Transforming Growth Factor- β and Nuclear Factor E2-related Factor 2 Regulate Antioxidant Responses in Airway Smooth Muscle Cells

Role in Asthma

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Rationale: Aberrant airway smooth muscle cell (ASMC) function and overexpression of transforming growth factor (TGF)- β , which modulates ASMC proliferative and inflammatory function and induces oxidant release, are features of asthma. Nuclear factor E2-related factor 2 (Nrf2) activates antioxidant genes conferring protection against oxidative stress.

Objectives: To determine the role of Nrf2 in ASMCs and its modulation by TGF- β , and compare Nrf2 activity in ASMCs from subjects with severe and nonsevere asthma and healthy subjects.

Methods: ASMCs were cultured from airways of subjects without asthma, and from airway biopsies from patients with severe and nonsevere asthma. We studied Nrf2 activation on antioxidant gene expression and proliferation, the effect of TGF- β on Nrf2 transcriptional activity, and the impact of Nrf2 activation on TGF- β -mediated proliferation and IL-6 release. Nrf2-antioxidant response elements binding and Nrf2-dependent antioxidant gene expression was determined in asthmatic ASMCs.

Measurements and Main Results: Activation of Nrf2 led to up-regulation of the antioxidant genes heme oxygenase (HO)-1, NAD(P)H:quinone oxidoreductase, and manganese superoxide dismutase, and a reduction in proliferation. TGF- β reduced Nrf2-mediated antioxidant gene transcription through induction of activating transcription factor-3 expression. Nrf2 activation attenuated TGF- β -mediated reduction in HO-1, ASMC proliferation, and IL-6 release. Nrf2-antioxidant response elements binding was reduced in ASMCs from patients with severe asthma compared with ASMCs from patients with nonsevere asthma and normal subjects. HO-1 expression was reduced in ASMCs from patients with both nonsevere and severe asthma compared with healthy subjects.

Conclusions: Nrf2 regulates antioxidant responses and proliferation in ASMCs and is inactivated by TGF- β . Nrf2 reduction may underlie compromised antioxidant protection and aberrant ASM function in asthma.

Keywords: asthma; airway smooth muscle; nuclear factor E2-related factor 2; transforming growth factor- β ; antioxidant

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Aberrant airway smooth muscle cell (ASMC) function and overexpression of transforming growth factor- β , which modulates ASMC proliferative and inflammatory function and induces oxidant release, are features of asthma. Nuclear factor E2-related factor 2 (Nrf2) activates antioxidant genes conferring protection against oxidative stress.

What This Study Adds to the Field

Nrf2 activation confers antioxidant protection and reduces proliferation in airway smooth muscle cells. Transforming growth factor- β inactivates Nrf2, thus providing a possible mechanism mediating the defective antioxidant protection and aberrant ASM function observed in asthma.

Increased airway smooth muscle cell (ASMC) proliferation and hypertrophy may contribute to increased airway smooth muscle (ASM) thickness and airway remodeling in asthma. ASM mass is particularly increased in patients with severe asthma compared with nonsevere asthma (1). ASMCs may also orchestrate airway inflammatory responses through the release of inflammatory cytokines (2). The airways of patients with asthma, particularly those with severe asthma, are exposed to increased levels of exogenous and endogenous reactive oxygen species (ROS) while at the same time antioxidant responses may be compromised (3, 4). ROS may mediate ASMC contraction, proliferation, and inflammatory mediator release through activation of redox-sensitive signaling pathways (5–9). Thus, endogenous ROS may provide a mechanism for aberrant ASM function in asthma.

The basic leucine zipper transcription factor, nuclear factor E2-related factor 2 (Nrf2), binds to common regulatory elements, termed “antioxidant response elements” (AREs), in the 5'-flanking regions of a wide range of antioxidant and detoxification genes triggering their activation. These include heme oxygenase (HO)-1, NAD(P)H:quinone oxidoreductase (NQO1), glutathione-S-transferases, superoxide dismutases, and catalase (10, 11). Under normal conditions Nrf2 is found in a complex with its inhibitor Kelch-like ECH-associated protein and the ubiquitin-ligase Cul3, where it is constantly targeted for proteosomal degradation. In the presence of oxidants, Nrf2 dissociates from the complex leading to an increase in its protein levels and thus activation of antioxidant genes. Nrf2 is also regulated at the level of cytoplasmic-nuclear shuttling and transcriptional activation (12). Nrf2 plays a protective role against vascular smooth muscle cell (VSMC) proliferation and

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reduces allergen-induced airway inflammation in mice (13–16). Thus, we postulated that Nrf2 may play an important role in regulating antioxidant defenses and proliferation in ASMCs.

