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Role of cyclooxygenase-2 in H5N1 viral pathogenesis and the potential use of its inhibitors

Key Messages

1. Cyclooxygenase-2 (COX-2), along with TNF- α and other proinflammatory cytokines, was hyperinduced in H5N1-infected macrophages in vitro and in epithelial cells of autopsied lung tissues of infected patients.
2. The COX-2 mediated amplification of the proinflammatory response is rapid, and the effects elicited by the H5N1-triggered proinflammatory cascade are broader than those arising from direct viral infection.
3. Selective COX-2 inhibitors suppress the H5N1-hyperinduced cytokines in the proinflammatory cascade.

Introduction

The highly pathogenic avian influenza viruses (H5N1) in poultry and wild birds and their repeated zoonotic transmission to humans has raised concerns about pandemics. The severity of H5N1-induced lung pathology may be due to increased viral replication and/or inflammatory responses.¹ In vitro, infection of macrophages and alveolar epithelial cells by H5N1 virus hyperinduces proinflammatory cytokines, compared to infection by H1N1 virus.^{2,3} Deficiency of cyclooxygenase-2 (COX-2) in the host response to influenza results in less severe disease.⁴ Moreover, COX-2 is hyperinduced in response to H5N1 infection, in addition to the proinflammatory cytokine cascade. Therefore, we aimed to investigate the role of COX-2 in H5N1 pathogenesis. We hypothesised that hyperinduction of COX-2 may play an important role in the pathogenesis of H5N1 virus infection.

Methods

This study was conducted from January 2007 to December 2008. The viruses used were A/Hong Kong/483/97 (H5N1 virus), a virus isolated from a patient with fatal H5N1 disease in Hong Kong in 1997, A/Vietnam/3212/04 (H5N1 virus), a virus isolated from a patient with H5N1 disease in Vietnam during 2004, and A/Hong Kong/54/98 (H1N1 virus).

Differentiated human macrophages or A549 cells were infected at an MOI=2. After 30 minutes of virus absorption, the virus inoculum was removed, and the cells were washed and then incubated in the corresponding medium.

To investigate the paracrine effects of virus-infected macrophages, supernatants of H5N1-, H1N1-, and mock-infected macrophages were collected at 6 hours post infection. The supernatants were filtered using a 100kDa filter (Millipore) to remove any virus and added to fresh (uninfected) macrophages or A549 cells. The macrophages or A549 cells were harvested for RNA to study COX-2 and cytokine gene expression.

To investigate the effects of selective COX-2 inhibitors on cytokine induction in macrophages or epithelial cells, the cells were pre-treated for 1 hour with nimesulide or NS-398 (Cayman) dissolved in a vehicle consisting of 0.1% DMSO prior to virus infection or application of filtered culture supernatant from infected macrophages. Cells were incubated in the presence of the selective COX-2 inhibitor or drug vehicle (as control) throughout the experiment.

Gene expression of COX-2 and cytokine was performed using real-time PCR. Total RNA was isolated and reverse transcribed. Transcript expression was monitored using a Power SYBR Green PCR master mix kit (Applied Biosystems) with specific primers. The fluorescence signals were measured in real-time during the extension step with MX3000P QPCR System (Stratagene). The ratio change in target gene relative to the β -actin control gene was determined by the $2^{-\Delta\Delta Ct}$ method.

The presence of COX-2 in autopsy lung tissues of H5N1-infected patients

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was detected by specific COX-2 antibody (Cayman) using the immunoperoxidase technique. For the co-localisation of COX-2 with macrophage and epithelial cell markers, the sections were incubated with CD68 (Dako) and AE1/AE3 (Dako) respectively, followed by incubation with FITC-conjugated donkey anti-mouse (Jackson Lab).

