Nox4 mediates TGF-β1-induced retinoblastoma protein phosphorylation, proliferation, and hypertrophy in human airway smooth muscle cells

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Asthma was originally defined as bronchodilator-reversible airways obstruction, but in a surprising number of patients, the condition is characterized by progressive fixed airways obstruction in severe asthma (51). Several mechanisms contribute to this enlargement, including bronchial smooth muscle hypertrophy (6), hyperplasia (67), and myocyte dedifferentiation with migration through the mucosa toward the luminal epithelium (19). In addition, the enlarged bronchial smooth muscle mass has functional consequences. Smooth muscle encircles airways as two opposing spirals that both constrict the lumen and shorten the bronchial length on contraction (29). Airways thickened by remodeling resist shortening, so that mechanical forces are directed toward constriction (34). This produces a greater reduction in luminal cross section when even normal force is applied (28). Furthermore, velocity of bronchial smooth muscle shortening is greater in asthmatics (38). Thus the abnormal mass of bronchial smooth muscle may also contribute to the increased airways hyperresponsiveness of asthma.

Among the growth factors and cytokines proposed to account for bronchial smooth muscle remodeling in asthma, TGF-β1 is the most intriguing. Elevated levels of TGF-β1 are present in the bronchoalveolar lavage fluid (53) and mucosal biopsies (45, 64) from asthmatics. While expressed by both epithelium (63) and airway smooth muscle itself (16), the likely source of TGF-β1 in asthma is eosinophils infiltrating the airway wall (13, 27). A growth factor with multitudinous potential, TGF-β1 is thought to have dual functions in asthma, simultaneously reducing the inflammatory response (47) while facilitating repair (46) of the injured airway. While it inhibits epithelial proliferation, TGF-β1 stimulates proliferation in cells of mesenchymal origin. In asthma, TGF-β1 can induce both proliferation (8, 15) and hypertrophy (8, 20) of airway smooth muscle cells (SMC) and decrease responsiveness of airway smooth muscle to β-agonist bronchodilators (48) while increasing hyperresponsiveness to contractile agents such as Bradykinin (32). Treatment with TGF-β1-blocking antibodies reduces allergen-induced airway SMC proliferation and remodeling in a murine model of asthma (43). Furthermore, the TGF-β1 promoter polymorphism C-509T, which enhances TGF-β1 gene transcription, is associated with severe asthma (52, 59). Thus TGF-β1 has a pivotal role in the bronchial smooth muscle mass enlargement contributing to asthmatic airway remodeling.

TGF-β1 signals through a transmembrane TGF-β superfamily of serine/threonine receptor kinases (12, 17, 40) that phos-
phorylate a number of Smad transcription factors that translocate to the nucleus, where they mediate TGF-β effects determined by which families of receptors and Smads are transactivated (12). TGF-β1 can also signal through mitogen-activated protein (MAP) kinases or cross talk between Smad 2/3 and MAP kinases (17). However, the distal signaling mechanisms by which TGF-β1/Smad or TGF-β1/MAP kinase signaling induce SMC proliferation and hypertrophy are not known. We (61) have recently reported that TGF-β1 stimulates proliferation of human pulmonary artery SMC (HPASMCC) by a redox-dependent process involving Smad 2/3-dependent but MAP kinase-independent expression of the Nox4 NAD(P)H oxidase with increased production of reactive oxygen species (ROS) and ROS-induced phosphorylation and activation of ERK 1/2.

We now report that Nox4 is also the major NAD(P)H oxidase in human airway SMC (HAWSMC). TGF-β1 stimulates Nox4 expression in these cells and increases ROS production. In turn, Nox4 mediates two complementary and interrelated effects of TGF-β1 on HAWSMC remodeling: 1) TGF-β1-induced, redox-dependent phosphorylation of retinoblastoma protein (pRb) and cdc2 kinase, facilitating HAWSMC proliferation; and 2) TGF-β1-induced phosphorylation of eukaryotic translation initiation factor 4E (eIF-4E) binding protein-1 (4E BP1), promoting HAWSMC hypertrophy.

MATERIALS AND METHODS

Procurement of human bronchial tissue. Tissue collection was approved by the University of Utah Institutional Review Board. Segments of main stem human bronchus from donors without cardiopulmonary disease were collected at the time of thoracic organ procurement usually within 8 h of the declaration of clinical brain death. At the time of organ procurement, hearts of donors were still beating, and their major organs were adequately perfused to maintain viability required for subsequent transplantation. On acquisition, bronchial segments were placed in ice-cold normal saline and transported to the laboratory where they were dissected and digested for cell culture.

Cell culture. HAWSMC were obtained by collagenase/elastase (Roche Biochemicals, Indianapolis, IN) digestion of donor bronchial rings. Briefly, the adventitia was physically removed and the cleaned bronchus was incubated in collagenase (1 mg/ml) for 20 min at 37°C, after which the epithelial and submucosal layers were removed by scraping with the blunt edge of a scalpel. The smooth muscle tissue was dissected away from the cartilaginous bronchial rings, minced in a solution containing collagenase A (2 mg/ml) and elastase Grade II (0.5 mg/ml), and incubated at 37°C in a shaking water bath for 1 h. The digest was vigorously pipetted several times to release the cells and incubated for an additional hour. Undigested tissue was removed by straining through sterile gauze. The cells were centrifuged at 1,000 g for 5 min, and the pellet was washed with sterile phosphate-buffered saline (PBS) and then resuspended in SMC growth medium (SMGM: Cascade Biologicals, Portland, OR) and plated on gelatin-coated dishes unless stated otherwise. The initial culture is referred to as the low-passage HAWSMC was obtained using the Amaxa Nucleofector (Amaxa, Basle, Switzerland). High transfection efficiency (>90%) of low-passage HAWSMC was obtained using the Amaxa Nucleofector system (Gaithersburg, MD), which allows nonviral gene transfer directly into the nucleus using proprietary Nucleofector solutions. We employed Nucleofector solution MC 59 optimized for primary mammalian SMC from various organs and the electrical parameters supplied by program A-33. Adenoviral gene transfer was used to express the dominant negative (dn) Rac1 protein (Ad-N17Rac1; a generous gift from Dr. Beata Wojciak-Strojnicki, Harvard College School of Medicine, London) in HAWSMC to determine the role of the small GTPase Rac1 in Nox4-dependent ROS production. Ad-N17Rac1 at multiplicity of infections (1:1,000 or 1:2,000) was added to HAWSMC. Experiments were evaluated 16–18 h after infection.

