidized difference spectrum of 558. The $O_2^\cdot-$ generating capacity is fully contained within the cytochrome. Based on studies of subjects with chronic granulomatous disease, at least two cytosolic peptides (p47 and p67) are also essential. It is generally believed that these components have to interact with the membrane-bound cytochrome to induce oxidase activity. Several other cytosolic components that appear to participate in the activity of the phagocyte NADPH oxidase have been identified, and include a small G protein (rac-1 or rac-2), rho-GDI, and p40phox (30). In neutrophils, the oxidase is activated by assembly of the cytosolic enzyme complex is present and exerts a variety of functions in the vascular (34, 35) and fibroblast (4) oxidases. Recent immunohistochemical studies have suggested expression of gp91 in vascular smooth muscle cells (32), but this has not been a consistent finding (36). Because the substrate recognition site, the flavin adenine dinucleotide binding site, and the heme binding site are all contained in gp91, it is likely that it is this subunit that determines the unique properties of the enzyme. Therefore, our finding of similar serum-induced growth rates (Fig. 8) and $O_2^\cdot-$ generation for airway smooth muscle in wild-type and gp91phox null mice would suggest distinct structural differences between the $O_2^\cdot-$-generating oxidases of tracheal myocytes and phagocytic cells.

Although the exact enzymatic source of growth promoting reactive oxygen species is unclear, a prominent effect of oxidant-stimulated mitogen-activated kinase cascades (8, 9) is the promotion of growth by rapid induction of early response genes, which include transcription factors that regulate the expression of other genes that are critical for cellular proliferation. The induction of c-fos gene expression is one of the earliest nuclear responses to a wide variety of growth and differentiation factors (37–39). Products of the fos gene family now include c-Fos, Fos-B, Fra-1, and Fra-2. Fos family proteins dimerize with other proteins of the Jun family to form the transcription factor AP-1, which activates transcription of a variety of target genes leading to initiation of DNA synthesis and eventually to mitosis (40, 41). The extent of transcriptional activation or repression conferred upon AP-1 response elements is a function of the particular heterodimers that are formed and the cell type
in which they are expressed (40). Fos proteins can also interact with the c-AMP-responsive element. In fibroblasts, c-Fos and Fos-B mediate cell cycle progression by acting at c-AMP-responsive elements in conjunction with cyclic-AMP responsive element protein/cyclic-AMP responsive element modulator transcription factors to promote cyclin D1 expression (42), and in vascular smooth muscle, c-Fos interacts with a c-AMP-responsive element in the cyclin A promoter to cooperate with the transcription factor E2F in the initiation of cyclin A expression (43). Antisense c-fos RNA inhibits cell proliferation (44) and reverses phenotypic transformation (45). Microinjection of antibodies to c-Fos, Fos-B, or Fra-1 alone only partially blocks cell cycle reentry, but inhibiting all three genes for these proteins together effectively abolishes cell cycle progression (44). In addition, combined c-fos−/−, fosB−/− mice are approximately 50–60% smaller at 6 weeks than wild-type mice, and their fibroblasts have dramatically reduced proliferation that is at least in part from a failure to induce cyclin D1 following serum-stimulated cell cycle reentry (46). Thus, fos gene family products are a whole a pivotal important for cell growth.

There is evidence that fos may play a similarly significant role in airway wall remodeling. Mitogenesis of airway smooth muscle is also associated with early c-fos expression (47–50), and levels of c-Fos are increased in biopsies of asthmatic airways (51, 52). Serum stimulation strongly increased both expression of c-Fos protein and proliferation of airway smooth muscle cells, and both of these events were significantly inhibited by antioxidants. However, despite the previously demonstrated importance of c-Fos in mitogenesis, additional studies will be needed to demonstrate that antioxidant-mediated reductions in c-Fos protein expression and airway smooth muscle proliferation are functionally related. Expression of fos products may also be important in the kinetics of matrix deposition within the remodeling airway wall. Fibroblasts from c-fos−/− mice suffer impaired AP-1-dependent basal and mitogen-stimulated expression of matrix metalloproteinases, including stromelysin and collagenase (53). In the vessel wall, migration of smooth muscle cells into areas of active remodeling is critically dependent upon the activity of metalloproteinases needed to digest away obstructing extracellular matrix proteins (54). Although not yet studied in the airway, matrix metalloproteinases may perform similar functions to allow airway myocytes to migrate into sites of active remodeling.

