

ORIGINAL**Mechanisms of matrix metalloproteinase-9 upregulation and tissue destruction in various organs in influenza A virus infection**

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Abstract : Severe influenza is characterized clinicopathologically by multiple organ failure, although the relationship amongst virus and host factors that influence this morbid outcome and the underlying mechanisms of action remain unclear. The present study identified marked upregulation of matrix metalloproteinase (MMP)-9 and pro-inflammatory cytokine tumor necrosis factor alpha (TNF- α) in various organs after intranasal infection of influenza A WSN virus. MMP-9 and TNF- α were upregulated in the lung, the site of initial infection, as well as in the brain and heart. The infection-induced MMP-9 upregulation was inhibited by anti-TNF- α antibodies and by anti-oxidative reagents pyrrolidine dithiocarbamate and N-acetyl-L-cysteine, which inhibit activation of nuclear factor kappa B (NF- κ B), as well as by nordihydroguaiaretic acid, which inhibits activation of activator protein 1 (AP-1). In addition, MMP-9 upregulation via TNF- α was also suppressed by inhibitors of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase 1/2 and p38, and partly by a c-Jun N-terminal kinase inhibitor. These results indicated that the influenza-induced MMP-9 upregulation in various organs is mediated through MAPK-NF- κ B- and/or AP-1-dependent mechanisms. Strategies that neutralize TNF- α as well as inhibitors of MAPK-NF- κ B- and/or AP-1-dependent pathways may be useful for suppressing the MMP-9 effect and thus preventing multiple organ failure in severe influenza. *J. Med. Invest.* 57 : 26-34, February, 2010

Keywords : matrix metalloproteinase, transcription factor, signal transduction, influenza A virus

INTRODUCTION

Matrix metalloproteinases (MMPs) are an important group of zinc enzymes responsible for degradation of extracellular matrix components such as collagen and proteoglycans during normal embryogenesis, tissue remodeling, and in many disease

processes such as arthritis, cancer, tumor metastasis, periodontitis, and osteoporosis (1). We reported recently that Sendai virus, a member of the *Paramyxoviridae* family, markedly upregulates MMP-9 and matrix degradation in rat lungs and lung L2 cells (2). The virus causes severe respiratory illness in rodents, similar to influenza virus pneumonia in humans. MMP-9 is an important player in many physiological processes such as development, wound healing, angiogenesis, and inflammation. Inflammatory cells, including T cells and macrophages, produce MMP-9 under pathological conditions (1, 3, 4). In addition, MMP-9 degrades type IV collagen,

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a major component of the basement membrane of endothelial cells, and is responsible for maintaining the integrity of the blood-brain barrier (5).

It has been reported that serum MMP-9 concentrations are significantly higher in patients with influenza-associated encephalopathy and associated with poor prognosis compared to patients with uncomplicated influenza or in healthy controls (6), although MMP-9 levels in the lung and other organs after influenza virus infection have not been studied. MMP-9 levels appear to be regulated by a number of extracellular proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β) (7-11), and by intracellular signaling factors, such as mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NF- κ B), and activator protein 1 (AP-1) (12-15). TNF- α also directly activates MAPK family members, such as extracellular signal-regulated kinase (ERK, p42/p44 MAPK), c-Jun N-terminal kinase (JNK), and p38 MAPK (16, 17). Despite its characterized role in many physiological and pathological processes, mode of regulation of MMP-9 and the mechanisms of regulation in the lungs and the other organs after influenza A virus infection remain unclear. Furthermore, the pathological roles of MMP-9 in multiple organ failure associated with severe influenza and drugs for suppressing MMP-9 activity are also largely unknown.

The present study first demonstrated MMP-9 upregulation and tissue destruction in various organs after influenza A WSN virus infection in mice and elucidated the intracellular signaling pathways involved in this phenomenon. The results also demonstrated that inhibitors of MAPKs and transcription factors for MMP-9 upregulation are potential drug targets for the treatment of influenza pneumonia and associated multiple organ failure.

MATERIALS AND METHODS

Animals

Specific pathogen-free 1-week-old C57BL/6CrSlc mice with mothers were purchased from Japan SLC. All animals were treated in accordance with the animal care committee guidelines of the University of Tokushima.

Materials

U0126, SB203580, and SP600125 inhibitors were purchased from Calbiochem (San Diego, CA).

