Role of Xanthine Oxidase Activation and Reduced Glutathione Depletion in Rhinovirus Induction of Inflammation in Respiratory Epithelial Cells*

Alberto Papi 1, Marco Contoli 2, Pier Luigi Gasparini 3, Laura Bristot 4, Michael R. Edwards 5, Milvia Chicca 6, Marilena Leis 1, Adalberto Ciaccia 3, Gaetano Caramori 7, Sebastian L. Johnston 8, and Silvano Pinamonti 9,1

From the 4 Research Centre on Asthma and COPD and the 6 Department of Biology, University of Ferrara, 44100 Ferrara, Italy and the 8 Department of Respiratory Medicine, National Heart and Lung Institute, Wright Fleming Institute of Infection & Immunity & MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, Imperial College London, London, W2 1PG United Kingdom

Rhinoviruses are the major cause of the common cold and acute exacerbations of asthma and chronic obstructive pulmonary disease. We previously reported rapid rhinovirus induction of intracellular superoxide anion, resulting in NF-κB activation and pro-inflammatory molecule production. The mechanisms of rhinovirus superoxide induction are poorly understood. Here we found that the proteolytic activation of the xanthine dehydrogenase/xanthine oxidase (XD/XO) system was required because pretreatment with serine protease inhibitors abolished rhinovirus-induced superoxide generation in primary bronchial and A549 respiratory epithelial cells. These findings were confirmed by Western blotting analysis and by silencing experiments. Rhinovirus infection induced intracellular depletion of reduced glutathione (GSH) that was abolished by pretreatment with either XO inhibitor oxypurinol or serine protease inhibitors. Increasing intracellular GSH with exogenous H2S or GSH prevented both rhinovirus-mediated intracellular GSH depletion and rhinovirus-induced superoxide production. We propose that rhinovirus infection proteolytically activates XO initiating a pro-inflammatory vicious circle driven by virus-induced depletion of intracellular reducing power. Inhibition of these pathways has therapeutic potential.

Rhinoviruses (RV) 2 are the major cause of the commonest human acute infectious disease, the common cold (1). They are also associated with the majority of acute exacerbations of asthma (2, 3) and chronic obstructive pulmonary disease (COPD) (4, 5). No licensed effective antiviral is currently available for the treatment of the common cold (6, 7) and treatment of virus-induced asthma and COPD exacerbations is a major unmet therapeutic need (8). Understanding the mechanisms of virus-induced exacerbation of airway diseases is required to identify molecular targets for therapeutic intervention.

The mechanisms underlying virus-induced exacerbations of airway diseases are poorly understood. However, rhinoviruses are believed to directly infect airway epithelium inducing pro-inflammatory cytokine production (9–11). This leads to recruitment and activation of inflammatory cells, resulting in airway inflammation (12, 13). We have recently demonstrated that bronchial epithelial cells from asthmatic subjects have a deficient innate immune response to rhinovirus infection, responsible for: (i) increased virus replication (14, 15) that could account for increased and more persistent inflammatory responses (12); (ii) increased severity and duration of lower respiratory tract symptoms and reductions in lung function (16) in rhinovirus-induced asthma exacerbations.

Increased oxidative stress is implicated in induction of the acute airway inflammation during exacerbations of asthma and COPD (17). Oxidants are directly involved in inflammatory responses via signaling mechanisms, including the redox-sensitive activation of transcription factors such as NF-κB (18, 19).

Recent data indicate that rhinovirus and other respiratory viruses can alter cellular redox homeostatic balance toward a pro-oxidative condition (20–22). The molecular pathways responsible for such disequilibrium are virtually unknown. A recent study suggested NADPH oxidase involvement in rhinovirus-induced production of reactive oxygen species over a 6-h infection (23). In a previous study we documented that rhinovirus infection induces a rapid increase of intracellular superoxide anion (O2−), which occurs within 15 min after infection. This early pro-oxidative response was found to induce NF-κB activation and downstream pro-inflammatory molecule production (24).

O2− is a product of cellular metabolism and mainly originates from the activity of two enzyme systems: NADPH oxidase and xanthine dehydrogenase/xanthine oxidase (XD/XO) (25). Here we studied the molecular mechanisms by which rhinovirus induces rapid O2− production in respiratory epithelial cells. We also analyzed the mechanisms by which reducing agents can abolish rhinovirus-induced O2− production and thus can stabilize the intracellular redox state in respiratory epithelial cells following infection. Finally, we demonstrated that blocking the
activity of the system responsible for rhinovirus-triggered $O_2^\cdot$ generation inhibited rhinovirus-induced inflammatory mediator production in respiratory epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture

Ohio HeLa cells were obtained from the MRC Common Cold Unit, Salisbury, UK, and A549 cells, a type II respiratory cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Primary human bronchial epithelial cells (HBEC) were obtained by bronchial brushing from healthy volunteers, and cultured as previously described (14, 24, 26).

Virus Stocks

Rhinovirus type 16 (RV16, a major group rhinovirus) was obtained from the MRC Common Cold Unit. Viral stocks were prepared by infection of sensitive cell monolayers (Ohio HeLa, HeLa) as described elsewhere (24, 26). TCID$_{50}$/ml values were determined and the rhinovirus serotype was confirmed by neutralization with serotype-specific antibodies (ATCC) (27). For selected experiments rhinovirus type 1B (RV1B, minor group), obtained from the MRC Common Cold Unit, was used to evaluate whether the results were group/receptor restricted. For selected experiments filtration of the virus from inoculum, to remove viral particles, was performed as previously described (24, 26). Filtered virus stocks were used as negative control. Virus at a multiplicity of infection of 1 was used for all the experiments.

