Respiratory syncytial virus (RSV) is the leading cause of epidemic respiratory tract illness in children in the United States and worldwide. RSV infection of airway epithelial cells induces formation of reactive oxygen species (ROS), whose production mediates the expression of cytokines and chemokines involved in the immune/inflammatory responses of the lung. In this study, we have investigated the role of ROS in RSV-induced signal transducers and activators of transcription (STAT) activation and interferon regulatory factor (IRF) gene expression in human airway epithelial cells. Our results indicate that RSV replication induces IRF-1 and -7 gene transcription, a response abrogated by antioxidants. RSV infection induces binding of STAT to the IRF-1 γ-interferon-activated sequence (GAS) and IRF-7 interferon-stimulated responsive element (ISRE). STAT1 and STAT3 bind IRF-1 GAS, whereas STAT1, STAT2, IRF-1, and IRF-9 bind IRF-7 ISRE. Antioxidant treatment blocks RSV-induced STAT binding to both the IRF-1 GAS and IRF-7 ISRE by inhibition of inducible STAT1 and STAT3 tyrosine phosphorylation, suggesting that RSV-induced ROS formation is required for STAT activation and IRF gene expression. Although protein tyrosine phosphorylation is necessary for RSV-induced STAT activation, Janus kinase and Src kinase activation do not mediate this effect. Instead, RSV infection inhibits its intracellular tyrosine phosphatase activity, which is restored by antioxidant treatment. Pharmacological inhibition of tyrosine phosphatases induces STAT activation. Together, these results suggest that modulation of phosphatases could be an important mechanism of virus-induced STAT activation. Treatment of alveolar epithelial cells with the NAD(P)H oxidase inhibitor diphenylene iodonium abolishes RSV-induced STAT activation, indicating that NAD(P)H oxidase-produced ROS are required for downstream activation of the transcription factors IRF and STAT in virus-infected airway epithelial cells.

Respiratory syncytial virus (RSV) is an enveloped, negative-sense single-stranded RNA virus (1). Since its isolation, RSV has been identified as a leading cause of epidemic respiratory tract illness in children in the United States and worldwide. In fact, RSV is so ubiquitous that it will infect 100% of children before the age of 3. It is estimated that 40–50% of children hospitalized with bronchiolitis and 25% of children with pneumonia are infected with RSV, resulting in 100,000 hospital admissions annually in the United States alone (1). Although the mechanisms of RSV-induced airway disease and the associated long term consequences are largely unknown, the local inflammatory response is thought to play a fundamental role. Airway epithelial cells are the target of RSV infection, and they respond to the infection producing a variety of mediators involved in lung immune/inflammatory responses, such as cytokines, chemokines, interferons, and up-regulating adhesion molecules and major histocompatibility complex antigens on the cell surface (for review, see Ref. 2). Regulation of cellular responses to extracellular stimuli is a central aspect of host defense, and it is coordinated by intracellular networks in which different subsets of transcription factors are involved in the expression of diverse set of target genes, depending on the nature of the extracellular stimulus. RSV induces gene expression through the coordinate induction of multiple transcription factors that assemble in nucleoprotein complexes, defined “enhancersomes,” as we demonstrated for the chemokines interleukin-8 and RANTES (regulated upon activation, normal T-cells expressed and secreted) (3, 4).

The cellular signaling events leading to RSV-induced transcription factor activation are mostly unknown. Reactive oxygen species (ROS) are ubiquitous, highly diffusible and reactive molecules, produced as a result of reduction of molecular oxygen, including species such as hydrogen peroxide, superoxide anion, and hydroxyl radical, and they have been implicated in damaging cellular components such as lipids, proteins, and DNA. In the past few years, there has been increased recognition of their role as redox regulators of cellular signaling (for review, see Refs. 5 and 6). We have shown recently that RSV infection of airway epithelial cells rapidly induces ROS production (7). Pretreatment of airway epithelial cells with the antioxidant butylated hydroxyanisole (BHA), as well a panel of chemically unrelated antioxidants, blocked RSV-induced chemokine gene expression and protein secretion, through inhibi-
tion of interferon regulatory factor (IRF) activation. Antioxidant treatment inhibited de novo IRF-1 and -7 gene expression, protein synthesis, and IRF-3 nuclear translocation, indicating that a redox-sensitive pathway was involved in RSV-induced IRF activation (7).

