MINIREVIEW

NADPH Oxidases as Novel Pharmacologic Targets against Influenza A Virus Infection

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Received August 4, 2014; accepted October 8, 2014

ABSTRACT

Influenza A viruses represent a major global health care challenge, with imminent pandemics, emerging antiviral resistance, and long lag times for vaccine development, raising a pressing need for novel pharmacologic strategies that ideally target the pathology irrespective of the infecting strain. Reactive oxygen species (ROS) pervade all facets of cell biology with both detrimental and protective properties. Indeed, there is compelling evidence that activation of the NADPH oxidase 2 (NOX2) isoform of the NADPH oxidase family of ROS-producing enzymes promotes lung oxidative stress, inflammation, injury, and dysfunction resulting from influenza A viruses of low to high pathogenicity, as well as impeding virus clearance. By contrast, the dual oxidase isoforms produce ROS that provide vital protective antiviral effects for the host. In this review, we propose that inhibitors of NOX2 are better alternatives than broad-spectrum antioxidant approaches for treatment of influenza pathologies, for which clinical efficacy may have been limited owing to poor bioavailability and inadvertent removal of beneficial ROS. Finally, we briefly describe the current suite of NADPH oxidase inhibitors and the molecular features of the NADPH oxidase enzymes that could be exploited by drug discovery for development of more specific and novel inhibitors to prevent or treat disease caused by influenza.

Introduction

Influenza A viral infections cause significant global morbidity and mortality and are a tremendous economic burden worldwide, with annual medical costs in the United States being approximately $90 billion (World Health Organization, 2009). Influenza A viruses can cause incapacitating respiratory illness in humans and severe, often fatal respiratory and systemic complications from pandemic influenza, such as the 1918 strain and the highly pathogenic influenza A virus subtype (H5N1) (Doherty et al., 2006). Annually, influenza epidemics are responsible for ~5 million cases of severe illness and 200,000–500,000 deaths worldwide (World Health Organization, 2009). In the last century, up to 100 million deaths occurred worldwide as a result of the four major pandemics (World Health Organization, 2009).

Currently, two therapeutic options exist to combat seasonal and pandemic influenza A virus outbreaks (Osterholm, 2005): the use of antivirals [e.g., the neuraminidase inhibitors zanamivir (Relenza) and oseltamivir (Tamiflu)] (Moscona, 2005a; Moscona, 2008) and strain-specific vaccination. The antivirals exert their therapeutic actions by preventing release of influenza virus progeny from infected cells and transmission of the virus. However, certain strains of influenza A viruses are developing resistance to these compounds (Moscona, 2005b). Current vaccines bolster host immune responses to surface influenza A virus glycoproteins but are limiting, as they are effective against only current circulating viruses. However, the global population is at continual risk because of the long lag time (~6 months) for vaccine production and the ongoing threat of new pandemic strains.

The adverse effects of severe influenza infections are believed to be due to inappropriate and heightened airways and lung inflammation (La Gruta et al., 2007). This outcome of an immune response is mounted by the host against influenza virus as a result of respiratory epithelial cell and alveolar macrophage infection. The activation of airway epithelium and alveolar macrophages by internalized influenza virus results in a burst of cytokine release, including a number of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and interferon (IFN)-γ, as well as chemokines, including IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2) and MIP-1α (Doherty et al., 2006; Perrone et al., 2008). This process of cytokine and chemokine
release is crucial for the recruitment of additional inflammatory cells, including neutrophils and blood-borne monocytes that differentiate into macrophages, which are important for viral clearance. However, this early innate immune response sets off an inflammatory cascade that causes persistent trafficking of inflammatory cells into the lung and an excessive production of proinflammatory cytokines and chemokines in what is referred to as the cytokine storm (La Gruta et al., 2007), which has been considered the underlying contributor to lethal disease caused by H5N1 viruses and the 1918 pandemic strain (Tumpey et al., 2005). Thus, the recruitment and activation of infiltrating macrophages and neutrophils must be tightly regulated during influenza infection to promote viral clearance and to minimize excessive damage of surrounding lung tissue. A developing area of research implicates oxidative stress or an overproduction of ROS as a cause of lung tissue damage from influenza, which we propose might be a novel target for pharmacologic modulation (Fig. 1).

**Reactive Oxygen Species: General Properties.** ROS are generally formed from the one electron reduction of molecular oxygen, giving rise to the parent ROS specie superoxide anion (eq. 1). Superoxide anions act locally at their site of production and generally fail to cross cellular membranes (although they may pass through some ion channels) because they are negatively charged molecules. This localized property of superoxide compartmentalizes its oxidizing power, making it, along with its derivative [i.e., hypochlorous acid (HOCl)] ideal microbicidal molecules to kill invading pathogens within the confines of phagosomes of neutrophils and macrophages. This phagosomally located ROS production protects the cell from the potential damaging effects of ROS throughout the rest of the cell. An additional single electron reduction of superoxide produces hydrogen peroxide (H$_2$O$_2$) (eq. 2), which is uncharged and free to diffuse across cellular membranes:

$$2O_2 + 2e^- \rightarrow 2O_2^\cdot$$  \hspace{1cm} (1)  

$$2O_2^\cdot + 2H^+ \rightarrow H_2O_2 + O_2$$  \hspace{1cm} (2)

Both superoxide and hydrogen peroxide influence varied biologic targets, with superoxide preferentially reacting with iron sulfur clusters, found on many transcription factors, whereas H$_2$O$_2$ reacts with susceptible cysteine residues found on enzyme proteins such as peroxidases, kinases, phosphatases, and others (Salmeen et al., 2003; Imlay, 2008).

