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Central Role of Double-Stranded RNA-Activated Protein Kinase in Microbial Induction of Nitric Oxide Synthase

Kohsaku Uetani,*† Sandy D. Der, † Maryam Zamanian-Daryoush, † Carol de la Motte, ‡ Belinda Y. Lieberman,§ Bryan R. G. Williams,† and Serpil C. Erzurum*2†

NO synthase 2 (NOS2) is induced in airway epithelium by influenza virus infection. NOS2 induction late in the course of viral infection may occur in response to IFN-γ, but early in infection gene expression may be induced by the viral replicative intermediate dsRNA through the dsRNA-activated protein kinase (PKR). Since PKR activates signaling pathways important in NOS2 gene induction, we determined whether PKR is a component in the signal transduction pathway leading to NOS2 gene expression after viral infection of airway epithelium. We show that NOS2 gene expression in human airway epithelial cells occurs in response to influenza A virus or synthetic dsRNA. Furthermore, dsRNA leads to rapid activation of PKR, followed by activation of signaling components including NF-κB and IFN regulatory factor 1. NOS2 expression is markedly diminished and IFN regulatory factor 1 and NF-κB activation are substantially impaired in PKR null cells. Strikingly, NOS2 induction in response to LPS is abolished in PKR null cells, confirming a central role for PKR in the general signaling pathway to NOS2. The Journal of Immunology, 2000, 165: 988–996.

Influenza virus infection causes significant morbidity and mortality in human populations worldwide with a broad spectrum of clinical responses ranging from asymptomatic infection, to rhinitis, to viral pneumonia (1). Although factors dictating the severity of virus disease are complex, interaction between inherent viral properties and host cellular response ultimately determines disease outcome (1–3). The first site of viral contact with the host and main target of infection and inflammation is the airway mucosal epithelium. Epithelial cells at the airway mucosal surface have a variety of inflammatory and immune defense mechanisms to deal with viruses, including expression of cytokines with chemotaxant and proinflammatory functions (4–7), MHC class II molecules (8), ICAM-1 (9), and NO synthase 2 (NOS2)3 (6, 10).

NO produced by NOS2 has potent antiviral activity against a number of viruses (11–15). However, NO also contributes to inflammation and injury through formation of toxic reactive nitrogen intermediates (6, 10, 16). In this context, development of pneumonia in a murine model of influenza infection has been linked to the host NOS2 expression (10, 17). Although viral infection often activates NOS2 in murine models (6, 10), the signaling pathways and effector mechanisms are not known. Viral mechanisms regulating NOS2 expression in human airway epithelial cells have not been studied. IFN-γ, produced by lymphocytes in the airway mucosa, is likely involved in NOS2 induction later in the course of viral infection. However, NOS2 induction by virus early in infection may be mediated by proteins responsive to the viral replicative intermediate, dsRNA. Intracellular dsRNA formed during viral replication (18) binds to and activates a serine/threonine kinase, dsRNA-activated protein kinase (PKR), which has been implicated in signal transduction (19, 20). In this context, we hypothesize that PKR is a component of the signaling pathway to NOS2 gene induction after viral infection of human airway epithelium. In this study, we show induction of NOS2 gene expression in human airway epithelial cells by influenza A virus or synthetic dsRNA. Importantly, dsRNA leads to activation of PKR, followed by activation of signal transduction proteins including NF-κB and IFN regulatory factor 1 (IRF-1) in airway epithelial cells. A role for PKR in the signal transduction pathway of virus-induced NOS2 expression is conclusively demonstrated in experiments using cells genetically deficient in PKR (PKR−/−). Impairment of NOS2 induction in response to LPS is also found in PKR−/− cells, confirming a central role for PKR in the general signaling pathway to NOS2.

Materials and Methods

Virus
Influenza A/Japan/305/57, subtype H2N2 (Advanced Biotechnologies, Columbia, MD) was grown in Madin-Darby canine kidney cells and suspended in serum-free MEM with Earle’s salts. The virus stock contained 106.25 TCID50 (50% of tissue culture infectious dose) per ml.

Sampling of human airway epithelial cells
Human airway epithelial cells were obtained through bronchoscopy with a flexible fiberoptic bronchoscope (Olympus BS-IT10; Olympus Optical, Tokyo, Japan) from normal nonsmoking volunteers with no history of lung disease and no medications. Bronchoscopic brush samplings of airway epithelial cells were taken from second and third order bronchi (21). For some experiments, airway epithelial cells were isolated from surgical specimens of tracheas and mainstem bronchi as previously described (22). Informed consent was obtained under a protocol approved by the Institutional Review Board at the Cleveland Clinic Foundation.