Infections, Harvesting of Cells, Preparation of Cell Homogenates, and Preparation of Membrane and Cytosolic Fractions

Confluent A549 or HBEC cells were exposed to rhinovirus, medium alone, or filtered virus (f-RV) inoculum for different time intervals (20 min to 8 h). Cell layers were thereafter washed three times in cold phosphate-buffered saline (PBS) before harvesting by scraping. Harvested cells were centrifuged and the cell pellet was resuspended in phosphate buffer (10 mM, pH 7.2). Cell lysis was obtained by repeated (three times) freezing and thawing. For preparation of cytosolic fractions, the cell homogenate was then ultracentrifuged at 20,000 g for 30 min, the cell fragments pelleted, and the supernatant (cytosol) collected. Where indicated, to obtain the membrane fraction, the cell homogenates were centrifuged at 800 × g for 10 min to separate nuclei from cell membranes. Supernatants were harvested and again centrifuged at 2,000 × g, supernatants discarded, and membrane pellets diluted in 0.1 M sucrose solution. A final centrifugation at 11,000 × g for 20 min was performed at 4 °C. Pellets containing membranes were diluted in 100 μl of PBS buffer. Protein content was determined photometrically using the Bio-Rad protein assay (Bio-Rad).

Protease and XO Inhibition

In selected experiments cells were pretreated, before infection, as follows: 12 h (0.25 to 10 mM) GSH (Sigma), or 12 h (0.25 to 2.5 mM) H$_2$S (Acqua Breta, Riole Terme SpA, Ravenna, Italy) or 4 h (20 μM) oxyxpurinol (4,6-dihydroxyxyprazol (3,4-d)pyrimidine, Sigma), a permanent inactivator of xanthine oxidase (28). Where indicated, a 4-h pretreatment with antiproteases (0.625 μM) serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) or 1.25 μM serine and cysteine protease inhibitor leupeptin (Leu), or 1.25 μM aspartic protease inhibitor pepstatin (Pep), or 1.25 μM serine protease inhibitor aprotinin (Apr), or 1.25 μM metalloprotease inhibitor phanethanoline (Phe) or cysteine protease inhibitor E-64, all from Sigma), or diluent alone was performed (29–34). Diluent was made of phosphate buffer, pH 7.2, with a maximal final concentration of 0.4%. PMSF only was previously resuspended in ethanol before dilution in PBS. Antiproteases were removed immediately before infection.

Cytosolic Fractions

Homogenates, and Preparation of Membrane and Intracellular Xanthine Oxidase

Uric Acid Kinetics

Uric acid kinetics were performed at 1 h infection to evaluate the involvement of XD/XO in rhinovirus-induced $O_2^\cdot$ generation, as uric acid represents the other end product of xanthine degradation by XO. Uric acid kinetics were spectrophotometrically monitored at 293 nm in a Uvikon spectrophotometer (Kontron) according to standardized procedures (36, 37). Measurements were based on absorbance differences in the presence or absence of SOD, after 5 min of kinetics, when the kinetic slope of cytochrome $c$ reduction was steepest. Data were normalized per mg of protein.

NADPH Oxidase Assay

NADPH oxidase assay was performed at different time intervals (20 min to 3 h) to evaluate the involvement of this system in rhinovirus-induced $O_2^\cdot$ generation. Cells homogenates were centrifuged as described above to separate nuclei from cell membranes. To reconstitute NADPH oxidase, supernatants containing membranes were centrifuged again at 40,000 × g. The reaction mixture contained 200 μl of supernatant and 50 μl of diluted membrane pellet. After 2 min, 200 μM NADPH and 5 mM MgCl$_2$ were added in the presence or absence of the specific inhibitor of NADPH oxidase diphenylene iodonium chloride (0.92 μg/ml, Sigma) (38). Cytochrome $c$ was added to a concentration of 0.1 mM and PBS, pH 7.2, to a final volume of 0.5 ml, and the reduction kinetics were monitored for 15 min at 37 °C as previously described.
Western Blot Analysis for Xanthine Dehydrogenase/Oxidase

Whole cell proteins were extracted from A549 cells as previously described (39). At least 50 mg/lane of whole cell proteins were subjected to a 4–12% Tris glycine gel electrophoresis, and transferred to nitrocellulose filters by blotting. Filters were blocked for 45 min at room temperature in Tris-buffered saline (TBS), 0.05% Tween 20, 5% nonfat dry milk. The filters were then incubated with rabbit anti-human XD/XO (LS-C26419; from LifeSpan Biosciences) for 1 h at room temperature in TBS, 0.05% Tween 20, 5% nonfat dry milk at dilution of 1:500. Filters were washed three times in TBS, 0.5% Tween 20 and after being incubated for 45 min at room temperature with goat anti-rabbit antibody conjugated to horseradish peroxidase (Dako) in TBS, 0.05% Tween 20, 5% nonfat dry milk, at a dilution of 1:4000. After three further washes in TBS, 0.05% Tween 20 visualization of the immunocomplexes was performed using ECL as recommended by the manufacturer (Amersham Biosciences). As an internal control we reprobed each filter with an anti-human actin antibody (Santa Cruz Biotechnology). The 145- and 85-kDa bands of the XD/XO system ((full-length XD and respectively (40, 41)) and the 43-kDa (actin) band were quantified using densitometry with VisionWorks® LS software (UVP) and expressed as the ratio with the corresponding actin optical density value of the same lane.

Knockdown of Xanthine Dehydrogenase Expression

RNA interference was used to specifically suppress expression of XD in A549 cells. Cells were transfected in 6-well plates with small interfering RNA (siRNA) using siPORT™ NeoFX™ Transfection Agent (Applied Biosystem), as described by the manufacturer. The following siRNA (all from Ambion) were used: siRNAs for XD (s14918; target sequence: sense, GCAUGCUAUGAGAUUGAtt; antisense, UUUAUAGCAUCCUAUUUttg), siRNA for GAPDH (4390849) and nonsilencing siRNA (4390843). Total mRNA was extracted by using the RiboPure™ kit (Ambion) as per the manufacturer’s instructions. 1 μg of mRNA was used to perform the reverse transcription assay with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). XD mRNA expression was monitored by Real Time RT-PCR using the TaqMan® Gene Expression Assay (Applied Biosystem) specific for XD (catalog number Hs00166010_m1) following the manufacturer’s recommendations. The reaction was carried out in a RotorGene™ 6000 instrument (Corbett Life Science). Results were normalized to 18S rRNA (sense, 5’-CGC CGC TAG AGG TGA AAT TCT-3’; antisense, 5’-CAT TCT TGG CAA ATG CTT TCG-3’ 300 nm each, probe, 5’-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3’, 175 nm) and expressed as XD mRNA relative levels as compared with nonsilencing siRNA-transfected cells by using the RotorGene software (Corbett Research) and the two standard curve methods for relative quantitation (42).