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![Fig. 1. Schematic diagram depicting the major cell types in the lungs, sites of influenza A virus infection, and expression profile of the various Nox isoforms. Influenza A virus infects airway epithelial cells, which are major sites for virus replication and cytokine release. In addition, virus infects and activates sentinel alveolar macrophages, resulting in cytokine and chemokine release. Replication in alveolar macrophages is abortive for most influenza virus strains (with the exception of some H5N1 strains). The cytokine and chemokine burst is responsible for exacerbating lung inflammation by stimulating the recruitment of additional inflammatory cells such as neutrophils (Neut) and monocytes (Mono) from the bloodstream. Alveolar macrophages and blood-derived neutrophils and monocytes that differentiate into macrophages within the lung are major sources of NOX2 oxidase–dependent reactive oxygen species. Airway epithelial cells express predominantly NOX1 and DUOX, with lower levels of NOX2 and NOX4. Lung endothelial cells express NOX1, 2, 4, and 5. Note: This schematic focuses on innate immune cells and does not deliberately ignore the importance of plasmacytoid dendritic cells, as well as cells of the adaptive immune system, including T-lymphocytes and B-lymphocytes. These have been omitted for clarity only. ssRNA, single-stranded RNA.](image)
As with all signaling molecules, superoxide and H\(_2\)O\(_2\) levels are tightly controlled to prevent excessive levels causing inappropriate activation of redox-sensitive signaling pathways and oxidative damage. For example, the reaction between superoxide and H\(_2\)O\(_2\) gives rise to the highly reactive hydroxyl radical (OH\(^•\)) via the Haber-Weiss reaction (eq. 3) (Haber and Weiss, 1932), which is greatly enhanced in the presence of free transition metal ions (e.g., Fe\(^{2+}\), Cu\(^{2+}\)), known as the Fenton reaction when iron catalyzed (eq. 4) (Chance et al., 1979). Hydroxyl radicals are the most powerful oxidizing agents yet identified, and they react indiscriminately with most biologic molecules at near diffusion-limited rates:

\[
\text{O}_2\text{•}^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2 \quad (3)
\]

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{3+} \quad (4)
\]

A particularly relevant reaction occurring in inflammatory cells such as macrophages is that between superoxide and nitric oxide (NO\(•\)) (eq. 5). The second-order rate constant for this radical-radical reaction is \(10^{10} \text{M}^{-1} \text{s}^{-1}\), and therefore its generation is most probably limited only by the diffusion of the two substrates (Beckman et al., 1990). The product of this reaction is peroxynitrite (ONOO\(^{−}\)), which has a high degree of oxidizing potential, perhaps as high as hydroxyl radicals and has been implicated as a major mediator of tissue injury to virus infections (Szabo et al., 2007).

\[
\text{O}_2\text{•}^- + \text{NO}\text{•} \rightarrow \text{ONOO}^{−} \quad (5)
\]

**ROS Influence on Lung Pathology Caused by Influenza A Viruses.** Accumulating evidence from around the late 1980s suggests that ROS, including superoxide and its derivatives, promote lung injury and inflammation from influenza A virus infection (Akaïke et al., 1996; Imai et al., 2008). The first study implicating superoxide demonstrated that inactivation of this radical by administration of pyran polymer conjugated superoxide dismutase (SOD) improved the mortality of mice against a lethal influenza virus infection (Oda et al., 1989). From a therapeutic point of view, this finding was a vital observation, as it demonstrated that protection could occur if SOD was administered 5 days after infection. In keeping with a role for superoxide as a culprit molecule, it was subsequently shown that mice with selective overexpression of extracellular SOD displayed significantly less lung injury from influenza virus (Suliman et al., 2001). These beneficial effects of superoxide inactivation may be due to inhibition of its downstream derivative, peroxynitrite, which causes significant alterations in the activation of small molecule antioxidants such as glutathione, cysteine, and tetrahydrobiopterin, and enzymes SOD, glutathione reductase, and glutaredoxin (Szabo et al., 2007) by oxidation-dependent inactivation. Indeed, peroxynitrite generation is associated with epithelial cell apoptosis and lung injury from influenza A virus infection (Vlahos et al., 2011). Moreover, peroxynitrite suppresses Na\(^+\) channels on the apical membrane of airway epithelial cells (Lazrak et al., 2009), causing an imbalance in fluid homeostasis, leading to edema of the airways. In addition, manipulation of superoxide production will decrease H\(_2\)O\(_2\) levels which could result in alterations in the master transcription factor nuclear factor kappa B (NF-κB) and expression of proinflammatory genes that influence lung inflammation and damage (Rahman et al., 2002, 2006; Takada et al., 2003). We have recently shown that the antioxidant enzyme glutathione peroxidase-1, which converts H\(_2\)O\(_2\) into water and oxygen, protects against influenza A virus-induced lung inflammation and pathology (Yatmaz et al., 2013).

In support of this finding, mice treated with the SOD/catalase mimic EUK beginning 3 days postinfection with the 1918 influenza A virus resulted in significantly increased survival and reduced lung pathology without a reduction in viral titers (Kash et al., 2014). Immunohistochemical analysis showed a reduction in the detection of the apoptosis marker cleaved caspase-3 and the oxidative stress marker 8-oxo-2′-deoxyguanosine in lungs of EUK-207–treated animals compared with vehicle controls (Kash et al., 2014). Together, these findings highlight the pathologic role of H\(_2\)O\(_2\) in influenza infection.

The bulk of the experimental data thus far places ROS as culprit mediators of lung injury from influenza A virus infections. However, it is noteworthy that there is an emergence of literature that indicates ROS as possessing important antiviral effects against influenza viruses. In fact, the key study by (Strengert et al., 2014) showed that H\(_2\)O\(_2\) suppresses virus replication and release of virus by airway epithelial cells.

It is unquestionable that ROS promote lung injury from influenza A virus infections and that suppressing their levels, and therefore their effects, might be beneficial. There are several ways by which ROS can be manipulated; however, given that some of them (i.e., superoxide, hydroxyl radicals and peroxynitrite) are highly and indiscriminately reactive and will essentially react with substrates within their vicinity with high avidity, perhaps the most effective form of defense against them is to prevent their formation. There are many ways by which this might be achieved, but here we highlight two that have received the most attention:

1. Limit the formation of their parent ROS superoxide, H\(_2\)O\(_2\) and/or NO, although it is important to maintain the balance of good antiviral effects of ROS versus the detrimental effects of excessive ROS. This might be achieved by targeting specific sources of ROS.