Transforming growth factor (TGF)- β is highly expressed in the ASM of patients with asthma and increases ASMC proliferation and size together with ASMC-mediated inflammatory, profibrotic, and proangiogenic effects (17–21). TGF- β induces IL-6 release, and also increases intracellular ROS levels in ASMCs through up-regulation of NADPH oxidase 4 (Nox4) and inhibition of manganese superoxide dismutase (MnSOD) and catalase (22).

We hypothesized that Nrf2 activation in ASMCs increases antioxidant protection and suppresses their proliferation, and that TGF- β interferes with Nrf2 activity leading to compromised ASMC antioxidant responses and increased proliferation and inflammatory responses. We therefore examined the effect of Nrf2 activation and overexpression on antioxidant gene expression and proliferation, and also the effect of TGF- β on Nrf2 transcriptional activity, expression, ARE-binding, and antioxidant gene expression. Finally, we compared the Nrf2-ARE binding and the expression of HO-1 in ASMCs from healthy subjects and subjects with asthma. Because patients with severe asthma have the highest amount of ASM mass and are exposed to the greatest amount of oxidative stress, we examined patients with both severe and nonsevere asthma. Some of the results of these studies have been previously reported in the form of an abstract (23).

METHODS

ASMC Isolation and Culture

ASMCs were dissected from bronchi and tracheas of transplant donor lungs as described previously and cultured in Dulbecco's modified Eagle medium supplemented with L-glutamine, penicillin, streptomycin, amphotericin B, and 10% fetal bovine serum (FBS) (24). ASMCs were

also cultured from bronchial biopsies from patients with mild-to-moderate (termed "nonsevere") and severe asthma (see Table E1 in the online supplement), as classified previously (25). A more detailed description of the culture method is provided in the online supplement.

This study was approved by the local ethics Committee, and informed consent was obtained from each participant.

Adenoviral Gene Transduction

Adenoviral vectors expressing GFP (Ad-GFP) and wild-type Nrf2 (Ad-Nrf2) were obtained from Dr. Paul Evans, National Heart and Lung Institute. Cells were infected at the required multiplicity of infection (MOI), then serum-deprived and treated as indicated in individual experiments. A more detailed description is provided in the online supplement.

Small Interfering RNA Transfection

ASMCs were transiently transfected with nontargeting small interfering RNA (siRNA) or activating transcription factor (ATF)-3 siRNA (200 nM) (Dharmacon, Lafayette, CO) using Amaxa nucleofection (Lonza AG, Cologne, Germany). A more detailed description is provided in the online supplement.

DNA Synthesis

DNA synthesis was determined by bromodeoxyuridine incorporation using Cell Proliferation ELISA kit (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's instructions. A more detailed description is provided in the online supplement.

Real-time Polymerase Chain Reaction

Total RNA was isolated using RNeasy Mini Kit (Qiagen, West Sussex, UK) and reverse-transcribed with random primers and AMV reverse transcriptase (Promega, Southampton, UK). mRNA was quantified by real-time polymerase chain reaction (PCR) (Rotor Gene 3000; Corbett Research, Sydney, Australia) using SYBR Green PCR Master Mix

Figure 1. (A and B) Semiconfluent airway smooth muscle cells (ASMCs) were serum-deprived for 24 hours; pretreated with vehicle or sulforaphane (SFN) (2–4 μ M) for 1 hour; and then incubated with serum-free medium or medium containing 2.5% fetal bovine serum (FBS). DNA synthesis was determined by measuring bromodeoxyuridine (BrdU) incorporation 48 hours after treatment (A). p21^{Waf1} and p27^{Kip1} expression was determined 24 hours after treatment in whole-cell protein extracts by Western blotting and normalized to β -actin expression (B). (C and D) ASMCs were incubated with adenoviral vectors expressing GFP (Ad-GFP) and wild-type nuclear factor E2-related factor 2 (Ad-Nrf2) (multiplicity of infection 250) for 18 hours, serum-deprived for 6 hours, and then incubated with serum-free medium or medium containing 2.5% FBS. DNA synthesis was determined by measuring BrdU incorporation 72 hours after treatment (C). p21^{Waf1} and p27^{Kip1} expression was determined 72 hours after treatment in whole-cell protein extracts by Western blotting and normalized to β -actin expression (D). * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with vehicle control or Ad-GFP. Bars represent mean $\pm$ SEM of six ASMC (A and C) and four ASMC donors (B and D).