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3 Abbreviations used in this paper: NOS2, NO synthase 2; PKR, dsRNA-activated protein kinase; IRF-1, IFN regulatory factor 1; TCID50, 50% of tissue infectious dose; MEF, mouse embryonic fibroblasts; poly IC, polyinosinic-polycytidylic acid.

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Cell culture and treatments

Human airway epithelial cells obtained by bronchial brushing were cultured in serum-free Lechner and LaVeck media (LH8C, Biofluids, Rockville, MD) on plates precoated with coating media containing 29 μg/ml collagen (Vitrogen, Collagen Corp., Palo Alto, CA), 10 μg/ml BSA (Biofluids), and 10 μg/ml fibronectin (Calbiochem, La Jolla, CA) for 5 min (21). The cells were passaged at 60–80% confluence by dissociation from plates with 0.02% trypsin (E-PET, Biofluids) which was neutralized with soybean trypsin inhibitor (Biofluids). Primary cultures of passage 0–2 were used in experiments. The epithelial nature of primary and cultured cells was confirmed by immunocytochemical staining as previously described (23). A549 cells, an epithelial cell line derived from lung adenocarcinoma (American Type Culture Collection, Manassas, VA), were cultured in MEM (Life Technologies, Gaithersburg, MD) with 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (23). Confluent monolayers of human airway epithelial cells and A549 in 35-mm diameter dishes were exposed to virus stock diluted in LH8C medium and serum-free MEM, respectively.

Mice homozygous for a targeted deletion of PKR were generated as previously described (20). Mouse embryo fibroblasts (MEF) derived from wild-type PKR+/+ or PKR−/− mice (129/SvEv × C57Bl/6) were isolated and cultured in DMEM (Life Technologies) with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (20). Human IFN-γ was a gift from Genentech (South San Francisco, CA) or purchased from R & D Systems (Minneapolis, MN). Recombinant human IL-1β and TNF-α were purchased from Genzyme (Cambridge, MA). Polynosinic-polycytidylic acid (poly IC) and LPS (Escherichia coli serotype O55:B5) were from Sigma (St. Louis, MO). Recombinant murine IFN-γ was from Genzyme.

Immunofluorescent detection of influenza

Confluent monolayers of human airway epithelial cells and A549 grown on 12-mm-diameter coverslips of shell vial system (BioWhittaker, Walkersville, MD) were infected at a concentration of 5 × 10^4.25 TCID50/ml with influenza A. At 24 h postinfection, cells were fixed for 30 min at −20°C with cold methanol, washed with PBS, and incubated for 1 h at room temperature with monoclonal anti-influenza A Ab (1:400 dilution) (Chemi-con International, Temecula, CA). After washing with PBS, cells were incubated for 1 h at room temperature with a FITC-labeled goat anti-mouse Ab (1:100 dilution) (Biosource, Camarillo, CA). Samples were analyzed by immunofluorescent microscopy.

RNA extraction and Northern analysis

Total RNA was extracted and evaluated by Northern analysis as previously described using 32P-labeled 1.9-kb NOS2 cDNA probe (pCCF21) or, as a control, a 2-kb γ-actin cDNA probe (pHFGγ-κ1) and a 1.5-kb 18S cDNA probe (HHCS65, American Type Culture Collection) (21, 23) and then subjected to autoradiography. RNA from A549 cells stimulated with 100 U/ml IFN-γ, 10 ng/ml TNF-α, and 10 U/ml IL-1β for 8 h was used as a positive control (23). Expression of Nos2 mRNA relative to γ-actin or 18S mRNA was accomplished using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western analysis

Cell lysate was prepared by freeze/thaw of A549, human airway epithelial cells, and MEF; cultured for the indicated times with IFN-γ, TNF-α, IL-1β, and/or poly IC; and isolated in lysate buffer (3 mM DTT, 5 μg/ml aprotinin, 0.1 μM leupeptin, and 0.1 mM PMSF). Protein concentration was measured by bicinchoninic protein assay (Pierce). To specifically identify NF-kB and IRF-1 proteins in binding complexes, G-4 μg of rabbit anti-phospho-p38 polyclonal Ab, rabbit anti-IRF-1 or anti-IRF-2 Ab (Santa Cruz Biotechnology) were added to the binding reaction mix and incubated for 30 min at 4°C before adding the 32P-labeled oligonucleotide. The reaction products were analyzed by electrophoresis on a 4% polyacrylamide gel with 0.25×22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA buffer for NF-kB or 4% polyacrylamide gel containing 50 mM Tris-HCl (pH 7.5), 0.38 M glycerine, and 2 mM EDTA for IRF-1. The gels were dried and analyzed by autoradiography.