N-acetyl-L-cysteine (NAC) was purchased from Nacalai Tesque (Kyoto, Japan), pyrrolidine dithiocarbamate (PDTC) from Wako (Osaka, Japan), and nordihydroguaiaretic acid (NDGA) from Sigma (St. Louis, MO). Rabbit anti-phosphokinase, anti-p42/p44 MAPK, anti-p38 MAPK, and anti-SAPK/JNK antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit antibodies specific for TNF- α , laminin, fibronectin, and collagen IV were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-TNF- α antibody was also purchased from Sigma (St. Louis, MO).

Viral infection

Mice were anesthetized with ketamine before intranasal inoculation of 100 plaque-forming units (PFU) of chicken egg-grown IAV/A/WSN/33(H1N1) virus in 6 μ l of saline or with saline as the vehicle control. The mice were then subjected to experiments at various times after infection. At -1 h before infection, some mice received intraperitoneal injections of anti-TNF- α monoclonal antibody (350 mg/kg) or specific inhibitors: U0126 (0.25 mg/kg), SB203580 (0.25 mg/kg), SP600125 (0.25 mg/kg), NAC (10 mg/kg), PDTC (10 mg/kg), and NDGA (2.5 mg/kg). The antibodies and inhibitors were injected once daily for 3 days, and the mice were sacrificed at 4 days postinfection. Virus titers were determined in Madin-Darby Canine Kidney (MDCK) cells as reported previously (18).

Western blot analysis

Mice tissues were homogenized with 3 volumes of Tris-HCl, pH 6.8, containing 2% SDS and 0.5 M NaCl, and then centrifuged at 12,000 \times g for 30 min. The protein concentrations of the extracts were measured by BCA protein assay (Bio-Rad Laboratories, Hercules, CA) and equal amounts (30 μ g protein) were subjected to SDS-PAGE under nonreducing conditions to detect MMP-9 and under reducing condition to detect the other proteins. After transfer to Immobilon transfer membrane (Millipore, Bedford, MA) and blocking with 5% skim milk in 0.02 M Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20, the membranes were probed with individual antibodies overnight at 4°C. After incubation for 1 h with the goat anti-rabbit IgG conjugated with horseradish peroxidase, immunoreactive bands were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Gelatin zymography

Tissue extracts were prepared as described above and equal amounts (50 µg protein) were subjected to electrophoresis on 10% gelatin zymogram gels (Invitrogen Life Technologies, Carlsbad, CA) as reported previously (19). The gels were then renatured in 2.5% (w/v) Triton X-100 for 30 min at room temperature and incubated overnight in substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂, and 0.02% w/v NaN₃) at 37°C according to the instructions provided by the manufacturer. Finally, the gels were stained with 0.05% Coomassie Blue R-250 buffer for 15-30 min, destained with water, photographed for lysis band intensity, and dried for storage.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from the infected mice and serum was separated by centrifugation at 2,000×g for 10 min at 4°C. The levels of TNF-α in the serum were measured by ELISA according to the protocol provided by the manufacturer (BD Biosciences, Franklin Lakes, NJ).

Total RNA extraction and reverse transcription (RT)-PCR

Total RNA was isolated from mouse lungs using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and reverse transcribed using universal primers of influenza virus and SuperScript III RT kit (Gibco BRL, Gaithersburg, MD) for cDNA synthesis. The following primer pairs were used to amplify influenza virus non-structure protein 1 (NS1) gene segments, a region highly conserved in various subtypes and genotypes of influenza A virus, (sense, 5'-CAGCACTCTCGGTCTGGACAT-3', and antisense, 5'-TCCTTCAGAATCCGCTCCACTA-3'). RT-PCR was initiated at 95°C for 15 min followed by 40 cycles of 15-sec denaturation at 95°C, 30-sec annealing at 58°C and 30-sec extension at 72°C. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Statistical analysis

Results are presented as mean ± SEM (from three independent experiments). Differences between groups were examined for statistical significance by Student's *t*-test. Differences were considered significant when the *P* values were < 0.05.

