Reactive Oxygen Species (ROS) Homeostasis in Influenza Virus Infection

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Abstract: Cellular oxidation is responsive to external and internal stimulation and is generated via signal molecules in defense mechanisms through networks of cell proliferation, differentiation, intracellular detoxification, bacterial infection, and immune reactions. Oxidative stress is not necessarily harmful per se; it depends on the balance between oxidation and antioxidation cascades, which are induced according to stimuli and can maintain oxygen homeostasis. The reactive oxygen species (ROS) that are generated during influenza virus (IV) infection have critical effects on both the virus and host cells. In this review, we outline the link between viral infection and ROS production, using IV as an example. We introduce the current state of knowledge on the molecular relationship between cellular oxidation mediated by ROS production and various effects of IV infection. We also summarize the potential anti-IV agents that act by targeting oxidative stress.

Keywords: antioxidation; aryl hydrocarbon receptor; cellular oxidation; nuclear factor E2-related factor 2; reactive oxygen species
1. Introduction

Influenza viruses (IVs) have been found in pandemics and seasonal epidemics and are serious threats to humans [1‒4]. Currently, vaccination is a key strategy against IV infection; however, effective vaccines may not be produced that precisely target new and emerging IV strains. Several anti-influenza drugs, including adamantanes, target the IV M2 protein and inhibit virus uncoating, whereas neuraminidase (NA) inhibitors block virus release from host cells [5,6]. However, because of resistance to antiviral agents, additional anti-influenza drugs should be developed to control IV outbreaks.

Reactive oxygen species (ROS) are often generated during IV infection [7], thus promoting apoptosis, lung injury (LI), and inflammation/allergy [7‒10]. Inhibitors of NADPH oxidase 2 (NOX2), an enzyme that is responsible for ROS production, are useful to protect mammals against severe IV infection [10]. These studies indicate the crucial roles of ROS in IV infection, which may have implications for therapy. In this review, we summarize ROS generation and redox control of the host cells upon IV infection and discuss how ROS can influence IV replication. We also describe the potential therapeutics against IV infection through modulating ROS and antioxidation of host cells and list their merits for clinical use.

1.1. ROS generation and antioxidation system in cells

Mitochondria are the target organelle of oxidation-reduction reactions [11]. Mitochondria play major roles not only in the production of adenosine triphosphate but also in the generation of ROS during oxidative phosphorylation [12]. The hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (HO•), and superoxide anions (O$_2$•–) produced during oxidation are identified as ROS [13]. Intracellular ROS are produced mainly by enzymes, such as mitochondrial nicotinic adenine dinucleotide phosphate (NADPH) oxidase (NOX) [14], xanthine oxidase (XO) [15,16], cytochrome p450 (CYP) [17,18], and other mitochondrial respiratory complexes, polyamines, lipid-catabolizing enzymes, and dual oxidase (DUOX) family members.

The mechanisms underlying superoxide anion production and the single-electron reduction of 8-nitroguanosine into the respective anion radical via NADPH-cytochrome P450 reductase (POR) have been reported [7]. Any of the isoforms of nitrogen oxide synthase (iNOS), or even XO, subsequently transfers electrons to molecular oxygen [19].

The balance of redox reactions is maintained in normal cells by the antioxidant system, which includes enzymatic antioxidant systems, such as superoxide dismutases (SODs) and catalases (CATs), glutathione peroxidase (GPx), and glutaredoxins (GR), and a nonenzymatic system, which consists of anserine, carnosine, carotenoids, flavonoids, GSH, homocarnosine, melatonin, vitamin C, and vitamin E [20,21]. The decrease in GSH/GSSG, which is a cellular antioxidant index, is caused by the decrease in the level of GSH, which acts as a redox buffer within cells [22]. GSH is a scavenger of singlet oxygen and hydroxyl radicals, a detoxification molecule of hydrogen and lipid peroxide, and a cofactor of enzymes of detoxification. It also plays a key role in suppressing oxidative stress in several RNA viruses, including IVs [23].

Nuclear factor E2-related factor 2 (Nrf2) regulates the expression of enzymes that participate in the defense against oxidation [63]. In the presence of normal levels of ROS, the expression of Nrf2 is regulated by the Kelch-like ECH-associated protein 1 (Keap 1), which targets Nrf2 to ubiquitin-mediated degradation in the cytoplasm. In the presence of increased ROS production, the Nrf2 molecule dissociates from Keap 1 and translocates into the nucleus, where it binds to the antioxidant-response element (ARE) together with the small Maf transcription factors in the promoters of target genes that encode antioxidant enzymes. Notably, the promoter of the Nrf2 gene itself contains AREs [24] and amplifies the redox cascades via positive-feedback regulation.

The cytoplasmic aryl hydrocarbon receptor (AhR) protein binds to the ligands that have translocated into the nucleus and activates the expression of a large family of antioxidant molecules, i.e., the cytochrome p450 proteins (CYP1A1, CYP1A2, and CYP1B) [25], as well as several other antioxidation proteins, such as NAD(P)H quinone oxidoreductase 1 (NQO1), after the formation of heterodimers with Arnt. These enzymes catalyze primarily oxidative-detoxification reactions.

1.2. IV

The genomes of IVs consist of negative single-stranded RNAs that are associated with the viral
nucleoprotein (NP) and interact with heterotrimeric viral RNA-dependent RNA polymerases, i.e., polymerase basic protein 1 (PB1), PB2, and polymerase acidic protein (PA), to build the viral ribonucleoprotein (vRNP) complexes.