High Performance Liquid Chromatography (HPLC) Analysis of Intracellular GSH

A549 cells were cultured at 85% confluence, incubated with RV16, medium alone, or f-RV for different periods (20 min to 1 h), then trypsinized, collected, and harvested in cryovials with 1.2 ml of 3% metaphosphoric acid in sterile conditions to avoid GSH oxidation and finally frozen in liquid nitrogen until used. Cell homogenate was obtained and protein content was determined as previously described. Intracellular GSH concentration was evaluated by HPLC in a Kontron Instruments apparatus (Milan, Italy) equipped with a C18 hydrophobic column (5 μm particle size, 4.6 × 250 mm), a 420 pump (range 0.005–10 ml/min), a 425 gradient former, and an injection valve with a 20-μl sampling loop. Elution was carried out at room temperature in isocratic gradient (75% methanol and KH2PO4 buffer, pH 3, 1 ml/min speed). GSH was analyzed at 200 nm by a 432 UV detector (Kontron) with an IBM integrated software PC Pack. Homogenates of cells were centrifuged at 40,000 × g for 20 min at 4 °C and the supernatant collected and concentrated on Amicon Ultra 10,000 centrifugal filter devices (Millipore, Bedford, MA) to a final volume of about 300 μl. Samples were analyzed without derivatization against standards of pure lyophilized GSH (Biomedica Foscama), diluted in 1 ml of normal saline solution. The final concentration was obtained by serial dilutions of the lyophilized product. In selected experiments cells were pretreated, before infection, as previously described, for 12 h with GSH, H2S, (0.25 to 2.5 mM), or 4 h with oxypurinol (20 μM). Where indicated, a 4-h pretreatment with protease inhibitors (PMSF, 0.625 μM; Leu, 1.25 μM; Pep, 1.25 μM; Apr, 1.25 μM; E-64, 1.25 μM) or diluent were performed. All protease inhibitors were removed immediately before infection.

Rhinovirus Replication

Titration Assay in a Sensitive Cell Line—Rhinovirus replication was evaluated by titration assay in a sensitive cell line (HeLa) (9). Cells were seeded in a 96-well plate. Where indicated, subconfluent cells were treated with the highest concentration used in the study for each of the tested compounds for the time intervals previously specified (4 h for Leu, Pep, PMSF, Apr, E-64, oxypurinol; 12 h for H2S and GSH) or diluent alone before the infection. Cells were exposed for 1 h to 10-fold serial dilution of RV16, from not diluted down to 10−8 (4 wells per condition). After a 1-h infection, virus unbound to cultured cells was removed and fresh medium added. The cells were incubated in 4% minimal essential medium (Invitrogen) at 37 °C for 5 days, fixed in methanol, and stained with 0.1% crystal violet. The cytopathic effect was evaluated by visual assessment and assessment of the continuity of the monolayer. For each experiment TCID90/ml values were calculated (27).

TaqMan® Real-time PCR—A549 cells were seeded in 6-well plates at 1.7 × 105 cells/ml. Where indicated, subconfluent cells were treated with the highest concentration used in the study for each of the tested compounds for the time intervals previously specified (4 h for Leu, Pep, PMSF, Apr, E-64, and oxypurinol; 12 h for H2S and GSH) or diluent alone before the infection. Cell lysates were harvested at 4 and 8 h following the infection. Total RNA was extracted from cell lysates by using a commercially available kit (RNasy Kit, Qiagen) following the manufacturer’s recommendations. Viral RNA in cell lysates was measured by TaqMan RT-PCR. For this purpose 2 μg of total RNA were used for cDNA synthesis (Omniscript RT kit, Qiagen).
Rhinovirus Activation of Intracellular Xanthine Oxidase

FIGURE 1. Rhinoviruses-induced production of superoxide anion (O2•-) in A549 respiratory epithelial cells and HBEC. The production is independent of the NADPH oxidase system. In panels A, B, and E, confluent cells were exposed to live RV16 (closed circles), medium alone (open circles), or RV16 physically removed by filtration (f-RV, dash line, diamonds) for different time intervals (20 min to 8 h). O2•- production was evaluated in cytosolic fractions of A549 cells (A, n = 7) and HBEC (E, n = 5) and in total A549 cell homogenates (B, n = 7) by SOD-inhibitable cytochrome c reduction kinetics. C, O2•- production by a mixture of cytosolic and membrane fraction from A549 cells was evaluated at different intervals following exposure to live RV16 (black circles), RV16 physically removed by filtration (open circles), medium alone (diamonds), without (continuous line) or with (dashed line) NADPH added to the reaction mixture. The addition of NADPH did not affect RV16-induced cytosol O2•- production (n = 5). D, O2•- production was not changed when a specific inhibitor of NADPH oxidase (diphenylene iodonium chloride) was added to the reaction mixture (RV16 physically removed by filtration (open circles), medium alone (diamonds) or live RV16 infection (black circles), without (continuous line) or with (dashed line) NADPH) confirming lack of involvement of NADPH oxidase (n = 5). F and G, O2•- production evaluated in the cytosolic fraction of HBEC (f, n = 5) and A549 cells (G, n = 7) exposed to live RV18 (closed circles), medium alone (open circles), or RV1B physically removed by filtration (dash line, diamonds). Newly generated O2•- was measured in each sample and expressed as micromolar and normalized per mg of protein (***, p < 0.001; *, p < 0.05 compared with medium alone treated cells and f-RV inoculated cells).

