The IκB Kinase Is a Key Factor in Triggering Influenza A Virus-induced Inflammatory Cytokine Production in Airway Epithelial Cells*

Daniela Bernasconi†, Carla Amici‡, Simone La Frazia‡, Angela Ianaro§, and M. Gabriella Santoro¶¶

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Influenza A viruses continue to represent a severe threat worldwide, causing large epidemics and pandemics responsible for thousands of deaths every year. Excessive inflammation due to overabundant production of proinflammatory cytokines by airway epithelial cells is considered an important factor in disease pathogenesis. Here we report that influenza A virus induced IκB kinase (IKK) activity in human airway epithelial A549 cells, resulting in persistent activation of nuclear factor-κB (NF-κB), a critical regulator of the inflammatory response. Although lung epithelial cells are highly sensitive to stimulation of the IKK/NF-κB pathway by influenza virus infection, NF-κB was not activated in several non-pulmonary cells permissive to the virus, indicating a cell-specific response. Moreover, NF-κB was not essential for virus replication but triggered the expression of proinflammatory cytokines in infected lung cells and was directly responsible for production of high levels of interleukin-8, a chemokine associated with inflammation-induced inflammation and airway pathology. We also report that 9-deoxy-Δ12,13,14-dihydro-prostaglandin D2, a cyclopentenone prostaglandin with therapeutic efficacy against influenza in preclinical studies, was a powerful inhibitor of influenza virus-induced IKK activity and interleukin-8 production by human pulmonary cells. The results identify IKK as an important factor in triggering influenza virus-induced inflammatory reactions in pulmonary epithelium, suggesting novel therapeutic approaches in the treatment of influenza.

Influenza, a highly contagious acute respiratory illness that affects all age groups, is responsible for an average of 36,000 deaths and 114,000 hospitalizations per year in the United States alone (1). The etiological agents of the disease, the influenza viruses or orthomyxoviruses, are enveloped, negative-stranded RNA viruses classified in three types (A, B, and C), of which the A type is clinically the most important. In recent history, highly pathogenic strains of influenza A virus with elevated case fatality rate have suddenly emerged (2). Influenza A virus replicates throughout the respiratory tract, where the viral antigen is found predominantly in the epithelial cells. The clinical responses range from mild disease to fatal viral pneumonia. Although the mechanisms underlying the expression of symptoms and the development of secondary complications that may result in respiratory failure are still not well understood, excessive inflammation due to overabundant production of proinflammatory cytokines and lung inflammatory infiltrates is considered an important factor in disease pathogenesis (2–4). Intense infiltration of inflammatory cells in the lung epithelial layer has been associated with the release of chemotactic cytokines, in particular, interleukin (IL)1-β, by virus-infected airway epithelial cells (5, 6). Interestingly, the expression of most cytokines induced by influenza A virus is regulated by the cellular nuclear factor-κB (NF-κB).

NF-κB is a critical regulator of the immediate early pathogen response, playing an important role in promoting inflammation and viral gene expression (7, 8). NF-κB normally exists as an inactive cytoplasmic complex bound to inhibitory proteins of the IκB family and is induced in response to a variety of pathogenic stimuli, including proinflammatory cytokines and viral infection. In the case of proinflammatory cytokines, induction requires activation of the IκB kinase (IKK) complex containing two catalytic subunits (IKKα and IKKβ) and the IKKγ regulatory subunit, which phosphorylates IkB, triggering their ubiquitination and proteasome-mediated degradation (6). Release of IκBs results in nuclear translocation of NF-κB and its binding to DNA at specific κB sites, rapidly inducing a variety of genes encoding, among others, cell adhesion molecules, inflammatory and chemotactic cytokines, cytokine receptors and enzymes that produce inflammatory mediators (9).

It is well known that infection with different DNA and RNA viruses, including orthomyxoviruses, can trigger NF-κB activation via different signaling mechanisms (7). In the case of influenza virus infection, however, little is known regarding both the signaling pathway utilized by the virus and the role of the nuclear factor in the infection process. In the present report, we show that influenza A virus induces a persistent (up to 48 h) activation of NF-κB in human airway epithelial A549 cells. Influenza A virus mimics proinflammatory cytokines inducing NF-κB by activation of the IκB kinase, followed by IκBα and IκBβ degradation. Interestingly, although lung cells are highly sensitive to influenza-induced NF-κB activation, NF-κB induction is not a general response of

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† To whom correspondence should be addressed. Tel.: 39-06-7259-4822; Fax: 39-06-7259-4821; E-mail: santoro@bio.uniroma2.it.
‡ To the Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome 00133, Italy and ¶¶ Department of Experimental Pharmacology, University of Naples Federico II, Via D. Montesano, Naples 49-80131, Italy.
the infected cell to influenza virus and was not detected in a variety of non-pulmonary cells permissive to the virus. Furthermore, IKK-mediated NF-κB activity is not essential for virus replication but triggers the expression of several proinflammatory cytokines and is directly responsible for inducing IL-8 production by infected lung cells.