TABLE 1. GENE EXPRESSION BY A GENE ARRAY IN AIRWAY SMOOTH MUSCLE CELLS EXPOSED TO TGF- β

Gene	% Inhibition by TGF- β (10 ng/ml) 48 Hours	P Value
Catalase	51	0.001
MnSOD	62	0.007
Extracellular-SOD	69	0.007
Glutathione-S-transferase A4	53	0.036
Glutathione-S-transferase M5	57	0.01
Glutathione-S-transferase M2	57	0.005
Glutathione-S-transferase M1	57	0.008
Glutathione peroxidase 3	70	0.038

Definition of abbreviations: MnSOD = manganese superoxide dismutase; SOD = superoxide dismutase; TGF = transforming growth factor.

Data expressed as % change compared with unstimulated controls. Data represent mean of three airway smooth muscle cell donors.

Reagent (Qiagen) and QuantiTect primer assays (Qiagen) for HO-1, NQO1, MnSOD, catalase, and IL-6. mRNA expression was normalized to 18S rRNA expression. A more detailed description is provided in the online supplement.

Western Blotting

Whole-cell protein extracts were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Protein expression was determined using anti-p21^{Waf1},

anti-p27^{Kip1} (Cell Signaling Technology, Danvers, MA), anti-Nrf2 (C-20; Santa Cruz Biotechnology, Heidelberg, Germany), or TATA binding protein anti- β -actin (AbCam, Cambridge, UK) antibodies. A more detailed description is provided in the online supplement.

ARE Reporter Assay

Cells were transfected with constructs expressing ARE-inducible firefly luciferase and constitutively active Renilla luciferase (SABiosciences, Frederick, MD), serum-deprived, and treated as indicated in individual experiments. Luciferase activity was determined by measuring luminescence. A more detailed description is provided in the online supplement.

Nrf2-ARE Binding Assay

Nrf2-ARE binding was determined using an ELISA-based TransAM Nrf2 Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. A more detailed description is provided in the online supplement.

IL-6 Assay

IL-6 was measured using ELISA according to the manufacturer's instructions (R&D Systems, Abingdon, UK).

Data Analysis

Data are expressed as mean $\pm$ SEM. Results were analyzed using one-way analysis of variance for repeated measures, followed by Dunnett *post hoc* test, unless otherwise specified. Statistical analysis was

Figure 2. (A and B) Confluent airway smooth muscle cells (ASMCs) were serum-deprived for 24 hours and then treated with transforming growth factor (TGF)- β (1 ng/ml) for 0.5–24 hours (A), or TGF- β (0.25–1 ng/ml) for 24 hours (B). Heme oxygenase (HO)-1 and NAD(P)H:quinone oxidoreductase (NQO1) mRNA was determined by real-time polymerase chain reaction (PCR) and normalized to 18S rRNA expression. (C and D) Confluent ASMCs were transfected with antioxidant response elements (ARE)-driven luciferase reporter vector for 18 hours, serum-deprived for 6 hours, and finally treated with TGF- β (0.25–1 ng/ml) for 20 hours (C) or pretreated with vehicle or sulforaphane (2–4 μ M) for 1 hour and then treated with TGF- β (0.25 ng/ml) for 20 hours (D). ARE-driven transcriptional activity was determined by measuring firefly luciferase activity and normalizing to Renilla luciferase activity. (E and F) Confluent ASMCs were serum-deprived for 24 hours and then treated with TGF- β (0.25–1 ng/ml) for 20 hours. Nuclear factor E2-related factor 2 (Nrf2) expression was determined in whole-cell extracts by Western blotting and normalized to β -actin expression (E). Nrf2-ARE binding was determined in nuclear extracts by an ELISA-based TransAM assay (F). Bars represent mean $\pm$ SEM of three ASMC (A and F), five ASMC (B), three to six ASMC (C), four ASMC (D), and four ASMC donors (E). * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with unstimulated control. ns = nonsignificant.