Confirmation of Rac1 inhibition by dnN17Rac1. Rac1 inhibition in HAWSMC by Ad-N17Rac1 infection was confirmed using a commercial Rac activation assay kit (Cell Biolabs, San Diego, CA). Rac activity was determined in HAWSMC with and without Rac activation by PMA (100 ng/ml for 10 min). In its active state (GTP-bound), Rac binds specifically to the p21-binding domain (PBD) of p21-activated protein kinase (PAK1) to control downstream signaling.
Fig. 1. Nox4 is the major Nox homolog present in human airway smooth muscle cells (HAWSMC) and induced by TGF-β1. A: TGF-β1 induces transcripts for Nox4 but no other NAD(P)H oxidase component. HAWSMC [passage 4 (p4)] were grown to 80% confluence with and without TGF-β1 (1 ng/ml) or fresh SMGM (growth), and the cells were incubated for 24 h. Real-time quantitative PCR of transcripts for the components of NAD(P)H oxidase and the Nox homologs of gp91phox (Nox2) were performed using the appropriate specific primers on 100 ng of cDNA transcribed from total RNA. The results from 1 set of 4 simultaneously studied, TGF-β1-stimulated cell cultures are shown, expressed as relative transcript levels normalized to GAPDH. This experiment was repeated 3 times. Treatment of HAWSMC with TGF-β1 substantially induced Nox4 but did not markedly affect constitutively high levels of p22phox or low transcript levels of p47phox and p67phox, the cytosolic components of the classical phagocyte NAD(P)H oxidase. B: Nox4 protein is present in primary cultures of HAWSMC but is progressively lost with passage in tissue culture. First passage HAWSMC (~10 days after establishment of culture) showed strong staining for Nox4 by Western blot (lane 1) compared with later passage HAWSMC (lane 2, p6). Immunoblots typically showed a doublet, with the major band at 65 kDa, the expected size for Nox4. Cells were grown in SMGM until 80% confluent, washed with PBS, and directly lysed in RIPA buffer plus a protease inhibitor cocktail. Immunoblots were performed using IgG-purified anti-rabbit Nox4 peptide antibody at a dilution of 1:1,000. Each lane was loaded with 20 μg of total cell lysate as determined by bicinchoninic acid (BCA) protein assay. After staining with Nox4 antibody, immunoblots were stripped and reincubated with antibody against β-actin (1:500) as a loading control. Results shown are representative of experiments from 3 donors. C: TGF-β1 induces expression of Nox4 protein in HAWSMC. HAWSMC (p4) at 80% confluence were grown in DMEM 0.2% FCS with and without TGF-β1 (1 ng/ml) for the indicated number of days. At each time point, the cells were washed with PBS, lysed, and immunoblotted as above. In these duplicate experiments, Nox4 and α-smooth muscle actin (act5a) showed induction by day 7 after treatment with TGF-β1. Immunoblots were stripped and reincubated with antibody against α-smooth muscle actin (act5a) as a loading control. Nox4 protein was also induced by TGF-β1 in 5 additional experiments.
Table 1. Nox and Duox transcript levels in TGF-β1-treated HAWSMC

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nox1</th>
<th>Nox2</th>
<th>Nox4</th>
<th>Nox5</th>
<th>Duox1</th>
<th>Duox2</th>
</tr>
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<tbody>
<tr>
<td>SMGM Absent</td>
<td>22</td>
<td>107±0.4</td>
<td>20×0.5</td>
<td>1×0.5</td>
<td>1×0.2</td>
<td></td>
</tr>
<tr>
<td>TGF-β1 Absent</td>
<td>3±1.8</td>
<td>30,644±0.1</td>
<td>8×0.9</td>
<td>3×0.7</td>
<td>6×0.3</td>
<td></td>
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Human airway smooth muscle cells (HAWSMC; passage 4) were grown to 80% confluence in smooth muscle cell growth medium (SMGM). The medium was changed to DMEM 0.2% FCS with TGF-β1 (1 ng/ml) or fresh SMGM, and the cells were incubated for 24 h. Real-time quantitative PCR of transcripts for the components of NAD(P)H oxidase and the Nox homologs of gp91phox (Nox2) were performed using Applied Biosystems primers (see MATERIALS and METHODS) on 100 ng of cDNA transcribed from total RNA. Results are expressed as Nox4 transcript levels normalized to GAPDH. These results are from a separate experiment than that displayed in Fig. 1A.

treated HAWSMC were negative for Nox2, Duox1, and Duox2 transcripts and had low levels of Nox2 and Nox5, with a Nox4 level fivefold greater (Table 1). In one of many experiments, TGF-β1 induced Nox4 up to 300-fold in HAWSMC (Table 1). This level of induction by TGF-β1 did not occur with any other homolog and is similar to the level of Nox4 transcript induction we (61) previously observed in HPASMC stimulated with TGF-β1. The low levels of Nox2 and Nox5 measured in untreated cells were reduced after addition of TGF-β1. There was no detectable Nox1 in either quiescent HAWSMC or after stimulation with TGF-β1 (Table 1).