Various antioxidation molecules are activated by IV infection, to ameliorate ROS damage in host cells. To examine the role of oxygen free radicals in hosts, SOD conjugated with a copolymer of pyran was administered to decay free radicals; this approach prevented infection with IV in mice [26]. GSH inhibits the expression of viral matrix proteins, IV replication, and the production of virion particles. Furthermore, GSH suppresses the upregulation of Fas, caspase activation, and apoptosis in infected cells [27]. However, IV infection disrupts redox balance by decreasing GSH production and promoting the propagation of their progeny, thus resulting in cell death [28]. The mechanism underlying the IV-induced downregulating of GSH remains unknown.

The replication of IVs is also affected by the Nrf2-regulated redox state [29]. The activation of the Nrf2/heme oxygenase 1 (HO-1) and toll-like receptor (TLR)/mitogen activated protein kinase (MAPK)/nuclear factor kappa B (NF-κB) signaling pathways is involved in IV replication and IV-related pneumonia [30,31]. In some cases, the aryl hydrocarbon receptor (AhR) also regulates redox genes, such as the NQO1 gene, to maintain the ROS balance in host cells [32].

At the molecular level, IVs induce oxidative stress via AhR in the cytoplasm, especially in the endoplasmic reticulum (ER) and mitochondria, which is followed by the production of ROS. Simultaneously, the AhR transcription factor is translocated into the nucleus to activate the phase I target genes, which encode detoxification enzymes, several antioxidation enzymes, and genes for the immunoregulatory and allergy related genes of anti-viral immunity [7,33,34]. If the extent of ROS production surpasses the level of antioxidation, the ROS balance was abrogated, and the IV-infected cells commit to apoptosis and necrosis for cell death. If the ROS generation is cancelled by the antioxidation, the redox balance is remained to commit the resistance of host cells to the IV infection and the anti-viral immunity.

Cellular proliferation and cellular apoptosis/necrosis, in addition to the immunity and allergy, are mostly dependent on the ROS balance [35]. Thus, ROS act as bifunctional reactors and exhibit both “good and bad” aspects in living cells, which might be similar in IV-infected host cells and the propagation of IV progeny [35].

2. ROS production in IV-infected cells

Increased ROS, activation of iNOS 2 for reactive nitrogen species (RNS) production, and higher level of nitrotyrosine have been detected in IV patients [9]. Some source of IV-infected ROS in host cells are summarized below.

2.1. PB1-F2 induces ROS production in host cells

PB1-F2 in influenza type A interacts with the adenine nucleotide translocator and the voltage-dependent anion channel 1 and inactivates matrix metalloproteinases, releases proapoptotic proteins, and induces cell death [36,37]. PB1-F2 is involved in the generation of mitochondrial ROS in alveolar epithelial cells by downregulating SOD1 [38]. In addition, H7N9 PB1-F2-induced ROS trigger inflammasome activation and IL-1β secretion, which is inhibited by Mitotempo, an inhibitor of mitochondrial ROS [39]. After viral infections, NOD-like receptor pyrin domain-containing-3 (NLRP3)/inflammasomes are activated to induce pyroptosis, a death pathway that is inherently associated with inflammation by activating caspase-1 and the secretion of cytokines from infected cells [38,39].

2.2. NOX and DUOX protein families

IV infection can induce cell death through viral PB1-F2, which targets mitochondria. Moreover, this cell death is conserved in influenza type A viruses, but not in influenza type B viruses [36,37]. In addition to the mitochondrial respiratory complex, ROS generated from NOX are involved in the pathogenesis of IV infection. The NOX family is found in cell and phagosome membranes and comprises several members: NOX1 to NOX5 and DUOX1 and DUOX2 [40]. An increase of NOX expression and downregulation of SOD1, SOD3, the
nuclear factor E2-related factor 2 (Nrf2), and catalase were also downregulated in H5N1-infected A549 cells [28]. Infection with H5N1 decreased SOD1 promoter activity, whereas the forced expression of SOD1 disrupted H5N1 virus replication in A547 cells.

NOX2 seems to be involved in the production of ROS during IV infection [8,10,11]. In a mouse model, Nox2-derived superoxide is critical for IV-induced pulmonary damage. Nox2\(^{-/-}\) knockout (KO) mice exhibit a milder airway inflammation and less apoptotic alveolar epithelium after IV infection than do wild-type mice [41]. The IV-mediated production of ROS and reactive nitrogen species (RNS) was decreased in Nox2\(^{-/-}\) KO mice [41]. Furthermore, the titer of active competent viruses and their associated inflammatory activities were decreased in these KO mice [42]. Therefore, NOX2 is a candidate anti-ROS therapy to manage IV infection [41].

NOX4 plays a role in ROS production in lung cancers and in IV-infected primary epithelium cells [42]. The production of superoxide anion in bronchoalveolar lavage fluid-derived fibroblasts from H3N2-infected Nox1\(^{-/-}\) KO mice was inhibited at day 7 postinfection; however, on day 3 (in the early stages of infection), it was unchanged compared with the level detected in the wild-type littermates [43]. Mice expressing an inactive Nox1 (Nox1\(^{+/}\)) exhibited a greater survival rate after an influenza type A virus challenge than control mice [44]. The adaptive immune response was altered after the IV challenge in these mice, such as a decrease in virus-specific CD8\(^{+}\) T cells in the lung, an increase in the number of virus-specific CD8\(^{+}\) T cells expressing CD127 (IL7 receptor) in the lung, and draining of lymph nodes. Thus, Nox1 may affect negatively the early adaptive immune response to IAV infection.

