Reactive Oxygen Species Induce Antiviral Innate Immune Response through IFN- λ Regulation in Human Nasal Epithelial Cells

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This study sought to explore the role of the IFN-related innate immune responses (IFN- β and IFN- λ) and of reactive oxygen species (ROS) after influenza A virus (IAV) infection for antiviral innate immune activity in normal human nasal epithelial (NHNE) cells that are highly exposed to IAV. Passage-2 NHNE cells were inoculated with the IAV WSN/33 for 1, 2, and 3 days to assess the capacity of IFN and the relationship between ROS generation and IFN- λ secretion for controlling IAV infection. Viral titers and IAV mRNA levels increased after infection. In concert with viral titers, we found that the generation of IFNs, such as IFN- β , IFN- λ 1, and IFN- λ 2/3, was induced after IAV infection until 3 days after infection. The induction of IFN- λ gene expression and protein secretion may be predominant after IAV infection. Similarly, we observed that intracellular ROS generation increased 60 minutes after IAV infection. Viral titers and mRNA levels of IAV were significantly higher in cases with scavenging ROS, in cases with an induced IFN- λ mRNA level, or where the secreted protein concentration of IFN- λ was attenuated after the suppression of ROS generation. Both mitochondrial and dual oxidase (Duox2)-generated ROS were correlated with IAV mRNA and viral titers. The inhibition of mitochondrial ROS generation and the knockdown of Duox2 gene expression highly increased IAV viral titers and decreased IFN- λ secretion. Our findings suggest that the production of ROS may be responsible for IFN- λ secretion to control IAV infection. Both mitochondria and Duox2 are possible sources of ROS generation, which is required to initiate an innate immune response in NHNE cells.

Keywords: influenza A virus; IFN- λ ; reactive oxygen species; mitochondria; Duox2

Influenza A virus (IAV) is a common pathogen of the human airway, and can cause severe viral infection or epidemics of respiratory disease. IAV contributes to global viral fatalities, and global mortality exceeded 20 million in the most lethal IAV pandemic (1). The virus is a prototype strain of the *Orthomyxoviridae* family, and

CLINICAL RELEVANCE

IFN- λ contributes to the main first-line defense against influenza A virus (IAV) infection, and may constitute a key element in therapies for acute IAV infection in nasal epithelia. Mitochondrial and Duox2-generated reactive oxygen species may be involved in the innate immune response against IAV infection and regulated IFN- λ induction to resist IAV replication in nasal epithelium.

possesses a segmented negative-strand RNA genome (1). IAV can infect macrophages and dendritic cells, but the primary targets of IAV are epithelial cells of the respiratory tract (2, 3). The innate immune system of the respiratory epithelium serves as a first line of defense against invading respiratory viruses. It senses microbial molecules, such as single-stranded and double-stranded viral RNA, and initiates the production of antiviral mediators such as IFN (4, 5). Secreted IFNs bind to their receptors and induce the expression of IFN-stimulating genes with antiviral activities via the Janus tyrosine kinase/signal transducers and activators of transcription proteins (JAK/STAT) signaling pathway (6). IFNs are defined by their ability to induce resistance to viral infection. The three distinct types of IFNs (Types I, II, and III) are classified according to their structural features, target receptors, and biological activities. Type I and Type III IFNs are directly produced in response to viral infection, and contribute to the clearance of viral infections in epithelial cells (7). Until now, Type I IFNs (IFN- α and IFN- β) were thought to play an exclusive role as early mediators of the innate immune response to viruses and as regulators of the subsequent response by the adaptive immune system (7–9). Recently, a group of proteins functionally similar to Type I IFNs was discovered and designated Type III (IFN- λ 1, IFN- λ 2, and IFN- λ 3) (8, 9). The induction, signaling, and biological activities of Type III IFNs are widely accepted as very similar to those of Type I IFNs, and Type I and Type III IFNs are directly produced in response to viral infections. However, evidence is emerging that the activation signaling of Type I and Type III IFNs is likely to be quite different, based on the unique distribution of their target receptors. In particular, the receptors for Type III IFNs are found primarily on epithelial cells (10, 11). A recent study verified that Type III IFNs are primarily responsible for protection against viral invaders in the respiratory tract, and play an important role in local antiviral innate immunity (10). However, the distinct mechanisms of Type III IFN regulation are not fully understood.

Reactive oxygen species (ROS) are highly diffusible and reactive molecules that are produced as a result of the molecular

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oxygen reduction of species such as hydrogen peroxide, superoxide anion, and hydroxyl radicals (12). The generation of ROS represents an important component of the host's arsenal to combat invading microorganisms. In addition, ROS possess a significant cell-signaling role in biological systems, capable of regulating the phenotype and function of immune cells (13, 14). Nicotinamide adenine dinucleotide phosphate-reduced oxidase (Nox) appears to be a particularly important enzyme for ROS generation in nonphagocytic cells, and is a more prominent ROS generator in the airway epithelium than xanthine oxidase, uncoupled nitric oxide synthase, or the mitochondrial respiratory reaction (15, 16). Mounting evidence suggests that intracellular ROS facilitate cellular damage or stress, and contribute to innate immune activation (16, 17). The deliberate production of ROS is widely appreciated as a critical component of chronic inflammation in the respiratory tract (15). However, few studies have demonstrated the role of ROS in a broad range of innate immune pathways.

In this study, we investigated whether Type III IFN plays a unique role in the antiviral defense mechanism, and we suggest that Type III IFNs are primarily responsible for protection against IAV infection in the nasal mucosa. We then describe the specific involvement of ROS in the antiviral immune response in nasal mucosa through the subsequent activation of Type III IFNs.