In HAWSMC at first passage (~10 days after establishment of culture), Nox4 protein was easily demonstrated on immunoblots as a doublet around 65 kDa (Fig. 1B, lane 1). This staining was progressively lost in culture during later passages (Fig. 1B, lane 2, passage 6). Treatment of HAWSMC (Cambrex; passage 6) with TGF-β1 (1 ng/ml DMEM 0.2% FCS) promoted reexpression of Nox4 within 7 days (Fig. 1C). We (61) observed a similar but not identical pattern of Nox4 expression in HPASMC with loss of protein expression during passage in tissue culture but more prompt return after stimulation with TGF-β1. Nox4 in HAWSMC was consistently identified as a doublet on the immunoblot with the affinity-purified peptide antibody we (61) developed or a commercial anti-Nox4 antibody (Santa Cruz Biotechnology). This doublet may reflect the translation of splice variants (23) or posttranslational modifications. Protein levels of p22phox were not affected by TGF-β1 treatment, and p47phox and p67phox were not detected by immunoblot under any condition (data not shown).

**Induction of Nox4 in HAWSMC is specific for isoforms of TGF-β and is not reproduced by other TGF-β superfamily members or growth factors.** Having ascertained that the effect of TGF-β1 on NAD(P)H oxidase in HAWSMC was confined to Nox4, we asked whether other members of the TGF-β superfamily or other known smooth muscle growth factors resulted in a similar Nox4 induction in this human cell type. Besides TGF-β, only activin A induced Nox4 transcript levels in HAWSMC (Fig. 2A). Although the increase in Nox4 transcripts after activin A was considerable, the level of induction was ~5× less than that from TGF-β1 and required a 50× greater concentration of agonist. Other TGF-β isoforms also induced Nox4 transcripts, with TGF-β3 proving significantly more active than either TGF-β1 or TGF-β2 (Fig. 2B). These results imply that, in HAWSMC, Nox4 induction is specific to isoforms of TGF-β signaling through Smad 2/3 (12, 40).

Nox4 induction by TGF-β1 is influenced by Smad3 and phosphatidylinositol 3-kinase (PI 3-K) signaling cascades. In HPASMC, we (61) have shown that the TGF-β1-induced increase in Nox4 transcripts is inhibited by transfection of expression plasmids containing constructs for dominant negative Smads 2 and 3. In HAWSMC, the TGF-β1-induced increase in Nox4 transcripts was inhibited by transfection of siRNA against Smad3 but not Smad2 (Fig. 2C). The Smad3-dependent nature of Nox4 regulation was confirmed by inhibition of TGF-β1 induction of Nox4 transcripts (Fig. 2D) with the specific inhibitor of Smad3 (Calbiochem), which has no activity against Smad2 (30). The type I TGF-β receptor binds to and activates PI 3-K (69). Therefore, we studied the effect of the specific PI 3-K inhibitor LY-294002 on Nox4 induction. LY-294002 significantly inhibited the TGF-β1-induced increase in Nox4 transcripts (Fig. 2E), suggesting that Nox4 may be regulated by cooperative cross talk between TGF-β1-activated Smad and PI 3-K signaling, similar to that previously demonstrated for molecular events important in TGF-β1-induced epithelial to mesenchymal transformation (4).

**TGF-β1 induces Nox4 expression that is localized to the perinuclear and nuclear regions of HAWSMC.** Nox4 has been immunohistochemically and functionally localized to the nucleus of human umbilical vein endothelial cells (HUVEC), where it plays a role in redox regulation of gene expression (33), and to perinuclear and nuclear regions of HPASMC (61). We determined Nox4 localization in HAWSMC using confocal microscopy. Compared with untreated but proliferating cultures (Fig. 3A, i), TGF-β1 treatment of HAWSMC induced strong expression of Nox4, detectable by either our affinity-purified peptide antibody (Fig. 3A, ii) or a different Nox4 antibody (gift from Dr. David Lambeth, Emory University) generated against amino acids 320–428 of Nox4 (Fig. 3A, iv). Similar to our (61) findings in HPASMC, Nox4 (green fluorescence) induced by TGF-β1 in HAWSMC localized to the ER (red fluorescence; Nox4 colocalization with red ER stain as yellow fluorescence in Fig. 3B, i) as well as perinuclear and nuclear regions of the cell but not to mitochondria (Fig. 3B, ii), oxsm (Fig. 3B, iii), or vinculin (Fig. 3B, iv). This is consistent with the possibility that Nox4 plays a role in transcription and protein synthesis.

**TGF-β1 induces redox-dependent proliferation of HAWSMC.** Perinuclear and nuclear localization and its induction by TGF-β1 suggested a potential role for Nox4 in TGF-β1-induced SMC proliferation (60). To explore this possibility, we first added TGF-β1 (1 ng/ml) to HAWSMC grown in DMEM 0.2% FCS. TGF-β1 induced increases in 1) the rate of BrdU incorporation (Fig. 4A), 2) cell proliferation as determined by the metabolic reduction of MTT to insoluble formazan (Fig. 4B), and 3) cell number compared with quiescent controls (Fig. 4C). TGF-β1 did not enhance the rate of cell proliferation when added to HAWSMC cultured with SMGM (Fig. 4D).

Next, we determined whether TGF-β1-induced HAWSMC proliferation was, like that stimulated by serum (9), inhibited by antioxidants. TGF-β1-induced HAWSMC proliferation was redox-dependent and inhibited when catalase was added to extracellular medium (Fig. 4E, proliferation determined by MTT reduction; Fig. 4F, proliferation determined by cell count).

**Nox4 induction increases ROS production by HAWSMC.** We then studied whether TGF-β1 induction of Nox4 is accompa-
ned by increased cellular production of ROS. As shown in Fig. 5A, TGF-β1 treatment (1 ng/ml) significantly increases intracellular ROS production. Both basal and TGF-β1-stimulated ROS production are significantly inhibited by pretreatment of cultures with the NAD(P)H oxidase flavoprotein inhibitor DPI (10 μM), further supporting the involvement of Nox4 flavoprotein as the source of TGF-β1-stimulated ROS in these cells.