Results

As early as 3 hours after infection, COX-2 was markedly up-regulated in H5N1-infected macrophages (Fig 1a). The less virulent H1N1 virus induced COX-2 mRNA with similar kinetics as H5N1 virus but at much lower magnitude. As the induction of COX-2 in virus-infected cells is abolished by pre-treatment of cells with cycloheximide (data not shown), its induction is likely to be due to cellular mediators rather than direct stimulation by the virus. In contrast to virus-infected macrophages, COX-2 mRNA was not up-regulated in H5N1- or H1N1-infected human alveolar epithelial (A549) cells (Fig 1b).

To determine whether COX-2 was induced in patients with H5N1 infection, immunohistochemical study for COX-2 protein was performed on autopsy specimens from three H5N1-infected patients. In all three specimens, there was evidence of cytoplasmic immunostaining for COX-2 in the respiratory bronchial epithelial cells and pneumocytes (Figs 1c and 1d). The COX-2 expression co-localised with cytokeratin antigen demonstrated expression of COX-2 in basal cells and ciliated cells of the respiratory epithelium (Fig 1e). COX-2 was identified in cytokeratin positive alveolar epithelial cells (Fig 1f) but was not co-expressed with macrophages as identified by the macrophage marker, CD68 (Fig 1g). In contrast, normal lung controls showed negative or very weak cytoplasmic staining for COX-2 (Fig 1h).

An *in vitro* model was developed to investigate the proinflammatory cascade mimicking the macrophage-epithelial cell interaction. Culture supernatants of influenza A virus-infected human macrophages were collected at 6 hours after infection, filtered to remove any infectious virus, and added to uninfected A549 cells. COX-2 expression was markedly up-regulated in epithelial cells challenged with supernatant from H5N1-infected macrophages; more so than in those challenged with supernatants from H1N1-infected cells (Fig 2a). Comparable induction of a number of cytokines, including TNF- α (Fig 2b to 2g) was also observed.

Selective COX-2 inhibitor (nimesulide) was used to further investigate the role of COX-2 in the proinflammatory response induced by H5N1 virus. Expression of all tested cytokine mRNAs were significantly suppressed by nimesulide at 600 μ M in both H5N1- and H1N1-infected macrophages (Fig 3a).

As the expression of viral M gene was also suppressed

by nimesulide in a dose-dependent manner (Fig 3b), it is uncertain whether the observed reduction in the induction of cytokines was partly or wholly due to suppression in viral replication. To further investigate the role of COX-2 within the proinflammatory cascade (distinct from any confounding effect of viral replication), virus-filtered supernatants from human macrophages infected with influenza A virus with or without nimesulide treatment were added to uninfected epithelial cells and the consequential effect on cytokine expression was determined. Supernatant from influenza A virus-infected macrophages which had been pre-treated with nimesulide induced lower levels of cytokine expression in epithelial cells than did supernatant from nimesulide untreated macrophages (Fig 3c). Furthermore, treatment of uninfected epithelial cells with nimesulide (Fig 3d) led to a suppression of the cytokines induced by treating these cells with supernatants from influenza A virus-infected macrophages.

Discussion

In the current study, we demonstrated that effects of the proinflammatory cascade was rapid, and the proinflammatory mediators induced were more diverse than those induced by direct influenza virus infection. Moreover, lethal H5N1 viruses induced the proinflammatory cascade to levels that were much higher than those induced by a seasonal H1N1 virus. This may explain, to certain extent, why H5N1 virus is so pathogenic in humans. Autopsy tissue from patients with H5N1 disease showed an extensive induction of COX-2 and TNF- α in epithelial cells. As virus antigen was scarce in these lung tissues showing COX-2 and TNF- α expression, our findings support the hypothesis that the cytokine cascade sustains itself even when viral replication has been largely controlled.⁵ As influenza virus infection of epithelial cells does not directly trigger COX-2 or TNF- α induction, the expression of these proinflammatory genes in the lung in the absence of significant viral replication is more likely due to a self-sustaining cytokine cascade rather than ongoing viral replication, at least in the later stages of the illness. Taken together, these findings support the hypothesis that excessively induced host inflammatory responses play a major role in contributing to the severity of human H5N1 disease.