DUOX2 is another source of ROS production during IV infection [45,46]. IV enhances the induction of DUOX2/DUOXA2 and a moderate reduction of DUOX1 [45]. This DUOX2/DUOXA2 induction is mediated by H1N1, but not by H3N2 [43]. DUOX2-induced ROS formation was triggered in the airway cells via type 1 and type 3 interferon (IFN) pathways, inducing the RIG-1-like receptor dsRNA helicase enzyme (RIG-1) and the melanoma-associated differentiation gene 5 (MDA5) in human nasal epithelium and mouse nasal mucosa [47]. The enhancement of ROS in mitochondria mediated by DUOX2 or NOX2 is indicative of mitochondrial dysfunction [48].

The generation of iNOS, nitric oxide, and 8-nitroguanosine represents IV-induced production of superoxide anion [9,49,50].

3. ROS in IV-induced tissue injury and cell death

Viral infection may alter oxidative stress and the antioxidation machineries. For example, Lin et al. reported that infection with H5N1 results in a higher level of ROS in A549 cells compared with infection with H1N1, accompanied by a significant reduction in the ration of reduced glutathione (GSH)/oxidized glutathione (GSSG) [28].

3.1. IV-induced cell injury in the lung

In humans, IV infection causes a contagious respiratory disease in which many alterations of biological functions are induced, such as apoptosis and necrosis [51], autophagy [52], inflammation [53], LI [54,55], DNA damage and oxidation in host cells [56], lipid peroxidation [57,58], and antioxidation reactions mediated by enzymes [59,60].

Oxygen radicals are produced in the lungs of mice infected with IV [61] and ROS play a critical role in the acute lung injury (ALI) that occurs in mice infected with the highly pathogenic avian IV type A (H5N1) [62]. Moreover, in some cases, infection with H5N1 induced a high viral load and a strong proinflammatory reaction [63]. This action of H5N1 increases mortality and generates a more pronounced oxidative stress than other IVs, such as the human influenza A virus (H1N1).

Recent studies have reported that ROS production exhibits a positive or negative function during IV infection [64]. In the former case, viral infection can generate moderate ROS levels, which play a critical role in biological reactions with few cellular damage events. In contrast, excessive ROS are the major cause of LI. Downstream ROS targets, such as NOX1, NOX2, NOX4, and DUOX2, are involved in apoptotic cell death in the epithelium and ALI [43,65]. After infection, IVs hijack the biological functions of host cells to enhance viral
Accordingly, the imbalance between the redox control against IV and the production of excess ROS results in tissue damage [65].

3.2. **Effect on the nervous systems**

IVs damage the central nervous system (CNS), leading to IV-associated encephalitis and encephalopathy [66–68]. Previous studies have suggested that IVs can infect astrocytes, which are the most abundant cells in the CNS and an integral part of the blood–brain barrier and induce a proinflammatory cytokine response and apoptosis [69,70]. Lin et al. reported that human astrocytes exhibit induction of the expression of several cytokines/chemokines, such as CXCL9-11, NF-κB, and p38MAPK phosphorylation, and receptors of neurotransmitters, such as the melanocortin 2 receptor, cholinergic receptor nicotinic gamma subunit, purinergic receptor, gamma-aminobutyric acid (GABA) A receptor α1, and epidermal growth factor receptor 2, which are involved in synaptic transmission and CNS disorders [71]. More recently, it was reported that H5N1 bound and cleaved HA with a specificity for alpha-2,3-linked sialic acids, allowing efficient binding of IVs and their efficient replication in CNS cells [72].

4. **IV-mediated ROS on cellular components**

These IV-mediated functions such as TLR family, inflammation, MMP, and ERs stress in host cells are triggered, at least in part, by regulation of Nrf2/ROS signaling and signaling pathways of PI3K/AKT, p38/JNK MAPK and NFκB.

4.1. **TLR family and membrane receptors**

Human IV infections, such as H1N1 and H3N2, increase the expression of TLR family members, including TLR 3, 7, 8, and 9; however, TLR2 and 4 are suppressed in this setting [73]. Another report showed that the expression of TRK2, 3, and 9 was correlated with H1N1 [74]. The upregulation of signaling molecules of IkB, P-MAPKs, and inflammatory cytokines (such as IL6, sTNFR-1, MCP-1, CXCL10, and IFN gamma) is closely related with the upregulation of TLRs, MyD88, IRAK4, and TRAF6 and with human, avian, and swine IVs [73–77].

TLR3−/− KO mice exhibit higher survival rates with lower viral titers, lower production of mediators of inflammation, and fewer pathological alterations in their lungs after IV infection than their wild-type counterparts [78,79]. TLR7 is also required for the efficient replication of IVs [80]. The inhibitors of the TLR7/8–MyD88 axis possibly also inhibit IV replication and control proinflammatory cytokines and matrix metalloproteases (MMPs) [75]. In contrast, the expression levels of TLR4 determined H1N1 entry and infection tropism via MyD88–p38 MAPK signaling [81]. Inactivated avian H5N1 can rapidly lead to the activation of the TLR4/TRIF/TRAF6/NF-κB axis [8]. TLR4−/− KO mice are refractory to H1N1-induced ALI, and a TLR4 antagonist decreases H1N1 viral titer and its lethality [80].