Fig. 2. Induction of Nox4 expression in HAWSMC is specific for isoforms of TGF-β and involves both Smad3 and phosphatidylinositol 3-kinase (PI 3-K) signaling. A: Nox4 is induced by TGF-β1 and to a lesser extent by activin A but not by other TGF-β superfamily members or growth factors. HAWSMC (p4) at 80% confluence were removed from SMGM and incubated with DMEM 0.2% FCS for 24 h. The cells were then treated with members of the TGF-β superfamily, namely TGF-β1 (1 ng/ml), activin A (50 ng/ml), bone morphogenic protein-7 (BMP-7; 50 ng/ml), and BMP-2 (50 ng/ml), for a further 24 h. In addition, HAWSMC were treated with smooth muscle cell (SMC) growth factors PDGF-BB (10 ng/ml), PDGF-AB (10 ng/ml), EGF (25 ng/ml), and thrombin (2 U/ml). Nox4 transcript level was measured by real-time quantitative PCR. Results are illustrative of 3 experiments with TGF-β superfamily members and 2 experiments with growth factors. B: all 3 isoforms induced Nox4 as determined by real-time quantitative PCR. HAWSMC (p4) at 80% confluence were removed from SMGM and incubated with DMEM 0.2% FCS for 24 h. The cells were then treated with TGF-β isoforms 1, 2, or 3 at 1 and 5 ng/ml for 24 h. The induction of Nox4 transcripts with TGF-β3 was significantly greater (*P < 0.001) at 1 ng/ml compared with TGF-β1 and TGF-β2 at 1 ng/ml. There was no difference in the degree of induction with all TGF-β isoforms between 1 and 5 ng/ml. Results represent the means of 4 replicate experiments. C: TGF-β1 induction of Nox4 is reduced by silencing Smad3. HAWMSC (p6) were transfected using an Amaxa system with 100 nM of target SmartPool siSmad2, siSmad3, or scrambled (siScr) control small interfering RNA (siRNA) and incubated overnight in full growth medium. The medium was changed to DMEM 0.2% FCS with and without TGF-β1 (1 ng/ml), and cells were incubated for a further 24 h. Induction of Nox4 transcripts as determined by real-time quantitative PCR was inhibited by siSmad3 but not by siSmad2 or siScr control. Results represent the means of 4 replicate experiments. *P < 0.001 for siSmad3 + TGF-β1 vs. siScr + TGF-β1. D: TGF-β1 induction of Nox4 is reduced by inhibiting Smad3. Confirmation that the Smad3 pathway is essential for TGF-β1 induction of Nox4 in HAWSMC was obtained using the specific inhibitor of Smad3 (SLS3), which has no activity against Smad2, p38 MAP kinase, ERK 1/2, or PI 3-K signaling. Real-time quantitative PCR demonstrated that SIS3 at 1 μM inhibited TGF-β1-induced Nox4 transcripts by 50%, whereas 3 μM SIS3 was sufficient to inhibit by >90%. Results represent the means of 4 replicate experiments. *P < 0.001 for 1 μM SIS3 + TGF-β1 and 3 μM SIS3 + TGF-β1 vs. TGF-β1 alone. E: TGF-β1 induction of Nox4 transcripts in HAWSMC was also reduced by the PI 3-K inhibitor LY-294002. HAWSMC (p6) at 80% confluence were removed from SMGM and incubated with DMEM 0.2% FCS for 24 h. Cells were then treated with TGF-β1 (1 ng/ml) with and without LY-294002 (10 μM) and incubated for 6 and 24 h. The PI 3-K inhibitor prevented TGF-β1 induction of Nox4 transcripts by >90% as measured by real-time quantitative PCR. Results represent the means of 4 replicate experiments. *P < 0.001 for TGF-β1 + LY-294002 at 6 and 12 h vs. TGF-β1 alone at 6 and 12 h.
To determine any role for cytosolic components of the classical phagocyte NAD(P)H oxidase in regulating ROS production by the HAWSMC Nox4 oxidase, we treated cultures with PMA, which induces the NAD(P)H-dependent oxidative burst in leukocytes through phosphorylation of p47phox and subsequent assembly of cytosolic p47phox and p67phox onto the p22phox membrane component to initiate and enhance electron flow from NAD(P)H (36). PMA failed to stimulate ROS production in either quiescent or TGF-β1-treated HAWSMC (Fig. 5B). In contrast, the amphibole AA stimulated ROS production in both quiescent and TGF-β1-treated HAWSMC (Fig. 5B), and AA-enhanced ROS production in TGF-β1-treated cells was significantly inhibited by DPI. We (61) observed a similar lack of response to PMA but stimulation of ROS production by AA in HPASMC.

Activation of the phagocytic NAD(P)H oxidase also depends on the small GTPase Rac (36). To explore a role for Rac in Nox4 activation, we studied cellular proliferation after infection with the adenoviral dnN17Rac1, which blocks Rac activation in HAWSMC (Fig. 5C). dnN17Rac1 had no effect on TGF-β1-induced HAWSMC proliferation measured by BrdU incorporation by HAWSMC (Fig. 5D). These results are identical to our (61) findings in HPASMC and suggest that Rac is also not essential for HAWSMC Nox4 regulation.

**TGF-β1 induces HAWSMC proliferation by a Nox4-dependent pathway.** The redox-dependent nature of TGF-β1-induced HAWSMC proliferation suggested that it might also be Nox4-dependent. To study this possibility, we inhibited Nox4 expression in HAWSMC using SmartPool Nox4 siRNA (siNox4). siNox4 totally inhibited the low Nox4 transcript levels in quiescent cultures, reduced TGF-β1 induction of Nox4 transcripts by ~90% compared with scrambled siRNA (siScr) controls (Fig. 6A), and reduced TGF-β1-induced expression of Nox4 protein (Fig. 6B, lane 4 vs. lane 2). There was no difference in lactate dehydrogenase (LDH) activity of medium treated with siScr vs. siNox4 (Fig. 6C), indicating that neither siNox4 nor the Amaxa electroporation were toxic. However, siNox4 completely prevented TGF-β1 stimulation of HAWSMC proliferation (Fig. 6D), a result identical to our (61) previous findings in HPASMC. Interestingly, LDH activity in the medium was significantly reduced in cells treated with TGF-β1, consistent with our observation that addition of TGF-β1 to HAWSMC cultured in DMEM 0.2% FCS is sufficient to stimulate cell growth and hypertrophy with no toxicity for at least 6 days compared with progressive cell toxicity in DMEM 0.2% FCS alone after 48 h (data not shown).