TagMan quantitative PCR was carried out using primers and probe for rhinovirus (sense, 5′-GTG AAG AGC CSC RTG TGC T-3′ 50 nM; antisense, 5′-GCT SCA GGG TTA AAG TTA GCC-3′ 300 nM; probe, 5′-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3′, 175 nM) and 18S rRNA (see above) (15). Reactions consisted of 2 μl of cDNA (cDNA for 18S was diluted 1:100), 12.5 μl of 2× QuantiTect Probe PCR Master Mix (Qiagen), primers, and probes at the final concentrations listed above and RNase-free water to a total volume of 25 μl. Reactions were performed on a Rotor-Gene™ 6000 instrument (Corbett Life Science). Viral RNA expressions were normalized to 18S rRNA and compared with standard curves and expressed as copies per μg of RNA.

Enzyme-linked Immunosorbent Assays for Chemokines

Subconfluent A549 cells were pretreated for 4 h with oxypurinol (20 μM) before RV16 or f-RV inoculum or medium alone treatment. After a 1-h infection unbounded virus was removed and fresh medium added. Supernatants were harvested at 4 h and levels of IL-8 and GRO-α were assessed using commercially available enzyme-linked immunosorbent assay kits (R&D System) following the manufacturer’s instructions. Detection limits for IL-8 and GRO-α enzyme-linked immunosorbent assay were ~10 and 15 pg/ml, respectively.

NF-κB Transcription Factor Activation

Nuclear extracts were prepared from A549 cells using the Nuclear Extract Kit (Active Motif). NF-κB activation was assessed in A549 cell nuclear extracts using the TransAM™ p65 Transcription Factor Assay Kit (Active Motif) following the manufacturer’s recommendations. Nuclear extract of Jurkat cells provided by the manufacturer (Active Motif) were used as positive controls.

Statistical Analysis

Group data were expressed as mean ± S.E. Analysis of variance was used to determine differences between groups. Paired or unpaired Student’s t tests were performed after the analysis of variance when appropriate. All experiments were carried out at least 5 times. Bonferroni adjustment was applied.
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**RESULTS**

Rhinovirus-induced \( O_2^- \) Production in Respiratory Epithelial Cells Is Cytosolic Not Membrane Associated—\( O_2^- \) production was evaluated by SOD-inhibitable cytochrome \( c \) reduction kinetics. In our previous study we found that RV16 infection rapidly induced intracellular \( O_2^- \) production, which was maximal at 1 h in A549 cells, a type II respiratory epithelial cell line (24, 35). That study evaluated intracellular \( O_2^- \) generation, i.e. cell membranes were precipitated before SOD-inhibitable cytochrome \( c \) reduction assay was performed. Because other workers had implicated NADPH oxidase in RV induction of reactive oxygen species (23) and because NADPH oxidase is a membrane bound system, we first sought to identify the cellular site of \( O_2^- \) production. In the search of cellular sources of RV16-induced \( O_2^- \) production, we first confirmed our previous findings of rapid induction of \( O_2^- \) by RV16 in membrane-free cytosolic fractions (Fig. 1A). We next investigated total cell homogenates, which included cell membranes (Fig. 1B) and found that \( O_2^- \) production was again rapidly increased after RV16 infection, detectable at 20–120 min in A549 total cell homogenates. Because \( O_2^- \) production (normalized per mg of protein) was almost doubled in the absence of cell membranes (see y axes of Fig. 1, A and B), whereas protein quantities were halved (14.8 \( \pm \) 1.9 mg/experiment versus 32.1 \( \pm \) 3.1 mg/experiment, in the absence and presence of cell membrane, respectively), we reasoned that the vast majority, if not all of RV16 early production of \( O_2^- \) came from the cytosolic fraction. To investigate membrane production, direct \( O_2^- \) production was evaluated in cell membranes obtained from A549 epithelial cells infected with RV16. No induction was observed (\( p = 0.82 \) versus unstimulated controls) at any time point (20, 60, 120, 180, 240, 300, 360, 420, and 480 min), indicating that the cytosol, not the membrane, is the site of RV16 induced \( O_2^- \) production.

**Rhinovirus Induction of \( O_2^- \) Is Independent of the NADPH Oxidase System**—The fact that \( O_2^- \) production occurs in cytosolic fractions and not cell membranes suggests that a membrane-bound enzyme system such as NADPH oxidase is not involved, however, this finding does not rule out such a possibility. We therefore investigated whether rhinovirus-induced \( O_2^- \) production could derive from the activity of NADPH oxidase in experimental conditions where both the cytosolic and membrane fractions of the homogenates were pooled together. The membrane fractions were added, together with the substrate of NADPH oxidase, NADPH, in the presence and absence of an inhibitor of the NADPH enzyme system, diphenylene iodonium chloride (38). NADPH addition would increase \( O_2^- \) production if production is NADPH oxidase-dependent. We found that both the addition of NADPH (Fig. 1C) and diphenylene iodonium chloride (Fig. 1D) to the reaction mixture did not change \( O_2^- \) production induced by RV16 infection, indicating that the NADPH oxidase system is not relevant to early \( O_2^- \) generation induced by RV16. Thus, from now on, intracellular \( O_2^- \) production and intracellular redox equilibrium will be solely evaluated in cytosolic fractions.