Finally, we have previously shown that cyclo pentene nonprostaglandins (PGs) of the A and J type block TNFα-induced NF-κB activation by direct inhibition and modification of the IKKβ subunit (10). We now show that 9-deoxy-3α,12,13,14-dihydro-PG D2 (Δ12-PG D2), a natural cyclo pentene metabolite of prostaglandin D2 that is physiologically present in human body fluids (11) and possesses therapeutic efficacy in a mouse model of lethal influenza A virus infection (12), is a powerful inhibitor of influenza A virus-induced IKK and NF-κB activities and that this effect results in the block of virus-induced IL-8 production by human pulmonary cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Treatments—Human A549 alveolar type II-like epithelial cells, 293 transformed primary embryonal kidney cells, SH-SY5Y neuroblastoma cells and HaCaT keratinocytes, and Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) were grown at 37 °C in a 5% CO2 atmosphere in F-12 medium (A549) and Dulbecco's modified Eagle's medium (293, SH-SY5Y, HaCaT, and MDCK) (Invitrogen), supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Δ12-PG D2, PGF2α (Cyagen Chemical Co., Ann Arbor, MD) were added to infected cells immediately after the 1-h adsorption period and maintained in the medium for the duration of the experiment. Controls received equal amounts of methyl acetate or ethanol diluent. Under the conditions described, Δ12-PG D2 did not affect cell viability as determined by vital dye exclusion technique or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma-Aldrich), as described previously (15). Complexes were analyzed by nonde-

Influenza Virus Preparation, Infection, and Titration—Influenza viruses A/WSN/33 (H1N1) (WSN) was grown in the allantoic cavity of 8-day-old embryonated eggs. After 48 h at 37 °C, the allantoic fluid was harvested and centrifuged at 5000 rpm for 30 min to remove cellular debris, and virus titers were determined by hemagglutination titration and plaque assay, according to standard procedures (12). One hemagglutina-
ting unit (HAU) corresponded to 108 plaque-forming units in this model. Confluent cell monolayers were infected with WSN for 1 h at 37 °C at a multiplicity of infection of 5 HAU/106 cells. After the adsorption period, the viral inoculum was removed, and cell monolayers were washed three times with phosphate-buffered saline. Cells were main-
tained at 37 °C in culture medium containing 2% fetal calf serum. Virus yield was determined at different times post-infection by hemagglutination titration, according to standard procedures (12). Western Blot Analysis and Enzyme-linked Immunosorbent Assay (ELISA)—Whole-cell extracts were prepared from mock-infected or WSN-infected cells as described previously (16). Equal amounts of protein (25 μg) from whole-cell extracts were separated by SDS-PAGE and blotted to nitrocellulose, and filters were incubated with polyclonal anti-IκBα antibody (Biomol, Plymouth Meeting, PA) or monoclonal anti-β-actin antibody (Imgenex, San Diego, CA), anti-control antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-IKKα antibody (BD Biosciences-PharMingen, San Jose, CA), followed by decoration with horseradish-labeled anti-mouse or anti-rabbit IgG antibodies (ECL; Amersham Biosciences) (15). Immunoreactive IL-8 was quantitated in cell supernatants by ELISA (17), using a double antibody and standards obtained from R&D Systems (Minneapolis, MN).