MATERIALS AND METHODS

Viruses and Reagents

Influenza A/WSN/33 virus (IAV WSN/33, H1N1) was used to infect nasal epithelial cells (American Type Culture Collection, Manassas, VA). Virus was grown in Madin-Darby canine kidney (MDCK) cells with virus growth medium, according to the standard procedure (18). After 48 hours at 37°C, the supernatant was harvested and centrifuged at 5,000 rpm for 30 minutes to remove cellular debris. The virus was titrated into MDCK cells, using a tissue-culture infectious dose assay. The virus stock was stored at -80°C until it was used for cell infection.

Anti- α -tubulin antibody and N^G -monoethyl-L-arginine (NMEA) were purchased from Calbiochem (San Diego, CA). Anti-mouse antibody for the nucleoprotein (NP) of IAV and short hairpin RNA (shRNA) containing lentiviral particles for IL-28R α , IL-10R β , Nox4, dual oxidase (Duox1), and Duox2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and N -acetyl-L-cysteine (NAC), mitoTEMPO, and allopurinol were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant IFN- λ 1 and IFN- λ 2 proteins were purchased from Invitrogen (Carlsbad, CA). Anti-mouse Duox2 antibody was provided by Dr. Yun Soo Bae (Ewha Women's University, Seoul, Republic of South Korea).

Cell Culture

The Institutional Review Board of the Chung-Ang University College of Medicine approved this study (Institutional Review Board number C20122095). Specimens were obtained from the middle nasal turbinate of five healthy volunteers. We cultured these specimens, using a system designed for normal human nasal epithelial (NHNE) cells (19, 20). Briefly, Passage-2 NHNE cells (1×10^5 cells/culture) were seeded in 0.5 ml of culture medium on Transwell clear culture inserts (24.5-mm, with a 0.45- μ m pore size; Costar Co., Cambridge, MA). Cells were cultured in a 1:1 mixture of basal epithelial growth medium and Dulbecco's Modified Eagle's Medium containing previously described supplements (19). Cultures were grown while submerged for the first 9 days. The culture medium was changed on Day 1, and every other day thereafter. An air-liquid interface (ALI) was created on Day 9 by removing the apical medium and feeding the cultures from the basal compartment only. The culture medium was changed daily after the initiation of the ALI. All experiments described here used NHNE cells at 14 days after the creation of the ALI.

Viral Inoculation

NHNE cells were either mock-infected (PBS) or inoculated with IAV (WSN/33, H1N1) at a multiplicity of infection (MOI) of 1. After a 2-hour

absorption period, the inoculum was aspirated, and cells were washed twice with culture medium. After washing, the culture medium was replaced, and cells were incubated at 37°C in 5% CO₂. At designated times after inoculation, the culture supernatant was collected for viral titration and ELISA. RNA and protein were extracted from cells to examine gene and protein expression.

Western Blot Analysis

NHNE cells were lysed with $\times 2$ lysis buffer (250 mM Tris-Cl, pH6.5, 2% SDS, 4% β -mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol). The cell lysate (30 μ g of protein) was electrophoresed in 10% SDS gels and transferred to polyvinylidene difluoride membranes in Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) for 1 hour at room temperature. The membrane was incubated overnight with primary antibody in Tween-Tris-buffered saline (TTBS; 0.5% Tween-20 in TBS). After washing with TTBS, the blot was incubated for 1 hour at room temperature with secondary anti-rabbit or anti-mouse antibody (Cell Signaling, Beverly, MA) in TTBS, and was visualized using an enhanced chemiluminescence system (Amersham, Little Chalfont, UK).

Real-Time PCR

Total RNA was isolated from NHNE cells infected with WSN/33 (H1N1) on Days 1, 2, and 3, using TRIzol (Invitrogen). The cDNA was synthesized from 3 μ g of RNA with random hexamer primers, using Moloney murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences, Waltham, MA, and Roche Applied Science, Indianapolis, IN). Commercial reagents (TaqMan Universal PCR Master Mix; PE Biosystems, Foster City, CA) were selected and conditions were set according to the manufacturer's protocol. The total reaction volume of 12 μ l contained 2 μ l of cDNA (reverse transcription mixture), oligonucleotide primers at a final concentration of 800 nM, and the TaqMan hybridization probe at 200 nM. The real-time PCR probe was labeled at the 5' end with carboxylfluorescein, and at the 3' end with the quencher carboxytetramethylrhodamine.

Primers for human IFNs (hIFNs) and Nox isoforms were purchased from Applied Biosystems (Foster City, CA) (hIFN- β 1, Hs01077958_s1; hIFN-L1, Hs00601677_g1; hIFN-L2/3, Hs04193049_gH; hNox1, Hs00246589_m1; hNox2, Hs00166163_m1; hNox3, Hs00210461_m1; hNox4, Hs00276431_m1; hNox5, Hs00225846_m1; hDuox1, Hs00213694_m1; and hDuox2, Hs00204187_m1). Real-time PCR was performed using the PE Biosystems ABI Prism 7700 Sequence Detection System. The thermocycler parameters included 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target mRNA levels were quantified using target-specific primer and probe sets for influenza A virus (H1N1) and IFN- β , IFN- λ 1, IFN- λ 2/3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All PCR assays were quantitative, and used plasmids containing the target gene sequences as standards. All probes were designed to span an intron, and did not react with genomic DNA. All real-time PCR data were normalized to the level of GAPDH (1×10^6 copies) to correct for variations between samples. All reactions were performed in triplicate, and the results were normalized against a housekeeping gene (GAPDH) as an endogenous control.