2. Regulate superoxide and H\(_2\)O\(_2\) metabolism by stoichiometric scavenging with expendable molecules, such as ascorbate, vitamin E, or others, or with specific antioxidant enzyme systems, such as SODs, catalase, glutathione peroxidases, thioredoxins, and such.

Both these scenarios have been exploited with pharmacologic inhibitors to varying degrees. However, this review focuses on point 1, namely, regulation of the production of superoxide and H\(_2\)O\(_2\) through inhibition of their major enzymatic sources: the NADPH oxidases.

**ROS-Generating Enzyme Systems in Mammalian Cells.** A number of enzyme systems transfer electrons to molecular oxygen to produce ROS, including NADH dehydrogenase and ubiquinone-cytochrome bc1 of the mitochondrial electron transport chain (Boveris and Cadenas, 1975; Turrens, 2003; Orrenius et al., 2007), nitrous oxide synthases (NOS) (Alp and Channon, 2004; Vasquez-Vivar et al., 1998; Vasquez-Vivar and Kalyanaraman, 2000), cyclooxygenases (Simmons et al., 2004), lipoxynegases (Wittwer and Hersberger, 2007), cytochrome P450 reductases (Zangar et al., 2004), and xanthine oxidase (McCord and Fridovich, 1968; Pacher et al., 2006). However, for all these systems, ROS production does not represent the main catalytic function of the enzyme but instead occurs as a by-product of another reaction (e.g., as in the mitochondrial electron transport chain) or from a dysfunctional variant of the enzyme.
SOD, NOX4, DUOX1, and DUOX2 enzymes generate H2O2 (Selemidis et al., 2013, 2014). DUOX 2 protect the host against these types of infections indicates the contrary. In fact, it appears that both NOX1 and NADPH oxidase isoforms, in particular NOX1 and DUOX, in- against primary influenza A virus infection, recent work on other 
T cells that express interferon-γ, TNF-α, or IL-2, was neither unaffected (Vlahos et al., 2011) or enhanced (Snelgrove et al., 2006). This gave rise to peroxynitrite production in the lungs, which contributed to the lung injury (Vlahos et al., 2011). In addition, NOX2-/- mice had substantially reduced airway inflammation and alveolar epithelial apoptosis after infection with low (HKx31) and high pathogenicity (PR8) influenza A viruses (Vlahos et al., 2011). Strikingly, inhibition of NOX2 oxidase resulted in reduced viral titers in the lungs (Snelgrove et al., 2006; Vlahos et al., 2011) after influenza A virus infection, even in the face of suppression of airway inflammation. Vital components of the adaptive immune system that clear influenza A viruses from the lungs were preserved after inhibition of NOX2 oxidase activity (Snelgrove et al., 2006; Vlahos et al., 2011). For instance, the degree of airway infiltration of influenza A virus–specific CD8+ T cells (i.e., CD8 “DbNP366” and DbPA224+ T cells), including CD8+ T cells that express interferon-γ, TNF-α, or IL-2, was neither unaffected (Vlahos et al., 2011) or enhanced (Snelgrove et al., 2006). The evidence thus far suggests that inhibition of NOX2 oxidase in vivo could lead to an improvement in phenotype against influenza A virus infections of mild to high pathogenic strains of virus. However, studies in mice deficient in the NOX1 oxidase have resulted in some conflicting results. For instance, NOX1 has been demonstrated to be protective to the host from influenza A virus infection (Selemidis et al., 2013). Indeed, NOX1 oxidase was shown to critically inhibit the early burst in lung proinflammatory cytokine expression, inflammation, and oxidative stress caused by influenza A virus infection. In contrast, a separate study has shown that deletion of NOX1 resulted in an improvement in survival to a lethal dose of the Puerto Rico (PR/8) strain of influenza (Hofstetter et al., 2013). It is unclear as to why data of these studies appear to conflict; however, it may be attributed to the differences in pathogenicity of the viruses used in each of the studies (i.e., a mild X-31 infection in the case of Selemidis et al., 2013 vs. a highly lethal dose of PR/8 in the Hofstetter et al., 2013 study), although this remains to be determined.
A key difference between NOX1 and NOX2 oxidases is that it is likely to underpin their distinct effects is their cellular localization pattern. NOX1 was found to be highly expressed in alveolar epithelial cells and vascular endothelial cells, whereas NOX2 is most highly expressed in inflammatory cells and, to a lesser degree, in epithelial and endothelial cells. An intriguing finding is that, DUOX2, which is highly expressed in airway epithelial cells and is the major source of H$_2$O$_2$ by these cells, like NOX1, appears to protect the host from influenza A virus infections. DUOX2 activity was associated with a marked reduction in influenza replication within epithelial cells, resulting in protection in the early phases of infection (Strengert et al., 2014). Overall, it appears that ROS have contrasting roles on influenza virus infections, depending on the cell type they are generated within. For instance, epithelial ROS by DUOX (and perhaps NOX1) are protective, whereas inflammatory cell NOX2 is detrimental. These contrasting roles of ROS not only highlight the pressing need for future studies using cell-specific inhibition of NADPH oxidase isoforms on the overall host antiviral response but also that the use of broad-based antioxidant approaches to scavenge ROS, indiscriminately of their source, might not be the most effective approach. Thus, selective enzyme inhibitors may prove to be the best approach. Here we suggest that, given the bulk of the evidence, that specific inhibitors of NOX2 oxidase might be the most effective approach to reduce the burden of the oxidative stress–dependent lung injury to influenza A virus infections.