RT-PCR—Total RNA was extracted using RNA-Be reagent (Invitrogen). RT-PCR was performed (1 μg of RNA) according to the manufacturer's protocols (Invitrogen). The sequences of TNFα, IL-1β, IL-6, IL-8, RANTES, and β-actin primers (MWG-Biotech, Ebersberg, Germany) were as follows: TNFα sense, 5′-TGAGGCACTGAAAGCTATGCT-3′; TNFα antisense, 5′-TTATCTCTGACCTGCACCGC-3′; IL-1β sense, 5′-GAGGATGATGAACTCAGGC-3′; IL-1β antisense, 5′-ACGCAGGAGCAGTACAGTT-3′; IL-6 sense, 5′-TGACTCCTTCTCACAAGCC-3′; IL-6 antisense, 5′-ATCCAGATTGGAAACATCCA-3′; IL-8 sense, 5′-ATTTGCACTGCTGTGTA-3′; IL-8 antisense, 5′-GGCCTGACCTCTCCTGTA-3′; RANTES antisense, 5′-CTGTCGCAAATCGCTGACTCT-3′; RANTES sense, 5′-GCGCTCAGGAGGCAAT-3′; and β-actin antisense, 5′-GCACCTTCTACAGCTTC-3′. Amplification was performed using the following parameters: 95 °C for 45 s, 61.2 °C for 45 s, and 72 °C for 1 min for 33 cycles for TNFα; 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min for 33 cycles for IL-1β; 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min for 28 cycles for IL-6; 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 1 min for 25 cycles for IL-8; 95 °C for 45 s, 63.5 °C for 45 s, and 72 °C for 1 min for 35 cycles for RANTES; and 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 1 min for 26 cycles for β-actin. PCR products were electrophoresed alongside DNA Molecular Weight Marker IX (Roche Applied Science) in 2% agarose gels and then stained with ethidium bromide.

RESULTS

Influenza A Virus Infection Induces NF-κB Activity in Human Airway Epithelial Cells via Stimulation of the IκB Kinase—As reported for several human viral pathogens, ortho-
myxoviruses are also able to activate NF-κB in infected cells (7, 18–19); however, the molecular mechanisms involved in NF-κB activation by influenza virus have not yet been elucidated. To investigate the effect of influenza A virus on NF-κB activity in pulmonary epithelial cells, confluent monolayers of human alveolar epithelium-like A549 cells were mock-infected or infected with WSN for 30 min. Endogenous IKK was immunoprecipitated with anti-IKKα antibodies and, kinase activity was determined in the presence of different concentrations of Δ12-PG D2 or PGF2α.

Statistical Analysis—Statistical analyses were performed using Student’s t test for unpaired data. Data are expressed as the mean ± S.D.; p values of <0.05 were considered significant.
FIG. 1. Influenza virus selectively induces NF-κB activity in human pulmonary A549 cells. A. A549 cells were mock-infected (U) or infected with influenza virus (WSN). At different times p.i., whole-cell extracts were assayed for NF-κB activity by EMSA (top panel). Positions of NF-κB-DNA (NF-κB) and non-specific protein-DNA (ns) complexes are indicated. Levels of IκBα, IκBβ, and β-actin (bottom panels) were determined by immunoblot analysis in the same samples. B, IKK activity was assayed in uninfected (U) and infected (WSN) cells at different times p.i. by kinase assay (KA, top panel). Endogenous IKK recovery was determined in the same samples by immunoblot analysis for IKKα (IB, middle panel). Levels of IKK activity quantified by PhosphorImager analysis are expressed as fold induction of the levels detected in uninfected control cells (bottom panel). Sections of fluorograms from native gels from a representative experiment are shown. Each experiment was repeated three times with similar results. C, confluent monolayers of human lung A549 cells, kidney 293 cells, neuroblastoma SH-SYSY cells, HaCaT keratinocytes, and canine kidney MDCK cells were mock-infected (–) or infected with WSN virus (5 HAU/10⁵ cells) for 1 h at 37 °C. Parallel samples were treated for 15 min with TNFα (25 ng/ml) (+) as a positive control of NF-κB activation. At different times p.i. (8, 16, and 24 h), whole-cell extracts were assayed for NF-κB activity by EMSA. Sections of fluorograms from native gels from a representative experiment are shown. Positions of NF-κB-DNA (NF-κB) and non-specific protein-DNA (ns) complexes are indicated. D, levels of NF-κB-DNA binding activities were quantified by PhosphorImager analysis and expressed as fold induction of the levels detected in uninfected control cells. A549 cells, 293 cells, x; SH-SYSY cells, o, HaCaT keratinocytes, □, canine kidney MDCK cells. E, in the same experiment, virus yield in the supernatant of WSN-infected cells was determined by standard hemagglutinin titration and expressed as HAU/ml. Data represent the mean ± S.D. of triplicate samples. Each experiment was repeated at least three times with similar results.
activation in other human non-pulmonary cell lines susceptible to viral infection, including SH-SY5Y neuroblastoma cells and HaCaT keratinocytes. Finally, no activation of NF-κB could be detected in MDCK cells, in which virus yield was higher than that in any of the other cell lines tested (Fig. 1, C–E). In addition, WSN infection did not alter NF-κB-dependent gene expression in MDCK cells transiently transfected with the luciferase reporter 2xNF-κB-LUC (15) up to 16 h p.i. (data not shown). These results indicate that activation of NF-κB is not required for influenza virus replication.

