

Practical Strategies for Targeting NF-kappaB and NADPH Oxidase May Improve Survival During Lethal Influenza Epidemics

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Abstract

The most foolproof way to promote survival in epidemics of potentially lethal influenza is to target, not highly mutable viral proteins, but rather intracellular signaling pathways which promote viral propagation or lung inflammation. NF-kappaB, activated in influenza-infected lung epithelial cells and macrophages, is one likely target in this regard, as it plays a role both in viral replication and in the excessive lung inflammation often evoked by influenza infection. Indeed, salicylates, which suppress NF-kappaB activation, have been shown to reduce the lethality of H5N1 avian-type influenza in mice. Another potential target is NADPH oxidase, as this may be a major source of influenza-evoked oxidant stress in lung epithelial cells as well as in phagocytes attracted to lung parenchyma. A number of studies demonstrate that oxidant stress contributes to overexuberant lung inflammation and lethality in influenza-infected mice. The documented utility of N-acetylcysteine, a glutathione precursor, for promoting survival in influenza-infected mice, and diminishing the severity of influenza-like infections in elderly humans, presumably reflects a key role for oxidative stress in influenza. The lethality of influenza is also reduced in mice pretreated with adenovirus carrying the gene for heme oxygenase-1; this benefit may be mediated, at least in part, by the ability of bilirubin to inhibit NADPH oxidase. It may be feasible to replicate this benefit clinically by administering biliverdin or its homolog phycocyanobilin, richly supplied by spirulina. If this latter speculation can be confirmed in rodent studies, a practical and inexpensive regimen consisting of high-dose salicylates, spirulina, and N-acetylcysteine, initiated at the earliest feasible time, may prove to have life-saving efficacy when the next killer influenza pandemic strikes.

Targeting Intracellular Signaling Pathways in Flu Therapy

The influenza epidemic of 1919 is estimated to have killed 20-40 million people, and there is considerable concern that avian flu (influenza A, H5N1), which has killed about half of the people who have managed to acquire it, might someday mutate to a strain that is readily transmitted from person to person, giving rise to another lethal pandemic. Efforts to protect against this eventuality have focused on vaccine production and the development of additional drugs that target the function of viral proteins. Unfortunately, influenza viruses are capable of mutating rapidly, implying that they can often quickly develop immunity to vaccines and drugs targeting their proteins.¹⁻³ Thus, some insightful researchers have suggested that it may be more fruitful to focus on human cellular signaling pathways that the virus exploits to proliferate and spread, or that contribute to the inflammatory pneumonitis that can make influenza infection lethal.⁴⁻⁶ In particular, attention has been drawn to the fact that an overexuberant inflammatory response in lung parenchyma is largely responsible for the lethality of “killer flus”.⁷⁻¹⁰ Indeed, the most lethal strains of influenza are characterized by increased capacity to induce proinflammatory cytokines in human macrophages.⁸ Thus, measures which dampen the influx

and activation of leukocytes during influenza infection may aid survival – even if they don't promote clearance of the virus.

Multiple Roles for NF-kappaB in Influenza Infection

One such potential target is NF-kappaB. This family of transcription factors is activated in influenza-infected pulmonary epithelial cells,^{4, 11-13} and this activation appears to contribute to the life cycle of the virus by aiding the caspase-mediated nuclear export of viral nucleoproteins.¹⁴ NF-kappaB also protects the virus by inhibiting the ability of type-1 interferons to induce antiviral proteins.¹⁵ Moreover, NF-kappaB activation evidently plays a role in the inflammatory response to influenza infection, as it promotes the transcription of a number of proinflammatory cytokines; thus, inhibitors of IkappaB kinase suppress the production of interleukin-8 in human epithelial cells infected with influenza A.⁹ Mazur and colleagues have recently shown that aspirin, which in high concentrations prevents NF-kappaB activation by blocking IkappaB kinase-beta activity,^{16, 17} suppresses the propagation of an Asian influenza virus in human cell cultures; this effect was not replicated by indomethacin, suggesting that inhibition of NF-kappaB, rather than cyclo-oxygenase, was responsible for aspirin's activity.⁶ Moreover, aspirin administered in drinking water or via aerosol reduced the lethality of this virus in mice. This study did not determine the extent to which a direct anti-viral effect, as opposed to anti-inflammatory activity, was responsible for this protection, although lung viral titers were indeed noted to be lower in mice receiving aerosolic aspirin. These findings suggest that prompt administration of oral or aerosolized aspirin, in high doses capable of influencing NF-kappaB activity, might improve survival during life-threatening influenza infections. A better option in this regard might be salicylate (clinically available in various forms such as sodium salicylate or salsalate), which is no less effective than its derivative aspirin in inhibiting NF-kappaB, but, owing to its weak and transient impact on cyclooxygenase activity, does not increase risk for gastrointestinal bleeding.¹⁸⁻²¹ The high oral doses of aspirin required for NF-kappaB suppression in contrast, would pose a considerable risk in this regard.