Intracellular ROS Assay

After stimulation with WSN/33 (H1N1) for 10, 30, 60, and 120 minutes, confluent cells were washed with RPMI (lacking phenol red) and incubated in darkness for 10 minutes in Krebs-Ringer solution containing 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR). Mitochondrial superoxide from the NHNE cells was measured with MitoSOX Red (Molecular Probes). Cells were incubated with 5 μ M DCF-DA for 10 minutes and with 5 μ M MitoSOX for 2 minutes, and then washed with 1 ml of Hanks' balanced salt solution at least five times to remove extracellular ROS. Transwell clear culture inserts were examined with a Zeiss Axiocvert 135 inverted microscope equipped with a $\times 20$ Neofluor objective and a Zeiss LSM 410 confocal attachment (Carl Zeiss, Minneapolis, MN). DCF and MitoSOX Red fluorescence levels were measured at an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm. Fluorescence intensities from seven randomly selected fields in each dish

were measured using the Carl Zeiss vision system (KS400, version 3.0). The seven values were averaged to obtain the mean relative fluorescence intensity, and the means were compared with each well. All experiments were repeated at least three times.

Cell Transfection with IL-28R α , IL-10R β , Nox4, Duox1, and Duox2 shRNAs

The expression of IL-28R α , IL-10R β , Nox4, Duox1, and Duox2 was suppressed using gene-specific shRNAs (lentiviral particles) that were purchased from Santa Cruz Biotechnology. The transfection rates for shRNAs were determined to be greater than 70% in NHNE cells. The cells were transfected with each shRNA, using oligofectamine reagent according to the manufacturer's instructions (Invitrogen). The shRNA ($10 \mu\text{l}$, 1×10^4 infectious units of virus) and oligofectamine ($1 \mu\text{g}$) were mixed individually with culture media. Transfection was then performed during a 48-hour period in 12-well NHNE cell plates. This procedure did not affect cell viability, and after 48 hours of transfection, cells were infected with IAV. This procedure was repeated with the control shRNA, which was also purchased from Santa Cruz Biotechnology. Endogenous IL-28R α , IL-10R β , Nox4, Duox1, and Duox2 gene expressions were partly suppressed using shRNA, and the suppression of endogenous gene expression was confirmed by real-time PCR or Western blot analysis for Duox2 protein expression.

Plaque Assay

Viral samples were serially diluted with PBS. Six-well plates of confluent monolayers of MDCK cells were washed twice with PBS, and infected in duplicate with $250 \mu\text{l}$ of each of the different virus dilutions. The plates were incubated at 37°C for 45 minutes to allow for viral adsorption. After adsorption, cells were overlaid with 1% agarose (Invitrogen) in complete Modified Eagle's Medium supplemented with L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin ($1 \mu\text{g}/\text{ml}$) and 1% FBS. The plates were incubated at 37°C , and 2 days after incubation, plaques were fixed with 10% formalin.

Quantification of Secreted IFN- β and IFN- λ Proteins

IFN- β and IFN- λ protein secretion was quantified using an ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's

instructions for NHNE supernatants. Assays demonstrated a range of 62.5–4,000 pg/ml.

Statistical Analyses

At least three independent experiments were performed with cultured cells from each donor, and the results are presented as the mean values $\pm$ SDs of triplicate cultures. Differences between treatment groups were evaluated according to ANOVA with a *post hoc* test. Differences were considered significant at $P < 0.05$.

RESULTS

NHNE Cells Were Susceptible to IAV Infection

NHNE cells were obtained from five healthy subjects to assess susceptibility to IAV. NHNE cells were infected with WSN/33 (H1N1) at MOI 1. Supernatants and cell lysates were harvested at 1, 2, and 3 days after infection (PI). We then measured mRNA levels of IAV using real-time PCR, and found that IAV mRNAs increased significantly until PI Day 3 (mean value of IAV mRNA, 4.2×10^5 on PI Day 2, and 6.5×10^5 on PI Day 3, $P < 0.05$; Figure 1A). We examined viral titers of IAV after infection by plaque assay, and found that viral titers also increased significantly from PI Day 2 (4.7×10^5 plaque-forming units [pfu]/ml). The peak titer of WSN/33 (H1N1) was 9.6×10^5 pfu/ml on PI Day 3 ($P < 0.05$, Figure 1B). Western blot analysis, using a polyclonal antibody of H1N1 NP, indicated that the NP of H1N1 increased in NHNE cells after WSN/33 (H1N1) infection until PI Day 3 (Figure 1C). These findings demonstrate the susceptibility of the nasal epithelium to WSN/33 (H1N1), and show that the mRNA level, NP expression, and viral titer of IAV increased until PI Day 3.

IFN-Related Immune Response Was Activated by IAV Infection

To evaluate changes in gene expressions after IAV infection, we performed gene expression analysis using the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA), which