**Rhinovirus Induction of \( O_2^- \) in Primary Bronchial Epithelial Cells Is Virus-specific and Serotype/Receptor Independent**—We previously demonstrated RV induction of \( O_2^- \) in A549 cells was

where indicated. A probability value of <0.05 was considered significant.
Rhinovirus Activation of Intracellular Xanthine Oxidase

![Image](396x60x603.png)

**FIGURE 3. Effects of protease inhibitors on rhinovirus-induced cytosolic superoxide anion \( (\mathrm{O}_2^-) \) production.** A, effects of serine protease inhibitors PMSF, leupeptin (Leu), pepstatin (Pep), aprotinin (Apr) on RV16-induced cytosolic \( \mathrm{O}_2^- \) production in A549 cells \( (n = 5) \). B (\( n = 7 \)) and C (\( n = 6 \)), effects of metalloprotease inhibitor phenanthroline (Phe) (B) or cysteine protease inhibitor E-64 (C) on RV16-induced cytosolic \( \mathrm{O}_2^- \) production in A549 cells. In A–C, where indicated (+) cells were pretreated for 4 h with protease inhibitors, then exposed, for 20 min (empty bars) or 1 h (filled bars) to RV16 or medium alone (in A, *** \( p < 0.001 \) versus all other conditions; B and C, *** \( p < 0.001 \) versus medium alone treated cells with or without inhibitor pre-treatment).

**Rhinovirus Activation of Intracellular Xanthine Oxidase (XD/XO) System**—In other experimental systems, the XD/XO enzymatic system is able to produce \( \mathrm{O}_2^- \) when it is converted to the oxidase form by proteolytic activity, exerted by a serine protease, which partially hydrolyzes the enzyme to its active form \( (40, 45) \). To investigate the mechanisms of rhinovirus induction of XD/XO, cells were next infected in the presence or absence of serine protease inhibitors, or with cysteine or metalloprotease inhibitors as controls. RV16 inoculation \( (20 \text{ min to } 1 \text{ h}) \) failed to generate \( \mathrm{O}_2^- \) in the cytosol when epithelial cells were pretreated for 4 h with protease inhibitors PMSF, leupeptin (Leu), pepstatin (Pep), or aprotinin (Apr), which all act as serine protease inhibitors \( (39, 40) \). Serine protease involvement was confirmed as neither the metalloprotease inhibitor phenanthroline \( (\text{Phe}, \text{Fig. 3B}) \) nor the cysteine protease inhibitor E-64 \( (\text{Fig. 3C}) \) \( (29–34) \) had any effect on rhinovirus induction of \( \mathrm{O}_2^- \). This data confirm the involvement of specific proteolytic mechanisms mediated by serine proteases in rhinovirus-induced activation of XO. To directly evaluate the effects of RV infection on XD/XO proteins, we performed Western blotting analysis using an antibody able to recognize XD/XO expression. The 145-kDa band corresponding to full-length XD was significantly reduced at 30 min \( (2\text{-fold}; \ p < 0.05) \) and at 1 h \( (4\text{-fold}; \ p < 0.01) \) after RV16 infection as compared with uninfected control cells \( (p < 0.05) \). Such a reduction was paralleled by 2-fold increased expression of the 85-kDa band, expected, uric acid was rapidly degraded by uricase confirming that the XD/XO system was activated by rhinovirus infection \( (\text{Fig. 2A}) \). Similar findings were observed in homogenate samples obtained from HBEC, in the same experimental conditions \( (\text{Fig. 2B}) \), confirming rhinovirus activation of XD/XO in primary cells. The fact that following RV1B infection the kinetics of uric acid production were identical \( (\text{Fig. 2C}) \) to those observed with RV16 \( (\text{Fig. 2A}) \) indicates that the effect is receptor independent. To further confirm the role of the XD/XO enzymatic system in \( \mathrm{O}_2^- \) production we performed experiments in the presence of oxypurinol, a permanent inactivator of the oxidase form of the enzyme \( (\text{XO}) \), the only form of the enzyme able to produce \( \mathrm{O}_2^- \) \( (28) \). At 1 h infection RV16-induced cytosol \( \mathrm{O}_2^- \) production was completely quenched when A549 cells were pretreated with oxypurinol \( (\text{Fig. 2D}) \). Similar findings were observed in homogenate samples obtained from HBEC in the same experimental conditions \( (\text{Fig. 2E}) \), as in both cases oxypurinol reduced \( \mathrm{O}_2^- \) production to levels observed with medium or diluent alone or inactivated virus.

**Rhinovirus Induces Proteolytic Activation of XD/XO Enzymatic System**—To confirm that \( \mathrm{O}_2^- \) is generated via the XD/XO enzyme system, we next investigated RV induction of uric acid, the other product, besides \( \mathrm{O}_2^- \), of XO degradation of the purine base xanthine. These experiments were conducted at 1 h after infection, i.e. at the peak of \( \mathrm{O}_2^- \) generation. Fig. 2A shows the kinetics of uric acid production, after addition of substrate xanthine, in cytosolic fractions of A549 cell homogenates after exposure for 1 h to RV16, f-RV16, or medium alone. Uric acid was detected only in RV16-infected samples. Its identification was confirmed by addition, after 15 min of the kinetic assay, of uricase, which degrades uric acid to allantoin \( (36, 37) \). As
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**Rhinovirus Depletes Intracellular Reduced GSH**—To investigate the consequences of RV-induced \( \text{O}_2^\cdot \) production on intracellular redox equilibrium, we evaluated whether the concentration of the intracellular reducing agent GSH is modified by RV16 infection. In A549 cells, as the duration of RV16 infection increased, endogenous stores of GSH were progressively reduced and complete depletion was observed at 1 h after infection (Fig. 5A). Similar results were found with RV1B (Fig. 5B). The HPLC absorbance peak for GSH is representatively shown for medium-treated cells in Fig. 5C and depletion of the HPLC peak in RV16-infected cells is representatively shown in Fig. 5D. As observed with \( \text{O}_2^\cdot \) induction, RV16-induced depletion of intracellular GSH at 1 h after infection was completely inhibited when A549 cells were pretreated with either oxypurinol or serine protease inhibitors, but not with metalloprotease inhibitor phenanthroline (Phe) or cysteine protease inhibitor (E-64) (Fig. 5E). Thus, RV16-induced depletion of intracellular GSH occurs via XO activation and subsequent cytosol \( \text{O}_2^\cdot \) production. The same findings were also observed when A549 cells were infected with minor group RV1B (Fig. 5F), to indicate that the effect was receptor independent.