Selective COX-2 inhibitors were able to attenuate the expression of a number of influenza A virus induced proinflammatory cytokines. Nimesulide was able to decrease the transcription of viral M gene, suggesting that nimesulide may also suppress viral replication. As the decrease in cytokine induction could be at least in part attributed to decrease in viral replication, to further demonstrate that COX-2 was indeed involved in mediating the induction of proinflammatory cytokines, we designed experiments to show that nimesulide reduced the induction of proinflammatory cytokines elicited by soluble factors derived from H5N1-infected macrophages. These experiments avoided the confounding effect of viral

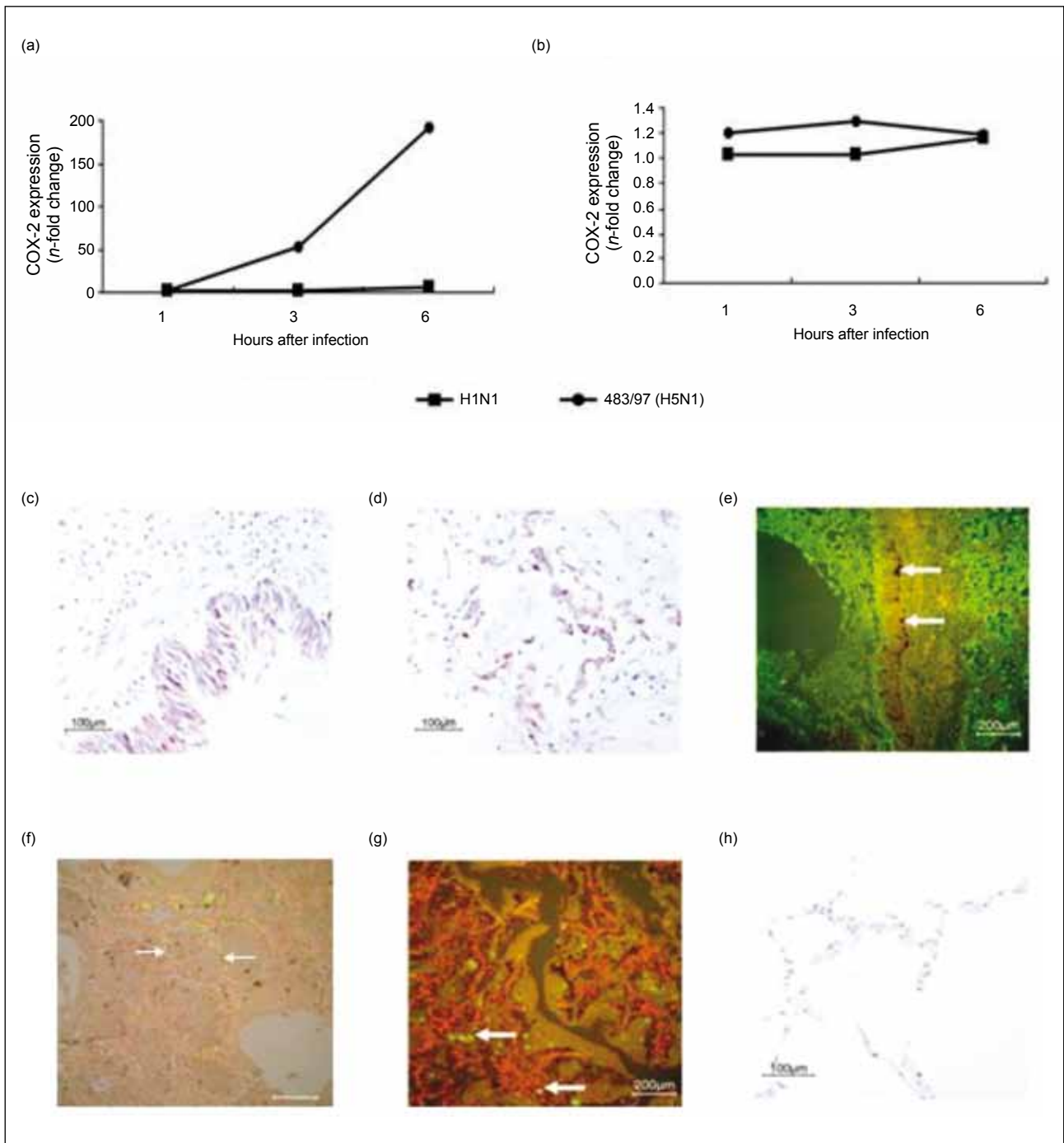


Fig 1. (a) In macrophages, cyclooxygenase-2 (COX-2) is markedly up-regulated by an H5N1 virus within 3 hours and further increased by 6 hours. An H1N1 virus induces a much weaker response. **(b)** In A549 epithelial cells, there is no induction of COX-2 by either virus. Data shown are fold change of COX-2 expression relative to mock-infected control after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown. COX-2 is expressed extensively in the lung epithelial cells of persons who died of H5N1 infection. Immunohistochemistry for COX-2 protein in H5N1-infected lungs shows strong cytoplasmic staining in **(c)** bronchial epithelial cells and **(d)** pneumocytes. **(e)** Double staining for COX-2 (DAB brown) and cytokeratin antigen (FITC green) in fatal H5N1 pneumonia demonstrates cytoplasmic staining of basal cells and ciliated cells (white arrows). COX-2 is expressed in **(f)** cytokeratin-positive alveolar epithelial cells (white arrows) but not in **(g)** macrophages as identified by the CD68 macrophage marker (FITC green) [white arrows]. **(h)** Representative data of two lung tissues from persons who died of non-respiratory causes show little or no staining for COX-2. Scale bar of double-stained images is 200 μ m.