Figure 1. NHNE cells were susceptible to influenza A virus (IAV) infection. Normal human nasal epithelial (NHNE) cells from five healthy volunteers were inoculated with WSN/33 (H1N1) for 1, 2, and 3 days at a multiplicity of infection (MOI) of 1. (A) Real-time PCR showed that the IAV mRNA level was elevated until postinfection (PI) Day 3, and was significantly higher from PI Day 2. (B) A plaque assay also showed that viral titers were significantly higher from PI Day 2. Results are presented as the means $\pm$ SDs from five independent experiments. * $P < 0.05$, compared with mock-infected cells. (C) Western blot analysis revealed that the expression of IAV nucleoprotein (NP) increased after infection until PI Day 3. The figure is representative of five independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

comprises more than 770,000 unique 25-mer oligonucleotide features constituting more than 28,000 genes, and in which each gene is represented on the array by 27 probes. The data were processed using the robust multiarray analysis algorithm, which performs a background correction, a normalization step, and a probe-level summary. On PI Day 2, NHNE cells were harvested for RNA purification and gene expression analysis. RNA was quantified and assessed for integrity, and equal quantities of NHNE total RNA were amplified and hybridized to oligonucleotide arrays. The resulting gene expression data were normalized directly to those cells that were mock-infected. Our analysis resulted in a list of 987 genes that showed significantly changed expression (> 3 -fold) after WSN/33 (H1N1) infection in NHNE cells. We also found 810 genes with mRNA levels that were significantly elevated after IAV infection on PI Day 2 (Figure 2). Among these, 111 genes were involved in immune function and cellular defense against external pathogens, such as genes for B-cell-mediated immunity, complement-mediated immunity, cytokine-mediated or chemokine-mediated immunity, granulocyte-mediated immunity, macrophage-mediated immunity, natural killer cell-mediated immunity, T cell-mediated immunity, and IFN-mediated immunity. Moreover, genes for nucleoside, nucleotide, and nucleic acid metabolism were elevated after WSN/33 (H1N1) inoculation. The analysis revealed that 11 genes (OAS1, OAS2, OAS3, IFIT1, IFIT2, CXCL10, MX1, IRF7, IRF9, STAT1, and STAT2) for the IFN-mediated immune response and signal transduction of IFN-stimulating genes were elevated significantly after WSN/33 (H1N1) infection (Table 1). These results demonstrate that IFN-mediated immunity could be activated in NHNE cells after IAV infection, instigating an innate immune response.

IFN- λ Was Preferentially Elevated after IAV Infection in NHNE Cells

Although Type I and Type III IFN secretions are induced at the same time after viral infection in the respiratory epithelium, the

production of IFN- β and IFN- λ in the nasal epithelium has not been comparatively investigated after IAV infection of the nasal epithelium. NHNE cells were inoculated with WSN/33 (H1N1), and IFN mRNA levels were measured at 1, 2, and 3 days after infection, using real-time PCR. The results showed that IFN- β , IFN- λ 1, and IFN- λ 2/3 mRNAs were elevated until 3 days after infection (Figure 3A). ELISA was performed on the supernatant to detect secreted proteins. Both IFN- β and IFN- λ proteins were increased after WSN/33 (H1N1) infection until 3 days (Figure 3B). At this virus dose (MOI 1), the mRNAs of IFN- λ were found to be significantly higher than those of IFN- β on PI Days 2 and 3. Mean mRNA levels of IFN- β were 7.4×10^3 on PI Day 2, and 1.4×10^4 on PI Day 3. Mean mRNA levels of IFN- λ 1 and IFN- λ 2/3 were 1.6×10^4 and 1.8×10^4 on PI Day 2, respectively, and 3.3×10^4 and 3.6×10^4 on PI Day 3, respectively ($P < 0.05$; Figure 3A). The protein concentrations of secreted IFN- β and IFN- λ were no different on PI Day 1, but the IFN- λ protein concentration in the supernatant of NHNE cells exceeded the IFN- β protein concentration from PI Day 2 ($P < 0.05$; Figure 3B). The mean concentration of IFN- β was 2.3×10^3 pg/ml on PI Day 2, and 2.8×10^3 pg/ml on PI Day 3. The mean concentration of IFN- λ was 5.4×10^3 pg/ml at PI Day 2, and 9.1×10^3 pg/ml on PI Day 3. Thus, the IFN- λ response to IAV infection appears to be much greater than that of IFN- β , and the induction of IFN- λ to control IAV infection may be more dominant in the nasal epithelium.

IFN- λ Was More Necessary for Resistance of IAV Infection than Was IFN- β

As a next step, we analyzed whether the control of IAV replication and susceptibility to infection were dependent on IFN- λ expression. For this experiment, NHNE cells were transfected with the shRNA of IFN-AR1, IL-28R α , and IL-10R β , which cause a functional loss of IFN- β and IFN- λ . Compared with their expression in cells transfected with control shRNA, endogenous *IFNAR1* gene expression was decreased by 75%, *IL28R α*

Figure 2. Gene analysis of molecules with elevated gene expression in IAV-infected NHNE cells. NHNE cells were inoculated with WSN/33 (H1N1) at MOI 1 for 2 days. In total, 810 gene mRNA levels were significantly elevated after IAV infection, with greater than 3-fold induction. The graph shows that most elevated gene expressions involved immunity and defense, signal transduction, and the metabolism of nucleosides, nucleotides, nucleic acids, and proteins. Among these genes, 111 (13.7%) were involved in immunity and defense in the nasal epithelium. The graph is representative of five independent experiments.

TABLE 1. GENE ANALYSIS AND QUANTIFICATION OF GENES FOR IFN-MEDIATED IMMUNE RESPONSE AND SIGNAL TRANSDUCTION OF IFN-STIMULATING GENES