**Increasing Intracellular GSH**

**XD Knockdown Reduces Rhinovirus-induced \( \text{O}_2^\cdot \) Production**—To further confirm the role of the XD/XO system in rhinovirus-induced \( \text{O}_2^\cdot \) production, we performed experiments in which XD expression in A549 cells was knocked down by siRNA. Transfection of XD siRNAs resulted in marked suppression of XD mRNA expression as compared with scrambled siRNA-transfected cells (Fig. 4D), with a peak of inhibition at 24 h. At this time point, XD knockdown suppressed RV16-induced \( \text{O}_2^\cdot \) production spectrophotometrically measured by SOD-inhibitable cytochrome c reduction kinetics (Fig. 4E). Control siRNAs had no effect either on XD mRNA expression (Fig. 4D) or on RV16-induced \( \text{O}_2^\cdot \) production (Fig. 4E).

**Inhibits Rhinovirus-induced Intracellular GSH Depletion and \( \text{O}_2^\cdot \) Production**—Because reduction of intracellular reducing power is "per se" a known mechanism of activation of XO (46, 47), we next investigated whether by increasing intracellular GSH with the reducing agents \( \text{H}_2\text{S} \) or exogenous GSH, we could block the activation of XO induced by RV infection. We first showed that pretreatment of A549 cells with \( \text{H}_2\text{S} \) and exogenous GSH increased intracellular GSH levels in a dose-dependent manner (Fig. 6A). A549 cells were then pretreated for 12 h with 2 mM \( \text{H}_2\text{S} \) to enhance intracellular GSH (Fig. 6B) and then infected for 1 h with RV16. No reduction of endogenous GSH was observed after a 1-h RV16 infection (Fig. 6C), confirming that enhancing intracellular GSH protected cells against virus-induced GSH depletion. Similar protection was observed with 10 mM exogenous GSH (data not shown) treatment before the infection. Pretreatment with either 2 mM \( \text{H}_2\text{S} \) or 10 mM exogenous GSH, not only increased intracellular GSH levels but also completely inhibited RV16 induced \( \text{O}_2^\cdot \) production at 1 h after infection, as assessed by SOD-inhibitable cytochrome c reduction assay.

**FIGURE 4.** Effect of rhinovirus on the XD/XO system and effect of XD knock-down on rhinovirus-induced superoxide anion production. A–C, Western blotting analysis of the XD/XO system. Where indicated (+) A549 cells were pretreated 4 h with protease inhibitors (cysteine protease inhibitor E-64 or the serine protease inhibitor aprotinin (Apr)), then exposed, for 30 min or 1 h to RV16 or medium alone. A, full-length XD (145 kDa band) and the 85-kDa band containing the C-terminal active site of the enzyme are shown on a representative film. Actin is shown as loading control. B and C, densitometric analyses of Western blotting assays are represented (n = 3, *, p < 0.05 versus medium alone treated cells). D, XD mRNA expression in A549 cells transfected with medium alone (white bars), GAPDH siRNA (gray bars), and XD siRNA (black bars) (n = 3, *, p < 0.05 versus all corresponding conditions). E, superoxide anion production following a 1-h RV16 infection in A549 cells transfected with medium alone (white bar), nonsilencing siRNA (bright gray bar), GAPDH siRNA (dark gray bar), and XD siRNA (black bar) (n = 3, *, p < 0.05 versus RV16-infected cells in all other experimental conditions).

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**containing the active site, at 1 h infection (p < 0.05) (Fig. 4, A and B). By measuring the XD/XO ratio we found a progressive significant reduction at 30 min and 1 h after RV16 infection as compared with uninfected control cells (3-fold; p < 0.05 and 10-fold; p < 0.01, respectively) (Fig. 4C). Moreover 4-h serine-protease inhibitor Apr, but not 4-h cysteine-inhibitor E64 pre-treatment, abolished XD cleavage (Fig. 4, A–C).**

**XD Knockdown Reduces Rhinovirus-induced \( \text{O}_2^\cdot \) Production**—To further confirm the role of the XD/XO system in rhinovirus-induced \( \text{O}_2^\cdot \) production, we performed experiments in which XD expression in A549 cells was knocked down by siRNA. Transfection of XD siRNAs resulted in marked suppression of XD mRNA expression as compared with scrambled siRNA-transfected cells (Fig. 4D), with a peak of inhibition at 24 h. At this time point, XD knockdown suppressed RV16-induced \( \text{O}_2^\cdot \) production spectrophotometrically measured by SOD-inhibitable cytochrome c reduction kinetics (Fig. 4E). Control siRNAs had no effect either on XD mRNA expression (Fig. 4D) or on RV16-induced \( \text{O}_2^\cdot \) production (Fig. 4E).
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**FIGURE 5. Effect of RV infection on intracellular reduced GSH concentration in A549 and modulation by oxypurinol or protease inhibitors.** Intracellular GSH was evaluated by HPLC in A549 cells incubated with RV16 (A, continuous line), RV1B (B, continuous line), or medium alone (A and B, dashed line) for 20 min to 1 h (n = 5, ***p < 0.001 versus control samples). C, representative HPLC profiles of intracellular GSH detection in A549 cells treated for 1 h with medium alone. D, representative HPLC profiles of intracellular GSH detection in A549 cells treated for 1 h with RV16. In panels C and D an arrow indicates the position of the GSH peak. E (n = 5), HPLC evaluation of intracellular GSH in A549 cells exposed for 1 h to medium alone (white bar), live RV16 (RV16), or RV16 physically removed by filtration (f-RV16, light gray bar). Where indicated A549 cells were pretreated for 4 h with oxypurinol (Oxy) or protease inhibitors PMSF, Leu, Pep, Apr, Phe, and E-64, then exposed for 1 h to medium alone (gray bars) or live RV16 (black bars). In panel F (n = 5) A549 cells were exposed to RV1B (***, p < 0.001).