NADPH Oxidase as a Potential Target

Another potential target in the treatment of influenza is NADPH oxidase. Influenza has been shown to induce oxidant stress in the cells it infects, an effect mediated at least in part by the viral hemagglutinin protein;¹¹ this evoked oxidant stress may contribute to activation of NF-kappaB.^{11, 12} Lung epithelial cells express several different isoforms of NADPH oxidase,²²⁻²⁴ and it seems likely that this enzyme complex is a major source of excess oxidant stress in influenza-infected cells – albeit xanthine oxidase may also contribute in this regard,²⁵ and a decrease in intracellular glutathione levels exacerbates the impact of oxidants in infected cells.^{26, 27} The phagocytic form of NADPH oxidase expressed by leukocytes infiltrating infected lung tissue also evidently contributes to total oxidative stress in infected lung parenchyma.

The increased oxidant stress evoked by influenza infection may contribute to the efficient viral propagation, while also playing a key role in inflammatory lung damage. Measures which boost glutathione levels in infected cells have been shown to decrease the transcription of late viral proteins.²⁶ Administration of N-acetylcysteine, which promotes glutathione synthesis by

increasing intracellular availability of free cysteine,²⁸ diminishes mortality in influenza-infected mice.²⁹ In a double-blind placebo-controlled study, supplementation with N-acetylcysteine (1200 mg daily) was found to decrease both the number and the severity of influenza-like episodes during the winter in initially healthy elderly volunteers.³⁰ (No attempts have been made to replicate this remarkable study, published in 1997.) In transgenic mice overexpressing extracellular superoxide dismutase, influenza infection evokes far less lung pathology than in wild-type mice.⁷ Induction of the inducible isoform of nitric oxide synthase also contributes notably to lung damage during influenza infection in mice, as such infection was less lethal and produced less lung pathology in mice genetically deficient in iNOS,³¹ in wild type mice, administration of the iNOS inhibitor L-NMMA reduced the lethality of influenza.³² This likely points to a role for peroxynitrite in influenza-associated lung pathology – implying that a reduction in superoxide production could provide protection by decreasing peroxynitrite formation. Reduced inflammation of lung parenchyma has also been noted during influenza infection in mice that lack the phagocytic form of NADPH oxidase or that are treated with a cell-permeable mimic of superoxide dismutase.¹⁰

Of particular interest is a study demonstrating that adenovirus-mediated transfer of heme oxygenase-1 (HO-1) cDNA reduces the lung damage and influx of leukocytes in influenza-infected mice, while markedly boosting survival (60% vs. 0% in controls).³³ HO-1 generates bilirubin, which recently has been shown to be a potent physiological inhibitor of NADPH oxidase activity.³⁴⁻³⁷ The possibility that concurrent generation of carbon monoxide also contributed to the observed protection merits consideration, but it is reasonable to suspect that the potent antioxidant activity of bilirubin contributed prominently to the protection afforded by HO-1 transfection. This conclusion is of particular interest in light of suggestions that oral administration of biliverdin, or of its homolog phycocyanobilin (PCB - richly supplied by spirulina), can mimic the NADPH oxidase-inhibitory activity of endogenous bilirubin.^{37,38} It would thus be of great interest to examine the impact of oral biliverdin, dietary spirulina, or PCB-enriched spirulina extracts on the course of influenza infection in mice.