**Influenza A Virus Induces NF-κB-dependent Cellular Gene Transcription and IL-8 Production in Human Pulmonary Epithelial Cells**—NF-κB is considered to be a critical regulator of the inflammatory response because it can rapidly induce transcription of a variety of genes encoding proinflammatory proteins, including several inflammatory and chemotactic cytokines (9). To investigate whether influenza virus-induced NF-κB could turn on proinflammatory gene expression in pulmonary epithelial cells, A549 cells were infected with WSN virus or mock-infected, and at 12, 24, and 48 h p.i., extracts were assayed for NF-κB activity by EMSA. In parallel samples, total RNA was harvested and analyzed for transcription of the NF-κB-dependent cytokines TNFα, IL-1β, IL-6, IL-8, and RANTES by RT-PCR. As shown in Fig. 2, activation of NF-κB by influenza virus persisted at high levels for 48 h in infected cells and was associated with an increase in the level of cytokine mRNAs, which was already evident at 12 h p.i. RNA levels of all cytokines examined continued to be elevated up to 48 h p.i., a time at which the virus-induced cytopathic effect is evident and cell viability decreases. Because the production of the chemotactic cytokine IL-8 has been associated with influenza virus-induced inflammation and airway pathology (5, 6, 22), we analyzed the role of NF-κB in WSN-induced IL-8 transcription in A549 cells. Cells were infected with WSN virus or mock-infected and then analyzed for NF-κB activity by EMSA, IL-8 transcription by RT-PCR, and IL-8 production by ELISA. As expected, NF-κB DNA binding activity and accumulation of IL-8 transcript were strongly induced by WSN infection (Fig. 3A). IL-8 level in uninfected cells was <0.5 ng/ml, whereas it was greatly increased (>8 ng/ml) in the supernatant of WSN-infected cells (Fig. 3B), indicating that the IL-8 mRNA is efficiently translated.

**NF-κB Is Required for Induction of IL-8 during Influenza A Virus Infection**—Previous studies have identified in the 5'-flanking region of the IL-8 gene consensus binding sites for NF-κB as well as AP-1, OCT-1, and NFAT-1 transcription factors (23, 24). To investigate whether influenza virus-induced IL-8 production was dependent on NF-κB activation by the virus, we utilized the IkBo super-repressor IkBo-AA, in which Ser23/26 residues critical for phosphorylation by IKK have been replaced by alanine (14). A549 cells were transiently transfected with the IkBo-AA expression vector or with an “empty” vector. After 18 h, cells were mock-infected or infected with WSN. Luciferase activity, determined at 24 h p.i. in duplicate samples, is expressed as relative light units/μg protein. Each experiment was repeated three times with similar results.
fected cells, 64 ± 0 HAU/ml), confirming that the nuclear factor is not required for influenza virus replication in lung cells. On the other hand, expression of IkBα-AA prevented IL-8 production by infected cells, indicating that activation of NF-κB is essential for WSN-mediated IL-8 induction. Inhibition of virus-induced IL-8 production was also detected after treatment of infected A549 cells with the proteasome inhibitor MG132, which prevents NF-κB activation by blocking IkBα degradation; moreover, as determined by RT-PCR, WSN infection did not induce IL-8 mRNA synthesis in 293 and SH-SY5Y cells, in which influenza virus was unable to activate NF-κB (data not shown).

We next examined the effect of WSN on IL-8 gene promoter activity, utilizing luciferase constructs containing the 133-bp sequence upstream from the transcription start site of the human IL-8 gene (IL-8-luc) or the same sequence mutated in the NF-κB site (IL-8-(NF-κB-mut)-luc). A549 cells were transiently co-transfected with IL-8-luc or IL-8-(NF-κB-mut)-luc reporter plasmids, together with empty or IkBα-AA vectors. After 18 h, transfected cells were infected with WSN and assayed for luciferase activity at 24 h p.i. As shown in Fig. 3D, WSN infection stimulated IL-8-luc promoter activity, whereas it had no effect on IL-8-(NF-κB-mut)-luc activity. Expression of IkBα-AA resulted in a significant decrease of WSN-induced IL-8-luc promoter activity, and it had no effect in cells co-transfected with the IL-8-(NF-κB-mut)-luc reporter. Taken together, these results confirm that NF-κB is essential for IL-8 induction during influenza virus infection.