The generation of NADPH and ROS requires the activation of TLRs, RIG-like receptors (RLRs), and NOD-like receptors (NLRs); moreover, these pattern-recognition receptors can promote innate immunity to protect host cells [73,82]. In addition, the mucosal defenses of the lungs against influenza type A can be followed by a single inhaled treatment comprising a synergistic combination of a TLR agonist (such as the diacylated lipopeptide ligand of TLR2/6, Pam2CSK4) and a CpG ligand for TLR9 (ODN362). These antiviral responses to viral burden attenuated the infectivity and enhanced survival potency via the protective responses afforded by ROS generation [83].

4.2. **MMP**

H3N2 infection induces the expression of MMP-9, but not of MMP-2, in Vero cells. MMP-2 production was increased in Madin–Darby canine kidney (MDCK) cells. Thus, the induction of MMPs is dependent on the epithelial cell type [84]. The expression of MMP-9 is increased in the lungs of a mouse model of IAV infection [85]. The immunopathological response to IV strains via the production of MMP-9 was compared between the human IV viruses H1N1 and H3N2 in mice and revealed that H1N1 induces high mortality and severe lung changes with G-1+ and CD11b+ cell infiltration and upregulation of CxCL6/GCP-2, CCL2/MCP-1, and the tissue inhibitor of metalloproteinase 1 (TIMP1) [86]. Infection with H1N1 upregulated the active and latent forms of MMP-9 in the lung and inhibitor of MMP-2 or MMP-9 reduced partially in lung pathology.
Both Gr-1+ and CD11b+ cells in H1N1-infected lungs produced ROS and RNS, indicating MMP expression is controlled by oxidative stress and antioxidation. The human influenza type A virus induced infiltration of neutrophils, which produced MMP-9 [87]. In contrast, MMP-9 production was not increased in human neutrophils by IV type A [88]. Thus, MMP-9 production in neutrophils is not controlled by IV per se. Other cell types, such as macrophages, might regulate IV-mediated MMP-9 production. H1N1 induced the expression of the MMP-9 gene and the cleavage of pro-MMP-2 into an active intermediate protein in human fetal membrane cells, resulting in the weakening of the membrane integrity and the degradation of the extracellular matrix [89].

4.3. Inflammation and ER stress

IV infection induces a robust production of cytokines, such as IFNs; interleukins (ILs); chemokines, such as CXCL10 and CCL5; tumor necrosis factors (TNFs); and ROS, which can promote the expression of inflammatory cytokines [90,91]. The generation of ROS is required in host cells after the activation of TLRs, which may be used by IVs to promote innate immunity functions in their hosts [8,63]. IVs trigger the production of proinflammatory cytokines/chemokines, such as CCL5/RANTES, CXCL10 (C-X-C motif chemokines), IL1β, IL6, IL8, and TNFα [92,93]. Some of these factors belong to the NF-κB signaling pathway, including IL2, IL6, IL8, MIP1α, MCP-1, and RANTES [94]. These issues were reviewed by other authors [95,96].

After IVs infect the host cells and the production of ROS/NOS surpasses the normal levels, events such as the production of oxidizing nitrogen oxides and peroxynitrite occur concomitantly. In turn, these events induce the oxidation or nitration of amino acid residues, lipid peroxidation, and DNA strand breaks, finally producing apoptotic signals in states of ER stress or of oxidative stress in mitochondria [97]. Thus, the generation of ROS is related to the cascades that commit the ER and mitochondria to apoptosis.

IV infection also induced ER stress and generated ROS in inflamed tissues [96,98–100]. IVs induce proteasome-dependent ER-associated degradation through the inositol-requiring enzyme 1/x-box binding protein 1 (IRE1/XBP1) signaling pathway and commitment to SOD1 downregulation, thus allowing ROS accumulation. ROS-mediated JNK or the IRE1-mediated JNK1 contributes to the control of IV infection and propagation [101].

4.4. Lipid oxidation

Malondialdehyde, F2-isoprostane, 7-ketocholesterol, and 7β-hydroxycholesterol were identified as lipid alterations induced by IV-mediated ROS [21,102]. IV infection induces oxidative stress, which is accompanied by an increase in the levels of lipid peroxidation [103] in the presence of vitamin E, conjugated dienes, and total malondialdehyde.

4.5. DNA oxidation on host cells

DNA is the most common target of ROS, which they modify to give, for example, 8'-hydroxy-2'-deoxyguanosine (8-OHdG) on the 8-position of dG in host DNA. 8-OHdG is produced via a transversion mutation of G to A in the host DNA and increases the risk of neoplasia [7,104].

5. Cellular signaling mediated by IV-induced ROS

The triggering of IV mediated these functions is related with the ROS generation and its redox regulation.

5.1. Nrf2 signaling pathway

Redox homeostasis is maintained by cellular enzymes such as SODs, CAT, and GPx, which participate in antioxidant defense systems. IV infection can decrease the production of antioxidation targets, such as HO-1 and NQO1. SOD, GR, CAT, and GPx are all downstream effectors of the Nrf2 pathway after IV infection. Thus, Nrf2 plays an important role in the redox regulation upon IV infection [24,105–108]. IVs activate the Nrf2/ARE antioxidation pathway via the nuclear translocation of Nrf2, followed by the transcriptional activation of Nrf2 target genes, such as HO-1 [108,109].