**NADPH Oxidases and Other Lung Conditions.** In addition to the disease caused by influenza A virus infection, NOX-generated ROS have been recognized to play key roles in the pathogenesis of a number of diverse chronic lung disorders that result in obstructive physiology, in particular asthma, cystic fibrosis, and emphysema (Griffith et al., 2009). Mice deficient in p47phox or NOX2 exhibit increased cigarette smoke-induced lung inflammation and emphysema despite decreased ROS production (Yao et al., 2008). The lung responses in p47phox- and NOX2-null mice were associated with increased production of proinflammatory cytokines and chemokines via a TLR4-NF-kB pathway, indicating that NOX2 may mediate anti-inflammatory functions by restraining TLR4 activation (Yao et al., 2008). However, another group reported that p47phox-null mice have less inflammation, IL-6, keratinocyte-derived chemokine, and monocyte chemotractant protein-1 in lung-lavage specimens after cigarette-smoke exposure compared with wild-type mice (Lagente et al., 2008). The differences observed by these groups may be due to variability in lung compartment sampling, cellular distributions, and chronicity of cigarette-smoke exposure.

**Molecular Description of NADPH Oxidases**

The purpose of this section is not to provide a comprehensive review of the molecular biology and biochemistry of the various NADPH oxidase family members, as this has been achieved by several reviews (Vignais, 2002; Groemping and Rittinger, 2005; Sumimoto et al., 2005; Sellemidis et al., 2008; Drummond et al., 2011; Harrison and Sellemidis, 2014). Instead, we highlight key molecular sites involved in NADPH oxidative activation, particularly those that are unique to specific NADPH oxidase isoforms, and may therefore be exploited by pharmacologic means.

**NOX2-Containing NADPH Oxidases.** NOX2-containing NADPH oxidases were the first identified enzymatic sources of superoxide and are expressed predominantly in phagocytic cells, including neutrophils and macrophages. The NOX2 catalytic domain, like all NOX homologs, harbors all the necessary redox-sensitive components for electron transfer from NADPH to molecular oxygen. The three-dimensional structure of this membrane protein has not been resolved, although the cytoplasmic C-terminal reductase domain of NOX2 has been predicted based on its sequence homology to the ferredoxin-NADP reductase family of enzymes (Taylor et al., 1993). In silico hydrophobicity plotting, random sequence peptide phage analysis, and epitope mapping have provided some topographic information about NOX2 and its molecular interactions with regulatory subunits (Rotrosen and Leto, 1990; Imajoh-Ohmi et al., 1992; DeLeo et al., 1995c; Burritt et al., 2001, 2003; Paclet et al., 2004). These studies predict NOX2 to be a 570 amino acid protein with six transmembrane-spanning domains and glycosylated at three asparagine residues (Asn132, Asn149, Asn240) (Harper et al., 1985; Parkos et al., 1987; Kleinberg et al., 1989; Wallachand Segal, 1997; Taylor et al., 2006). NOX2 consists of an N-terminal segment, six predictable transmembrane a helices, and a cytoplasmic C-terminal tail containing NADPH and FAD recognition sites (Babior and Kipnes, 1977; Taylor et al., 1993; Cheng et al., 2001) that carry electrons to one of two nonidentical heme moieties within the transmembrane segments of the NOX protein (Isogai et al., 1995; Doussiere et al., 1996; Diatchuk et al., 1997), which are held in place by four histidine residues (Finegold et al., 1996). The first heme group is positioned toward the cytoplasmic end of the molecule; the second heme is situated toward the outer face. This outer heme might be responsible for the ultimate facilitation of electron transfer to molecular oxygen to generate superoxide either extracellularly or within the phagosome. The precise role of the C-terminal heme remains to be fully determined, but it is likely that this moiety is involved in stabilizing the interaction between NOX2 and p22phox, an interaction that is critical for stability and maturation of the NOX2 protein within the membrane (Dinauer et al., 1991; Huang et al., 1995; DeLeo et al., 2000).

Although p22phox is the membrane-bonding partner of NOX1, NOX2, NOX3, and NOX4, it is not a partner of NOX5 (Cheng et al., 2001; Banfi et al., 2004). It possesses 195 amino acids, has a molecular weight of ~21 kDa, and hydrophobicity analysis of its amino acid structure indicates that p22phox most likely possesses a cytoplasmic N terminus (residues 1–90), two transmembrane a-helices (residues 91–106 and 112–127) connected by a short extracellular loop (residues 107–111) (Taylor et al., 2004; Zhu et al., 2006) and a C terminus that extends back into the cytoplasm (residues 128–195). The N terminus contains two regions (residues 6–11 and 65–90), which are essential for maturation of NOX2 into a fully glycosylated protein and its expression at the membrane. The most noticeable feature of the C-terminal end is a PRR containing a consensus motif surrounding Pro156, which plays a critical role in facilitating the association of p47phox with the membrane-bound cytochrome (Leto et al., 1994; Leusen et al., 1994; Sellemidis et al., 1994; Sumimoto et al., 1994; Groemping et al., 2003). The tandem SRC homology (SH3) domains of p47phox form a “sandwich” around the PRR of p22phox (Nobuhisa et al., 2006), but the interaction is further facilitated by a contact of one of the SH3 domains with an a-helix immediately
This additional contact contributes to full activation of the NOX2 oxidase, which is facilitated by direct interactions of the p47phox SH3 domains with specific proline and arginine residues within the PRR (especially P152, P156, and R158), as well as with certain residues in the adjacent a-helix domain of p22phox (Nobuhisa et al., 2006).