Effect of Δ12-PGJ2 on IL-8 Production Induced by Influenza Virus—Cyclopentenone prostaglandins PGA1 and 15-deoxy-Δ12,14-PGJ2 are potent inhibitors of TNFα-induced IKK activity (10). Because the natural cyclopentenone PG Δ12-PGJ2 was shown to possess therapeutic efficacy in a mouse model of lethal influenza A virus infection (12), we investigated the effect of Δ12-PGJ2 treatment on influenza virus-induced NF-κB activation and IL-8 production in human pulmonary cells. A549 cells were infected with WSN and then treated with Δ12-PGJ2 (20 μM) or control diluent. As previously reported in canine kidney cells (12), Δ12-PGJ2 also inhibited influenza virus replication in human cells (control, 56 ± 0; Δ12-PGJ2-treated, 6 ± 0 HAU/ml) and partially protected A549 cells by WSN-induced cytopathic effect. At different time intervals, whole-cell extracts were analyzed for endogenous IKK and NF-κB activities by kinase assay and EMSA, respectively. As expected, WSN infection induced IKK and NF-κB activities starting at 12 h p.i. (Fig. 4A). Treatment with Δ12-PGJ2 was effective in preventing WSN-induced IKK and NF-κB activities, resulting in a significant decrease of virus-induced IL-8 production (Fig. 4). Under the same conditions, Δ12-PGJ2 did not affect the activity of AP-1, another transcription factor involved in the control of IL-8 expression, which is induced during influenza virus infection (Fig. 4B). Treatment with non-cyclopentenone prostanoids of the E and F type had no effect on both WSN-induced NF-κB and AP-1 activities (Fig. 4A) and IL-8 production in A549 cells (data not shown).

We next investigated whether Δ12-PGJ2 could inhibit IKK activity in vitro. A549 cells were stimulated with TNFα for 30 min. Endogenous IKK was immunoprecipitated with anti-IKKα antibody, and kinase activity was determined in the presence of different concentrations of Δ12-PGJ2 or PGF2α as a control (Fig. 5B). IKK activity was inhibited in a dose-dependent fashion by Δ12-PGJ2, whereas PGF2α had no effect.

In a different experiment, A549 cells were transiently transfected with IL-8-luc or IL-8-(NF-κB-mut)-luc reporter plasmids. After 18 h, cells were infected with WSN and then treated with Δ12-PGJ2. Luciferase activity was determined at

Fig. 4. Δ12-PGJ2 inhibits WSN-induced IKK activity and NF-κB-dependent IL-8 production. Mock-infected (U) or infected (WSN) A549 cells were treated with Δ12-PGJ2 (20 μM) (+) or control diluent (−). A, at different time intervals, extracts were analyzed for NF-κB activity by EMSA (top panels) and for endogenous IKK activity and recovery by kinase assay (IKK-KA) and immunoblotting (IKK-IB), respectively (bottom panels). IKK and NF-κB activities in untreated (C) or Δ12-PGJ2-treated (D) infected cells quantified by PhosphorImager analysis are expressed as arbitrary units. B, at 24 h p.i., extracts were assayed for NF-κB and AP-1 activity by EMSA (top panels). NF-κB and AP-1 activities in uninflamed (U) and WSN-infected (C) cells quantified by PhosphorImager analysis are expressed as fold induction of the levels detected in uninflamed control cells (bottom panels). C, in the same experiment, supernatants from treated and control samples were analyzed for IL-8 production by ELISA at 24 h p.i. Data represent the mean ± S.D. of triplicate samples.

24 h p.i. As expected, WSN infection induced IL-8-luc promoter activity, whereas it had no effect on IL-8-(NF-κB-mut)-luc activity (Fig. 5C). In contrast to the cyclopentenone prostanoid 15-deoxy-Δ12,14-PGJ2 (25), treatment with Δ12-PGJ2 did not influence IL-8-luc promoter activity in uninfected cells (data not shown). In WSN-infected cells, Δ12-PGJ2 greatly reduced the activity of the wild type IL-8 promoter, whereas it had no effect on IL-8-(NF-κB-mut)-luc activity (Fig. 5C).