Figure 3. (A) Confluent airway smooth muscle cells (ASMCs) were serum-deprived for 24 hours and then treated with transforming growth factor (TGF- β) (1 ng/ml) for 0.5–24 hours. ATF-3 mRNA was determined by real-time polymerase chain reaction and normalized to 18S rRNA expression. (B–D) ASMCs were transfected with scramble or ATF-3 small interfering RNA (siRNA) (200 nM). Eighteen hours post-transfection cells were serum-deprived for 6 hours and then treated with TGF- β (0.25 ng/ml) for 24 hours. ATF-3 protein expression was determined in whole-cell protein extracts by Western blotting and normalized to β -actin expression. ATF-3 and heme oxygenase (HO)-1 mRNA expression was determined by real-time polymerase chain reaction and normalized to 18S rRNA expression. Bars represent mean $\pm$ SEM of four to nine ASMC donors (A) and four ASMC donors (C and D). The blot in B is representative of experiments on two ASMC donors. * P < 0.05, ** P < 0.01, and *** P < 0.001.

performed using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA).

RESULTS

Activation of Nrf2 Augments Antioxidant Gene Expression

Treatment with the Nrf2 inducer sulforaphane (2–4 μ M) for 4 hours increased Nrf2 protein expression by approximately fourfold (*see* Figure E2A), accompanied by a concentration-dependent increase in ARE-driven luciferase activity 18 hours after treatment (*see* Figure E2B). Furthermore, sulforaphane (2–4 μ M) increased HO-1 ($\sim$ twofold), NQO1 ($\sim$ twofold), and MnSOD mRNA ($\sim$ 50%), but not catalase mRNA levels (*see* Figure E2C). We also activated Nrf2 by transiently over-expressing wt-Nrf2 protein using adenoviral gene transduction. Ad-Nrf2 (MOI 250) increased Nrf2 protein expression 72 hours after treatment (*see* Figure E2D), whereas it led to an increase in HO-1 ($\sim$ fivefold) and NQO1 ($\sim$ 40%) mRNA after 30 hours, and a more delayed increase in MnSOD mRNA ($\sim$ eightfold) 72 hours after treatment. Catalase mRNA expression was not affected (*see* Figure E2E).

Activation of Nrf2 Leads to Reduced ASMC Proliferation

We studied the effect of Nrf2 activation on ASMC proliferation both at baseline and in response to mitogen stimulation (2.5% FBS). Sulforaphane (2–4 μ M) inhibited baseline and FBS-induced DNA synthesis in a concentration-dependent manner, reaching a maximum inhibition of approximately 30% and approximately 60%, respectively (Figure 1A). Sulforaphane also concentration-dependently induced p21^{Waf1} expression ($\sim$ three-fold maximal increase) 24 hours after treatment both under serum-free and FBS-stimulated conditions. However, sulforaphane led to a nonstatistically significant reduction in p27^{Kip1} expression both under serum-free and FBS-stimulated conditions. Ad-Nrf2 (MOI 250) also inhibited the rate of DNA synthesis both under serum-free ($\sim$ 30%) and FBS-dependent conditions ($\sim$ 25%) (Figure 1C). Moreover, Ad-Nrf2 led to an increase in p21^{Waf1} expression both under serum-free ($\sim$ 2.8-fold) and FBS-stimulated conditions ($\sim$ 1.8-fold). However, p27^{Kip1} expression was not affected by Ad-Nrf2 under serum-free conditions but it was inhibited under FBS-stimulated conditions (Figure 1D).

TGF- β Inhibits Antioxidant Gene Expression and Nrf2 Transcriptional Activity

TGF- β (10 ng/ml) inhibited the expression of glutathione-S-transferases A4, M1, M2, and M5, glutathione peroxidase 3, MnSOD, and catalase 48 hours after treatment, as determined by a gene microarray, suggesting that TGF- β inhibits the expression of a wide range of antioxidant genes in ASMCs (Table 1). To investigate whether the reduction in antioxidant gene expression by TGF- β occurs as a result of Nrf2 inactivation, the effect of TGF- β (1 ng/ml) on the mRNA levels of two ARE-driven genes, NQO1 and HO-1, was determined. TGF- β induced an early induction of HO-1 mRNA, peaking after 2 hours of stimulation, followed by a late inhibition 24 hours after stimulation. NQO1 mRNA expression was also attenuated 24 hours after treatment (Figure 2A). The inhibitory effect on HO-1 and NQO1 mRNA at 24 hours was also apparent at a TGF- β concentration of 0.25 ng/ml (Figure 2B). This was accompanied by an inhibition of ARE-driven luciferase activity (Figure 2C). The inhibition of ARE-dependent transcription by TGF- β (0.25 ng/ml) was also completely prevented by sulforaphane (2–4 μ M) (Figure 2D).