The transcriptional activator Myc is important for TGF-β1 induction of Nox4. When TGF-β1 inhibits growth, it rapidly downregulates expression of the proto-oncogene c-myc, with precipitous fall in levels of its protein product, the early-response transcription factor Myc (58). In contrast, Myc levels increase as early as day 1 in HAWSMC stimulated to proliferate by TGF-β1 (1 ng/ml in DMEM 0.2% FCS) and remain...

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*Fig. 3.* Nox4 induced by TGF-β1 is localized to perinuclear regions of the cell. A: confocal microscopy confirmed that TGF-β1 increased Nox4 protein in HAWSMC (p4). Cells were grown on Thermofax coverslips and fixed with 4% freshly prepared paraformaldehyde. Nox4 was localized by staining with primary antibody (Ab) and identified by secondary Alexa Fluor 488-conjugated secondary antibodies, which emit green fluorescence. A, i: proliferating HAWSMC in SMGM showed low levels of diffuse Nox4 staining with affinity-purified Nox4 antibody (1:200 dilution). A, ii: HAWSMC grown for 7 days in DMEM 0.2% FCS + 1 ng/ml TGF-β1 showed greatly increased Nox4 staining with affinity-purified Nox4 peptide antibody (1:200 dilution). Staining appears more intense around the nucleus. A, iii: HAWSMC were treated to the same conditions as in A, ii, except the antibody was preincubated with blocking peptide (10 μg/ml) for 1 h before it was added to HAWSMC. A, iv: HAWSMC grown for 7 days in DMEM 0.2% FCS + 1 ng/ml TGF-β1 showed greatly increased Nox4 staining with Nox4 antibody raised against amino acids (AA) 320–428 (1:200 dilution). We observed a similar staining pattern as that obtained with the peptide antibody. Magnification is ×10. B: Nox4 localizes within the endoplasmic reticulum (ER) of HAWSMC (p5). Affinity-purified Nox4 peptide antibody was identified by Alexa Fluor 488-conjugated secondary antibodies, which fluoresce green. Mitochondria, osma, and vinculin were localized with mouse monoclonal antibodies and identified with Alexa Fluor 568-conjugated secondary antibodies, which emit red fluorescence. ER was localized using a proprietary stain, antibodies and identified with Alexa Fluor 568-conjugated secondary antibodies, which fluoresce green. Magnification is ×60.
Nox4 mediates TGF-β1-stimulated retinoblastoma protein phosphorylation

Elevated through day 6 of culture (Fig. 7A). Treatment of HAWSMC with (Z,E)-5-(4-ethylbenzylidine)-2-thioxothiazolidin-4-one (Calbiochem), an inhibitor of Myc-Max interaction (70), significantly inhibits TGF-β1 induction of Nox4 transcripts (Fig. 7B). In contrast, neither TGF-β1 nor inhibition of Myc has any effect on levels of p22phox transcripts (Fig. 7C). This suggests that TGF-β1-induced Nox4 expression is partially under the control of transcriptional activation provided by the binding of Myc-Max heterodimers to the E-box CACAGTG consensus sequence (2).

Nox4 influences redox-mediated cell cycle regulation. It is noteworthy that within a single culture, Nox4 was present in the nuclei and/or perinuclear region of some cells but absent in others (Fig. 3B, iv). We therefore hypothesized that this might imply Nox4 influences regulation of the cell cycle. In addition to decreasing expression of c-myc, TGF-β1-induced growth inhibition is also dependent on sustaining pRb in the hypophosphorylated state, facilitating its complexation to members of the E2F transcription factor family, thereby repressing transcription (41). In contrast, TGF-β1 treatment of HAWSMC, which induces proliferation (Fig. 4, A–C), increases phosphorylation of pRb (a regulator of G1 to S transition) at both Ser807/811 and Ser780 (Fig. 8A, lane 2) and also increases phosphorylation of cdc2 kinase (with cyclin B, a regulator of G2 to M transition) at Tyr15 (Fig. 8A, lane 2). Silencing of Nox4 by transfection of siNox4 decreased baseline phosphorylation of both pRb and cdc2 (Fig. 8A, lane 3) and prevented an increase in pRb and cdc2 phosphorylation in HAWSMC from stimulation with TGF-β1 (Fig. 8A, lane 4). Levels of pRb were unaffected by either TGF-β1 or silencing of Nox4, and equal loading of the immunoblot was confirmed by equal staining with unstimulated controls. *P < 0.05 vs. quiescent cells; †P < 0.05 vs. quiescent cells treated with TGF-β1 alone; ‡P < 0.01 vs. cells treated with TGF-β1 alone. Results represent the means of 3 experiments.

Fig. 4. TGF-β1 induces redox-dependent proliferation of quiescent HAWSMC. Proliferation of HAWSMC (p4) was determined using 3 methods. A: the rate of incorporation of bromodeoxyuridine (BrdU) into quiescent (DMEM 0.2% FCS) was significantly increased on addition of TGF-β1 for 15 h compared with unstimulated controls. *P < 0.05 vs. quiescent cells. B: stimulation of HAWSMC in DMEM 0.2% FCS with TGF-β1 (0.5–10 ng/ml) for 72 h significantly increased cell proliferation compared with untreated cells as determined by the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan (see MATERIALS AND METHODS). †P < 0.01 vs. quiescent cells. C: stimulation of HAWSMC in DMEM 0.2% FCS with TGF-β1 (1–5 ng/ml) significantly increased cell number compared with unstimulated controls. *P < 0.05 vs. quiescent cells. D: TGF-β1 (0.1–5 ng/ml) did not increase cell proliferation (MTT assay) when added to HAWSMC grown in DMEM with 10% FCS (Growth). E and F: TGF-β1-induced HAWSMC proliferation is significantly inhibited by antioxidant treatment with catalase (Cat). HAWSMC were preincubated with 100 or 500 U/ml catalase added to media (DMEM 0.2% FCS) 30 min before stimulation with TGF-β1 (1 ng/ml). Proliferation was determined after 72 h by MTT reduction (E) and by cell count (F). †P < 0.01 vs. quiescent cells; ‡P < 0.05 vs. cells treated with TGF-β1 alone; ‡P < 0.01 vs. cells treated with TGF-β1 alone. Results represent the means of 3 experiments.
Transfection of siNox4, but not siScr, inhibited TGF-β1-induced hypertrophy (Fig. 9B).