DISCUSSION

Despite large immunization programs, viral influenza remains a serious source of morbidity and mortality throughout
IKK Mediates Influenza-induced Inflammation in the Lung

The block of the IKK/NF-κB pathway is responsible for inhibition of WSN-induced IL-8 expression by Δ12-PGJ2. A, mock-infected (U) or infected (WSN) A549 cells were treated with 20 μM Δ12-PGJ2, PGE2, PGF2α, or control diluent (–). At 24 h p.i., extracts were analyzed for NF-κB activation by EMSA. B, A549 cells were stimulated with TNFα (100 ng/ml) for 20 min or left untreated (– TNFα). Endogenous IKK was immunoprecipitated with anti-IKKα antibody, and kinase activity was determined in the presence of different concentrations of Δ12-PGJ2, PGE2, no compound (control), or methyl acetate (diluent) at the concentration corresponding to the one used for 200 μM Δ12-PGJ2. IKK activity in PGE2-treated (⊙) or Δ12-PGJ2-treated (●) samples was quantified by PhosphorImager analysis and expressed as a percentage of untreated control. C, A549 cells were transiently transfected with IL-8-luc or IL-8-(κB-mut)-luc reporters or mock-transfected. After 18 h, cells were infected with WSN and treated with Δ12-PGJ2 (20 μM) or control diluent. Luciferase activity, determined at 24 h p.i. in duplicate samples, is expressed as relative light units/μg protein. Each experiment was repeated three times with similar results.

FIG. 5. The block of the IKK/NF-κB pathway is responsible for inhibition of WSN-induced IL-8 expression by Δ12-PGJ2. A, mock-infected (U) or infected (WSN) A549 cells were treated with 20 μM Δ12-PGJ2, PGE2, PGF2α, or control diluent (–). At 24 h p.i., extracts were analyzed for NF-κB activation by EMSA. B, A549 cells were stimulated with TNFα (100 ng/ml) for 20 min or left untreated (– TNFα). Endogenous IKK was immunoprecipitated with anti-IKKα antibody, and kinase activity was determined in the presence of different concentrations of Δ12-PGJ2, PGE2, no compound (control), or methyl acetate (diluent) at the concentration corresponding to the one used for 200 μM Δ12-PGJ2. IKK activity in PGE2-treated (⊙) or Δ12-PGJ2-treated (●) samples was quantified by PhosphorImager analysis and expressed as a percentage of untreated control. C, A549 cells were transiently transfected with IL-8-luc or IL-8-(κB-mut)-luc reporters or mock-transfected. After 18 h, cells were infected with WSN and treated with Δ12-PGJ2 (20 μM) or control diluent. Luciferase activity, determined at 24 h p.i. in duplicate samples, is expressed as relative light units/μg protein. Each experiment was repeated three times with similar results.

The results described here indicate that viral replication is independent from the ability of the virus to activate NF-κB in infected cells. In fact, the block of WSN-induced NF-κB activation by expression of a dominant-negative form of the inhibitory protein IkBa (IkBa-AA super-repressor) did not affect virus replication in pulmonary cells. Moreover, unexpectedly, whereas it potently induced NF-κB activity in human lung cells, WSN virus was unable to activate NF-κB in a variety of non-pulmonary human cells permissive to the virus, including

the world and a significant cause of illness and death among people with immune deficiency associated with aging or different clinical conditions (1). Pandemics, such as the one in 1918, have been responsible for the death of millions of people, and the possibility of the sudden emergence of highly pathogenic strains of influenza A virus with elevated case fatality rate continues to represent a severe threat to human health (2, 26). Antiviral chemotherapy with amantadine and rimantadine reduces the duration of symptoms of clinical influenza, but major side effects and the emergence of drug-resistant variants have been described (1, 18). Novel sialic acid analogs, zanamivir and oseltamivir, have prophylactic and therapeutic effects (18). However, also due to the extremely high mutation rate of influenza viruses, the disease is by no means under control, and novel therapeutic approaches are needed, especially in view of the possible emergence of new virulent strains. Because severe lung inflammation has been associated with the development of secondary complications that may result in respiratory failure during influenza (2, 4), in addition to the development of new antiviral agents, an understanding of the molecular mechanisms responsible for virus-induced inflammatory responses in the lung, could provide novel tools for therapeutic intervention during influenza virus infection.