Results

Induction of Nos2 in human airway epithelial cells by influenza A or synthetic dsRNA viral mimic

Nos2 mRNA is present in A549 cells infected with influenza A as early as 8 h after infection (Fig. 1). Because intracellular dsRNA is produced during the viral replication cycle, and appears to be the active component in viral infection that stimulates gene expression in infected cells (30, 31), poly IC, a synthetic dsRNA, was used to simulate the viral-infected state and to study dsRNA-viral mechanisms regulating cellular gene expression. Accordingly, we tested whether dsRNA would induce Nos2 expression in human airway epithelial cells. Cells exposed to poly IC express Nos2 mRNA maximally between 8 and 24 h, returning to near baseline at 48 h
NF-κB increases the expression of the genes for many cytokines, enzymes, and adhesion molecules in the course of inflammation, including NOS2 (33). On the other hand, IRF-1 is essential for NOS2 induction in murine macrophages and mediates transcriptional activation via specific cis-acting elements resident in the promoters of IFN-stimulated genes (34, 35). To determine the involvement of NF-κB and IRF-1 in dsRNA viral induction of NOS2, whole cell extracts for EMSA were prepared from human airway epithelial cells stimulated for 1 h with IFN-γ, poly IC, or TNF-α. Poly IC and TNF-α induce NF-κB activity (Fig. 5A), whereas IFN-γ has little effect on basal NF-κB activity. To detect IRF-1 induction and activation, human airway epithelial cells were stimulated with IFN-γ, TNF-α, poly IC, or IL-1β for 4 h and evaluated by Western analysis and EMSA. IRF-1 protein is induced by IFN-γ, and to a lesser degree by poly IC (Fig. 5B). Neither TNF-α nor IL-1β induce IRF-1 protein. Both IFN-γ or poly IC result in the activation of IRF-1 DNA binding (Fig. 5C). In contrast to IRF-1, IRF-2 activation is not detected in human airway epithelial cells by any of the stimuli in our experimental
systems. Although IFN-γ activates STAT-1α in human airway epithelial cells (23), poly IC does not (data not shown). These data suggest that NOS2 expression in human airway epithelial cells by dsRNA or IFN-γ is likely mediated through the activation of NF-κB and/or IRF-1. Loss of NOS2 expression by poly IC in PKR−/− cells indicates a crucial role for PKR in the signal transduction pathway of dsRNA viral-induced NOS2 expression. Moreover, loss of NOS2 induction by LPS, and decreased NOS2 induction by IFN-γ + poly IC or IFN-γ + LPS in PKR−/− cells suggests an important role for PKR in the signaling pathway to

FIGURE 2. Immunodetection of influenza A virus in vitro and in vivo. A, Primary human airway epithelial cells; B, A549 cells 24 h infection with influenza A (5 × 10^4.25 TCID50/ml); C, epithelial cells obtained by nasopharyngeal swab from a child with influenza pneumonia. Original magnification, ×200.
NOS2 expression by a wide variety of microbes. On the basis of these findings, we reasoned that PKR signaling to NOS2 gene expression might be through activation of NF-κB and/or IRF-1 pathways.

Impaired NF-κB and IRF-1 activation in PKR knockout cells

To confirm the involvement of NF-κB and/or IRF-1 in PKR-mediated NOS2 expression, EMSA were performed on whole cell extracts of PKR+/+ and PKR−/− MEF stimulated with IFN-γ, LPS, or poly IC for 4 h. NF-κB is prominently activated by poly IC but is reduced in PKR−/− cells in contrast to wild-type cells (Fig. 6A). However, IRF-1 activation by poly IC or LPS also was impaired in PKR−/− cells (Fig. 6B), whereas comparable levels of constitutive IRF-2 binding activity are similar in both PKR+/+ and PKR−/− cells. IRF-1 and IRF-2 were verified in complexes by using Abs that specifically supershift the DNA protein band (Fig. 6B). These results suggest that PKR plays an essential role in the signaling events leading to NF-κB and IRF-1 activation and subsequently NOS2 induction.