In these experiments uric acid production was similarly suppressed (Fig. 6E). Thus, the results indicated that increasing GSH intracellular storage with exogenous GSH or H₂S completely inhibited rhinovirus-induced XO activation and O₂⁻ production.

**XO Inhibition Reduces Rhinovirus-induced Chemokine Production—**RV infection of bronchial epithelial cells induces the expression of several proinflammatory cytokines, including many that are involved in neutrophil chemotraction and activation (i.e. IL-8 and Gro-α). Neutrophil inducing cytokines are not effectively suppressed by the currently available asthma therapies, i.e. steroids or long acting β agonists (10). Because the induction of these mediators occurs through oxidative sensitive pathways (e.g NF-κB signaling activation (17)), we evaluated whether oxypurinol inhibition of XO-mediated O₂⁻ generation affects rhinovirus-induced IL-8 and Gro-α production in A549 respiratory epithelial cells. Significant induction of IL-8 and Gro-α was apparent at 4 h post-RV16 infection (Fig. 6, A and B). Oxypurinol pre-treatment significantly reduced both IL-8 (Fig. 7A) and Gro-α RV16-induced production (Fig. 7B), whereas diluent had no effect. These data confirmed that inhibition of XO was effective in suppressing rhinovirus-induced neutrophil chemokine production.

**Effect of Oxypurinol on NF-κB Activation, Modulation by Oxypurinol—**To assess whether rhinovirus infection activates NF-κB and whether this is mediated by O₂⁻ production, we measured activated NF-κB in nuclear extracts in A549 cells following RV16 infection with or without a 4-h oxypurinol pretreatment. p65 nuclear concentration was 40.7 ± 6.3 pg/μl in unstimulated conditions. In accordance with previous data (24, 26) we found that 30 min RV16 infection significantly induced p65 nuclear translocation (2-fold versus unstimulated; p < 0.05). Four h pre-treatment with 20 μM oxypurinol significantly inhibited RV16-induced NF-κB activation (p < 0.05 versus RV16-infected cells not pretreated) (Fig. 7C). These data indicate that rhinovirus-induced O₂⁻ is involved in the activation of the NF-κB-dependent pro-inflammatory pathways.

**Effect of Tested Compounds on Rhinovirus Replication and Infection—**Control experiments were performed to assess whether any of the antioxidant approaches used above had any antiviral activity that could provide an alternative explanation for our findings. HeLa cells were infected with RV16 for 1 h, i.e. until the peak of O₂⁻ generation was reached and intracellular GSH fully depleted, in the presence or absence of antiproteases, oxypurinol or GSH, or H₂S pretreatment for the time intervals previously specified for each compound. The chosen concentrations for each compound were the highest utilized in the present study. No difference was found in the progression of RV16-
induced cytopathic effects measured on a daily basis by visual assessment (data not shown). At 5 days there was no difference in virus yields expressed as mean TCID50/ml values between treated and control samples for all tested compounds (Fig. 8A). These results were confirmed by TaqMan PCR assay showing progressive rhinovirus replication (i.e. increased rhinovirus RNA), which was not affected by antiprotease pretreatment (data not shown at 4 h infection; data at 8 h represented in Fig. 8B).

DISCUSSION

In this study we found that O2\textsuperscript{\textcircled{.}} generation induced by rhinovirus infection is initiated by proteolytic activation of the XD/XO enzyme system. Consequently, newly generated O2\textsuperscript{\textcircled{.}} leads to progressive depletion of intracellular GSH storage, a condition that can further activate the XD/XO system. Inhibition of XO activity completely abolished rhinovirus-induced O2\textsuperscript{\textcircled{.}} production and intracellular GSH depletion, as did a variety of serine protease inhibitors. We also found that by enhancing intracellular GSH storage with exogenous H2S or GSH rhinovirus infection was rendered unable to induce O2\textsuperscript{\textcircled{.}} production and to affect intracellular GSH levels.

Several studies have described oxidant generation following respiratory virus infection both in vitro and in vivo (20, 48). In some studies a role for virus-induced oxidants in the production of inflammatory responses/mediators has been identified (49). However, the molecular mechanisms regulating generation of oxidant species by viruses in biological systems have never been fully investigated.

The findings of the present study indicate a complex mechanism of oxidant induction following activation of XO induced by rhinovirus infection (Fig. 9). A “vicious circle” would represent the final scenario where activation of XO, initiated immediately after infection via proteolysis of XD to XO, is thereafter implemented via a non-proteolytic mechanism mediated by oxidative consumption of intracellular reducing capacity via depletion of GSH stores. Depletion of intracellular reducing agents is a known mechanism of activation of XO and O2\textsuperscript{\textcircled{.}} production (46, 47). The involvement of rhinovirus-induced oxidants in GSH consumption was confirmed by the finding that, when XO activation was inhibited, rhinovirus infection did not result in GSH depletion. The sequence of events represented in Fig. 9 is supported by the timing of the different steps involved, with O2\textsuperscript{\textcircled{.}} production being rapidly induced 20 min after infection (Fig. 1), whereas GSH depletion is undetectable 20 min after infection and thereafter progressively increases for 40 min, being complete at 60 min after infection (Fig. 5, A and B). The fact that in a reducing environment no uric acid was produced and that the intracellular concentration of GSH was unchanged after rhinovirus infection confirms the inverse relationship between GSH intracellular concentra-
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FIGURE 7. Effect of oxypurinol pre-treatment on RV16-induced chemokine (IL-8 and GRO-α) production and on NF-κB transcription factor activation. A (n = 5) and B (n = 5), A549 cells were exposed for 1 h to medium alone (Medium), RV16 physically removed by filtration (f-RV16) or live RV16 (RV16). Where indicated cells were pretreated for 4 h with oxypurinol (Oxy+RV16) or diluent (Diluent+RV16) before the infection. Supernatants were harvested at 4 h and levels of IL-8 (panel A) and GRO-α (panel B) were assessed (***, p < 0.001). C, A549 cells were exposed for 20 min to RV16 with (dark gray bar) or without (black bar) 20 μM oxypurinol (Oxy) after a 4 h pretreatment and NF-κB activation was assessed in nuclear extracts (n = 3, *, p < 0.05 versus medium alone).