We determined the effect of TGF- β on Nrf2 protein expression in whole-cell and nuclear extracts and on Nrf2-ARE

binding. Treatment with TGF- β (0.25–1 ng/ml) for 20 hours had no effect on the expression of Nrf2 in whole-cell extracts (Figure 2E). Nrf2 protein was predominantly found in the nucleus, but TGF- β (0.25–1 ng/ml) for 20 hours neither modulated its nuclear expression (*see* Figure E4) nor its binding to ARE consensus sequences (Figure 2F).

Role of ATF-3 in TGF- β -induced Inhibition of Nrf2-Dependent Antioxidant Gene Expression

To explore the possibility that the inhibitory effect of TGF- β is mediated through transcriptional mechanisms, we measured the expression of the Nrf2 repressor ATF-3. TGF- β (1 ng/ml) caused a time-dependent increase in ATF-3 mRNA peaking at 4 hours and remaining elevated at 24 hours (Figure 3A). Transfection with ATF-3 siRNA (200 nM) strongly reduced TGF- β (0.25 ng/ml)-mediated ATF-3 mRNA (Figure 3C) and protein expression (Figure 3B) and prevented TGF- β (0.25 ng/ml)-mediated inhibition of HO-1 mRNA (Figure 3D). Thus, ATF-3 is directly involved in the inhibitory effect of TGF- β on Nrf2-mediated antioxidant gene expression.

Effect of Nrf2 Activation on TGF- β -induced Inhibition of Antioxidant Gene Expression

TGF- β (0.25 ng/ml)-induced inhibition of HO-1 mRNA was prevented by sulforaphane (2–4 μ M) (Figure 4A) and also by Ad-Nrf2 (MOI 250) (Figure 4D). On the contrary, TGF- β -induced

inhibition of MnSOD mRNA was not affected by either sulforaphane or Nrf2 overexpression (Figures 4C and 4F), whereas the inhibition of catalase was partially prevented by sulforaphane at 4 μ M, but not Nrf2 overexpression (Figures 4B and 4E). Nrf2 protein expression was elevated 2 to 24 hours after treatment with sulforaphane, indicating that Nrf2 was activated during the time of TGF- β stimulation (data not shown). Thus, inhibition of Nrf2 activity underlies TGF- β -mediated inhibition of HO-1 but not of catalase and MnSOD expression.

Effect of Nrf2 Activation on TGF- β -induced Proliferation and IL-6 Release

TGF- β (0.25–1 ng/ml) alone had no effect on baseline rate of DNA synthesis but TGF- β (0.25 ng/ml) increased DNA synthesis in the presence of 2.5% FBS by approximately twofold 72 hours after treatment (*see* Figure E3). Sulforaphane (2–4 μ M) prevented TGF- β -induced DNA synthesis in a concentration-dependent manner, reaching almost complete inhibition at the highest concentration (Figure 5A). Ad-Nrf2 (MOI 250) also reduced TGF- β -induced DNA synthesis, but to a lesser extent than sulforaphane (Figure 5B). Sulforaphane (2–4 μ M) inhibited TGF- β (0.25 ng/ml)-induced IL-6 mRNA (Figure 5C) and protein release (Figure 5E) in a concentration-dependent manner. In agreement with these findings, Ad-Nrf2 (MOI 250) strongly suppressed the effect of TGF- β on IL-6 mRNA (Figure 5D) and IL-6 protein release (Figure 5F).

Figure 4. Confluent airway smooth muscle cells (ASMCs) were serum-deprived for 24 hours, pretreated with vehicle or sulforaphane (2–4 μ M) for 1 hour, and then stimulated with transforming growth factor (TGF)- β (0.25 ng/ml) for 24 hours (A–C). Alternatively, ASMCs were incubated with adenoviral vectors expressing GFP (Ad-GFP) and wild-type nuclear factor E2-related factor 2 (Ad-Nrf2) (multiplicity of infection 250) for 18 hours, serum-deprived for 24 hours, and then stimulated with TGF- β (0.25 ng/ml) for 24 hours (D–F). Heme oxygenase (HO)-1, catalase, and manganese superoxide dismutase (MnSOD) mRNA expression was determined by real-time polymerase chain reaction and normalized to 18S rRNA expression. Bars represent mean $\pm$ SEM of five ASMC (A–C) and four ASMC donors (D–F). * P < 0.05, ** P < 0.01. ns = non-significant.