Reference Sequence Number	Gene	Gene Identification Number	Fold Change
NM_002534.2	OAS1	4938	6.55
NM_016817.2	OAS2	4939	9.87
NM_001032409.1	OAS1	4938	6.61
NM_001548.3	IFIT1 (IP10)	3434	61.29
NM_002535.2	OAS2	4939	7.33
NM_001547.4	IFIT2	3433	9.34
NM_006187.2	OAS3	4940	9.31
NM_001565.2	CXCL10	3627	6.03
ILMN_1662358	MX1	4599	6.82
NM_001032409.1	OAS1	4938	6.62
NM_006084.4	IRF9	10379	6.02
NM_004029.2	IRF7	3665	11.66
NM_004029.2	IRF7	3665	10.56
NM_007315.2	STAT1	6772	4.94
NM_005419.2	STAT2	6773	3.03
NM_139266.1	STAT1	6772	5.50

by 85%, and *IL10R β* by 82% after each shRNA transfection. This result demonstrates that the shRNAs induced significant silencing of their respective genes, and resulted in a functional loss of IFN- β and IFN- λ during subsequent experiments. The level of IAV mRNA was significantly higher in cells transfected with IL-28R α (1.4×10^6) and IL-10R β shRNAs (1.0×10^6), compared with cells transfected with control shRNA (1.9×10^5) and IFN-AR1 shRNA (3.1×10^5) ($P < 0.05$; Figure 3C). The viral titer of WSN/33 (H1N1) was also significantly elevated after transfection with IL-28R α shRNA (9.6×10^5 pfu/ml) and IL-10R β shRNA (8.0×10^5 pfu/ml), compared with the cells transfected with control shRNA (2.6×10^5 pfu/ml) and IFN-AR1 shRNA (2.8×10^5 pfu/ml) ($P < 0.05$; Figure 3D). The cells transfected with IFN-AR1 shRNA demonstrated a relatively normal secretion of IFN- λ , whereas cells transfected with IL-28R α and IL-10R β shRNAs exhibited an intact response of IFN- β against IAV infection.

To compare IFN- β -dependent and IFN- λ -dependent protective effects against IAV infection, NHNE cells were treated with recombinant IFN- β (1,000 units) and IFN- λ (IFN- λ 1, 10 ng/ml; IFN- λ 2, 10 ng/ml) 1 hour before WSN/33 (H1N1) infection. The increased mRNA level (5.7×10^5) and viral titer (4.9×10^5 pfu/ml) of IAV on PI Day 2 were attenuated after treatment with IFN- β (mRNA level, 2.9×10^5 pfu/ml; viral titer, 2.7×10^5 pfu/ml) and IFN- λ (mRNA level, 2.1×10^4 pfu/ml; viral titer, 1.0×10^4 pfu/ml) ($P < 0.05$; Figures 3E and 3F). However, the IAV mRNA level and viral titer were more completely reduced in cells treated with IFN- λ .

These findings indicate that IFN- λ is preferentially elevated to resist IAV infection, and thus exogenous IFN- λ treatment may be more effective at controlling IAV infection in the nasal epithelium. The loss of IFN- λ secretion may lead to more severe IAV infection. This further suggests that treatment with exogenous IFN- λ in the nasal epithelium could be effective at suppressing IAV infection.

Intracellular ROS Generation Was Induced after IAV Infection

ROS are known to be involved in the pathogenesis of chronic inflammatory diseases in the respiratory tract. Recently, an alternative role for ROS regarding the induction of antiviral immunity in the airway epithelium was reported (12, 24). To test ROS-induced antiviral immunity in NHNE cells, WSN/33 (H1N1) was inoculated in NHNE cells for 10, 30, 60, and 120 minutes.

Intracellular ROS was then measured using a fluorescence-based assay with 2',7'-DCF-DA as a probe and laser-scanning confocal microscopy. The infection of cells with WSN/33 (H1N1) resulted in a time-dependent increase in DCF fluorescence intensity, with the maximum increase at PI Minute 60 (32.1 ± 1.6), compared with control intensity (5.4 ± 1.3). By the 120-minute time point, fluorescence had diminished (Figure 4A).

The pretreatment of cells with an ROS scavenger (10 μ M, NAC) 1 hour before infection with WSN/33 (H1N1) suppressed IAV-induced ROS generation (31.0 ± 2.3 versus 10.8 ± 1.4 , $P < 0.05$) at PI Minute 60 (Figure 4B). Interestingly, ROS scavenging significantly increased the IAV mRNA level (1.3×10^5 versus 6.0×10^5 pfu/ml, $P < 0.05$; Figure 4C) and viral titer (8.0×10^5 versus 6.9×10^6 pfu/ml, $P < 0.05$; Figure 4D), compared with cells that were only infected with WSN/33 (H1N1). These results indicate that IAV infection in the nasal epithelium generated intracellular ROS within 1 hour of IAV infection, and IAV-induced ROS generation was involved in the antiviral activity of the nasal epithelium. Hence, antioxidant treatment diminished ROS generation and subsequently aggravated IAV infection in the nasal epithelium.

ROS Scavenging Inhibited IAV-Induced IFN- λ Activation

Although the induction, signaling, and biological activities of IFN- λ are widely accepted as very similar to those of Type I IFNs, the mediators for the induction of IFN- λ secretion have not been comparatively investigated. We investigated whether ROS generation after IAV infection could lead to the induction of IFN- λ transcription and secretion in the nasal epithelium. After NHNE cells were infected with WSN/33 (H1N1) for 2 days, IFN- λ 1 and IFN- λ 2/3 mRNA levels were significantly higher than those of mock-infected cells (IFN- λ 1, 1.6×10^5 versus 1.2×10^2 , $P < 0.05$, Figure 5A; IFN- λ 2/3, 1.7×10^5 versus 3.4×10^3 , $P < 0.05$, Figure 5B). The treatment of cells with NAC (10 μ M) for 1 hour before infection with WSN/33 (H1N1) suppressed IAV-induced IFN- λ 1 (1.7×10^4 versus 3.9×10^3 , $P < 0.05$; Figure 5A) and IFN- λ 2/3 (1.8×10^4 versus 3.8×10^3 , $P < 0.05$; Figure 5B) gene expressions. ELISA results showed that IAV-induced IFN- λ secretion also comparatively decreased in cells that were treated with NAC (Figure 5C). IFN- β gene expression and secreted protein levels were not significantly changed after ROS scavenging (data not shown). These results indicate that ROS may be responsible for the activation of IFN- λ , which is the preferential IFN used against IAV infection in the nasal epithelium.