The IRF proteins belong to a family of transcription factors involved in the regulation of important immune/inflammatory genes, including RANTES and interleukin-15, as well as of genes necessary to mount an effective antiviral response, such as interferons (8). IRF-3 is constitutively expressed, but IRF-1 and -7 are virus-inducible (8). Here we investigated the unknown mechanism of antioxidant inhibition of virus-induced IRF-1 and -7 activation. Our results indicate that RSV infection of alveolar epithelial cells induces IRF-1 and -7 gene transcription, an effect abrogated by BHA treatment. RSV infection induces members of the signal transducers and activators of transcription (STAT) family to bind the IRF-1 γ-interferon-activated sequence (GAS) and the IRF-7 interferon-stimulated responsive element (ISRE) of transcription factors belonging to the STAT1 and STAT3 binding site. Gas-6 transgenic mice have reduced STAT activation and induction of IRF promoter, Janus kinase inhibitors AG490, AG82, protein phosphatase (PP)1, PP2, and DPI were tested for mycoplasma contamination by PCR analysis and were used if negative. Viral pools were routinely generated in a reaction, which includes the source of phosphatase plus the tyrosine-phosphorylated form of STAT1 and STAT3. Membranes were stripped and reprobed with antibodies to STAT1 and STAT3. For secondary detection, we used a horseradish-coupled anti-rabbit or antimouse antibody, in the enhanced chemiluminescence assay (Amersham Biosciences).

**Experimental Procedures**

**Reagents**—Antibodies anti-STAT1 (sc-592X), anti-STAT2 (sc-476X), anti-STAT3 (sc-482X), anti-STAT5 (sc-1656X), anti-tyrosine-phosphorylated STAT1 (sc-8384), anti-tyrosine-phosphorylated STAT3 (sc-8059), anti-IRF-1 (sc-497X), and anti-IRF-9 (sc-496X) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The chemical inhibitors AG490, AG82, protein phosphatase (PP)1, PP2, and DPI were purchased from Calbiochem.

**RSV Preparation**—The A2 strain of RSV was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (9). The virus titer was determined by a methyl-β-D-galactoside reporter assay, No contaminating cytokines were found in these sucrose-purified virals preparations (10). Viral pools were routinely generated in Hep-2 cells and used as a source of virus for infection studies.

**Cell Culture and Infection of Epithelial Cells with RSV**—A549, human alveolar type IIlike epithelial cells (ATCC, Manassas, VA), were maintained in F12K medium containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell monolayers were infected with RSV at a multiplicity of infection of 1 (unless otherwise stated), as described previously (11). An equivalent amount of a 20% sucrose solution was added to uninfected A549 cells, as a control. When BHA or other inhibitors were used, cells were pre-treated with the compound for 1 h and then infected in the presence of that compound. Because BHA was diluted in ethanol and the other inhibitors in dimethyl sulfoxide, equal amounts of either ethanol or dimethyl sulfoxide were added to untreated cells, as a control. The total number of cells and cell viability, following treatment, were measured by trypan blue exclusion.

**Northern Blot**—Total RNA was extracted from control and infected A549 cells by the acid guanidinium thiocyanate-phenol chloroform method. 20 μg of RNA was fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized to a radiolabeled 180-base-pair protein cDNA at 42 °C. After exposure, membranes were stripped and rehybridized with a β-actin probe.

**DNA binding assays**—Nuclear extracts of uninfected and infected A549 cells were prepared using hypotonic/nondenaturing conditions, as described previously (12). DNA binding reactions contained 10–15 μg of nuclear proteins, 5% glycerol, 12 μM HEPES, 80 mM NaCl, 5 mM dithiorthreitol, 5 mM MgCl₂, 0.5 mM EDTA, 1 μg of poly(dI-dC), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 μl. The nuclear proteins were incubated with the probe for 15 min at room temperature and then fractionated by 4% PAGE. The buffer (2 mM Tris-Cl, 2 mM boric acid, 0.25 mM EDTA, pH 8) at 120 volts. After electrophoretic separation, gels were dried and exposed for autoradiography with Kodak XAR film at −70 °C using intensifying screens. The gel mobility supershift, commercial antibodies against specific transcription factors were added to the binding reactions and incubated on ice for 1 h prior to fractionation on 4% PAGE.