Protective Potential of Combination Regimens

If NADPH oxidase proves to be a useful target in influenza therapy, it is reasonable to expect that jointly targeting both this enzyme complex *and* NF-kappaB would produce a more substantial impact on the survivability of potentially lethal influenza strains. If this strategy showed promising results in mice, joint administration of ample doses of spirulina and salicylate, initiated as soon as feasible during infection, might be expected to have life saving potential during dangerous flu epidemics. This would be a reasonably feasible and inexpensive strategy, as spirulina and salicylate could be stockpiled in anticipation of such an epidemic. Inclusion of N-acetylcysteine in this regime – also inexpensive and readily available - might further boost its protective efficacy, and it would of course be reasonable to use this regimen in conjunction with any drugs targeting viral proteins that would seem likely to have efficacy. In this regard, administration of N-acetylcysteine in conjunction with antiviral drugs (ribavirin or oseltamivir) produces a more substantial reduction in influenza lethality in mice than either agent administered alone.^{39, 40}

References

- (1) Fleming DM. Managing influenza: amantadine, rimantadine and beyond. *Int J Clin Pract* 2001 April;55(3):189-95.
- (2) Gubareva LV, Webster RG, Hayden FG. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. *Antiviral Res* 2002 January;53(1):47-61.
- (3) Alexander ME, Bowman CS, Feng Z, Gardam M, Moghadas SM, Rost G, Wu J, Yan P. Emergence of drug resistance: implications for antiviral control of pandemic influenza. *Proc Biol Sci* 2007 July 22;274(1619):1675-84.
- (4) Ludwig S, Planz O, Pleschka S, Wolff T. Influenza-virus-induced signaling cascades: targets for antiviral therapy? *Trends Mol Med* 2003 February;9(2):46-52.
- (5) Nimmerjahn F, Dudziak D, Dirmeier U, Hobom G, Riedel A, Schlee M, Staudt LM, Rosenwald A, Behrends U, Bornkamm GW, Mautner J. Active NF-kappaB signalling is a prerequisite for influenza virus infection. *J Gen Virol* 2004 August;85(Pt 8):2347-56.
- (6) Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, Puthavathana P, Silberzahn T, Wolff T, Planz O, Ludwig S. Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. *Cell Microbiol* 2007 July;9(7):1683-94.
- (7) Suliman HB, Ryan LK, Bishop L, Folz RJ. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. *Am J Physiol Lung Cell Mol Physiol* 2001 January;280(1):L69-L78.
- (8) Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y, Peiris JS. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* 2002 December 7;360(9348):1831-7.
- (9) Bernasconi D, Amici C, La FS, Ianaro A, Santoro MG. The IkappaB kinase is a key factor in triggering influenza A virus-induced inflammatory cytokine production in airway epithelial cells. *J Biol Chem* 2005 June 24;280(25):24127-34.
- (10) Snelgrove RJ, Edwards L, Rae AJ, Hussell T. An absence of reactive oxygen species improves the resolution of lung influenza infection. *Eur J Immunol* 2006 June;36(6):1364-73.
- (11) Pahl HL, Baeuerle PA. Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B. *J Virol* 1995 March;69(3):1480-4.
- (12) Knobil K, Choi AM, Weigand GW, Jacoby DB. Role of oxidants in influenza virus-induced gene expression. *Am J Physiol* 1998 January;274(1 Pt 1):L134-L142.
- (13) Flory E, Kunz M, Scheller C, Jassoy C, Stauber R, Rapp UR, Ludwig S. Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and

- involves oxidative radicals and activation of IkappaB kinase. *J Biol Chem* 2000 March 24;275(12):8307-14.
- (14) Wurzer WJ, Ehrhardt C, Pleschka S, Berberich-Siebelt F, Wolff T, Walczak H, Planz O, Ludwig S. NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J Biol Chem* 2004 July 23;279(30):30931-7.
 - (15) Wei L, Sandbulte MR, Thomas PG, Webby RJ, Homayouni R, Pfeffer LM. NFkappaB negatively regulates interferon-induced gene expression and anti-influenza activity. *J Biol Chem* 2006 April 28;281(17):11678-84.
 - (16) Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994 August 12;265(5174):956-9.
 - (17) Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 1998 November 5;396(6706):77-80.
 - (18) Morris HG, Sherman NA, McQuain C, Goldlust MB, Chang SF, Harrison LI. Effects of salsalate (nonacetylated salicylate) and aspirin on serum prostaglandins in humans. *Ther Drug Monit* 1985;7(4):435-8.
 - (19) Whittle BJ, Hansen D, Salmon JA. Gastric ulcer formation and cyclo-oxygenase inhibition in cat antrum follows parenteral administration of aspirin but not salicylate. *Eur J Pharmacol* 1985 October 8;116(1-2):153-7.
 - (20) Rosenkranz B, Fischer C, Meese CO, Frolich JC. Effects of salicylic and acetylsalicylic acid alone and in combination on platelet aggregation and prostanoid synthesis in man. *Br J Clin Pharmacol* 1986 March;21(3):309-17.
 - (21) Zambraski EJ, Atkinson DC, Diamond J. Effects of salicylate vs. aspirin on renal prostaglandins and function in normal and sodium-depleted dogs. *J Pharmacol Exp Ther* 1988 October;247(1):96-103.
 - (22) Ranjan P, Anathy V, Burch PM, Weirather K, Lambeth JD, Heintz NH. Redox-dependent expression of cyclin D1 and cell proliferation by Nox1 in mouse lung epithelial cells. *Antioxid Redox Signal* 2006 September;8(9-10):1447-59.
 - (23) Goyal P, Weissmann N, Grimminger F, Hegel C, Bader L, Rose F, Fink L, Ghofrani HA, Schermuly RT, Schmidt HH, Seeger W, Hanze J. Upregulation of NAD(P)H oxidase 1 in hypoxia activates hypoxia-inducible factor 1 via increase in reactive oxygen species. *Free Radic Biol Med* 2004 May 15;36(10):1279-88.
 - (24) Amara N, Bachoual R, Desmard M, Golda S, Guichard C, Lanone S, Aubier M, Ogier-Denis E, Boczkowski J. Diesel exhaust particles induce matrix metalloprotease-1 in human lung epithelial cells via a NAD(P)H oxidase/NOX4 redox-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 2007 July;293(1):L170-L181.