The highly pathogenic avian H5N1 IV reduces the levels of phosphorylated Nrf2 in the nucleus and downregulates the expression of fibronectin to a greater extent than does the human H1N1 IV [110]. Several studies found no changes in the levels of SOD in IV-infected cells [111]; however, other studies found a contradictory lower level of SOD1 caused by protease degradation [27,112]. Increased expression of SOD1 was
reported in patients with asymptomatic IV infection [113]. Decreased levels of SOD1 have been found in children infected with H1N1 [114]. Therefore, whether SOD1 is a marker of IV infection remains uncertain.

Similar controversial findings have been reported for other antioxidant enzymes, such as CAT and indolamine-2,3-dioxygenase (IDO). IDO scavenges superoxide anion for oxidation or for converting tryptophan into kynurenine [115]. The IDO level is unaltered and the CAT levels are reduced in IV-infected cells in vitro [111]. In contrast, in infected mice, both IDO and HO-1 are induced and CAT is unchanged [116]. CAT- and peroxiredoxin-6 (PRDX-6)-deficient mice infected with H1N1 exhibit depletion of IV-permissive bronchial Clara cells and/or alveolar type 2 (AT-2) cells [109]. Similar studies reported the induction of other enzymes, such as GPX3 and HO-1. Other Nrf family members, such as Nrf1, bind to the ARE in the promoter regions of redox-related genes, although to a lesser extent than that observed for Nrf2. In contrast, Nrf3 does not behave similarly [117].

5.1. The p38 MAPK signaling pathway

p38 MAPK plays a vital role in cell proliferation, differentiation, development, and death. For example, phosphorylated p38 translocated into the cell nucleus and upregulated cytokines/chemokines under oxidative stress. p38 MAPK signaling is sensitive to oxidants and is involved in IV replication [107,108]. Thus, it is a critical mediator of oxidation-induced apoptosis, to increase ROS and COX-2 production [118,119].

5.2. The NF-κB signaling pathway

NF-κB plays a key role in the activation of the immune system. The NF-κB complex comprises five proteins, namely Rel A (p65), c-Rel, Rel B, p50, and p52. The NF-κB p50/p65 heterodimer associated with IκBa is related to the outcome of oxidative stress [120,121]. After phosphorylation of p65 at Ser 276, NF-κB antagonizes Nrf2 and suppresses the transcription of ARE-dependent genes by recruiting histone deacetylase 3 to the ARE [121]. Thus, inhibition of NF-κB activity may benefit Nrf2-mediated antioxidation and the suppression of IV-induced inflammation.

5.3. The PI3K/AKT signaling pathways

IVs can modulate several oxidative-stress- and redox-activated signaling pathways, such as those involving NF-κB, MAPK, and PI3K/AKT [122‒125], to promote viral replication and pathogenesis [125–128]. Therefore, the modulation of these signaling pathways may attenuate IV-induced pulmonary damage [127,128].

6. Activation of AhR augments IV virulence

TCDD-treated and IV-infected mice exhibit activation of AhR in the lungs and a decrease in survival, which suggests a relationship between the susceptibility to viral respiratory infections and exposure to environmental toxin ligands [129]. In this model, increased iNOS levels in endothelial cells of virus-infected mice and an increased number of neutrophils around pneumocytes were observed after AhR activation, which requires a nuclear transport signal and intact DNA-binding domains within AhR [130]. The activation of the AhR, which occurs via kynurenine mediation, regulates the production of IFNβ negatively after IV infection, which allows virus propagation [131]. IV infection can increase kynurenine production by upregulating the expression of indoleamine-2,3-dioxygenase (IDO1), which is a key enzyme in the kynurenine biosynthesis pathway [132].

In addition to the increased pulmonary neutrophilia and iNOS levels resulting from IV infection, mice treated with TCDD, which activates AhR only transiently, exhibited a diminished IV-specific CD8+ T-cell response [133]. This suggests that the prolonged AhR activation induced by the environmental pollutant
TCDD correlates with increased respiratory IAX infection. Furthermore, Boule et al. [134] compared the effects of four AhR agonists, TCDD, 3,3′,4,4′,5-pentachlorobiphenyl-126 (PCB126) 2-(1H-indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE), and FICZ, on the immune response in mice infected by IVs. Treatment with TCDD, PCB, and ITE decreased the virus-specific IgM/IgG levels and the number of helper T cells and CD8+ cytotoxic T cells but increased the number of regulatory T cells. However, FICZ alone decreased the levels of virus-specific IgG and the CD8+ T-cell response and increased the number of helper T cells. These studies suggest that harnessing AhR activity is critical for modulating the host cell immunity to IV infection.

Regarding ROS production and AhR activity in vivo, singlet molecular oxygen (1O2) is not fully characterized in mammals, but its role is well established in plants, bacteria, and fungi. Although the mammalian enzyme myeloperoxidase mediates the production of 1O2, the physiological role of 1O2 in mammals (other than photosensitization of the skin by the UVA component of solar radiation) has not been established [135]. A recent report by Stanley et al. [136] showed that 1O2 plays a critical role in redox regulation in atrial relaxation and in controlling blood pressure in mammals during inflammation accompanied by endothelial IDO1 expression. Thus, 1O2 is an important ROS in the fields of biology and medicine.