RESULTS

Influenza A WSN virus infection induces TNF-α in various tissues and serum, while inhibitors of NF-κB and AP-1 inhibit the induction

We have reported the kinetics of viral replication in the lungs of mice and rats after intranasal instillation of influenza A virus with a peak on day 4 postinfection (20-22). Fig. 1A shows the time courses

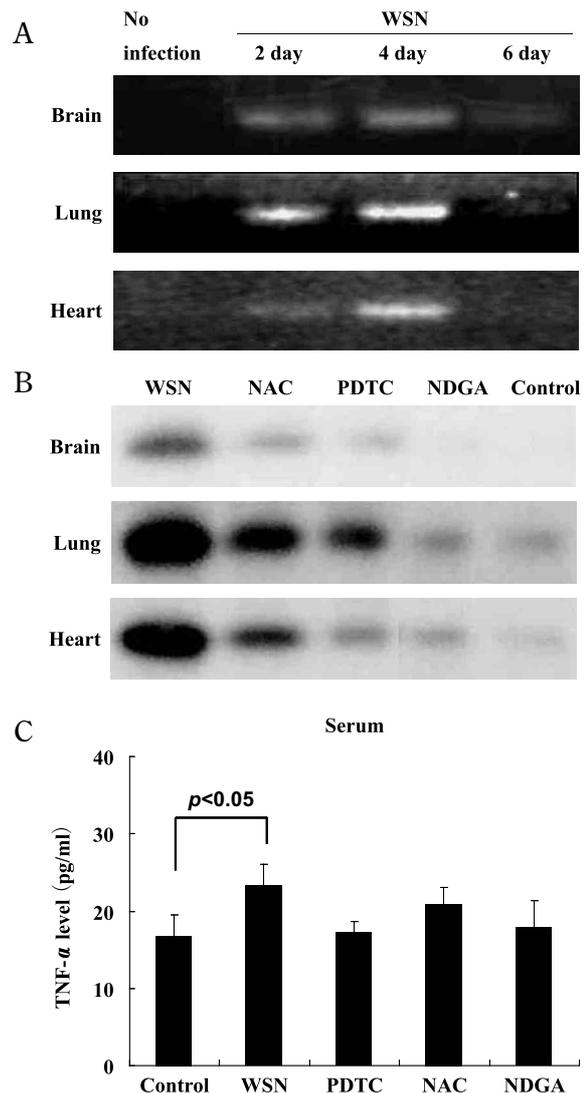


Fig. 1 Kinetics of influenza A WSN viral replication in mice, upregulation of TNF-α in various tissues and inhibition of the upregulation by inhibitors of NF-κB and AP-1.

(A) Detection of influenza A WSN virus NS1 gene in the lung, heart and brain of mice during 2-6 days postinfection by RT-PCR. Right side columns show the levels without infection. (B) TNF-α expressions in the extracts (30 µg) of brain, lung, and heart of mice at 4 days postinfection were analyzed by western blotting. PDTC, NAC, and NDGA were administered at infection -1 h and then once daily for 3 days. Representative example of three experiments with similar results. (C) Serum levels of TNF-α were measured by ELISA at 4 days postinfection. Data are mean ± SEM of three independent experiments. *P* < 0.05 was considered statistically significant, analyzed by using Student's *t*-test.

of viral replication in various organs monitored by viral NS1 gene after intranasal instillation of influenza A WSN virus. Levels of viral RNA in the lungs, the site of initial virus infection, were the highest with a peak at day 4 postinfection, and were under detection at day 6 in all organs. Influenza virus infection also induces a significant increase in levels of proinflammatory cytokine TNF- α , which affects host survival by initiating and/or promoting various immunological and inflammatory responses (23-25). TNF- α levels were analyzed by western blotting and ELISA in the brain, lung, heart and serum of mice on day 4 after influenza A WSN virus infection (Fig. 1B and C). The infection markedly upregulated TNF- α in mouse tissues, particularly in lung and heart. TNF- α levels in the blood were also slightly but significantly increased. Since TNF- α activity is associated with activation of transcription factors NF- κ B and AP-1 (25), we pretreated mice 1 h before infection and then once daily after infection for 3 days with anti-oxidative reagents, PDTC and NAC, to suppress NF- κ B (26, 27), and with NDGA to suppress AP-1 (28). The treated tissues showed significantly lower expression of TNF- α in the brain, lung, and heart at day 4 postinfection. NDGA was particularly potent at suppressing TNF- α in all tissues tested, and also tended to mildly suppress the production of TNF- α in serum, although not significantly.