Figure 5. (A and B) Semiconfluent airway smooth muscle cells (ASMCs) were serum-deprived for 24 hours, pre-treated with vehicle or sulforaphane (2–4 μ M) for 1 hour, and then incubated with medium containing 2.5% fetal bovine serum (FBS) or 2.5% FBS and transforming growth factor (TGF)- β (0.25 ng/ml) for 72 hours (A). Alternatively, ASMCs were incubated with adenoviral vectors expressing GFP (Ad-GFP) and wild-type nuclear factor E2-related factor 2 (Ad-Nrf2) (multiplicity of infection 250) for 18 hours, serum-deprived for 6 hours, and then incubated with medium containing 2.5% FBS or 2.5% FBS and TGF- β (0.25 ng/ml) for 72 hours (B). DNA synthesis was determined by measuring bromodeoxyuridine (BrdU) incorporation. (C–F) Confluent ASMCs were serum-deprived for 24 hours, pretreated with vehicle control or sulforaphane (2–4 μ M) for 1 hour, and then stimulated with TGF- β (0.25 ng/ml) for 24 hours (C and D). Alternatively, ASMCs were incubated with Ad-GFP or Ad-Nrf2 (MOI 250) for 18 hours, serum-deprived for 6 hours, and then stimulated with TGF- β (0.25 ng/ml) for 24 hours (E and F). IL-6 mRNA expression was determined by real-time polymerase chain reaction, normalized to 18S rRNA expression, and expressed as fold change with respect to unstimulated control. IL-6 release was determined by ELISA. * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with vehicle or Ad-GFP control. # P < 0.05, ## P < 0.01, and ### P < 0.001 compared with TGF- β and vehicle or Ad-GFP-treated cells. Bars represent mean $\pm$ SEM of five ASMC donors (B and C), four ASMC donors (A, D, and F), and three ASMC donors (E).

Nrf2 Expression, ARE Binding Activity, and HO-1 Expression in ASMCs from Patients with Asthma

ASMCs cells dissected from bronchoscopic biopsies or transplant donor lungs of healthy subjects and subjects with nonsevere or severe asthma were grown to confluence and Nrf2 expression and Nrf2-ARE binding were assessed in whole-cell protein extracts. Nrf2 protein expression was similar in ASMCs from patients with nonsevere and severe asthma compared with healthy subjects (Figures 6A and B). However, in severe asthmatic ASMCs there was a reduction in Nrf2-ARE binding compared with healthy and nonsevere asthmatic ASMCs, whereas in patients with nonsevere asthma there was a small increase compared with healthy ASMCs (Figure 6C). HO-1 mRNA and protein expression was significantly lower both in nonsevere and severe asthma ASMCs compared with healthy ASMCs (Figures 7A and B). In view of our data showing Nrf2-dependent induction of p21^{Waf1}, we compared p21^{Waf1} expression in whole-cells extracts from the three groups. Although there were no significant differences between the groups (*see* Figures E5A and E5B), p21^{Waf1} expression was significantly correlated with Nrf2-ARE binding (*see* Figure E5C).

DISCUSSION

We have shown that Nrf2 activation by sulforaphane or by Nrf2 overexpression led to an increase in expression of the antioxidant enzymes HO-1, NQO1, and MnSOD and to inhibition

of proliferation with increased expression of the cyclin-dependent kinase inhibitor p21^{Waf1} in ASMCs. TGF- β inhibited Nrf2 transcriptional activity, with attenuation of HO-1 and NQO1 expression, by inducing the expression of the transcriptional repressor ATF-3. Equally, activation of Nrf2 prevented TGF- β -mediated inhibition of HO-1 expression, and partially inhibited TGF- β -induced ASMC proliferation and IL-6 release. Nrf2-ARE binding and HO-1 expression was found to be reduced in ASMCs from subjects with severe asthma compared with ASMCs from subjects with nonsevere asthma and healthy subjects. HO-1 expression was also reduced in ASMCs from subjects with nonsevere and severe asthma compared with healthy subjects suggesting that that Nrf2 may also be inactivated in nonsevere asthmatic ASM, but through a transcriptional mechanism. This may be mediated by TGF- β , which is elevated in asthmatic ASM (26).