**Western Immunoblot**—Nuclear proteins were prepared as described previously, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (3). Membranes were blocked with 5% albumin in TBS-Tween and incubated overnight with antibody recognizing the tyrosine-phosphorylated form of STAT1 and STAT3. Membranes were stripped and reprobed with antibodies to STAT1 and STAT3. For secondary detection, we used a horseradish-coupled anti-rabbit or antimouse antibody, in the enhanced chemiluminescence assay (Amersham Biosciences).

**Intracellular Tyrosine Phosphatase Assay**—Total intracellular tyrosine phosphatase activity was performed using the tyrosine assay kit from Promega, Madison, WI, according to manufacturer’s protocol. This buffer, a nondenaturing assay that separates the active enzyme from the substrate generated in a reaction, which includes the source of phosphatase plus a tyrosine phosphopeptide, by measuring the absorbance of a molybdate-malic acid green-phosphate complex. Briefly, total cell lysates from A549 cells, control and infected with RSV, in the absence or presence of 400 μM BHA, were prepared using radioimmune precipitation assay buffer (20, described previously (3)). Excess free phosphate was removed from cell lysates using the provided spin columns and 3 μg of samples were mixed with 100 μM tyrosine phosphoprotein substrate and incubated at 30 °C for 20–60 min. Reactions were stopped adding a molybdate dye solution, color was allowed to develop for 15–20 min, and absorbance was read at 600 nm with a plate reader. Phosphatase
RESULTS

BHA Inhibits RSV-induced IRF Transcription—We have recently shown that RSV infection of A549 cells, type II-like alveolar epithelial cells, induced IRF-1 and -7 gene expression, and antioxidant treatment greatly diminished RSV-induced steady-state IRF mRNA levels (7). Treatment of A549 cells with 400 μM BHA almost completely abolished the expression of IRF-1 and IRF-7 genes following RSV infection, without significantly affecting viral replication, as determined by levels of RSV N protein expression (Fig. 1). Antioxidant treatment slightly reduced cell viability (a decrease of ∼10% in BHA-treated cells versus untreated, data not shown) without reducing the amount of virus released by infected cells, as shown previously (7). Previous studies, investigating interferon-α and -β stimulation of IRF-1 and -7 gene induction, have demonstrated that IRF gene expression is controlled at the transcription level (14–17). To determine whether IRF-1 gene transcription was increased after RSV infection, A549 cells were transiently transfected with a construct containing 1.3 kb of the human IRF-1 promoter linked to the luciferase reporter gene (13). The day after, cells were infected with RSV for various lengths of time and harvested to measure luciferase activity. As shown in Fig. 2A, RSV infection induced a time-dependent increase in IRF-1 promoter activation, which started between 3 and 6 h postinfection, peaked at 12 h, and started to decrease at 24 h. To determine whether the antioxidant effect of BHA influenced IRF gene transcription, A549 cells were transiently transfected with the IRF-1 promoter, infected with RSV in the absence or presence of 400 μM BHA, and harvested 12 h later to measure luciferase activity. We have shown previously that a 400 μM concentration of BHA is able to block ∼90% of RSV-induced ROS production without interfering with cell viability (7). As shown in Fig. 2B, treatment with BHA completely abolished RSV-induced luciferase activity, suggesting that the antioxidant inhibits IRF-1 gene expression, at least partially, by interfering with gene transcription.

To investigate IRF-7 gene transcription following RSV infection, we performed similar studies using A549 cells transiently transfected with a construct containing 1.7 kb of the human IRF-7 promoter linked to the luciferase reporter gene (14). As with IRF-1, RSV infection induced a time-dependent increase in luciferase activity, which started around 3 h postinfection, peaked at 6 h, and then declined after 12 h (Fig. 3A). Similarly, BHA treatment completely abolished RSV-induced luciferase activity, indicating that antioxidant treatment also inhibits IRF-7 gene transcription (Fig. 3B). It is important to notice that, although BHA treatment inhibited RSV-induced IRF-1

| BHA | RSV |
|-----|-----|
| --  | --  | +  |
| --  | +   | +  |

FIG. 1. Northern blot of IRF mRNA in RSV-infected A549. A549 cells were infected with RSV for 15 h in the absence or presence of 400 μM BHA. 20 μg of RNA extracted from control and infected cells were fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized to radiolabeled IRF-1, IRF-7, and RSV N protein cDNA probes. Membranes were stripped and hybridized with a radiolabeled β-actin probe to show equal loading of the samples.