Discussion

Human airway epithelium, a primary site of viral infection, expresses NOS2 in response to influenza A virus infection. Influenza viruses are enveloped viruses with a segmented, single-stranded RNA genome, which generate dsRNA during replication (1, 18). Viral dsRNA has been isolated from influenza-infected lungs, which is able to mimic effects of virus when injected into animals (18, 36, 37). Here, we show that dsRNA is an effector molecule that mediates NOS2 expression in human airway epithelial cells, and we identify for the first time an important downstream target gene for PKR. Previously, there was no information on the viral/dsRNA signaling pathways and effector mechanisms to gene expression in primary human airway epithelial cells. This study provides definitive evidence that PKR is physiologically important in human airway cells and influences downstream NOS2 induction.
IFN-γ also induces NOS2 expression; however, the kinetics of NOS2 induction by dsRNA and IFN-γ are different, suggesting distinct mechanisms of gene regulation. Furthermore, we show that PKR is essential for dsRNA induction of NOS2 using cells genetically deficient in PKR.

PKR is important for host antiviral mechanisms, as evidenced by impaired antiviral responses in mice with a homozygous targeted deletion in the PKR gene (20). First identified as a component of IFN-inducible cellular antiviral defenses, PKR exhibits two distinct kinase activities after activation by dsRNA, autophosphorylation/activation and phosphorylation of substrates (38). One antiviral effect mediated by PKR is the phosphorylation of eukaryotic

**FIGURE 5.** dsRNA activation of NF-κB and IRF-1 in human airway epithelial cells. **A**, NF-κB activation was analyzed by EMSA of whole cell extracts (5 μg protein/lane) from human airway epithelial cells nonstimulated (NS, lane 1) or stimulated for 1 h with IFN-γ (1000 U/ml, lane 2), TNF-α (10 ng/ml, lanes 3 and 4), or poly IC (100 μg/ml, lanes 5 and 6). Anti-p65 polyclonal Abs were added to the binding reactions as indicated to verify NF-κB family binding complexes (lanes 4 and 6). **B**, Western analyses (50 μg protein/lane) of IRF-1 in human airway epithelial cells nonstimulated (NS, lane 1) or stimulated for 4 h with IFN-γ (1000 U/ml, lane 2), TNF-α (10 ng/ml, lane 3), poly IC (100 μg/ml, lane 4), or IL-1β (10 U/ml, lane 5). **C**, Activation of IRF-1 was determined by EMSA (5 μg/lane) of whole cell extracts from human airway epithelial cells nonstimulated (NS, lane 1) or stimulated for 4 h with IFN-γ (1000 U/ml, lane 2), TNF-α (10 ng/ml, lane 3), poly IC (100 μg/ml, lane 4), or IL-1β (10 U/ml, lane 5). Anti-IRF-1 or IRF-2 Ab were added to the binding reactions as indicated to verify binding complex proteins (lanes 6 and 7). Results shown are representative of three separate experiments.

**FIGURE 6.** Impaired activation of NF-κB and IRF-1 in PKR−/− MEF. **A**, NF-κB activation determined by EMSA of whole cell extracts (5 μg protein/lane) from PKR+/+ and PKR−/− MEF nonstimulated (NS, lanes 1 and 5) or stimulated for 4 h with IFN-γ (100 U/ml, lanes 2 and 6), LPS (500 ng/ml, lanes 3 and 7), or poly IC (100 μg/ml, lanes 4, 8, and 9). IRF-1 activation was analyzed by EMSA similar to the method in A. Anti-p65, IRF-1, or IRF-2 Ab were added to binding reactions to confirm binding complex proteins. Similar results were obtained in three separate experiments.
initiation factor-2α, effectively restricting viral protein translation and subsequent replication (38). In addition to effects on translation, PKR regulates transcriptional events by phosphorylation of proteins related to signal transduction pathways. For example, PKR is required for the activation of NF-κB in immortal cell lines in response to different stimuli (19). In this study, dsRNA-induced PKR autophosphorylation and substrate phosphorylation in human airway epithelial cells provide conclusive evidence of a functional PKR pathway in these cells. These findings provide strong support that NF-κB activation by dsRNA in human airway epithelial cells is most likely due to PKR activation and phosphorylation of the inhibitor of NF-κB (IκB). Thus, PKR appears to mediate signal transduction in human airway epithelial cells in part through NF-κB. In addition, PKR may have impact on the signaling pathways through transcriptional and/or posttranslational effects on IRF-1 (32). For example, expression of IRF-1 protein in cells does not manifest functional DNA-binding activity unless a phosphorylation signal is provided (39), potentially by PKR (40, 41). In support of this concept, IRF-1 protein is induced and activated by dsRNA in human airway epithelial cells.