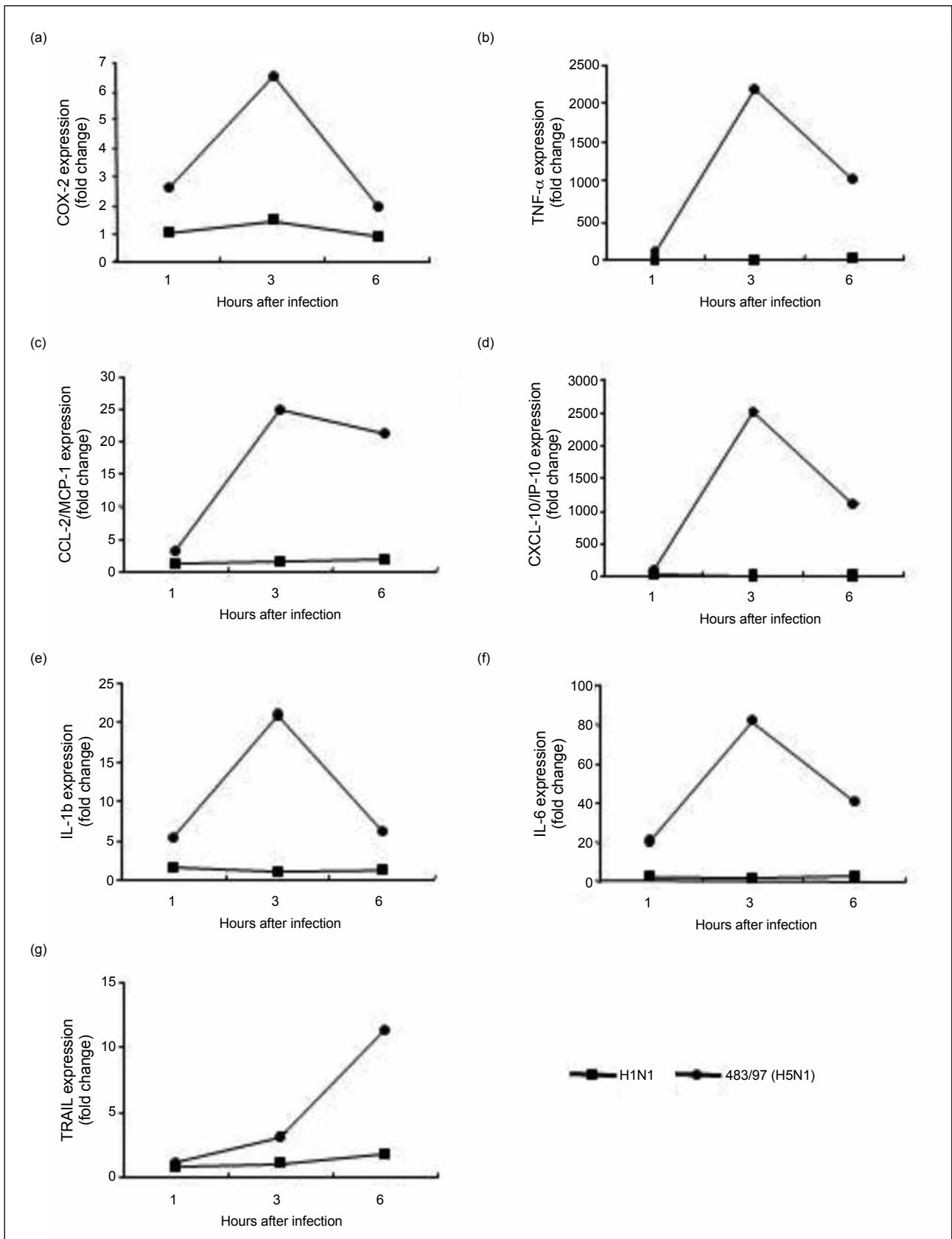