FIG. 2. IRF-1 promoter activation after RSV infection. A, time course. A549 cells were transiently transfected with the IRF-1 promoter plasmid and infected with RSV, multiplicity of infection of 1. At different times postinfection, cells were harvested to measure luciferase activity. Uninfected plates served as controls. B, effect of BHA. A549 cells were transfected with the IRF-1 promoter plasmid, infected with RSV for 12 h, in the absence or presence of 400 μM BHA, and harvested to measure luciferase activity. For each plate luciferase was normalized to the β-galactosidase reporter activity. Data are expressed as the mean ± S.D. of normalized luciferase activity.
and -7 promoter activation, it did not affect the basal level of transcription of both genes, indicating that BHA affects only the inducible component of gene transcription. We have also shown previously that BHA does not affect expression of constitutively transcribed cellular genes, such as β-actin (7).

RSV-induces STAT Activation Is Redox-sensitive—Several studies have investigated the promoter elements involved in the regulation of IRF-1 gene expression and have identified the GAS as a major site necessary for inducible transcription (15). To determine whether RSV infection produced changes in the abundance of DNA-binding proteins recognizing the IRF-1 GAS, EMSAs were performed with nuclear extracts prepared from A549 cells, control or infected with RSV for various lengths of time. As shown in Fig. 4A, a single nucleoprotein complex (C3) was formed from nuclear extracts of control cells on the IRF-1 GAS probe. RSV infection induced the appearance of two other complexes, C1 and C2 (better resolved on longer electrophoresis as in Fig. 4C), starting between 1 and 3 h postinfection, peaking around 3–6 h, and declining after 12 h of infection. The sequence specificity of the different complexes was examined by competition with unlabeled oligonucleotides in EMSA (Fig. 4B). C1 and C2 were competed by the wild type but not by the mutated oligonucleotide, indicating GAS-specific binding. IRF-1 GAS has been shown to bind transcription factors of the STAT family (18). To determine the composition of the RSV-inducible complexes, we performed supershift assays using a panel of antibodies reacting with the different members of STAT family of transcription factors. The anti-STAT1 antibody induced the disappearance of C2 and the appearance of a supershifted band, whereas the anti-STAT3 antibody caused a reduction of C1 and the appearance of a supershifted band (supershifted complexes are indicated by the arrows), as shown in Fig. 4C. Antibodies recognizing STAT2, STAT5, and IRF-9 did not induce changes in binding or mobility shifts. These data indicate that STAT1 and -3 are the major components of the RSV-inducible IRF-1 GAS complexes.

To determine whether BHA-induced inhibition of IRF-1 gene transcription was the result of changes in STAT binding to the IRF-1 GAS, we performed EMSAs using nuclear extracts prepared from A549 cells, control or infected with RSV for 3 h, in the absence or presence of BHA. As shown in Fig. 5, RSV
infection induced a significant increase in STAT binding, which was completely abolished by treatment with BHA.

IRF-7 gene transcription is controlled mainly through activation of the promoter ISRE site, which binds transcription factors of the STAT and IRF families (14). To determine whether inhibition of RSV-induced IRF-7 transcription by antioxidant treatment was also the result of changes in abundance of proteins binding to the IRF-7 ISRE, we performed EMSAs using nuclear extracts of A549 cells, control and infected with RSV for various lengths of time, in the presence or absence of BHA. As shown in Fig. 6A, a single nucleoprotein complex (C3) was formed from nuclear extracts of control cells on the IRF-7 ISRE probe. RSV infection induced the appearance of three other complexes, C1, C2, and C4, starting around 3 h postinfection, with a progressive increase in binding intensity at 6 h and a decline after 12 h of infection. Antioxidant treatment greatly reduced binding of the RSV-inducible complexes to the ISRE probe at all time points. The sequence specificity of the different complexes was examined by competition with unlabeled oligonucleotides in EMSA (Fig. 6B). C1, C2, and C4 were competed by the wild type but not by the mutated oligonucleotide, indicating binding specificity. Super-shift assays showed that anti-IRF-1 antibody induced a reduction of C4 and appearance of supershifted bands (indicated by the arrows), whereas the anti-STAT1, -2, and IRF-9 antibodies caused the disappearance or reduction of C1 and, in case of anti-STAT2, the appearance of a faint supershifted band (Fig. 6C). These data indicate that IRF-1 is the major component of the RSV-inducible C4, and STAT1, STAT2, and IRF-9, which together form the ISGF3 complex, are the major components of C1. We were not able to identify the composition of C2.