MMP-9 upregulation by viral infection is mediated through TNF- α , MAPK pathways, and activation of NF- κ B and AP-1

We reported previously that Sendai virus, a *Paramyxovirus*, upregulates MMP-9 expression in the lung and in lung L2 cells (2). The present study demonstrated that influenza A WSN virus, an *Orthomyxovirus*, also upregulates MMP-9 expression markedly in the lung and moderately in the brain and heart at 4 days after infection (Fig. 2). To elucidate the relationship between the infection-induced upregulation of TNF- α and MMP-9, and to clarify the mechanisms involved, we administered anti-TNF- α antibodies, anti-oxidative reagents (PDTC, NAC, and NDGA), and inhibitors of MAPK signaling pathways intraperitoneally at 1 h before infection and then once daily for 3 days. MMP-9 in the brain, lung, and heart was analyzed by gelatin zymography at 4 days postinfection. The upregulation of MMP-9 in the lung and brain was almost completely suppressed to preinfection basal levels by all treatments, while the upregulated activities in the heart were

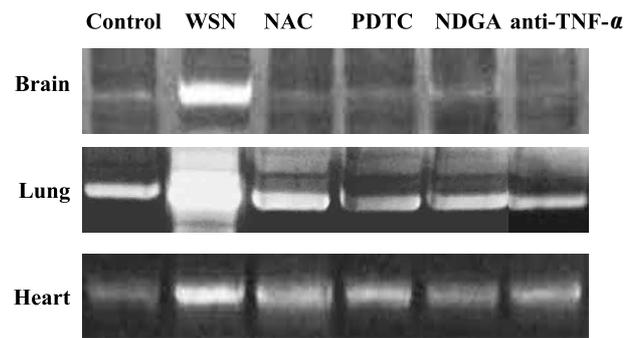


Fig. 2 Upregulation of MMP-9 by influenza A WSN virus infection and its inhibition by treatment with anti-TNF- α antibodies and by inhibitors of NF- κ B and AP-1. The levels of MMP-9 expression in the brain, lung, and heart of mice at 4 days postinfection were analyzed by gelatin zymography. Representative example of three experiments with similar results.

partly decreased. These results indicate that MMP-9 upregulation by influenza A WSN virus infection is mediated mainly through TNF- α and activation of NF- κ B and AP-1.

Phosphorylation of MAPKs by viral infection and effects of MAPK inhibitors on MMP-9 upregulation

Human immunodeficiency type-1 virus Tat upregulates MMP-9 in human astrocytes via TNF- α production and MAPK-NF- κ B-dependent mechanisms (29). To investigate whether influenza A WSN virus modulates MMP-9 upregulation through MAPK signaling pathways, we measured phosphorylated levels of p38 MAPK, ERK1/2, and JNK in cytoplasmic extracts of lung, brain, and heart by western blotting using the appropriate phosphorylation-specific antibodies. At 4 days postinfection, there was a marked increase in phosphorylated p38 MAPK and ERK1/2, and a slight or no increase in JNK phosphorylation in all mouse tissues examined (Fig. 3A).

In the next step, the effects of specific inhibitors for ERK (U0126), JNK (SP600125), and p38 (SB203580) (30-32) on MMP-9 upregulation by influenza A virus infection were examined. Treatment with U0126 and SB203580 efficiently suppressed the upregulated activities of MMP-9 to basal levels in all tissues at 4 days postinfection, while SP600125 had a partial effect (Fig. 3B). These results suggest MAPK-NF- κ B and/or AP-1 signaling pathways are predominant mediators of MMP-9 upregulation by influenza A WSN virus infection.

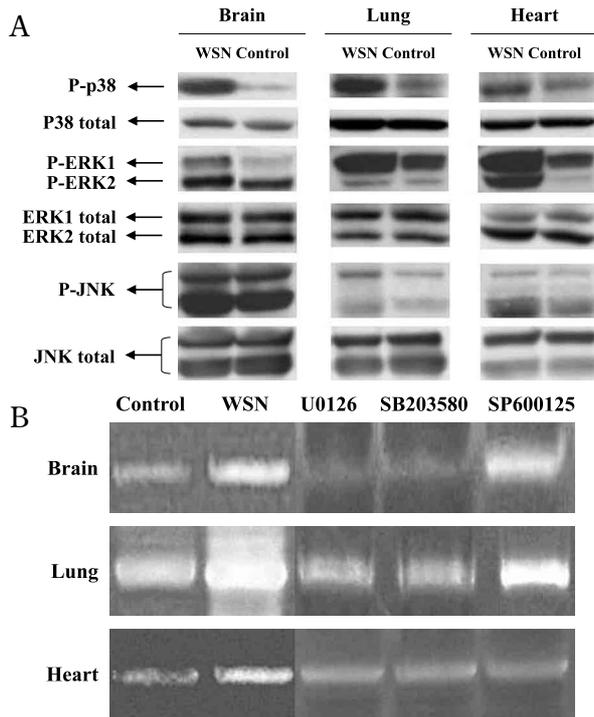


Fig. 3 Phosphorylation of p38, ERK1/2, and JNK MAPKs by influenza A WSN virus infection in the brain, lung, and heart of mice, and the effects of inhibitors of MAPK signaling pathways on the MMP-9 upregulation.