TGF-β1 has been recently reported to promote HAWSMC hypertrophy by stimulating phosphorylation of 4E BP1 (20). This translational repressor binds tightly to eIF-4E but releases on phosphorylation, allowing eIF-4E to form an active complex with eIF-4G and initiate mRNA translation, thereby increasing cellular content of protein. To determine if 4E BP1 phosphorylation is redox sensitive, we transfected HAWSMC with either siNox4 or siScr and treated cultures with TGF-β1. TGF-β1 prominently stimulated 4E BP1 phosphorylation at 24 h (Fig. 9C, lanes 7 and 8 with TGF-β1 compared with lanes 5 and 6 without). TGF-β1-induced 4E BP1 phosphorylation was significantly inhibited by siNox4 (Fig. 9C, lanes 3 and 4 with siNox4 compared with lanes 7 and 8 with siScr). TGF-β1-induced 4E BP1 phosphorylation in HAWSMC was also reduced by treatment with antioxidants and NAD(P)H oxidase inhibitors (data not shown). This suggests that the remodeling effects of TGF-β1 on HAWSMC to stimulate both proliferation and hypertrophy are interrelated and mediated in part by induction of Nox4, with redox-signaled increases in both cell number and size.

**DISCUSSION**

We hypothesized that TGF-β1, acting through the Nox4 NAD(P)H oxidase, plays a major role in asthmatic airway remodeling. The major goals of this study were to determine the effects of TGF-β1 on cultured HAWSMC and identify important components the NAD(P)H oxidase of this cell. We have shown that Nox4 is the major Nox membrane homolog in HAWSMC (Fig. 1A and Table 1) and that it is present in freshly cultured cells but progressively lost with each passage (Fig. 1B). Nox4 transcript expression is substantially induced (Fig. 1B and Table 1) and that it is present in freshly cultured cells but progressively lost with each passage (Table 1 and Fig. 1A). Moreover, TGF-β1 significantly stimulated the rate of incorporation of BrdU into quiescent HAWSMC. This increase was not affected by dominant negative N17Rac1 adenoviral infection at a MOI of 1:1,000 or 1:2,000 (shaded bars). Virus alone did not significantly alter the rate of BrdU incorporation compared with quiescent controls (open bars). *P < 0.01 vs. quiescent cells. Results represent the means of 4 experiments for ROS production and 2 experiments for dnN17Rac1.
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for facilitating the Nox4-dependent phosphorylation of pRb and cdc2 (Fig. 8A) that we observed in TGF-β1-stimulated HAWSMC. This is the first report to suggest that Nox4 may play a functional role influencing cell cycle transition through G1/S (phosphorylation of pRb) and G2/M (phosphorylation of cdc2).

TGF-β1 promotes SMC proliferation by two additive mechanisms: 1) autocrine secretion of PDGF (accounting for 10–40% of TGF-β1-induced growth); and 2) a second mechanism enhancing proliferative responses to other growth factors (60). Recently, we (61) suggested an explanation for this second mechanism: TGF-β1 stimulates phosphorylation of Nox4 by a redox-dependent process dependent on expression of the Nox4 NAD(P)H oxidase with increased production of ROS and ROS-induced phosphorylation and activation of ERK 1/2. Nox4 was also the primary active Nox homolog in HPASMC. As in HAWSMC, Nox4 expression was lost during HPASMC culture but reversed by TGF-β1 (61). These (61) and our present findings in HAWSMC suggest that the major signaling Nox oxidase in the lung may be Nox4.

We (61) have previously shown in HPASMC that TGF-β1 induces Nox4 expression by Smad 2/3-dependent but MAP kinase-independent signaling. In HAWSMC, transfection of siRNA against Smad3 but not Smad2 (Fig. 2C) and inhibition of Smad3 with the specific inhibitor of Smad3 (SIS3) (Fig. 2D) both inhibited TGF-β1 induction of Nox4, indicating that Smad3 is the predominant Smad involved in Nox4 regulation by TGF-β1. In addition, the PI 3-K inhibitor LY-294002 also significantly reduced TGF-β1 induction of Nox4 (Fig. 2E). This suggests the possibility that PI 3-K may also play a role in TGF-β1-stimulated gene regulation (4, 69) of Nox4.

A number of growth factors stimulate HAWSMC proliferation by inducing intracellular production of ROS (9, 49, 50). Proliferation from growth factor-induced ROS (7, 57) is mediated in part through transient oxidative inactivation of signaling pathway phosphatases (44), augmenting tyrosine and serine-threonine kinase signaling cascades. This sequence of events might explain TGF-β1-induced pRb phosphorylation. pRb, along with p107 and p130, are pocket proteins that function in binding members of the E2F transcription factor family, thereby inhibiting E2F-mediated transactivation, and repressing E2F-mediated transcription and G1/S cell cycle progression (14). During the cell cycle, pRb is minimally phosphorylated in Go, increasingly phosphorylated through G1, and maintained in a hyperphosphorylated state until late mitosis. Whereas hypophosphorylated pRb binds and inhibits the E2F transcriptional domain, pRb phosphorylation releases E2F to allow gene expression mediating entry into S phase. pRb is phosphorylated by D cyclins acting in concert with cdk4 or cdk6 kinases in early and mid-G1 and by E cyclins acting with cdk2 kinase in late G1 with maintenance of pRb in the phosphorylated state by cyclin A- and cyclin B-cdk complexes during S and G2 (14). pRb is subsequently reactivated by dephosphorylation at the end of mitosis by proteinase serine-threonine phosphate-1 (PP1) and to a lesser extent by protein serine-threonine phosphatase-2A (PP2A) (56, 68). Both PP1 and PP2A are sensitive to inhibition by reversible oxidation of cysteine residues and/or metal ions essential for activity of the enzyme (26, 31, 37). Thus an increase in ROS produced by TGF-β1-stimulated induction of Nox4 expression might serve to tip the delicate balance between cdk-mediated phosphorylation and dephosphorylation by reversibly inhibiting PP1 and...