Mitochondria and Duox2 Generate Intracellular ROS to Resist IAV Infection

To identify the sources of IAV infection-induced intracellular ROS generation, we first used NMEA (the NO synthase inhibitor), allopurinol (the xanthine oxidase inhibitor), and mito-TEMPO (the inhibitor of the mitochondrial respiratory chain reaction), each of which inhibits a specific enzyme involved in intracellular ROS generation. After pretreating NHNE cells with 10 μ M NMEA, 100 μ M allopurinol, and 500 μ M mito-TEMPO, NHNE cells were infected with WSN/33 (H1N1) for 2 days. We then performed real-time PCR to evaluate changes in IAV gene expression. NMEA and allopurinol did not significantly change IAV gene expression (Figure 6A). In contrast, inhibition of the mitochondrial respiratory chain reaction increased IAV gene expression. The IAV mRNA level was 4.7×10^5 on PI Day 2, but pretreatment with mitoTEMPO increased IAV mRNA level by 1.4×10^6 ($P < 0.05$; Figure 6A).

To determine which Nox isoforms are involved in intracellular ROS generation after IAV infection, we performed real-time

Figure 3. IFN-λ was preferentially induced to control IAV infection in the nasal epithelium. NHNE cells were inoculated with WSN/33 (H1N1) for 1, 2, and 3 days at MOI 1. (A) Real-time PCR revealed that IFN-β, IFN-λ1, and IFN-λ2/3 mRNAs were elevated until PI Day 3, and IFN-λ mRNA levels were significantly higher compared with the mRNA level of IFN-β from PI Day 2 in NHNE cells. (B) The supernatant was assayed by ELISA for measuring the secreted IFN-β and IFN-λ concentrations after IAV infection. The IFN-λ protein concentration in the NHNE cell supernatant exceeded IFN-β protein concentrations from PI Day 2. Results are presented as the means $\pm$ SDs from five independent experiments. * $P < 0.05$, comparing mRNA and protein levels of IFN-λ with those of IFN-β. NHNE cells were transfected with the short hairpin RNA (shRNA) of IFNAR1, IL-28Rα, and IL-10Rβ for the functional loss of IFN-β and IFN-λ. (C) Real-time PCR showed that the amount of IAV mRNA was significantly higher in cells transfected with IL-28Rα and IL-10Rβ shRNAs, compared with cells transfected with control (cont) and IFNAR1 shRNA. (D) The plaque assay revealed that viral titers were also significantly elevated after transfection with IL-28Rα and IL-10Rβ shRNAs after infection, compared with cells transfected with control and IFNAR1 shRNA. NHNE cells were treated with recombinant IFN-β (1,000 U) and IFN-λ (IFN-λ1, 10 ng/ml; IFN-λ2, 10 ng/ml), simultaneous with WSN/33 (H1N1) inoculation. Real-time PCR (E) and a plaque assay (F) showed that the increased mRNA levels and viral titers of IAV at 2 days after infection (PI) were completely attenuated after treatment with recombinant IFN-λ. Results are presented as the means $\pm$ SDs from five independent experiments. * $P < 0.05$, compared with the level of IAV-infected cells.

PCR to measure IAV mRNA levels after the knockdown of Nox gene expression in NHNE cells. We found that *Nox4*, *Duox1*, and *Duox2* mRNAs increased significantly after IAV infection by PI Day 1, and *Nox1*, *Nox3*, *Nox4*, and *Nox5* mRNAs were minimally induced by IAV infection in NHNE cells (data not shown). Based on these findings, we focused on *Nox4*, *Duox1*, and *Duox2* as possible Nox subtypes that are involved with IAV infection-induced ROS generation in the nasal epithelium.

Real-time PCR was performed to verify IAV mRNA levels after the knockdown of *Nox4*, *Duox1*, and *Duox2*. The results

showed that IAV mRNA levels in the cells transfected with *Duox2* shRNA (2.6×10^6) before WSN/33 (H1N1) infection were significantly higher after infection, compared with cells infected with only WSN/33 (H1N1) (7.2×10^5) and compared with cells transfected with control shRNA (7.9×10^5) before IAV infection (Figure 6B).

Endogenous *Nox4* gene expression was decreased by 79%, *Duox1* by 78%, and *Duox2* by 85%, after each shRNA transfection. We found no significant changes in IAV mRNA levels in cells transfected with *Nox4* and *Duox1* shRNAs for the inhibition of endogenous *Nox4* and *Duox1*.

Figure 4. Intracellular reactive oxygen species (ROS) generation was induced in the nasal epithelium after IAV infection. NHNE cells were infected with WSN/33 (H1N1) for 10, 30, 60, and 120 minutes (min), and then intracellular ROS generation was measured with 2',7'-dichlorofluorescein diacetate (DCF-DA). (A) After IAV infection, the DCF fluorescence intensity was elevated at 30 and 60 minutes after infection (PI) in NHNE cells. ROS scavenging using the antioxidant *N*-acetyl-L-cysteine (NAC; 10 μ M) attenuated intracellular ROS generation (B), and increased the viral mRNA level (C) and viral titer (D) in NHNE cells. The fluorescence intensity data are representative of five independent experiments. PCR and plaque assay data are presented as means $\pm$ SDs from five independent cultures. * P < 0.05, compared with the level of IAV-infected cells.