NOS2 is subject to predominantly transcriptional regulation (42). The molecular basis for induction of the human NOS2 gene is only partially understood (33, 43). In contrast, regions in the murine macrophage NOS2 promoter essential for conferring inducibility of NOS2 to LPS and IFN-γ have been well defined (44, 45). An NF-κB element at positions −76 to −85 bp relative to the transcription start point binds members of the NF-κB/Rel family of proteins in response to LPS (46), and further upstream an IFN-stimulated response element site binds IRF-1 on stimulation of RAW 264.7 cells with IFN-γ (34).

Originally identified as a transcriptional activator of IFN-β as well as IFN-inducible genes (39), IRF-1 is essential for NOS2 activation in murine macrophages (34, 35). However, the role of IRF-1 in human NOS2 gene expression is unknown. This study provides the first clear evidence of IRF-1 expression and activation in human airway epithelial cells by IFN-γ or poly IC. The promoter of the IRF-1 gene contains an NF-κB binding site and an IFN-γ-activated sequence (47). Thus, IFN-γ induction of IRF-1 is likely mediated through STAT-1α activation and binding to promoter elements. Interestingly, TNF-α or IL-1β activate NF-κB, but do not induce IRF-1 expression. Thus, NF-κB activation alone is not sufficient for IRF-1 induction in human airway epithelial cells. In the context that dsRNA does not activate STAT-1α, the mechanism of IRF-1 induction by dsRNA is unclear but may be mediated through a specific dsRNA virus-inducible element in coordination with NF-κB. Importantly, these studies demonstrate that NOS2 expression parallels IRF-1 expression, with IRF-1 expression preceding NOS2 mRNA accumulation. Collectively, the characteristics of these responses strongly support that the expression of NOS2 in human airway epithelial cells may depend, as it does in other cell types, on induction of IRF-1. Although NF-κB activation is not sufficient for NOS2 expression in human airway epithelial cells, studies of the 5′-flanking region of the human NOS2 gene reveal that NF-κB binding sites are critical for cytokine inducibility of the gene in human epithelial cell lines (33). Thus, we cannot exclude the possibility that dsRNA induction of NOS2 may be mediated in part through PKR activation of NF-κB (19).

To assess the role of PKR in signaling pathways essential for activation of NOS2, MEF derived from mice with homozygous deletions for PKR were studied. Although NOS2 expression is regulated in a cell- and species-specific manner, investigation of mechanisms of PKR signaling to NOS2 in MEF are justified by similarity of dsRNA induction of NOS2 in airway epithelial cells.

FIGURE 7. Mechanisms of viral-induced NOS2 expression in human airway epithelial cells. Virus infection and replication in human airway epithelial cells result in formation of dsRNA which binds to and activates PKR through autophosphorylation (P). PKR subsequently phosphorylates IκB, which leads to NF-κB activation. dsRNA also induces de novo synthesis and activation of IRF-1 through PKR. IRF-1 in cooperation with other factor(s), such as NF-κB, induces NOS2 gene expression.
and MEF and by the definitive model system. These studies provide conclusive evidence that PKR is essential in the signaling pathway for dsRNA induction of NOS2. Similar to previous studies, we show that PKR contributes significantly to activation of signaling pathways including NF-κB and IRF-1, which are important for NOS2 gene expression (31, 32). Interestingly, PKR is also essential for LPS induction of NOS2 in murine cells, confirming a central role for PKR in microbial-induced signaling pathway to NOS2. On the other hand, continued NOS2 expression in PKR−/− cells exposed to a combination of IFN-γ and LPS or poly IC, albeit at lower levels than in PKR+/+ cells, points out the possibility of inducible alternative signaling pathways to NOS2, which are independent of PKR.

On the basis of these data, we suggest a model for regulation of NOS2 in the airway early in the course of viral infection (Fig. 7). Upon infection of human airway epithelial cells, the viral replicative intermediate dsRNA binds to and activates PKR, which leads to activation of NF-κB. dsRNA also induces de novo synthesis and activation of IRF-1 through PKR activation. IRF-1 in cooperation with other factor(s), such as NF-κB, leads to NOS2 gene induction. Soon, release of IFN-γ by activated lymphocytes in the virus-infected airway induces prolonged NOS2 gene expression in human airway epithelium. Increases in airway NOS2 expression early in viral infection may be antimicrobial and limit spread of virus. However, IFN-γ-mediated high level NOS2 expression that persists for days to weeks most likely contributes to airway inflammation and injury and associated clinical respiratory symptoms.

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