Fig. 6. Nox4 is essential for TGF-β1 stimulation of HAWSMC proliferation. HAWSMC (p4) were transfected using an Amaxa system with 100 nM Smart-Pool siNox4 or scrambled (siScr) control siRNA and incubated overnight in SMGM. The medium was changed to DMEM 0.2% FCS with and without TGF-β1 (1 ng/ml), and the cells were incubated for a further 24 h. A: Nox4 transcript levels were determined by real-time quantitative PCR. Induction of Nox4 by TGF-β1 was reduced by >90% with siNox4 (100 nM). Results are representative of 2 experiments. B: siNox4 treatment inhibited TGF-β1-induced Nox4 protein expression determined by immunobots. After staining with affinity-purified Nox4 peptide antibody, immunobots were stripped and reincubated with antibody against ERK 1/2 as a loading control. C: siNox4 treatment was not toxic, as determined by the release of lactate dehydrogenase (LDH). In addition, TGF-β1 (1 ng/ml) treatment for 24 h resulted in a significant reduction of extracellular LDH, consistent with it being protective for quiescent HAWSMC. Results represent the means of 3 experiments. *P < 0.005 vs. siScr; † P < 0.005 vs. siNox4. D: siNox4 reduced TGF-β1-stimulated proliferation as determined by MTT reduction. siNox4 at 50 and 100 nM were equally effective at inhibiting TGF-β1-induced growth. Results represent the means of 3 experiments. * P < 0.01 vs. siScr + TGF-β1.

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BP2A, phosphatases important for maintaining pRb in the hypophosphorylated state. This explanation for Nox4-dependent pRb phosphorylation in TGF-β1-treated HAWSMC (Fig. 8A) is supported by the ability of the H2O2 scavenger catalase to prevent TGF-β1-stimulated pRb phosphorylation (Fig. 8B).

HAWSMC hypertrophy in response to TGF-β1 was also a Nox4-mediated, redox-sensitive event (Fig. 9). TGF-β1-stimulated significant HAWSMC hypertrophy by day 6 (Fig. 9A) that was prevented by siRNA gene silencing of Nox4 (Fig. 9B). Furthermore, transfection of siNox4 significantly reduced TGF-β1-induced Thr37/46 phosphorylation (Fig. 9C) of 4E-BP1, an event necessary for SMC hypertrophy (20).

In vascular SMC, this process is stimulated by growth factors through ROS-mediated 4E-BP1 phosphorylation at Ser65 (54), possibly by way of oxidant inactivation (26, 31, 37) of PP2A, the phosphatase regulating this site (18). In addition, phosphorylation of cdc2 at Tyr15, which inhibits cdc2/cyclin B kinase activity, delays mitosis, and prolongs S phase, is regulated by cdc25B (35), a tyrosine phosphatase that can be oxidatively inactivated at its catalytic cysteine (11). Induction of hypertrophy by TGF-β1 might also be facilitated by Nox4-mediated pRb phosphorylation, the maintenance of which also contributes to the prolongation of S phase, allowing for the protein synthesis required for cellular enlargement (14). In addition, proliferation itself requires protein synthesis, making it dif-

Fig. 7. The transcriptional activator Myc is essential for TGF-β1 induction of Nox4. A: TGF-β1 (1 ng/ml) increases levels of Myc protein product of the proto-oncogene c-myc in quiescent HAWSMC (DMEM 0.2% FCS). Increased Myc protein was observed as early as day 1 and was maintained through 6 days. Immunoblots were performed with Myc antibody (1:500), stripped, and then reprobed with antibody against β-actin as a loading control. Results are representative of 2 experiments. Prolif, proliferating cells stimulated with 10% FCS. B: TGF-β1 induction of Nox4 transcripts is decreased by (Z,E)-5-(4-ethylbenzylidine)-2-thioxothiazolidin-4-one, an inhibitor of the interaction of Myc with its heterodimeric partner protein, Max. Quiescent (DMEM 0.2% FCS) cells were treated with TGF-β1 (1 ng/ml) in the presence of inhibitor. Cells were incubated for 24 h, real-time quantitative PCR was performed, and Nox4 transcript levels were normalized to GAPDH. Results represent the means of 4 experiments. *P < 0.05 vs. quiescent cells; †P < 0.01 vs. TGF-β1 alone. C: levels of p22(phox) transcripts normalized to GAPDH and real-time quantitative PCR are not affected by either stimulation of HAWSMC with TGF-β1 (1 ng/ml) or inhibition of Myc. Experiments were performed analogously to those above and represent means of 4 experiments.

Fig. 8. TGF-β1 progression of the HAWSMC cell cycle is Nox4- and ROS-dependent. A: TGF-β1 (1 ng/ml) induces phosphorylation (lane 2) of retinoblastoma protein (pRb), a regulator of G1 to S transition, at Ser807/811 and Ser780 and increases phosphorylation (lane 2) of cdc2 (with cyclin B as a regulator of G2 to M transition at Tyr15). siNox4 reduced both basal (lane 3) and TGF-β1-stimulated (lane 4) phosphorylation of pRb and cdc2. Neither TGF-β1 nor siNox4 treatment affected pErk 1/2, or β-actin protein levels. Immunoblots were performed with 10 μg of HAWSMC cell lysate protein per lane using phospho-specific antibodies (1:1,000). Immunoblots were then stripped and reprobed with antibodies to pRb, Erk 1/2, and β-actin as loading controls. Data shown are representative of immunoblots from 2 lots of HAWSMC from different donors (cdc2 phosphorylation) or 3 experiments (pRb phosphorylation) using the 2 lots of cells plus 1 experiment using HAWSMC established in our laboratory from donor airway tissue. B: the TGF-β1-induced increase in pRb phosphorylation is prevented by antioxidant treatment with 500 units but not 100 U/ml catalase. Quiescent (Q) HAWSMC (DMEM 0.2% FCS) were preincubated with 100 or 500 U/ml bovine catalase added to media 30 min before stimulation with TGF-β1 (T; 1 ng/ml) for 24 h. pRb phosphorylation was studied with phospho-specific antibodies to pRb phosphorylated at Ser807/811. Immunoblots were then stripped and reprobed with an antibody to Erk 1/2 as a loading control. In the absence of TGF-β1, catalase at either concentration had no effect on pRb Ser807/811 phosphorylation.
cult to determine whether the primary influence of Nox4-mediated ROS is on proliferation or hypertrophy.