The p47phox subunit (∼47 kDa; 390 amino acids) contains two SH3 domains, which are conserved regions and well known for protein-protein interactions, an autoinhibitory region (AIR) in the C-terminal end, a Phox homology (PX) domain (Ponting, 1996; Bao et al., 2001), and a PRR similar to p22phox in the N-terminal end (Fig. 2). Under resting conditions, p47phox is incapable of binding to p22phox because the AIR of p47phox prevents, via an intramolecular interaction, the SH3 domains from interacting with the PRR of p22phox (Ago et al., 2003) as determined by X-ray crystallographic analysis. Furthermore, and perhaps less well defined, is another intramolecular inhibitory interaction between the carboxy terminal SH3 domain of p47phox and the PX domain (Ago et al., 2001), thought to prevent the binding of the PX domain with membrane-bound phosphoinositides, which presumably acts as a further hindrance to its association with the membrane during resting conditions (Ago et al., 2001; Kanai et al., 2001). In the presence of an appropriate stimulus, such as phorbol myristate acetate, serine residues on p47phox become phosphorylated (El Benna et al., 1996; Inanami et al., 1998; Fontayne et al., 2002) unleashing the autoinhibition (Ago et al., 1999; Groemping et al., 2003; Yuzawa et al., 2004), and the SH3 and PX domains, which associate with targets on p22phox (Finan et al., 1994; Leto et al., 1994; McPhail, 1994; Sumimoto et al., 1994) and the plasma membrane, respectively. P47phox also binds at multiple sites with NOX2 (DeLeo et al., 1995c), and this interaction is also essential in the assembly of the oxidase. On this point, interference of the molecular interaction between NOX2 and p47phox with short peptide mimetics of this region inhibits NADPH oxidase (Rey et al., 2001) (see later discussion), and this approach has given rise to one of the most selective inhibitors of NOX2 oxidases (i.e., gp91dstat), which is effective at suppressing NOX2 oxidase activity as it prevents p47phox and the ternary complex it forms with two additional subunits within the cytosol, p67phox and p40phox, to interact with NOX2 and form an entire active enzyme complex.

P67phox is a 59.8-kDa protein composed of 526 amino acids and contains at its N-terminal end four tetratricopeptide repeat (TPR) motifs and two SH3 domains, one located centrally and the second at the C-terminal end. A specific activation domain in p67phox docks to a specific region on NOX2, leading to a conformational change in NOX2 and induction of electron flow through the complex (Han et al., 1998; Nisimoto et al., 1999). Thus, p67phox is commonly referred to as an activator subunit.

P40phox is a 339-amino-acid protein with a molecular weight of 39 kDa, and it possesses an N-terminal PX domain, one SH3 domain, and an acidic OPR/PC/AID (OPCA) motif or PBI domain — a class of domains shared by p67phox. p40phox binds to p67phox via the Phox and Bem1 (PBI) domains (Nakamura et al., 1998; Ago et al., 2001; Noda et al., 2003) and with plasma membrane phosphoinositides via its PX domain (Ellson et al., 2001; Honbou et al., 2007; Bissonnette et al., 2008), analogous to p47phox. X-ray crystallography and nuclear magnetic resonance predict that p67phox serves as a link between p47phox and p40phox by interacting with both of these regulatory subunits. Interestingly, the SH3 domain of p40phox may interact directly with the PRR regions of p22phox and as such may act as an alternative organizer to p47phox in NOX2.
activation (Tamura et al., 2007), although the relevance of this direct p40phox-p22phox interaction on oxidase activation is still unknown.

Rac1 or Rac2 belongs to the Rho family of small GTPases, whose activity is governed by the guanine nucleotide they are associated with and are essential for oxidase function (Abo et al., 1991; Knaus et al., 1991; Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002; Dinauer, 2003; Werner, 2004). Rac is inactive when bound by GDP and active when associated with GTP (Bourne et al., 1990, 1991), the interconversion of which is regulated by guanine-nucleotide exchange factors. Rac is recruited to the membrane independently of the cytosolic ternary complex (Heyworth et al., 1994; Dorseuil et al., 1995; Gorzalczyz et al., 2002) where GDP is replaced by GTP, allowing Rac to bind to the TPR motifs in the N-terminal region of p67phox (Diekmann et al., 1994; Nisimoto et al., 1997; Koga et al., 1999; Lapouge et al., 2000). The precise mechanism by which Rac influences NADPH oxidase activity is an ongoing matter for debate. One suggestion states that the binding of Rac with p67phox induces a conformational change in p67phox that allows it to associate with NOX2, leading to superoxide production (Sarfstein et al., 2004). Rac also tethers covalently to plasma membrane lipids in a process that is dependent on a posttranslational modification of the protein involving addition of an isoprenoid group (i.e., geranyl-geranylation) at the cysteine residue within the C terminal end motif consisting of C-Ali-Ali-Xaa (Didsbury et al., 1990; Kinsella et al., 1991; Ando et al., 1992; Kreck et al., 1996). One of the most commonly prescribed classes of drugs used to alleviate symptoms of cardiovascular disease (the statins) suppresses the synthesis of isoprenoid derivatives such as geranyl-geranyl-pyrophosphate, thus inhibiting Rac from binding to the plasma membrane. Statins are powerful inhibitors of NADPH oxidase-derived superoxide production, which may partially explain their clinical efficacy (Liao, 2004).

**NOX1-Containing NADPH Oxidases.** The first homolog of the gp91phox subunit of the prototypical phagocytic oxidase was identified nearly 25 years after the discovery of NADPH oxidases in phagocytic cells and termed Mox1 (for mitogen oxidase 1) (Suh et al., 1999). Later renamed NOX1, this protein consists of 564 amino acids, has a molecular mass of 65 kDa (Cheng et al., 2001), and shares 56% homology with NOX2. It possesses some structural similarities to NOX2, but it possesses different tissue distribution (Suh et al., 1999; Banfi et al., 2000). NOX1 is expressed in colon and airway epithelium (Banfi et al., 2003; Szanto et al., 2005), stomach (Suh et al., 1999), placenta (Cui et al., 2006), uterus (Suh et al., 1999), as well as vascular smooth muscle cells (Cheng et al., 2001), and analogous to phagocytes, may play a role in cell defense at those sites (Cheng and Lambeth, 2004; Kawahara et al., 2004). The similarities between NOX1 and NOX2 include the C-terminal cytoplasmic tail containing the NADPH binding sites, FAD moieties and two heme regions as well as six putative transmembrane spanning regions. Like NOX2, NOX1 is complexed and stabilized with p22phox at the protein level (Ambasta et al., 2004; Kawahara et al., 2005) and is tightly and efficiently regulated by several modulatory protein subunits. Indeed, NOX1-p22phox heterodimers are primarily inactive but produce superoxide in the presence of homologs of p47phox and p67phox called NOXO1 (nox-organizer 1) and NOXA1 (nox-activator 1), respectively (Ago et al., 2003; Banfi et al., 2003; Geiszt et al., 2003; Cheng and Lambeth, 2004).