In other cells, activation of c-fos by H2O2 (55) or hormones such as angiotensin II that stimulate cellular H2O2 production (56) is regulated by both transiently enhanced transcription and stabilization of mRNA. In the present investigation, serum
treatment increased c-fos mRNA levels in airway smooth muscle, and antioxidants did not affect this response (Fig. 3). However, antioxidant treatment markedly reduced levels of c-Fos protein (Fig. 2), suggesting interruption at a posttranscriptional level. Protein half-life appeared substantially unchanged by antioxidant treatment (Fig. 4A), but catalase and NAC reduced incorporation of c-fos mRNA into heavier polyribosomes associated with endoplasmic reticulum (Fig. 4B), in which message is actively translated. This would suggest the existence of a novel redox-sensitive trans-acting mechanism regulating ribosomal translation of c-fos mRNA. Antioxidants have been previously shown to inhibit posttranscriptional expression of macrophage tissue factor (57), and redox-sensitive RNA-binding proteins have been reported to regulate translation of catalase (58), manganese superoxide dismutase (59), erythropoietin (60), thymidylate synthase (61), c-Myc (61), and p53 (61) mRNAs. One potential explanation for redox regulation of c-fos translation would be through an RNA-binding protein similar to iron-responsive element-binding protein 1, with suppression of c-fos translation when the binding protein is attached, but unfettered translation with detachment of the binding protein from message. The RNA binding activity of iron-responsive element-binding proteins is regulated in part at cysteine 437, the oxidation of which reduces iron-responsive element-binding protein 1 binding to the 5'-untranslated region of ferritin mRNA, releasing it from translational blockade (62). The 5'-untranslated region of rat c-fos contains two iron-responsive element loop sequences, CAGUGN (where N is any nucleotide other than G) (62), at bases 45–50 and 87–91, but these are not flanked by complementary bases necessary for stem formation (63). Nevertheless, a similar c-fos-binding protein redox-regulated at a cysteine critical for binding would be compatible with facilitation of ribosomal translation during the prooxidant stress of serum stimulation and inhibition by a reducing environment.

Whereas others have studied airway smooth muscle prolif-
eration in response to individual mitogens, such as histamine (47), endothelin (49), or PDGF (64, 65), we have previously found that endothelin, histamine, and PDGF individually are weak stimuli for cellular proliferation compared with FBS (18). Fetal serum contains a complex mixture of mitogens more reflective of the complex environment to which asthmatic airway smooth muscle is exposed in vivo. The interactions of various mitogens on cellular oxidant generation may be complicated. In fibroblasts, insulin, insulin-like growth factor-I, acidic fibroblast growth factor, and the AA homodimer of PDGF all stimulated NADPH-dependent generation of H$_2$O$_2$, whereas the basic isof orm of fibroblast growth factor and the BB homodimer of PDGF antagonized it (66). Also, as a consequence of submucosal edema and increased submucosal vascular permeability, the asthmatic airway normally contains elevated levels of many serum components (10–13). We therefore chose to perform most of our experiments with serum as the mitogenic stimulus.

Previously, asthma was defined as episodic reversible airways obstruction, but it is now appreciated that patients with chronic severe asthma can suffer irreversible obstruction of airways (67, 68). This complication develops from architectural remodeling of the airway wall, resulting in increased smooth muscle mass (67) from both hyperplasia and hypertrophy (69), remodeling of the airway wall, resulting in increased smooth muscle proliferation (68). This complication develops from architectural remodeling of the airway wall, resulting in increased smooth muscle proliferation (68).

Probuloc treatment dramatically reduced proliferation of airway smooth muscle in response to serum stimulation (Fig. 9). Probucol has been previously shown to blunt the mitogenic effect of H$_2$O$_2$ for vascular smooth muscle (71) and has recently advanced in the treatment of chronic severe asthma.

Acknowledgment—We gratefully acknowledge the technical assistance of Dr. Francois Villinger (Department of Pathology, Winship Cancer Center, Emory University, Atlanta, GA).

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