STAT proteins are constitutively expressed and, in unstimulated cells, are located in the cytoplasm. Upon activation, they are phosphorylated on specific tyrosine residues, a post-translational modification necessary for dimerization and nuclear translocation, both of which are required for DNA binding (for review, see Ref. 19). To determine whether RSV infection of A549 cells induced STAT1 tyrosine phosphorylation, we performed Western blot analysis of nuclear proteins extracted from A549 cells uninfected or infected for various lengths of

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**Fig. 5. EMSA of IRF-1 GAS binding complexes in response to antioxidant treatment.** Nuclear extracts were prepared from A549 cells, control and infected with RSV for 3 h, in the absence or presence of 400 μM BHA, and used for binding to the IRF-1 GAS in EMSA. Shown are the inducible complexes formed on the probe in response to RSV infection.

**Fig. 6. EMSA of IRF-7 ISRE-binding complexes in response to RSV infection.** A, effect of antioxidant treatment. Nuclear extracts were prepared from A549 cells, control and infected with RSV for various lengths of time, in the absence or presence of 400 μM BHA, and used for EMSA. The time (in h) after RSV infection is indicated at the top. B, competition assay. Nuclear extracts of A549 cells infected for 6 h were used in the EMSA. 2 pM unlabeled wild type (WT) or mutated (MUT) competitors were included in the binding reaction as indicated at the top. C, supershift interference assay. Nuclear extracts of A549 cells infected for 6 h were used in the EMSA in the presence of pre-immune serum (PI), anti-STAT1, -2, -3, and anti-IRF-1 and -9 antibodies. Arrows indicate supershifted complexes.
RSV-induced STAT and Tyrosine Phosphatases

As shown in Fig. 7A, RSV infection induced a time-dependent increase in tyrosine phosphorylation and nuclear translocation of STAT1, starting between 1 and 3 h postinfection. To determine whether BHA inhibition of RSV-induced STAT binding to the IRF-1 GAS was the result of inhibition of STAT phosphorylation, we performed Western blot analysis of nuclear proteins extracted from A549 cells uninfected or infected for various lengths of time, in the presence or absence of BHA, using anti-STAT1 phosphotyrosine-specific antibodies. As shown in Fig. 7B, RSV-induced STAT1 phosphorylation was completely blocked by BHA treatment. The same blot was stripped and reprobed with an anti-STAT1 antibody, showing two forms of nuclear STAT. The first form is present in uninfected and infected cells and is not affected by antioxidant treatment. The second form is RSV-inducible and disappears following BHA treatment, likely representing the tyrosine-phosphorylated STAT1. RSV infection also induced a time-dependent increase in the abundance of nuclear phosphorylated STAT1. RSV infection also induced a time-dependent increase in tyrosine phosphorylation and nuclear translocation of STAT1, starting between 1 and 3 h postinfection. To determine whether BHA inhibition of RSV-induced STAT binding to the IRF-1 GAS was the result of inhibition of STAT phosphorylation, we performed Western blot analysis of nuclear proteins extracted from A549 cells uninfected or infected for various lengths of time, in the presence or absence of BHA, using anti-STAT1 phosphotyrosine-specific antibodies. As shown in Fig. 7B, RSV-induced STAT1 phosphorylation was completely blocked by BHA treatment. The same blot was stripped and reprobed with an anti-STAT1 antibody, showing two forms of nuclear STAT. The first form is present in uninfected and infected cells and is not affected by antioxidant treatment. The second form is RSV-inducible and disappears following BHA treatment, likely representing the tyrosine-phosphorylated STAT1. RSV infection also induced a time-dependent increase in the abundance of nuclear phosphorylated STAT1, as shown in Fig. 8A. Antioxidant treatment completely abolished RSV-induced STAT3 phosphorylation, as shown in Fig. 8B. The same blot was stripped and reprobed with an anti-STAT3 antibody, showing that RSV-induced STAT3 nuclear translocation was greatly reduced by BHA treatment. Together, these data indicate that RSV-induced ROS formation is involved in STAT activation and IRF gene expression.