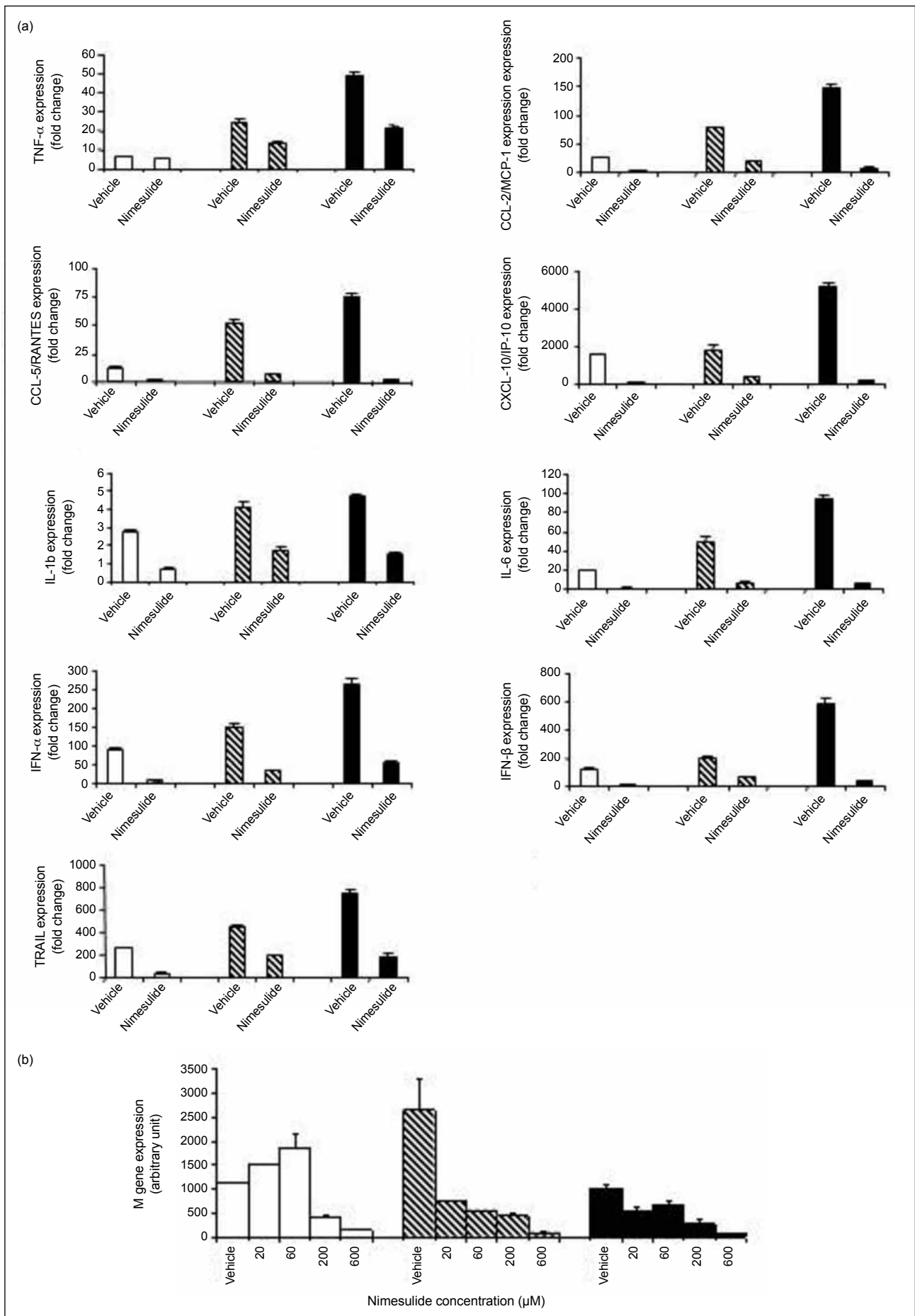