Like p47phox, NOXO1 protein possesses an N-terminal PX domain, two tandem SH3 domains, and a C-terminal PRR that serves as a binding site for SH3 domains (Ago et al., 2001, 2003); however, they differ in that NOXO1 is only ~41 kDa and lacks an AIR (Ago et al., 1999), and this distinctive feature allows the SH3 domains of NOXO1 to interact with p22phox, even in the absence of stimulation (Ago et al., 2003). Also, like p47phox, the PX domain of NOXO1 associates with membrane phospholipids, and the identification of four alternative splice variants of NOXO1 (Cheng and Lambeth, 2005; Ueyama et al., 2007), which vary in the nature of their PX domain, influences the subcellular localization of NOX1 and ROS production (Opitz et al., 2007).

Similar to p67phox, NOXA1 (molecular size ~51 kDa) contains four TPRs that associate with Rac and an activation domain for NOX1 binding, which are required for full NOX1 activity (Cheng et al., 2006; Miyano et al., 2006; Ueyama et al., 2007). Also, like p67phox, NOXA1 contains only a single C-terminal SH3 domain that associates with NOXO1 (Ago et al., 2003). These structural differences between NOXO1 and p47phox, and between NOXA1 and p67phox, are likely to account for the major functional differences between NOX1- and NOX2-containing oxidases. NOX1 oxidase is likely to be constitutively active, whereas NOX2 requires activation by phosphorylation of p47phox.

**NOX4-Containing NADPH Oxidases.** NOX4 is a 578-amino-acid protein that shares 39% homology with NOX2 (Geiszt et al., 2000; Shiose et al., 2001), but in addition to its expression in kidney cortex, NOX4 is expressed in endothelial cells (Hanna et al., 2004), smooth muscle cells (Lassegue et al., 2001; Wingerle et al., 2001; Paravicini et al., 2002; Bengtsson et al., 2003), heart (Cheng et al., 2001; Byrne et al., 2003), pancreas (Cheng et al., 2001), and osteoclasts (Yang et al., 2001) but in low amounts in phagocytic cells. As for NOX1 and NOX2, NOX4 possesses the same series of electron transporting moieties, including the NADPH binding site, FAD, and heme groups. Also, NOX4 may undergo alternative splicing, giving rise to at least four splice variants that appear not to possess some of these important moieties. Similar to NOX1 and NOX2, the full size NOX4 forms a heterodimer with p22phox that promotes its activity and stability (Ambasta et al., 2004; Martyn et al., 2006); but, unlike NOX1 and NOX2, the PRR region of p22phox is not necessary for NOX4 oxidase activity. Additional molecular interactions that p22phox make with organizer proteins like p47phox and NoxO1 are not crucial for NOX4 oxidase-dependent ROS production (Kawahara et al., 2005). Rac does not appear to regulate NOX4 activity, as neither constitutively activates Rac1, or a dominant negative Rac1 influences NOX4 activity in epithelial cells (Martyn et al., 2006), although in renal mesangial cells, the activation of NOX4 by angiotensin II (Ang II) has been suggested to be dependent on Rac (Gorin et al., 2003). Given that activity of NOX4-containing oxidases is not dependent on at least the currently identified activator and organizer subunits, which appear to be critical for activity of NOX1- and NOX2-containing oxidases, NOX4 may serve as an important constitutively active ROS generating system, whose overall ROS output is perhaps governed by its expression level (Wingler et al., 2001) and posttranslational modification.

**NOX5.** NOX5 is a 737-amino-acid protein expressed in human lymphoid tissues, testes, and spleen (Banfi et al., 2001) and...
vascular endothelial cells (BelAiba et al., 2007). It possesses the same redox active domains and NADPH binding sites as NOX1 to NOX4, but it also possesses a unique, cytosolic N-terminal Ca\textsuperscript{2+} binding domain with calmodulin-like EF-hand motifs that render this oxidase highly sensitive to Ca\textsuperscript{2+} (Banfi et al., 2001) and to phosphorylation that increases Ca\textsuperscript{2+} sensitivity (Jagnandan et al., 2007; Fulton, 2009) . NOX5 exhibits only 22–27% homology to NOX1–NOX4 (Banfi et al., 2001), but unlike those NOX proteins, NOX5 does not require p22phox for activation (Kawahara et al., 2005) or, indeed, any of the other known regulatory subunits. Five splice variants have been identified to date, named NOX5a, NOX5ß, NOX5γ, NOX5δ, and NOX5s, that differ in the sequence of their Ca\textsuperscript{2+} binding regions as well as their tissue distribution (BelAiba et al., 2007). NOX5s lacks the Ca\textsuperscript{2+} binding regions and is constitutively active (BelAiba et al., 2007). Recent evidence shows that NOX5 is regulated by protein kinase C isoforms (Pandey and Fulton, 2011).

**DUOX Enzymes.** Like NOX5, DUOX1 and DUOX2 do not require p22phox for activity, and they also possess EF hands, which render them Ca\textsuperscript{2+}-dependent enzymes. Distinguishing characteristics in DUOX1 and DUOX2 versus other members of the Nox family are that they contain seven membrane-spanning domains and a peroxidase-like domain in their extracellular N-terminal region.