Sulforaphane induces dissociation of Nrf2 from its inhibitor protein Kelch-like ECH-associated protein leading to increased protein stability of Nrf2 and consequently ARE-driven gene transcription (27). Sulforaphane increased Nrf2 protein levels, ARE-driven luciferase activity, and antioxidant gene (HO-1, NQO1, and MnSOD) expression in ASMCs. Because sulforaphane may have potential off-target effects, we also transiently overexpressed Nrf2 levels using adenoviral vectors (28, 29). This led to a similar profile of expression of antioxidant genes as sulforaphane indicating that pharmacologic and molecular

Figure 6. Airway smooth muscle cells (ASMCs) cultured from bronchoscopic biopsies and transplant airways taken from healthy subjects ($n = 5-6$) or bronchoscopic biopsies from patients with nonsevere ($n = 6$) and severe asthma ($n = 6-7$) were grown to confluence in medium containing 10% fetal bovine serum, and whole-cell protein was extracted. (A and B) Nuclear factor E2-related factor 2 (Nrf2) protein expression was determined in whole-cell protein extracts by Western blotting and normalized to β -actin expression. To ensure that all membranes were equally exposed to antibodies, substrate, and X-ray film a control sample (c) was run in each of the gels. (C) Nrf2-antioxidant response elements (ARE) binding was determined in whole-cell protein extracts by an ELISA-based TransAM assay. Data were analyzed using Mann-Whitney test.

activation of Nrf2 both led to antioxidant response. However, the time-course of induction of HO-1 and NQO1 mRNA by Nrf2 overexpression was different from that of MnSOD, indicating that Nrf2 may activate MnSOD gene expression through a different mechanism.

In addition, Nrf2 activation led to accumulation of the cyclin-dependent kinase inhibitor p21^{Waf1} and reduction in DNA synthesis. Sulforaphane was more effective than Ad-Nrf2 in inhibiting DNA synthesis and inducing p21^{Waf1}, which may reflect differences between the two modes of Nrf2 activation. In line with our findings, Nrf2 activation by curcumin and sulfasalazine leads to HO-1-mediated up-regulation of p21^{Waf1} and inhibition of proliferation in VSMCs (13, 30), whereas superoxide ions inhibit p21^{Waf1} expression and promote VSMC proliferation (31), suggesting that Nrf2 may prevent ASMC proliferation by increasing antioxidant enzyme expression. In contrast, Nrf2 augments the proliferation of cancerous lung and liver epithelial cells through inhibition of p21^{Waf1} expression, suggesting that the effect of Nrf2 on cell cycle may be cell-specific (32-34).

We have previously reported that TGF- β disturbs oxidant-antioxidant enzyme balance in ASMCs by inducing Nox4 expression and reducing MnSOD and catalase expression (22). We now show that TGF- β reduces ARE-driven luciferase activity and the expression of the Nrf2-dependent genes HO-1 and NQO1, in line with findings in mammary epithelial cells (35). However, TGF- β caused an early increase in HO-1 mRNA expression, consistent with findings in VSMCs where TGF- β -mediated up-regulation of HO-1 protein occurred 4- to 2

hours after treatment, through activation of Nrf2. However, Churchman and coworkers (36) show that HO-1 levels return to baseline after 24 hours of treatment, whereas we show strong inhibition of HO-1 at this time point. Because we have shown that NQO1 does not show a similar early induction in response to TGF- β , this effect could be gene-specific. Moreover, in ASMCs TGF- β may trigger an early activation of Nrf2 followed by inhibition of its activity after prolonged treatment, reflecting the complexity and cell-specificity of TGF- β -mediated signaling. Interestingly, activation of Nrf2 prevented TGF- β -mediated inhibition of HO-1 but not of catalase and MnSOD, indicating that other mechanisms may be involved in the down-regulation of the latter antioxidant enzymes by TGF- β , including PPAR- γ coactivator-1 α (PGC-1 α) and forkhead box transcription factors (FoxOs) (37).