In addition to endogenous gene suppression, we confirmed that Duox2 protein expression was also completely suppressed after Duox2 shRNA transfection (Figure 6C).

Cells treated with mitoTEMPO (6.9 ± 0.5) at 1 hour before infection, and transfected with Duox2 shRNA (9.7 ± 1.1) for 2 days when Passage-2 cells were seeded, exhibited significantly suppressed IAV infection-induced intracellular ROS generation (28.2 ± 1.7 , P < 0.05; Figure 6D). In addition, IAV infection-induced mitochondrial ROS generation (25.9 ± 3.5) was inhibited in cells whose mitochondrial respiratory chain reaction (8.5 ± 1.9) was suppressed, and also in cells where Duox2 expression was knocked down (10.6 ± 1.3 , P < 0.05; Figure 6E). These results suggest that IAV infection induces intracellular ROS through the mitochondrial respiratory chain reaction and Duox2 in NHNE cells, and that suppression of the mitochondrial respiratory chain reaction and Duox2 expression may aggravate IAV infection.

IAV Infection-Induced IFN- λ Expression May Be Mediated by Mitochondrial and Duox2-Generated ROS

To determine whether mitochondria and Duox2 promote a significant induction of IFN- λ after IAV infection, cells were

treated with mitoTEMPO, transfected with Duox2 shRNA, and inoculated with WSN/33 (H1N1). On PI Day 2, the IFN- λ mRNA level and the amount of secreted protein were analyzed by real-time PCR and ELISA. Real-time PCR showed that IAV infection-induced IFN- $\lambda 1$ (2.7×10^4) and IFN- $\lambda 2/3$ (1.8×10^4) mRNA levels decreased significantly in cells with an inhibited mitochondria respiratory chain reaction (IFN- $\lambda 1$, 4.6×10^3 ; IFN- $\lambda 2/3$, 6.7×10^3 ; P < 0.05) and with knocked-down Duox2 gene expression (IFN- $\lambda 1$, 7.4×10^3 ; IFN- $\lambda 2/3$, 4.6×10^3 ; P < 0.05; Figures 7A and 7B). IAV infection also increased the secreted IFN- λ concentration (7.7×10^3 pg/ml) on PI Day 2 in NHNE cells. However, the IFN- λ concentration was decreased when intracellular ROS generation was inhibited by mitoTEMPO shRNA (0.7×10^3 pg/ml) or Duox2 shRNA (1.5×10^3 pg/ml, P < 0.05; Figure 7C). IAV infection-induced intracellular ROS generation through the mitochondrial respiratory chain and Duox2 activation may mediate IFN- λ gene expression and protein secretion in NHNE cells.

DISCUSSION

We found that IFN- λ is the predominant IFN produced by NHNE cells in response to IAV, and we characterized mitochondrial and

Figure 5. Intracellular ROS mediated IFN-λ regulation after IAV infection in the nasal epithelium. NHNE cells were treated with NAC for 1 hour, and then inoculated with WSN/33 (H1N1) for 2 days at MOI 1. Real-time PCR showed that the pretreatment of cells with NAC (10 μM) suppressed IAV-induced IFN-λ1 (A) and IFN-λ2/3 (B) gene expressions. (C) ELISA results showed that IAV-induced IFN-λ secretion also comparatively decreased in cells treated with NAC. Results are presented as the means ± SDs from five independent experiments. **P* < 0.05, compared with the level of IAV-infected cells.

Duox2-generated ROS as mediators of IFN-λ regulation after IAV infection. Both IAV-induced ROS generation and ROS-mediated IFN-λ secretion limited IAV replication and dissemination in the nasal epithelium.

Although Type I IFNs, such as IFN-α and IFN-β, have been well documented as early mediators of the innate immune response to viruses, and as regulators of the subsequent activation of the adaptive immune system that contribute to the clearance of viral infection (21–27), recent studies have indicated a unique role for IFN-λ in antiviral defense (7, 28–30). In addition, evidence is emerging that IFN-λ is a necessary component of the respiratory viral infection response, and that a deficiency of IFN-λ is related to an exacerbation of respiratory infections in pneumonia and asthma (28). Our results showed that IFN-λ gene expression and secreted protein were more highly induced after IAV infection than was IFN-β gene expression in NHNE cells. Moreover, we found that blocking the IFN-λ-related immune response through the knockdown of IFN-λ receptors aggravated IAV infection, even when the IFN-β-related immune response remained relatively intact. We propose that the higher levels of IFN-λ produced in the course of IAV infection constitute the primary antiviral defense in NHNE cells, and that IFN-λ regulates the innate immune mechanism to resist IAV infection. Some studies reported evidence that IFN-λ is the primary cytokine for mediating an antiviral defense against rhinovirus in the lungs (29, 30). IFN-λ was also shown to be the predominant IFN induced by IAV in the mouse lung, and a much larger burst of IFN-λ was observed in an *in vivo* model after IAV infection (10). IFN-λ receptor expression has been shown to be limited in epithelial cells (28), and IFN-λ contributes to the main first-line defense against respiratory viral infections in nasal epithelial cells

via the retinoic acid-inducible gene-1-dependent pathway (31). Taking these findings together, IFN-λ has been demonstrated to play a newly discovered role in protecting respiratory and nasal epithelia from viral infection, and IFN-λ provides an IFN-β induction-independent therapeutic strategy against viral infection.