FIGURE 8. Effect of tested compounds on rhinovirus replication and infectivity. Where indicated cells were treated for 4 h (oxypurinol (Oxy), serine protease inhibitor PMSF, serine and cysteine protease inhibitor Leu, aspartic protease inhibitor Pep, serine protease inhibitor Apr, metalloprotease inhibitor Phe, cysteine protease inhibitor E-64 (E64), diluent (Diluent)) or 12 h (H2S, GSH) with the highest concentration used in the study for each of the tested compounds before the infection. Rhinovirus infectivity and replication were evaluated by performing titration assays (A, n = 3) in a sensitive cell line (HeLa) and (B, n = 3) by TaqMan RT-PCR for rhinovirus RNA at 8 h after the infection.

Moreover, at variance with Kaul and colleagues (23), we employed oxypurinol, which completely inactivates XO by direct binding to the enzyme active site (28), and not allapurinol, which only partially inhibits the enzyme. Treatment with oxypurinol completely abolished rhinovirus-induced O2− generation, thus confirming the specificity of our findings on XO activation. These and other differences between the two studies, in particular different samples for analyses (intracellular versus extracellular compartments) and different timing are likely explanations for the different results reported. In the experimental setting evaluating the functional effect of oxypurinol on cytokine production, to exclude nonspecific interference, we tested the effect of oxypurinol on IL-1β-induced IL-6 production. This is a pathway for which mechanisms other than oxidant generation are considered relevant (52, 53). We found that IL-1β significantly induced IL-6 in a dose-dependent manner and that oxypurinol does not affect IL-6 production induced by 4-h IL-1β stimulation (data not shown).

Pretreatment with all four serine antiproteases investigated, but not the cysteine and metalloprotease inhibitors phenanthroline and E-64, also completely prevented O2− production in response to rhinovirus infection. Previous studies have documented that limited proteolysis of XD with serine proteases converts the enzyme to the active XO form (40). With the exception of phenanthroline and E-64, all antiproteases here utilized can act as serine protease inhibitors, or serine-cysteine protease inhibitors (29, 30, 32). Pepstatin, an inhibitor of aspartate proteases, may also act on serine proteases because of active site target similarities (31). The fact that phenanthroline and E-64, two protease inhibitors for which no serine protease inhibitory activity is documented, do not prevent rhinovirus-induced O2− production and intracellular GSH depletion supports the serine specificity of the pathway of activation of XD to XO following rhinovirus infection.

Not only activity but also the amount of XD protein was found to be decreased after rhinovirus infection with a parallel increase of the proteolytic fragment containing the active site of protein and XO activation. Previous studies have documented GSH depletion following viral infections (50), however, the underlying mechanisms were not described. The involvement of a NADPH oxidase-like enzyme in rhinovirus-induced oxidative stress has been previously described (23). In contrast to the study by Kaul and colleagues (23), our study focuses strictly on the early oxidative events occurring within cells immediately after rhinovirus infection (with a peak at 1 h after infection). Also, by using a method able to specifically detect newly generated O2−, i.e. the SOD-inhibitable cytochrome c reduction assay (51), we directly evaluated O2− intracellular production, and not oxidative stress in general.
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the enzyme. Further confirmation comes from silencing experiments showing that XD knockdown suppresses rhinovirus-induced $O_2^-$ production.

Control experiments were performed to evaluate the effects of the compounds used in the study on rhinovirus replication, to assess whether they have antiviral activities that could provide an alternative explanation for our findings. Virus binding to host cells, virus cell entry, and infectivity would be the most relevant events in our experimental conditions, where the mechanisms analyzed begin a few minutes after the infection has started (24). By using HeLa cells we were able to examine the effects of the tested compounds independently from the up-regulatory effect of rhinovirus on its own receptor (ICAM-1) observed in respiratory epithelial cells (26). Indeed, ICAM-1 surface expression decreases on rhinovirus-infected HeLa cells in parallel with the appearance and severity of cytopathic effects (data not shown). Moreover, we further exclude any direct anti-RV16 effect of the proteases used in the present study by assessing viral replication by TaqMan Real Time PCR (Fig. 8B). Taken together these experiments demonstrated that the tested compounds at the highest concentrations used in our experimental setting had no effect on rhinovirus replication and infectivity.

Although we cannot exclude the involvement of rhinovirus 3C proteolytic enzyme in XD/XO activation, we believe this possibility is unlikely to be relevant in our experimental conditions where oxidant activation was already detectable at 20 min and peaked at 1 h after infection, whereas 2–4 h infection is required for rhinovirus 3C protease to be produced (54).

Despite the effort and resources expended, no antiviral drugs are currently marketed for the prevention or treatment of rhinovirus infection (6). In the absence of effective anti-viral therapies, development of therapies that blocked the inflammatory responses to infection would be a major advance. Our demonstration of the molecular mechanisms by which rhinovirus activates oxidant generation, a crucial step in the complex inflammatory response to infection (23, 24) could open new possibilities in the search for therapeutic targets for future intervention. In particular, the documentation that: (a) exogenous interventions able to increase intracellular reducing agent storage can block rhinovirus-mediated activation of the vicious circle that leads to sustained production of $O_2^-$, and (b) inhibition of XO, with protease inhibitors or specific inhibitors (oxypurinol) inhibits $O_2^-$ and pro-inflammatory mediator production, indicate promising options for the development of treatment for rhinovirus-induced diseases including the common cold and exacerbations of asthma and COPD.

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