The nuclear transcription factor NF-κB is undeniably recognized as a critical regulator of the inflammatory response (27). Once activated, NF-κB rapidly triggers the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, such as cyclooxygenase 2, the inducible form of nitric-oxide synthase, cell adhesion molecules, and a variety of proinflammatory and chemotactic cytokines, including IL-8, which has been associated with lung inflammatory pathology (5, 6, 28). Interestingly, cytokines that are stimulated by NF-κB, such as TNFα and IL-1β, are also potent NF-κB inducers, thus establishing a positive auto-regulatory loop that can amplify the inflammatory response and lead to chronic inflammation (27). Activation of NF-κB has been reported after infection with different viruses, including the immunodeficiency virus HIV-1, hepatitis B and C viruses, herpes viruses, and influenza viruses (20, 29–32). Different viruses have evolved different strategies to modulate the NF-κB pathway, most of which converge on activation of the IkB kinase IKK (7). In the case of orthomyxoviruses, whereas it has been recently reported that expression of different structural influenza viral proteins may trigger IKK activity (19), there was no direct evidence that in vivo infection with influenza virus could stimulate IKK function.

In the present report we show that influenza A virus infection strongly induces IKK activity in human pulmonary A549 cells. Stimulation of IKK function, determined by kinase assay, was maximal at 12 h after infection, leading to rapid degradation of NF-κB, IkBa and IkBβ, and resulting in a strong activation of NF-κB. Interestingly, in lung cells NF-κB DNA-binding activity was found to persist at high levels (over 4-fold above control) up to 48 h after infection, suggesting that the virus may interfere with the NF-κB auto-regulatory loop.

NF-κB activation during viral infection has been interpreted in some cases as a protective response of the host to viral pathogens, whereas in other cases it has been shown that the virus utilizes the factor to enhance its replication (7). Triggering of NF-κB activation is particularly relevant during infection with viruses that harbor NF-κB inhibitory proteins IkBa and IkBβ, and resulting in a strong activation of NF-κB. Interestingly, in lung cells NF-κB DNA-binding activity was found to persist at high levels (over 4-fold above control) up to 48 h after infection, suggesting that the virus may interfere with the NF-κB auto-regulatory loop.

NF-κB activation during viral infection has been interpreted in some cases as a protective response of the host to viral pathogens, whereas in other cases it has been shown that the virus utilizes the factor to enhance its replication (7). Triggering of NF-κB activation is particularly relevant during infection with viruses that harbor NF-κB binding sites in their promoters. In this case, induction of the transcription factor results in transactivation of the κB-containing viral primer and in enhanced viral transcription. This has been documented in the case of HIV-1 infections (20, 33) and, more recently, in the case of herpes simplex infections (15, 34). In the case of influenza viruses, little is known thus far regarding the role of NF-κB in the infection process. NF-κB activation has been commonly interpreted as a marker of the cellular antiviral response to influenza infection; however, it has been recently suggested that, on the contrary, NF-κB activity promotes both virus infectivity (35) and virus propagation via induction of proapoptotic factors in the infected cells (21).
kidney 293 cells, SH-SY5Y neuroblastoma cells, and HaCaT keratinocytes. In addition, induction of NF-κB DNA binding activity was not detected in canine kidney MDCK cells, which are commonly used for studies on influenza virus replication. These results also indicate that lung epithelial cells are particularly sensitive to NF-κB induction by influenza virus. The mechanism responsible for the highly sensitive response of the IKK/NF-κB pathway to influenza virus infection in pulmonary cells is not known and needs to be investigated.

Because NF-κB does not appear to be essential for influenza virus replication, we speculated that the dramatic and persistent activation of the nuclear factor observed in lung cells could induce cellular proinflammatory gene transcription. We then investigated the effect of WSN infection on the expression of NF-κB-dependent cytokines TNFα, IL-1β, IL-6, IL-8, and RANTES by RT-PCR. The results indicate that activation of NF-κB by influenza virus was associated with an increase in the level of all cytokine mRNAs. Cytokine RNA levels started to increase at 12 h p.i. and continued to be elevated up to 48 h p.i. In the case of IL-8, levels of the chemokine were found to be greatly increased in the supernatant of WSN-infected cells, indicating that the IL-8 mRNA is efficiently translated. Instead, IL-8 expression was not induced in other types of cells, in which NF-κB was not activated following WSN infection.