- (25) Akaike T, Ando M, Oda T, Doi T, Ijiri S, Araki S, Maeda H. Dependence on O₂- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J Clin Invest* 1990 March;85(3):739-45.
- (26) Nencioni L, Iuvara A, Aquilano K, Ciriolo MR, Cozzolino F, Rotilio G, Garaci E, Palamara AT. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. *FASEB J* 2003 April;17(6):758-60.
- (27) Cai J, Chen Y, Seth S, Furukawa S, Compans RW, Jones DP. Inhibition of influenza infection by glutathione. *Free Radic Biol Med* 2003 April 1;34(7):928-36.
- (28) N-acetylcysteine. *Altern Med Rev* 2000 October;5(5):467-71.
- (29) Ungheri D, Pisani C, Sanson G, Bertani A, Schioppacassi G, Delgado R, Sironi M, Ghezzi P. Protective effect of n-acetylcysteine in a model of influenza infection in mice. *Int J Immunopathol Pharmacol* 2000 September;13(3):123-8.
- (30) De FS, Grassi C, Carati L. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. *Eur Respir J* 1997 July;10(7):1535-41.
- (31) Akaike T, Okamoto S, Sawa T, Yoshitake J, Tamura F, Ichimori K, Miyazaki K, Sasamoto K, Maeda H. 8-nitroguanosine formation in viral pneumonia and its implication for pathogenesis. *Proc Natl Acad Sci U S A* 2003 January 21;100(2):685-90.
- (32) Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A* 1996 March 19;93(6):2448-53.
- (33) Hashiba T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Tsuburai T, Matsuse T, Ishigatubo Y. Adenovirus-mediated transfer of heme oxygenase-1 cDNA attenuates severe lung injury induced by the influenza virus in mice. *Gene Ther* 2001 October;8(19):1499-507.
- (34) Lanone S, Bloc S, Foresti R, Almolki A, Taille C, Callebert J, Conti M, Goven D, Aubier M, Dureuil B, El-Benna J, Motterlini R, Boczkowski J. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB J* 2005 November;19(13):1890-2.
- (35) Matsumoto H, Ishikawa K, Itabe H, Maruyama Y. Carbon monoxide and bilirubin from heme oxygenase-1 suppresses reactive oxygen species generation and plasminogen activator inhibitor-1 induction. *Mol Cell Biochem* 2006 October;291(1-2):21-8.
- (36) Jiang F, Roberts SJ, Datla S, Dusting GJ. NO modulates NADPH oxidase function via heme oxygenase-1 in human endothelial cells. *Hypertension* 2006 November;48(5):950-7.
- (37) McCarty MF. "Iatrogenic Gilbert syndrome" - a strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin. *Medical Hypotheses* 2007;accepted for publication.
- (38) McCarty MF. Clinical potential of spirulina as a source of phycocyanobilin. *J Medicinal Food* 2007;in press.

- (39) Ghezzi P, Ungheri D. Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. *Int J Immunopathol Pharmacol* 2004 January;17(1):99-102.
- (40) Garozzo A, Tempera G, Ungheri D, Timpanaro R, Castro A. N-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection. *Int J Immunopathol Pharmacol* 2007 April;20(2):349-54.