**RSV Infection Inhibits Tyrosine Phosphatase Activity, Which Is Restored by Antioxidant Treatment**

—— A number of receptor-associated and nonreceptor-associated tyrosine kinases have been shown to phosphorylate STAT proteins (for review, see Ref. 19). Among them, the best characterized ones are the JakS. JakS are a family of tyrosine kinases which includes Jak1, 2, 3, and Tyk2. Jak1, Jak2, and Tyk2 are ubiquitously expressed, whereas Jak3 is tissue-specific. Jak1 can form heterodimers with Jak2, Jak3, or Tyk2, depending on the activating stimulus (20). To determine whether RSV infection induced Jak activation, we measured Jak2 and Tyk2 phosphorylation by Western blot. We did not detect significant levels of Jak phosphorylation at early time points of infection, from 1 to 6 h, when RSV-induced STAT activation occurs, although there was increased Jak2 and Tyk2 tyrosine phosphorylation following stimulation of A549 cells with interferon-γ and -β, respectively (data not shown). Furthermore, an inhibitor of Jak2 activation, AG490, did not block RSV-induced STAT binding to the IRF-1 GAS or activation of the IRF-1 promoter (data not shown). We also investigated kinases belonging to the Src family, which have been shown to mediate receptor-independent STAT protein tyrosine phosphorylation (21, 22), using the broad spectrum Src kinase family inhibitors PP1 and PP2 (23). Similar to the results of AG490, neither PP1 or PP2 inhibited STAT activation and IRF-1 promoter induction following RSV infection, suggesting that other molecules might be involved. However, tyrosine phosphorylation is an important event in regulating RSV-induced STAT activation because treatment of A549 cells with a generic tyrosine kinase inhibitor, AG82, significantly reduced STAT binding to the IRF-1 GAS (Fig. 9A) and IRF-1 promoter induction (Fig. 9B), following RSV infection. Protein phosphorylation is a reversible and dynamic process, which requires not only protein kinases but also protein phosphatases (PPs). H2O2 treatment has been shown to induce an increase in the overall intracellular levels of protein tyrosine phosphorylation, and it has been suggested that one of the mechanisms mediating the biological effects of H2O2 treatment is inhibition of PPs (24). To determine whether RSV infection alters PP function, we assayed total intracellular protein-tyrosine phosphatase activity in cells uninfected or infected with RSV, in the absence or presence of antioxidant. As shown in Fig. 10, RSV infection significantly decreased protein-tyrosine phosphatase activity in the first 12 h of infection, with maximal inhibition at 3 h postinfection, when there is significant RSV-induced STAT phosphorylation, which was completely reversed by BHA treatment. Protein-tyrosine phosphatase activity returned to control levels at 24 h postinfection. To confirm the possibility that inhibition of tyrosine phosphatase activity in airway epithelial cells can lead to STAT activation, we determined STAT1 phosphorylation, by Western blot analysis, in A549 cells treated with sodium pervanade, a derivative of the tyrosine phosphatase inhibitor sodium orthovanadate. As shown in Fig. 11, there was a significant induction of tyrosine-phosphorylated STAT1 shortly after cellular treatment with the inhibitor, suggesting that modulation of phosphatases can indeed be an important mechanism of virus-induced STAT activation.
NAD(P)H Oxidase Is Involved in RSV-induced STAT Activation

An important source of inducible intracellular ROS, generated in response to a variety of stimuli, is the membrane-bound NAD(P)H oxidase system (for review, see Ref. 25). To test whether RSV-induced STAT activation was mediated through ROS production by this enzyme complex, we treated RSV-infected A549 cells with DPI, a known NAD(P)H oxidase inhibitor. As shown in Fig. 12, DPI completely abolished RSV-induced STAT binding to the IRF-1 GAS, indicating that the NAD(P)H oxidase system is an important enzyme for the generation of ROS and subsequent activation of STAT following RSV infection. Because DPI is not completely specific for NAD(P)H oxidase, we investigated the effect of inhibitors of nitric oxide synthase (\(N\)-nitro-L-arginine methyl ester), and mitochondrial electron transport (rotenone and antimycin A) on STAT activation. Although \(N\)-nitro-L-arginine methyl ester and rotenone did not have any effect on STAT binding to the IRF-1 GAS, antimycin A partially reduced STAT activation, probably by virtue of reducing the overall ROS intracellular load (data not shown).