**Pharmacology of Current NADPH Oxidase Inhibitors.** The evidence presented herein provides a strong rationale for the use of pharmacologic inhibitors of NOX2-containing NADPH oxidase to alleviate oxidative stress and its associated pathologies in influenza infection. Several compounds are commonly purported as being inhibitors of NADPH oxidase, and these have been the covered in recent comprehensive review articles (Selemidis et al., 2008; Brandes et al., 2010; Drummond et al., 2011). We have previously proposed that perhaps the most effective and safest way of suppressing NOX2 oxidase activity would be to inhibit the association of p47phox with NOX2 (Vlahos et al., 2012). As mentioned, NOX2 oxidase relies on the organizer subunit p47phox for full activity, although the enzyme can still be partially activated in its absence (Drummond et al., 2011). Given the importance of preserving some NADPH oxidase activity in immune cells, p47phox represents a clinically safer target than other enzyme subunits (i.e., p22phox and p67phox) that are absolutely required for activity.

Of the currently available inhibitors, gp91dsstat and apocynin are the most likely to be specific for the phagocytic NOX2 acting to suppress the actions via interactions with p47phox. The synthetic peptide NOX2ds-tat (also known as Gp91ds-tat) was designed to penetrate cells and prevent the assembly of the NOX2 oxidase complex (see later discussion). This peptide inhibits NOX2 oxidase activity in vitro and reduces parameters of cardiovascular disease in a mouse model of hypertension (Rey et al., 2001). Being a peptide, however, it is unlikely to be orally active or to display a suitable pharmacokinetic profile to have widespread use as a clinical drug.

Apocynin inhibits the association of membrane bound NOX2 with p47phox in a mechanism that appears to be enhanced in the presence of the MPO enzyme and H\textsubscript{2}O\textsubscript{2}, which renders the compound a selective inhibitor of the phagocytic NOX2 oxidase (Heumuller et al., 2008) (given the high amounts of H\textsubscript{2}O\textsubscript{2} generation and expression of MPO by these cells), and suitable for suppressing the oxidative stress caused by inflammatory cells in response to influenza. Indeed, apocynin administration to mice in vivo resulted in a significant reduction in inflammatory cell superoxide production and airway inflammation, and it enhanced the clearance of virus from the lungs after low pathogenic (i.e., HKx31 virus) or moderate pathogenic (i.e., PR8 virus) virus infection in mice (Vlahos et al., 2012). This protective effect of apocynin was achieved with only a 50% suppression of superoxide production by inflammatory cells; therefore, apocynin might be useful in suppressing oxidative stress to influenza while preserving some phagocytic function.

**Exploiting Novel Interfaces in the NOX2 Oxidase Enzyme Complex to Unravel NOX2-Selective Inhibitors**

We suggest that selective targeting of the p47phox organizer protein of NOX2 oxidase, which contributes to the pathophysiology of influenza disease, could be achieved via a drug discovery approach that begins by unraveling key p47phox interactions with NOX2 and other protein subunits that are exclusive to NOX2 oxidase complexes (Fig. 2). The best characterized protein–protein and protein–lipid interactions involving p47phox include its interactions with the p67phox or NOXA1 subunits, p22phox, and the NOX2 catalytic subunit, as well as with phospholipids in the biologic membrane to which the enzyme complex is fastened.

**The p47phox-p67phox Interface.** As mentioned in the previous section, p47phox interacts directly with an SH3 domain on p67phox via a Pxpp-binding motif contained within a PRR near its C terminus. Moreover, further contacts with the SH3 domain of p67phox are made by a domain on p47phox that lies distal to the PRR on its C terminus (amino acids 368–390), and these contacts are important for increasing the affinity of the protein–protein interaction (KA ~ 20 nM) and for conferring binding partner specificity (Kami et al., 2002). Therefore, disruption of the interaction of the PRR of p47phox and the SH3 domain of p67phox represents a possible tactic for preventing the constitutive association of the two subunits in resting cells and for the translocation of the ternary complex to the membrane complex, thereby inhibiting NADPH oxidase activation. A succession of synthetic peptides based on the PRR of p47phox was shown to prevent the binding of p47phox to p67phox, thus providing proof of principle that this interaction may represent a future target for selective NOX2 oxidase inhibitors (Finan et al., 1994).

**Intramolecular Interactions of the p47phox Auto-inhibitory Region with its SH3 and Phox Homology Domains.** In resting cells, p47phox is folded in on itself such that the bis-SH3 and Phox homology (PX) domains of the protein are concealed owing to the intramolecular interactions between its AIR and bis-SH3 domain (Groemping et al., 2003; Yuzawa et al., 2004) and also between its bis-SH3 domain and PX domains (Hiroaki et al., 2001; Marcoux et al., 2010). Serine residues within the AIR are phosphorylated by protein kinase C (PKC) or phosphoinositide 3-kinase (P13K) (Huang and Kleinerberg, 1999; Ago et al., 1999, 2003; Hoyal et al., 2003), which weakens the interaction of the AIR with the bis-SH3 domain, allowing p47phox to assume an open conformation whereby its bis-SH3 and PX domains are free to interact with the p22phox PRR and membrane phospholipids, respectively (El-Benna et al., 2009). Therefore, prevention of phosphorylation of the AIR could lock p47phox in its closed
state and thereby prevent its interaction with the NOX2-p22phox components of the NADPH oxidase enzyme. Indeed, inhibitors of PKC might reduce phosphorylation of the AIR of p47phox, although inhibition of PKC is likely to have additional consequences. Perhaps a more selective approach involves modification of the sulfhydryl side groups of the regulatory serine residues present within the AIR, which may be the mechanism by which apocynin achieves its widely reported inhibitory effects on NADPH oxidase.

The p47phox–NOX2 Subunit Intermolecular Interface. P47phox makes multiple contacts with the NOX2 subunit, including contact on the first predicted intracellular loop (amino acids 86–93) of NOX2 and on two further domains on the cytosolic C-terminal tail (amino acids 450–457 and 554–564) (DeLeo et al., 1995b). Elegant studies looking for regions on p47phox that interact with the NOX2 subunit identified only one site situated within the AIR (amino acids 323–342) (De Leo et al., 1995a, 1996), which may indicate a single point of contact for the NOX2 subunit on p47phox. Short peptides that correspond to any one of the three p47phox-interacting domains on the NOX2 subunit acted as competitive inhibitors of NADPH oxidase activation in both cell-free systems and in electroporamealized neutrophils (Rotrosen and Leto, 1990; DeLeo et al., 1995b). Follow-up to this work showed that the addition of a human immunodeficiency virus transactivator of transcription (tat) peptide sequence to the NOX2 subunit–containing cell-free system resulted in a compound that was effective at inhibiting NOX2 oxidase activity.