7. Possible anti-influenza therapies

Antioxidant genes, which can be upregulated by Nrf2, play a critical role in the elimination of ROS/RNS; therefore, enhancement of Nrf2 activity and inhibition of AhR activity have been proposed as approaches to ameliorate the IV-associated pathology. For example, the downstream target of Nrf2, SOD conjugated with a copolymer of pyran was administered to decay free radicals; this approach prevented infection with IV in mice [26].

7.1. Inhibition of AhR activity

AhR activation during IV infection disrupts host immunity and causes increased lung inflammation and mortality in mice [102,134,138,139]. The suppression of AhR activity is assumed to attenuate IV-induced lung damage. The level of IV-induced IFNβ is increased in AhR-deficient cells and mice, thus leading to the suppression of viral replication [138]. Several AhR antagonists, such as CH-223191 and Stem Regenin 1, have been identified; however, their therapeutic value against IV-infection-induced LI is unclear. Because AhR responds differentially to diverse intrinsic and extrinsic ligands and affects multiple types of immune cells [139], a careful examination of the advantages and disadvantages of these AhR antagonists is required to assess their value in the treatment of IV infection.

7.2. N-acetyl l-cysteine (NAC)

NAC is a precursor of intracellular cysteine and GSH in mammals. NAC resists IV infection through mechanisms including the inhibition of IV replication, the production of proinflammatory cytokines, and the prevention of IV-induced apoptosis [140–143]. NAC suppresses viral replication and the expression of IV-mediated inflammatory factors, such as TNFα, IL-6, and IL-1β [141]. In addition, cellular damage in the lungs suppresses TLR4 [141]. However, Garigliany et al. insisted that NAC was strain dependent and lacked inhibitory activity against some IVs. NAC was effective for A/PR/8 and H5N1, but not for murinized swine H1N1 IV [144]. The synergistic use of NAC and antiviral drugs as a combination therapy provided effective protection against IV infection in mice [140,142].

7.3. Glutathione

The antiviral activity of GSH may be involved in inhibiting the synthesis of viral proteins [145]. A higher level of GSH might interfere with the formation of disulfide bonds, thus preventing the correct folding of viral proteins.
hemagglutinin (HA), followed by the alteration of its transport and its insertion into host cell membranes [146]. Furthermore, a derivative of GSH, N-butanoyl glutathione (GSH-C4), which is a cell-permeable chemical compound [147], diminishes IV replication by maintaining the immature monomeric HA in the ER and inhibiting the targeting of mature membrane glycoproteins, which is achieved by an increase in GSH levels [20, 148].

GSH inhibits the viral apoptosis induced by IVs and the production of IV particles. It also depresses the expression of viral matrix proteins, caspase cascades, and Fas induction [27]. Moreover, nutritional supplements that induce GSH may afford resistance to the major pathogenic processes of H5N1 [149]. Bakuchiol, a phenolic isoprenoid, activates the Nrf2 pathway and blocks IV infection, which suggests that bakuchiol has antiviral activity [96]. Overexpression of Nrf2 or addition of sulforaphane and EGCG decreases the replication and protein synthesis in IVs [145,150] (Figure 1). Moreover, Nrf2 knockdown increases the entry and replication of IVs and enhances IV-induced pulmonary cell injury. Nrf2 is also a factor in the outcome of IV-infected mice after exposure to cigarette smoke [151]. Nrf2-deficient mice exhibit more severe bronchial inflammation, permeability damage in the lungs, mucus hypersecretion, and higher mortality rates after IV infection and cigarette smoke exposure than did wild-type mice. Taken together, these results suggest that the Nrf2-mediated antioxidant pathway plays a critical role in suppressing IV-induced LI under oxidative conditions, such as cigarette smoke exposure [149,151–153].

7.4. Other small molecules

Isoprenoid phenols (baicalein and biochanin) prevent the replication of the highly pathogenic avian H5N1 virus by repressing ROS production [154]. EGCG and catechin exhibited an antiviral activity that involved antioxidant activity [155,156]. Quercetin decreases the production of superoxide in alveolar macrophages during IV infection [58]. Quercetin also has antiviral activity by inhibiting HA2 during IV infection [157]. A biflavonoid isolated from Garcinia kola seeds (kolaviron) exhibited a strong anti-IV activity that occurred via antioxidative activity [158].

7.5. Nontargeted inhibition (NF-κB, p38 MAPK, and PI3K/AKT inhibitors)

The effects of the dietary flavonoid kaempferol on H9N2-mediated acute LI included the repression of oxidative stress and inflammatory responses via the downregulation of NF-κB [77]. Kaempferol inhibits the NF-κB and MAPK pathways, leading to an increase in SOD activity, the attenuation of ROS levels, and H9N2-induced acute LI [77]. Some ROS scavengers, such as polyphenols, may modulate the NF-κB and MAPK pathways and upregulate GSH biosynthesis through Nrf2 activation [139,159–163]. The immune-regulatory properties of NAC and IV-induced pneumonia are related to the inhibition of the p38 MAPK pathway [164]. Thus, NF-κB activation and the p38 MAPK and p13K/AKT pathways may serve as biomarkers of oxidative stress [165]. These cascades are also involved in the nontargeted signaling induced by IV.
Figure 1. Model of infection of influenza viruses in normal host cells and cells overexpressing Nrf2. Infection by influenza viruses led to the production of reactive oxygen species (ROS). Antioxidation through the Nrf2 transcription factor and viral replication are illustrated. The addition of sulforaphane (SFN) or epigallocatechin gallate (EGCG) inhibits the viral infection of IV via activation of the antioxidation pathway.