DISCUSSION

Lower respiratory tract infections caused by RSV are characterized by profound cellular inflammation of the airway mucosa, which contributes to disease manifestations including air flow limitation, lung atelectasis/emphysema, and hypoxemia (26). The mechanisms that regulate airway inflammation in viral respiratory infections are not fully understood. However, airway epithelial cells represent a major initiator of pulmonary host defense and inflammatory reactions by their ability to synthesize and secrete soluble mediators, upon injury or infection, which are important for the recruitment and activation of immune/inflammatory cells. Indeed, airway epithelial cells infected with RSV produce a variety of these proinflammatory molecules, such as cytokines and chemokines (2).

The intracellular signaling events leading to RSV-induced gene expression are mostly unknown. Free radicals and ROS species have recently been shown to function as second messengers influencing a variety of molecular and biochemical processes, including expression of a number of genes (for review, see Ref. 5). Virus-induced ROS generation has been linked to NF-\(\kappa\)B activation and gene expression in influenza virus and human immunodeficiency virus-infected cells (27). We have shown recently that antioxidant treatment of airway epithelial cells blocked RSV-induced RANTES gene expression and protein secretion, through inhibition of de novo IRF-1 and -7 gene expression and protein synthesis and IRF-3 nuclear translocation (7). In the present study we have investigated the mechanism of inhibition of IRF induction by antioxidant treatment. We show that RSV infection of alveolar epithelial cells

A

| AG82 | 10 \(\mu\)M | 50 \(\mu\)M |
| RSV | + | + | + | + |

B

| Time (m):  | 0 | 15' | 30' | 0 | 15' | 30' |
|-----------|---|-----|-----|---|-----|-----|
| p-STAT1   |   |     |     |   |     |     |
| STAT1     |   |     |     |   |     |     |

Fig. 11. Inhibition of tyrosine phosphatases induces STAT1 phosphorylation. Cytoplasmic (Cyto) and nuclear extracts (NE) were prepared from A549 cells, control and treated with 100 \(\mu\)M sodium pervanadate for 15 and 30 min, and assayed for phosphorylated STAT1 by Western blot. Membrane was stripped and reprobed with an antibody recognizing total STAT1.