ROS appear to promote inflammation through the activation of signaling proteins, and the extreme toxicity of ROS is capable of eliciting significant immunopathology in surrounding tissues (32). In the respiratory tract, ROS are regarded as one of the pathologic components of chronic inflammatory airway diseases, such as asthma, pneumonia, and chronic obstructive pulmonary disease (33). Based on this knowledge, some researchers suggested that a faster clearance of ROS could reduce lung damage and improve lung function. However, ROS have recently been shown to function as a messenger influencing a variety of immunologic processes, and to enhance host immunity through the prevention of pathogen-induced proinflammatory cytokines (33–36). ROS generation by exogenous pathogens has also been established in respiratory epithelial cells, and the modulation of ROS was reported to be important for respiratory virus-induced innate immune mechanisms (12, 24). In the present study, IAV infection increased intracellular ROS in NHNE cells within 1 hour. ROS scavenging aggravated IAV replication, and the viral titer was higher in cells where ROS were cleared by antioxidant introduction. ROS were also involved in the induction of IFN-λ gene expression and protein secretion, and ROS scavenging reduced IFN-λ regulation after IAV infection in NHNE cells. With the understanding that ROS may play a major role in cellular damage or inflammatory signaling, the present findings suggest that the absence of ROS could lead to accelerated IAV infection by impeding IFN-λ production and secretion in NHNE cells. Although

Figure 6. Mitochondria and Duox2 are involved in intracellular ROS generation after IAV infection in the nasal epithelium. After pretreating NHNE cells with 10 μ M *N*^G-monoethyl-L-arginine (NMEA), 100 μ M allopurinol, and 500 μ M mitoTEMPO (all from Sigma-Aldrich), NHNE cells were infected with WSN/33 (H1N1) for 2 days. (A) Pretreatment with mitoTEMPO only induced IAV mRNA levels after WSN/33 (H1N1) infection. (B) Real-time PCR showed that IAV mRNA levels in cells transfected with Duox2 shRNA before WSN/33 (H1N1) infection were significantly higher after infection. (C) NHNE cells were treated with mitoTEMPO and transfected with control shRNA and Duox2-specific shRNA. (D and E) The efficiency of Duox2 knockdown was monitored by Western blot analysis. The cells treated with mitoTEMPO and transfected with Duox2 shRNA significantly suppressed IAV infection-induced (D) DCF-DA fluorescence intensity and (E) MitoSOX red fluorescence intensity. The fluorescence intensity data are representative of five independent experiments, and the results are presented as the means $\pm$ SDs from five independent experiments. * P < 0.05, compared with the level of IAV-infected cells or cells transfected with control (cont) shRNA.

Figure 7. Mitochondria-induced and Duox2-induced intracellular ROS regulated IFN-λ gene expression and protein secretion after IAV infection in the nasal epithelium. NHNE cells treated with mitoTEMPO and transfected with Duox2 shRNA significantly suppressed IAV-induced (A) IFN-λ1 and (B) IFN-λ2/3 gene expressions. (C) ELISA results showed that IAV-induced IFN-λ secretion also comparatively decreased in cells treated with mitoTEMPO and transfected with Duox2 shRNA. The results are presented as the means ± SDs from five independent experiments. **P* < 0.05, compared with the level of IAV-infected cells or cells transfected with control (cont) shRNA.

little is known about the regulatory mechanisms behind IFN-λ in the nasal epithelium, we demonstrated that intracellular ROS generation rapidly increased after IAV infection and mediated IFN-λ induction to control IAV replication. Therefore, further research on the enzymatic sources of ROS may promote a greater understanding of IFN-λ regulatory mechanisms, and may suggest better therapeutic strategies for acute IAV infection in NHNE cells.

The deliberate production of ROS by Nox has been widely appreciated as a critical component in the lungs (15, 37–39), and evidence points to Duox as the main Nox isoform that generates ROS in the apical portion of bronchial epithelial cells (40, 41). We also previously reported that Duox is the most abundant Nox subtype in NHNE cells (16). Recent studies suggest that Duox may participate in innate host defense, and it also appears to be involved in epithelial signaling pathways for the production of Th1 mediators (42). In fact, Duox1 and Duox2 are thought to produce hydrogen peroxide as a substrate for peroxidase-mediated antibacterial action in bronchial epithelial cells (40, 41). We suggest here that Duox2 plays an important role in host innate immunity against IAV infection in NHNE cells, based on the finding that the suppression of Duox2-generated intracellular ROS or reduced Duox2 expression inhibited a component of the innate immune response (i.e., IFN-λ), leading to enhanced IAV infection.

We additionally demonstrated that mitochondria are involved in IAV infection-induced intracellular ROS generation in NHNE cells. Mitochondria are critical organelles that are implicated in the induction of cell-death pathways, and consequently, mitochondrial ROS also comprise a very potent element in immune interactions with pathogens (43). Mitochondria induce

ROS-regulated IFN-λ expression after IAV infection in NHNE cells. Furthermore, inhibition of the mitochondrial respiratory chain reaction reduced IFN-λ secretion and aggravated IAV infection. Based on these results, we conclude that IAV infection also triggered intracellular ROS generation in the mitochondria within 1 hour, and then intracellular ROS mediated IFN-λ regulation in NHNE cells.

We did not conclusively prove an interaction between Duox2 and mitochondria as the main cellular sources of ROS after IAV infection. However, we assumed that both Duox2 and mitochondria are involved in the rapid generation of intracellular ROS within the first hour after IAV infection in NHNE cells.

In conclusion, these results indicate that IFN-λ contributes to the main first-line defense against IAV infection in NHNE cells, and may be a key element in therapy for acute IAV infection in the upper airway tract. Mitochondrial and Duox2-generated ROS regulated IFN-λ induction to limit IAV replication in NHNE cells.

Author disclosures are available with the text of this article at www.atsjournals.org.

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