NAD(P)H oxidase has been extensively studied in systemic vascular SMC (36), but important components in HAWSMC have been less explored. We (9) previously demonstrated p22^{phox} in early passage HAWSMC. Among its potential regulatory components are necessary for Nox4 activity. We have found that TGF-β1 prominently induces both Nox4 (Fig. 1 and Table 1) and ROS (Fig. 5A) in HAWSMC in a manner unaffected by stimulation with PMA (Fig. 5B) and that Nox4-dependent, TGF-β1-induced HAWSMC proliferation (Figs. 4 and 6) is not inhibited by dnN17Rac1 (Fig. 5D). This indicates that as long as p22^{phox} is available (3), Nox4 is sufficient for O_2^- production without need for membrane or cytosolic regulatory elements and that Nox4 activity, at least in HAWSMC and HPASMC (61), is primarily regulated by its transcription.

This scenario would make Nox4 similar to inducible nitric oxide synthase, which is exclusively regulated by transcription and substrate availability (5). Alternately, stimulation of ROS production in HAWSMC (Fig. 7B), HPASMC (61), and rat glomerular mesangial cells (22) by AA may suggest a role for TGF-β1-stimulated activation of phospholipase A_2 (24) in regulation of Nox4 enzymatic activity.

Induction of Nox4 gene expression has been demonstrated in response to vascular injury (62), the hyperglycemia of diabetes (21), angiotensin II (66), FCS (66), and now TGF-β1 (Ref. 61; Fig. 1 and Table 1), but the intracellular signaling events leading to an increase in Nox4 transcript expression are poorly understood. TGF-β1 normally arrests epithelial cell division by switching off the proto-oncogene, c-myc, along with its protein product, Myc, and rapidly switching on cyclin-dependent kinase inhibitors such as p15^{INK4b} (58). By contrast, TGF-β1

Fig. 9. TGF-β1 induction of HAWSMC hypertrophy is Nox4-dependent. A: addition of TGF-β1 (1 ng/ml) to quiescent HAWSMC (DMEM 0.2% FCS) induced a significant increase in HAWSMC cell size by day 6 but not by day 3. In contrast, PDGF-AB (20 ng/ml) showed no induction of hypertropy at either day. The hypertrophy index was determined as described in MATERIALS AND METHODS. Results represent the means of 3 experiments. *P < 0.05 vs. quiescent cells. B: HAWSMC transfected with a scrambled siRNA control (siScr) showed a significant increase in cell size on addition of TGF-β1 for 4 days. This TGF-β1-induced increase in cell size was significantly reduced by siNox4. Results represent the means of 3 experiments. †P < 0.005 vs. cells treated with siScr alone. *P < 0.01 vs. cells treated with siScr + TGF-β1. C: TGF-β1-stimulated phosphorylation of eukaryotic translation initiation factor 4E binding protein-1 (4E BP1) is inhibited by silencing Nox4. TGF-β1 treatment of HAWSMC for 24 h stimulates phosphorylation of 4E BP1 at Thr37/46 in cells treated with siScr (lanes 7 and 8) compared with lanes 5 and 6, but TGF-β1-stimulated Thr37/46 phosphorylation is prevented by siNox4 (lanes 3 and 4 with siNox4 + TGF-β1 compared with lanes 7 and 8 with siScr + TGF-β1). HAWSMC (p4) were transfected with siNox4 or siScr using the Amaxa system, as described in Fig. 6. The medium was changed to DMEM 0.2% FCS with or without TGF-β1 (1 ng/ml) for 24 h, protein was harvested, and immunoblots were performed using phospho-specific antibodies for 4E BP1 phosphorylated at Thr37/46 (1:1,000). Immunoblots were then stripped and reprobed for β-actin as a loading control. Data shown are representative of immunoblots from 2 experiments. Densitometry of bands is displayed beneath gels. *P < 0.05 siNox4 + TGF-β1 vs. siScr + TGF-β1.
stimulates division in cells of mesenchymal origin such as aortic SMC (60), HPASM (61), and HAWSMC (Fig. 6), accompanied by an increase in Myc (Fig. 7A). We found that blocking Myc-Max interaction significantly inhibited TGF-β1 induction of Nox4 transcripts (Fig. 9B). This raises the possibility that Nox4 is also a Myc target gene, although the influence of Myc on Nox4 transcript expression might be one of a general nature from overall activation of transcriptional machinery (2).

At present, Nox4-derived ROS may have many undefined functions in HAWSMC. This work represents the first demonstration of Nox4-mediated functions for TGF-β1 in stimulating proliferation (Fig. 6) and hypertrophy (Fig. 9) of HAWSMC and is also the first report to suggest a functional role for Nox4 in influencing oxidant-mediated phosphorylation of pRb and cdc2 (Fig. 8) and cell cycle transition. TGF-β1 is elevated in the asthmatic airway (45, 53, 64), and ROS production is important in both proliferation (9, 49, 50) and hypertrophy (54) of SMC. Therefore, a TGF-β1- and Nox4-mediated signaling relationship may play a pivotal pathogenic role in the development of bronchial smooth muscle remodeling in asthma.

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