NAD(P)H Oxidase Is Involved in RSV-induced STAT Activation—An important source of inducible intracellular ROS, generated in response to a variety of stimuli, is the membrane-bound NAD(P)H oxidase system (for review, see Ref. 25). To test whether RSV-induced STAT activation was mediated through ROS production by this enzyme complex, we treated RSV-infected A549 cells with DPI, a known NAD(P)H oxidase inhibitor. As shown in Fig. 12, DPI completely abolished RSV-induced STAT binding to the IRF-1 GAS, indicating that the NAD(P)H oxidase system is an important enzyme for the generation of ROS and subsequent activation of STAT following RSV infection. Because DPI is not completely specific for NAD(P)H oxidase, we investigated the effect of inhibitors of nitric oxide synthase (\(N\)-nitro-L-arginine methyl ester), and mitochondrial electron transport (rotenone and antimycin A) on STAT activation. Although \(N\)-nitro-L-arginine methyl ester and rotenone did not have any effect on STAT binding to the IRF-1 GAS, antimycin A partially reduced STAT activation, probably by virtue of reducing the overall ROS intracellular load (data not shown).
induces IRF-1 and -7 gene transcription in a time-dependent manner; however, antioxidant treatment is completely blocked following treatment. RSV infection induces STAT protein phosphorylation, nuclear translocation, and binding to the IRF-1 and -7 promoters, an event necessary for IRF-inducible transcription, as seen with interferon stimulation (14, 15). Antioxidant treatment completely abolishes RSV-induced STAT activation by blocking tyrosine phosphorylation. Therefore, preventing STAT translocation and DNA binding to both the IRF-1 GAS and IRF-7 ISRE promoter sequences. Oxidative stress has been traditionally linked to activation of the transcription factors NF-κB and AP-1 and the ras/rac mitogen-activated protein kinase pathway (for review, see Refs. 5 and 6). However, recent studies have shown that activation of the JAK-STAT pathway is also redox-sensitive. Simon et al. (28) demonstrated that STAT1 and STAT3 are activated in response to H₂O₂ in fibroblasts, and JAK-STAT activation following stimulation with angiotensinogen II and oxidized low-density lipoprotein is inhibited by antioxidant treatment (29, 30). Little is known about the role of ROS in virus-induced STAT activation. Very recently Gong et al. (31) have shown that the hepatitis C virus NS5A protein alters intracellular calcium levels, triggering ROS formation and nuclear translocation of NF-κB and STAT3, which is completely inhibited by the use of antioxidants. Similarly, the hepatitis B virus X protein induces ROS formation through association with an outer mitochondrial anion channel, an event that leads to NF-κB and STAT3 activation, which is sensitive to antioxidant treatment, as well as manganese superoxide dismutase overexpression (32). In this study, we have shown that RSV-induced STAT activation also seems to be ROS-dependent because it is completely blocked by antioxidant treatment. The mechanism involved in ROS formation and STAT activation, following RSV infection, is not clear yet. We investigated possible tyrosine kinase signaling complexes leading to STAT activation in alveolar epithelial cells following RSV infection. The use of standard JAK2 and Src kinase inhibitors failed to block RSV-induced STAT activation, and we did not find consistent activation of Tyk2 at early time points of infection, suggesting that alternative pathways could be involved, such as the mitogen-activated protein kinase kinase, MEKK1, which has been shown to regulate STAT3 activation (33). However, we found that RSV infection significantly inhibited protein-tyrosine phosphatase activity, which was restored by antioxidant treatment. Very little information is available regarding virus-induced regulation of protein-tyrosine phosphatase activity and their role in signaling pathways activated by viral infections. However, exogenous H₂O₂ treatment has been shown to induce an increase in the overall intracellular levels of protein tyrosine phosphorylation, triggering activation of multiple signaling molecules and transduction factors, ultimately leading to gene expression (24), and it has been suggested that one of the mechanisms mediating the biological effects of H₂O₂ treatment is inhibition of protein phosphatases (24). More specifically, two studies have shown that H₂O₂ inhibits specific protein-tyrosine phosphatases, whereas antioxidants can increase protein-tyrosine phosphatase activity (34–36). It is possible that virus-induced ROS activate STAT through an imbalance between cellular tyrosine kinases and phosphatases, resulting in increased net phosphorylation and therefore activation. Interestingly, we have shown that airway epithelial cell treatment with pervanadate, a derivative of the tyrosine phosphatase inhibitor orthovanadate, can induce STAT1 phosphorylation. Similar results have been reported in other cell types, and STAT activation as well as subsequent STAT-driven gene expression occurs in a ligand-independent manner, which does not require JAK1, JAK2, or Tyk2 activity (37–39).

Intracellular ROS can be generated by different systems, including the NAD(P)H oxidase system, the mitochondrial electron transport chain, and enzymes such as xanthine oxidase, and cyclooxygenase plays an important role in the regulation of intracellular signaling cascades in various cell types including fibroblasts, endothelial cells, myocytes, smooth muscle cells, etc. (for review, see Ref. 40). In our study, treatment of RSV-infected alveolar epithelial cells with DPI, an inhibitor of the NAD(P)H oxidase complex, completely abolishes virus-induced STAT activation, suggesting that the NAD(P)H oxidase system is an important enzyme for the generation of ROS and subsequent activation of STAT, following RSV infection. Indeed, angiotsenin II-induced STAT activation occurs via the NAD(P)H oxidase system (41), and Rac1, a small GTP-binding protein that is part of the NAD(P)H complex in nonphagocytic cells, has been shown to regulate STAT3 activity (42). Because DPI is not completely specific for NAD(P)H oxidase, current studies are in progress to define better the role of NAD(P)H complex in RSV-induced ROS formation and STAT activation, by looking at membrane translocation of components of the oxidase system and by interference with its activation overexpressing dominant negative mutants of Rac1.

In summary, our study indicates that RSV-induced ROS formation, occurring likely via the NAD(P)H oxidase system, is involved in STAT activation, and subsequent IRF-1 and -7 gene expression. Induction of intracellular tyrosine phosphatase activity, following viral infection, could potentially be a very important mechanism involved in activation of molecules regulated by tyrosine phosphorylation-like STAT proteins. Identification of the molecular mechanisms involved in RSV-induced gene expression is fundamental for developing strategies to modulate the inflammatory responses associated with RSV infection of the lung.

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