Fig 2. (a) Cyclooxygenase-2 (COX-2) expression is markedly up-regulated by the supernatants collected from H5N1-infected macrophages, peaking at 3 hours post-challenge. Supernatants collected from H1N1-infected macrophages cause only a small rise in COX-2 expression. Comparable findings are observed for other cytokines; (b) TNF- α , (c) CCL-2/MCP-1, (d) CXCL-10/IP-10, (e) IL-1b, (f) IL-6, and (g) TRAIL. Data shown are fold change of gene expression relative to mock-infected control after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown.



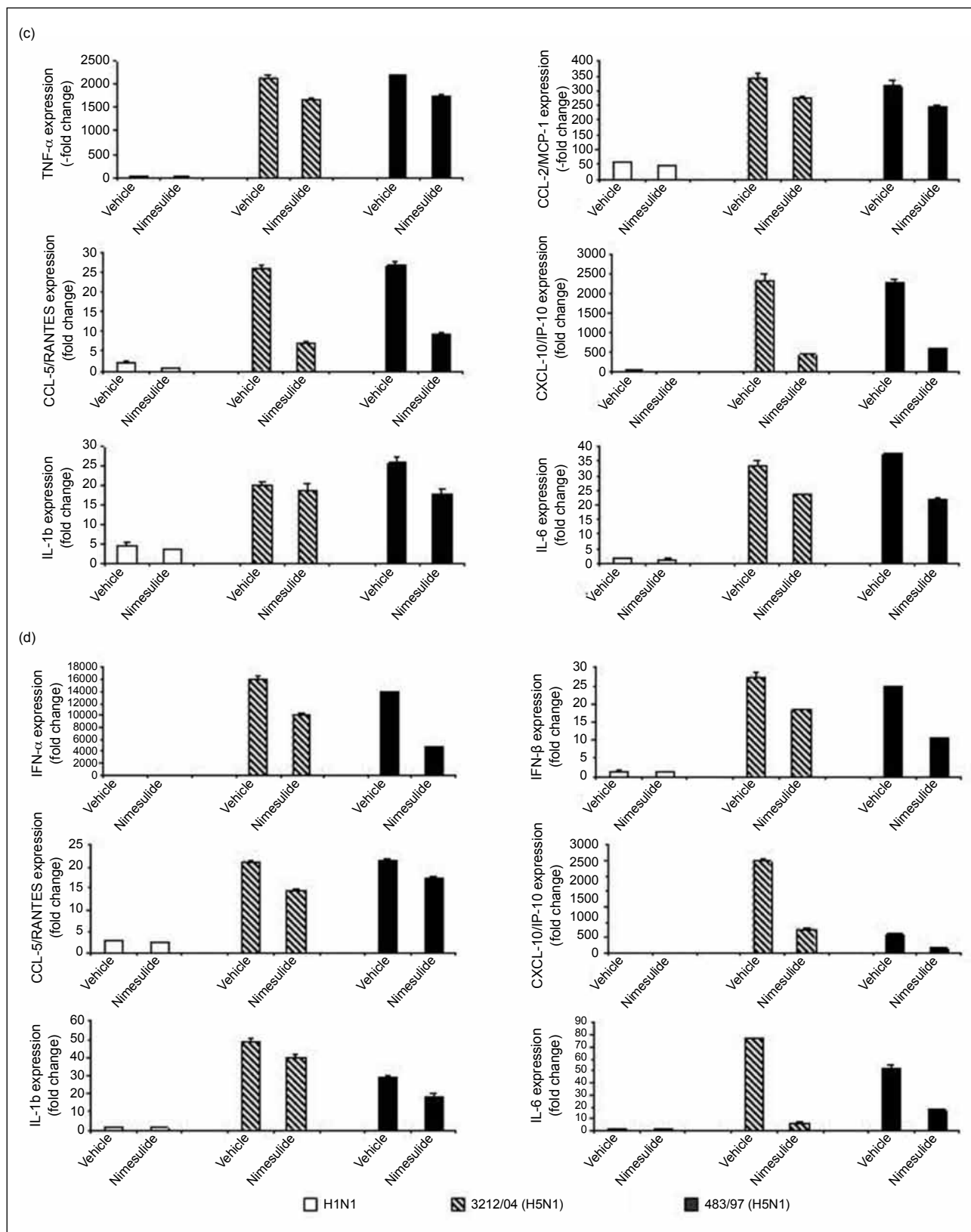


Fig 3. (a) A panel of cytokines tested show attenuation in expression level at 600 μ M nimesulide after influenza A infection in macrophages. **(b)** Viral M gene transcription in influenza A-infected macrophages is attenuated by nimesulide in a dose-dependent manner. Cytokine expression is attenuated within the proinflammatory cascade by nimesulide. **(c)** Cytokine expression is attenuated in epithelial cells challenged with supernatants from influenza A-infected macrophages, which have been treated with 200 μ M nimesulide. **(d)** Treating epithelial cells with 200 μ M nimesulide show similar attenuation in cytokine expression induced by supernatants from influenza A-infected macrophages. Data shown are fold change of gene expression relative to corresponding mock after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown.

infection, as the macrophage supernatants were filtered to remove the virus. The current paradigm is that COX-2 is induced by cytokines.⁵ We demonstrated that COX-2 drove and maintained the proinflammatory cascade via a complex positive feedback loop during H5N1 infection.

At present, early antiviral therapy by oseltamivir is the mainstay for managing patients with H5N1 disease. However, the clinical response to antiviral therapy has been variable. This can be attributed to a number of factors, including delayed commencement of therapy, development of antiviral resistance, and poor bio-availability of the oral drug in severely ill patients. The cytokine cascade was maintained even in the absence of significant virus infection in the lung. Thus, in addition to antiviral therapy, interventions to selectively modulate this cascade may be helpful. One such potential intervention is the inhibition of the COX-2 pathway, which may attenuate the proinflammatory cascade and possibly the pathology associated with it. Such an approach may be more beneficial than attenuating the action of a single cytokine such as TNF- α using direct antagonists. COX-2 inhibitors are either already registered for clinical use or undergoing late phase clinical trials, and may also have the added benefit of inhibiting viral replication.

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