As indicated above, the production of the chemotactic cytokine IL-8 has been associated with influenza virus-induced inflammation and airway pathology (5, 6, 22), suggesting that NF-κB activation could be one of the events responsible for virus-induced pathogenicity in the lung. However, apart from κB sites, consensus binding sites for different transcription factors, including AP-1, OCT-1 and NFAT-1, have been identified in the 5′-flanking region of the IL-8 gene (23, 24). We then analyzed the role of NF-κB in WSN-induced IL-8 transcription in A549 cells, inducing a functional knock-down of NF-κB by using the dominant-negative form of IκBα. Expression of IκBα-AA prevented both WSN-induced NF-κB activation and IL-8 production in A549 cells, indicating that activation of NF-κB is essential for IL-8 induction. Similar results were obtained in experiments in which the IκBα-AA was utilized to block NF-κB function in A549 cells expressing luciferase constructs containing the 133-bp sequence upstream from the transcription start site of the human IL-8 gene (IL-8-luc) or the same sequence mutated in the NF-κB site. In these cells, expression of IκBα-AA resulted in a significant decrease of WSN-induced IL-8-luc promoter activity, and it had no effect in cells co-transfected with the mutated reporter. Moreover, inhibition of WSN-induced IL-8 production was also detected after treatment of infected A549 cells with the proteasome inhibitor MG132, which prevents WSN-induced NF-κB activation by blocking IκB degradation. Taken together, these results indicate that NF-κB is essential for IL-8 induction during influenza virus infection and suggest that the block of NF-κB function could result in inhibition of virus-mediated inflammatory reactions. Inhibition of NF-κB has also been recently suggested to decrease cell susceptibility to influenza virus infection by an as yet unknown mechanism (35).

We have shown that cyclopentenone prostanooids are potent inhibitors of NF-κB activation by direct inhibition and modification of the IKKβ subunit (10, 16). In addition, we have shown that the natural cyclopentenone prostanooid 12-15-PGJ2 possesses therapeutic efficacy in a mouse model of lethal influenza A virus infection (12). We then investigated the effect of 12-15-PGJ2 treatment on influenza virus-induced NF-κB activity and IL-8 production in human pulmonary cells. 12-15-PGJ2 was found to be a powerful inhibitor of WSN-induced IKK and NF-κB activities and caused a significant decrease of virus-induced IL-8 production, whereas treatment with non-cyclopentenone prostanooids of the E and P type had no effect. The fact that 12-15-PGJ2 did not affect the activity of AP-1, another transcription factor involved in the control of IL-8 expression and induced during influenza virus infection (18), indicated that the NF-κB pathway is the main target for the inhibitory activity of the prostanoiod in influenza-infected cells. Furthermore, 12-15-PGJ2 was found to greatly reduce the activity of the wild type IL-8 promoter, whereas it had no effect on the κB-mutated reporter in WSN-infected cells. Finally, the fact that 12-15-PGJ2 was able to directly inhibit IKK function in vitro kinase assays identifies IKK as the target of the prostanoiod in lung cells.

Cyclopentenone prostanooids are known to possess a potent antiviral activity against several RNA viruses in vitro and in vivo (12, 36–39). The mechanism of the antiviral activity is distinct from any other known antiviral agent because cyclopentenone PGs act on the host cell by inducing a cellular defense response, which involves the synthesis of cytoprotective heat shock proteins (39–41). We have now shown that, in the case of influenza A viruses, in addition to the previously described antiviral activity, 12-15-PGJ2 is able to prevent IKK-mediated virus-induced inflammatory reactions that could be involved in the pathogenesis of the disease. It should be pointed out that IKK inhibition could also alter normal host cell functions because the host utilizes NF-κB to trigger defense mechanisms against invading viruses (7, 42, 43). However, the fact that a protective activity of cyclopentenone PG has been shown during influenza A virus infection in preclinical studies (12, 36) argues against this hypothesis.

The results described here identify IKK as an important factor in triggering influenza A virus-induced inflammatory reactions in pulmonary epithelium, indicating a possible involvement of the IKK/NF-κB pathway in the pathogenesis of influenza. They also suggest that cyclopentenone prostanooids or prostanooid-derived molecules could be good candidates for a novel class of antiviral drugs inhibiting both influenza virus replication and virus-induced inflammatory reactions.

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