Nrf2 inactivation by TGF- β in ASMCs was not accompanied by changes in Nrf2 expression, nuclear translocation, or binding to ARE. We show that TGF- β increases the mRNA expression of ATF-3, a basic leucine-zipper DNA binding protein and a known repressor of Nrf2. ATF-3 blocks Nrf2-mediated transcription by displacing the Nrf2 coactivator, CREB binding protein, from the ARE-binding complex (38). Indeed, inhibition of TGF- β -mediated ATF-3 expression by siRNA prevented the suppression of HO-1 by TGF- β . Our data indicate that ATF-3 mediates the suppressive effect of TGF- β on Nrf2-dependent antioxidant genes, in agreement with a study in mammary epithelial cells (38). The induction of DNA synthesis by TGF- β was inhibited by sulforaphane and to a lesser extent by Nrf2

Figure 7. Airway smooth muscle cells (ASMCs) cultured from bronchoscopic biopsies and transplant airways taken from healthy subjects ($n = 5$) or bronchoscopic biopsies from patients with nonsevere ($n = 6$) and severe asthma ($n = 7$) were grown to confluence in medium containing 10% fetal bovine serum, and whole-cell protein and RNA were extracted. (A) Heme oxygenase (HO)-1 mRNA expression was determined by real-time polymerase chain reaction and normalized to 18S rRNA expression. (B) HO-1 protein expression was determined in whole-cell protein extracts by Western blotting and normalized to β -actin expression (C). To ensure that all membranes were equally exposed to antibodies, substrate, and X-ray film a control sample (c) was run in each of the gels. The protein expression in each patient sample was normalized to the expression in the control sample. Data were analyzed using Mann-Whitney test.

overexpression. TGF- β inhibited p27^{Kip1} expression but did not affect p21^{Waf1} expression (data not shown) suggesting that its effect on ASMC proliferation is not directly linked to p21^{Waf1}. Sturrock and coworkers (39) have demonstrated that TGF- β induces ASMC proliferation by increasing retinoblastoma protein phosphorylation in a ROS-dependent manner. Thus, activation of Nrf2-dependent antioxidant enzyme expression may prevent ROS-dependent Rb phosphorylation and hence inhibit TGF- β -mediated ASMC proliferation. Nrf2 activation also inhibited the induction of IL-6 release by TGF- β , possibly through inactivation of redox-sensitive inflammatory transcription factors by antioxidant enzymes. Thus, Nrf2 plays a protective role against TGF- β -mediated responses in ASMCs.

We examined Nrf2 function in ASMCs cultured from subjects with asthma. We studied mild-moderate patients with asthma whose disease is well controlled by corticosteroid treatment and patients with severe asthma who have uncontrolled asthma despite corticosteroid treatment (25). Nrf2 protein expression was not significantly different between the different groups, but Nrf2-ARE binding and HO-1 expression were reduced in ASMCs from subjects with severe asthma compared with healthy ASMCs. Thus, the defective Nrf2 binding observed in the ASMCs of subjects with severe asthma could be secondary to changes in post-translational modifications (40, 41). Although nonsevere asthmatic ASMCs showed increased Nrf2-ARE binding, their expression of HO-1 was also significantly reduced compared with healthy ASMCs. These data indicate that Nrf2 activity is also inhibited in nonsevere asthmatic ASMCs but at the transcriptional level. This mechanism may be mediated either by TGF- β , because its expression is known to be up-regulated

in asthmatic ASMCs, or through changes in the expression or activities of transcriptional repressors or post-translational modifications (26, 35, 42, 43). Nonetheless, we cannot exclude the possibility that an Nrf2-independent pathway may also be involved, because the HO-1 gene is also known to be activated by other transcription factors (44).

In view of our findings showing that p21^{Waf1} expression is increased by Nrf2, we determined p21^{Waf1} expression in ASMCs from patients with asthma. Although p21^{Waf1} expression was not significantly different between the three groups, Nrf2-ARE binding activity was found to correlate positively with p21^{Waf1} expression. This finding in conjunction with our data showing induction of p21^{Waf1} expression by Nrf2 suggests a possible role of Nrf2 in regulating p21^{Waf1} expression in ASMCs. However, because the regulation of p21^{Waf1} expression is known to be complex, other transcription factors may also be involved in the modulation of p21^{Waf1} levels in ASMCs (45). This could underlie the weak correlation between p21^{Waf1} expression and Nrf2 binding activity. In contrast, epithelial cells from subjects with nonsevere and severe asthma show attenuated proliferation and increased p21^{Waf1} levels, reflecting tissue-specific differences in proliferation in asthma (46).

We have demonstrated that Nrf2 plays a key role in ASMC biology by conferring antioxidant protection through activation of antioxidant and cytoprotective genes and inhibiting ASMC proliferation. We show for the first time that TGF- β reduces Nrf2 activity in ASMCs leading to reduced antioxidant gene expression and possibly defective ASM function in asthma. Furthermore, we show evidence of defective Nrf2 signaling and antioxidant responses in asthmatic ASMCs.

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