Figure 2. Schematic model of the mechanism of ROS production and antioxidation during infection with influenza viruses. During the life cycle of IVs, infection with these viruses causes oxidative-stress reactions in host cells, thus inducing Nox1/Nox4 and AhR and the production of ROS, followed by the replication of the viruses. Nrf2 and other signaling molecules, such as p38, AKT, and PI3K, are also involved in this ROS stress
and redox control. Oxidative stress also induces the translocation of the AhR transcription factor to the ER and mitochondria (MIT), to enhance ROS production. The antioxidation against ROS via the Nrf2 transcription factor leads to the prevention of cell damage at the initial phase; however, the excess of ROS causes apoptosis and other types of cellular death in infected host cells. The life cycle of IVs is summarized and the possible targets of drugs to treat IV infection are also indicated.

Table 1. Drugs and small molecules that prevent infection with influenza viruses.

<table>
<thead>
<tr>
<th>Thiol compounds and prodrugs</th>
<th>Effect on influenza virus infection</th>
<th>References</th>
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<tbody>
<tr>
<td>N-acetyl-L-cysteine (NAC)</td>
<td>Reduction of the cell population at the G0/G1 phase</td>
<td>Geiler et al. [141]</td>
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<td></td>
<td>Reduction of pro-inflammatory molecule production (CXCL8, CXCL10, CCL5, and IL-6)</td>
<td>Wu et al. [169]</td>
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<tr>
<td>Glutathione (GSH)</td>
<td>Affects viral mRNA export and decreases the expression of late viral proteins</td>
<td>Nencioni et al.[59]</td>
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<td>Inhibition of caspase activation and Fas upregulation</td>
<td>Cai et al. [27]</td>
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<td>GSH-C4</td>
<td>Inhibition of influenza virus HA maturation</td>
<td>Sgarbanti et al. [170]</td>
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<tr>
<td>PDTC (pyrrolidine dithiocarbamate)</td>
<td>Decrease in viral RNA synthesis</td>
<td>Uchide et al. [171]</td>
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<td>Hydroxyl antioxidants</td>
<td>Inhibition of apoptosis</td>
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<tr>
<td>NDGA (Nordihydroguaiaretic acid)</td>
<td>Inhibition of apoptotic DNA fragmentation and virus proliferation</td>
<td>Uchide et al. [172]</td>
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<tr>
<td>Thujaplicin</td>
<td>Inhibition of apoptosis, virus replication and release from the infected cells</td>
<td>Miyamoto et al. [173]</td>
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<tr>
<td>Resveratrol/ Vitisin A (tetramer of resveratrol)</td>
<td>Inhibition of the nuclear–cytoplasmic translocation of vRNP Downregulation of viral proteins Inhibition of protein kinase C activity Inhibition of virus-induced RANTES production, to decrease of the virus-stimulated phosphorylation of Akt and STAT1</td>
<td>Palamara et al. [174]; Huang et al. [175]</td>
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<td>Ambroxol</td>
<td>Stimulation of the release of pulmonary surfactants, mucus protease inhibitor, IgA, and IgG Suppression of the release of cytokines, TNF-α IFN-γ, and interleukin-12</td>
<td>Yang et al. [176]</td>
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<tr>
<td>Ascorbic acid</td>
<td>Inhibition of the entry of viruses Increase in the production of IFN-α/β at the initial stage of infection Inhibition of excessive CORT synthesis</td>
<td>Wang et al. [177]; Kim et al. [178]; Cai et al. [179]</td>
</tr>
<tr>
<td>Tert-buthyhydroquinone (tBHQ)</td>
<td>Inhibiting of ROS production and increase antioxidation</td>
<td>Antanasijevic et al. [186, 187]</td>
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<tr>
<td>Curcumin + Resveratrol</td>
<td>Scavenging of H2O2, HON, and ROON Inhibition of TLR 2/4, p38MAPK, and NFkB</td>
<td>Sharma et al. [180]; Barzegar et al. [181]; Dai et al. [182]</td>
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<tr>
<td>Chemical Name</td>
<td>Effect</td>
<td>References</td>
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<td><strong>Emodin</strong> (1,3,8-trihydroxy-6-methyl anthraquinone)</td>
<td>Inhibition of IA replication, IV pneumonia; Inhibition of TLR 4, p38/JNK, and NFkB</td>
<td>Dai et al. [183]</td>
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<tr>
<td><strong>Oxymatrine (OMT); C15H24N2O20, immunosuppressive reagent</strong></td>
<td>Antioxidant; Suppression of inflammation and viral infections; Hepatoprotective and immunosuppressive; Inducer of TLR4, p38 MAPK, NFkB, and PI3K/AKT</td>
<td>Dai et al. [184]</td>
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<tr>
<td><strong>Aurantiamide acetate (E17)</strong></td>
<td>Strong anti-inflammatory and antiviral effects</td>
<td>Zhou et al. [185]</td>
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<td><strong>4-PBA (4-phenyl butyrate)</strong></td>
<td>Inhibitor of ER stress</td>
<td>Jung et al. [148]</td>
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<td><strong>Kaempferol</strong></td>
<td>Inhibition of TLR4/MyD88-mediated signaling of NFkB and MAPK</td>
<td>Zhang et al. [77]</td>
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<td><strong>Apocynin</strong></td>
<td>Inhibitor of NOX2; Inhibition of ROS and IV-induced cytokine production</td>
<td>Ye et al. [206]</td>
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<td><strong>Flavonoids</strong></td>
<td>Effect on influenza virus infection</td>
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<td><strong>Dianthus (quercetin 3; isorhamnetin 3)</strong></td>
<td>Binding to IV polymerase membrane glycoproteins ROS inhibitor</td>
<td>Kim et al. [197]</td>
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<td><strong>Quercetin</strong></td>
<td>Protecting low-density lipoprotein against oxidation; Antithrombic, antivirus, and anti-inflammatory effects</td>
<td>Formica et al. [198]</td>
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<td><strong>Polyphenol</strong></td>
<td>Effect on influenza virus infection</td>
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<td><strong>Chlorogenic acid</strong></td>
<td>Antivirus and antihypertension effects; Protection of dopaminergic neurons against neuroinflammation</td>
<td>Zhao et al. [199]; Shen et al. [200]</td>
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<tr>
<td><strong>Chemicals</strong></td>
<td>Effect on influenza virus infection</td>
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<td><strong>Poly (aniline-co-pyrrole) polymerized nanoregulators (PASomes) with mPEG-b-pPhe (methoxy polyethylene glycol-block-polyphenylalanine copolymer)</strong></td>
<td>Inhibition of ROS production; Inhibition of viral replication and cell death</td>
<td>Kim et al. [210]</td>
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<tr>
<td><strong>Cholesterol conjugated gp91 of NOX2 oxidase gp91phox sequence linked to the human immunodeficiency virus-tat peptide (Cgp91de-TAT)</strong></td>
<td>Inhibitor of NOX2 oxidase; Inhibitor of ROS and inflammation</td>
<td>To et al. [209]</td>
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**[Against Influenza viruses]**