The p47phox PX–Membrane Phospholipid Interaction. The PX domain of p47phox is a conserved sequence of approximately 120 amino acids that is present in at least 100 other proteins, including p40phox and NOXO1 subunits (Ponting, 1996; Cheng and Lambeth, 2004, 2005). As mentioned, the p47phox PX domain contains a PRR that maintains p47phox in its inactive state in resting cells. The p47phox PX domain contains two highly basic pockets that bind to membrane phosphoinositides after unfolding of the protein (Kanai et al., 2001; Karathanassis et al., 2002), which are structurally distinct from the one that is present on the p40phox PX domain. The first pocket is comparatively large and unlikely to represent an appropriate target for small-molecule drugs and selectively binds to phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P2). The second pocket is much smaller and has strong affinities toward anionic phospholipids such as phosphatidic acid or phosphatidylserine (Karathanassis et al., 2002). Using immunoassaying, this splice variant of NOX2 was undetectable in other organs and tissues, including the heart, brain, kidney, and aorta, but it was highly expressed in the lungs (Harrison et al., 2012). Importantly, siRNA transfection to downregulate its expression resulted in a significant decrease in the NOX2-containing NADPH oxidase activity of macrophages, even though the expression of the full length NOX2 was unaffected (Harrison et al., 2012). These results suggest that NOXβ may represent a novel target for drugs to suppress macrophage-dependent NOX2 activity, which has been implicated in the pathobiology of influenza virus infections (Imai et al., 2008).

Inhibition of NOX2 Oxidase Priming in Neutrophils and Macrophages. Work from our laboratory has recently shown that influenza A virus infection of macrophages or activation of Toll like receptor 7 (i.e., TLR7) with imiquimod resulted in a significant elevation in the oxidative burst in macrophages (To et al., 2014). The virus per se had little to no effect on superoxide production; however, it substantially elevated the ability of phorbol dibutyrate to activate NOX2 activity and the oxidative burst. This observation is analogous to the “priming effect” characterized in human neutrophils whereby an initial exposure to TNF-α results in a greater degree of superoxide production to a second stimulus such as N-formyl-methionyl-leucyl-phenylalanine (El-Benna et al., 2008). This primed state for NOX2 oxidase has been described as a “ready-to-go” state, as it results in a higher and faster response to a second stimulus (Clark et al., 1990). How priming of NOX2 oxidase occurs has been elucidated, and it is believed to involve p47phox. As already mentioned, the multiple serine residues on p47phox become phosphorylated to induce conformational
changes in the protein that allow it and its associated subunits (i.e., p67phox and p40phox) to assemble with NOX2 to form a fully functional oxidase unit capable of ROS generation. However, for priming to occur, generally the priming agents cause partial phosphorylation of p47phox, in particular its serine 345 on the peptide sequence (Sheppard et al., 2005; El-Benna et al., 2008). This partial phosphorylation status of p47phox then results in a greater increase in phosphorylation of the remaining serine residues on p47phox on subsequent stimulation with the second stimulus and a greater superoxide response. We hypothesized that the priming effect of the virus demonstrated in macrophages may involve phosphorylation of serine 346 (mouse sequence differs from human sequence, which is serine 345) on the p47phox subunit, analogous to what occurs in human neutrophils (Sheppard et al., 2005; Dang et al., 2006; El-Benna et al., 2008). To this end, we custom designed in the same manner as Dang et al. (2006), a peptide inhibitor corresponding to amino acids 337–348 of p47phox tagged at the N terminus to a HIV-tat based peptide (YGRKKRRQRRR) to permit membrane translocation, which we anticipate will compete against serine 346 of p47phox for the substrate that causes phosphorylation in response to influenza A virus. Our study showed that this Ser346 peptide inhibitor significantly suppressed the influenza virus-dependent enhancement in the oxidative burst in macrophages (To et al., 2014). It remains to be determined whether this phenomenon of NOX2 priming occurs in vivo following influenza A virus infection. Certainly, as a starting point, peptide inhibitors that span over serine 345 (Ser346 in mouse) should be tested in vivo against influenza A virus-dependent enhancement in the oxidative burst in macrophages (To et al., 2014). It remains to be determined whether this phenomenon of NOX2 priming occurs in vivo following influenza A virus infection. Certainly, as a starting point, peptide inhibitors that span over serine 345 (Ser346 in mouse) should be tested in vivo against influenza A virus infections.

Conclusion

The primary focus of our mainstay therapies for treatment of disease caused by influenza A virus infections is to minimize virus infection and replication. However, with influenza viruses becoming resistant to antivirals and inprominent threats of a new pandemic strain, there is a serious need to develop novel pharmacologic approaches that ideally target lung injury irrespective of strain. There has been a strong movement toward understanding key features of the host immune response triggered by virus, such as the lung oxidative stress induced by NOX2-containing NADPH oxidase enzymes. Thus, inhibitors of NOX2 oxidase are likely to provide some protection against these types of infection; coupled with antivirals, this type of polypharmacy could alleviate both the host immunopathology and viral burden. However, this field is still in its infancy; in particular, there is a lack of knowledge as to which cells of the immune system are responsible for this ROS generation, although macrophages have been implicated. In addition, the potential roles of other ROS-generating enzymes (including NOX4, DUOX1, and DUOX2, and others) need to be elucidated; in particular, their spatial-temporal aspects are unknown. Finally, there is a dire need to establish the X-ray crystal structures of catalytic (i.e., NOX and DUOX) subunits, their splice variants, regulatory (i.e., p47phox and p67phox) subunits, and their key binding sites to facilitate the development of more specific inhibitors of these enzyme complexes.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Vlahos, Selemidis.


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