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<tr>
<th>Chemical Name</th>
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<tr>
<td><strong>Hydroxyl antioxidants</strong></td>
<td>Effect on influenza virus infection</td>
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<td><strong>Atorvastatin (Lipitor)</strong></td>
<td>Inhibition of HMG-CoA reductase</td>
<td>Episcopio et al. [208]</td>
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<td><strong>Clarithromycin (Biaxin)</strong></td>
<td>Inhibition of MCP-1 and MMP-9; Increases of IL6 and IFNgamma</td>
<td>Takahashi et al. [211]</td>
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<tr>
<td><strong>Flavonoids</strong></td>
<td>Effect on influenza virus infection</td>
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<td><strong>5,7,4'-Trihydroxy-8-methoxyflavone</strong></td>
<td>Inhibition of virus fusion with endosome/lysosome membranes</td>
<td>Nagai et al. [188–192]</td>
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<td><strong>Catechins</strong></td>
<td>Inhibition of HA and NA activity</td>
<td>Song et al. [156]</td>
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</table>
Conclusions

The pathophysiology of IV infection is concerned, at least in part, with the imbalance between oxidation and antioxidation systems, as well as with the state of AhR activation (Figure 2). In this review, we presented several examples of the effect of the IV–host interaction on the intracellular redox and ROS states. Based on this knowledge, several potential therapeutics for the treatment or prevention of IV infection are presented (Table 1). Heaton et al. [166] reported a CRISPR activation screening that aimed to identify a pan-avian IV inhibitor host factor; those authors isolated the glycosyltransferase B4GALNT2, which can modify glycans containing α-2,3-linked sialic acids. However, most of these proposed therapeutic strategies require validation using animal models of IV infection and human clinical trials. Although many studies have uncovered the multifaceted roles of the cellular redox system and of ROS activity in IV-infection-induced lung inflammation and injury, several questions remain unanswered that are important for illustrating the precise IV–host
interaction and that await further investigation. For example, both oxidative stress and virus replication result in chromatin remodeling, which largely affects gene expression, including that of Nrf2- and AhR-regulated genes, and determines the outcome of the virus–host interaction. Understanding the precise cross-talk between multiple chromatin modifiers, such as histone acetyltransferases/deacetylases and methyltransferases/demethylases, and the transcription factors Nrf2 and AhR upon IV infection is required to predict the consequences of virus infection accurately. Accordingly, a more specific and effective intervention for IV infection may be developed by targeting either ROS homeostasis or chromatin modifiers [167,168]. In conclusion, this review presents the notion that, in addition to virus components, the therapeutic treatment of IV infection may be achieved by targeting virus-induced ROS and redox-associated cellular responses, which may suppress IV propagation and reduce adverse inflammation in the host.

**Abbreviations**

ARE, antioxidant-response element; AP1, activated protein 1; AhR, aryl hydrocarbon receptor; CYP, cytochrome p450; DUOX, dual oxidase; EGCG, epigallocatechin gallate; FICZ, 6-formylindolo[3,2-b]carbazole; HIF, hypoxia response factor; IV, influenza type A virus; IDO, indoleamine-2, 3-dioxygenase; iNOS, inducible nitric oxide; ITE, 2-(1H-indole-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester; Keap 1, Kelch-like ECH-associated protein 1; NA, neuraminidase; NADPH, nicotine adenine dinucleotide phosphate; NOX, NADPH oxidase; NO, nitric oxide; Nrf2, nuclear factor E2 related factor 2; NP, nucleoprotein; PA, polymerase acidic protein; PB, polymerase basic protein; ROS, reactive oxygen species; SFN, sulforaphane; SOD, superoxide dismutase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; vRNP, ribonucleoprotein

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**Ethics approval and consent to participate:** Not relevant.

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