(A) Phosphorylation (P-) levels of ERK1/2, JNK, and p38 MAPK in the extracts (30 μ g) of brain, lung, and heart of the mice with and without influenza A WSN virus infection were detected by western blotting at 4 days postinfection. Equal lane loading was confirmed by detecting blots for total MAPKs. (B) Effects on MMP-9 upregulation by influenza A WSN virus infection at 4 days postinfection using specific inhibitors of ERK (U0126), JNK (SP600125), and p38 MAPK (SB203580) were analyzed by gelatin zymography as described in the Materials and Methods. Control column shows MMP-9 levels without viral infection. Representative example of three experiments with similar results.

Effects of inhibiting MMP-9 upregulation on lung inflammation after viral infection

MMP-9 plays an important role in inflammation and degradation of extracellular matrix (ECM) proteins. We therefore monitored the amounts of ECM proteins collagen IV, fibronectin, and laminin by western blotting in lung of mice after infection (Fig. 4A). Collagen IV and fibronectin, specific substrates of MMP-9, but not laminin, largely disappeared following influenza A WSN virus infection, but treatment of the mice with PDTC, NAC, or NDGA rescued the loss of collagen IV and fibronectin. The lungs of infected mice showed macroscopic lesions by day 4 postinfection (Fig. 4B). The inhibitor treatments with PDTC, NAC, and NDGA also restricted these pathological changes in the lungs of infected mice.

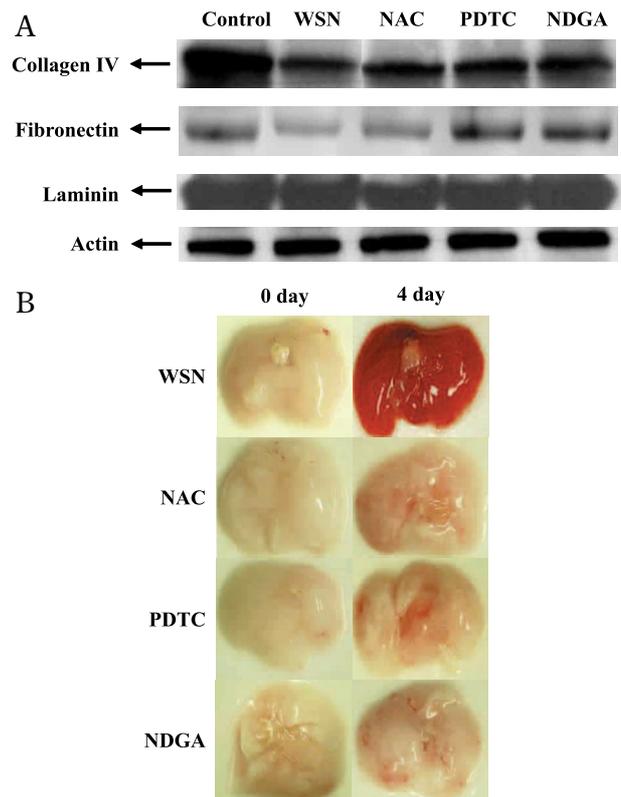


Fig. 4 Lung injury in mice after influenza A WSN virus infection and effects of inhibitors of NF- κ B and AP-1, PDTC, NAC, and NDGA, on the lung injury.

(A) ECM proteins (collagen IV, fibronectin and laminin) in the lungs of mice at 4 days postinfection, were analyzed by western blotting. (B) Lungs of mice before infection (at day 0) and at 4 days postinfection were removed and examined under the microscope after perfusion.

DISCUSSION

The present study reported several new observations: 1) influenza A WSN virus infection results in marked upregulation of proinflammatory cytokine TNF- α and matrix-degrading enzyme MMP-9 in the lung, as initial site of infection, as well as in the brain and heart; and 2) anti-TNF- α antibodies and inhibitors of AP-1, NF- κ B and MAPKs effectively suppressed MMP-9 upregulation *in vivo*. Considered together, these findings indicate that influenza A virus infection upregulates the expression of MMP-9 via TNF- α -mediated activation of MAPK-NF- κ B- and/or AP-1 pathways in mice organs. JNK signaling seems also to be partly involved.

Influenza A virus is the most common infectious pathogen in humans, causing significant morbidity and mortality particularly in infants and the elderly. Multiple organ failure is observed during the advanced stage of influenza pneumonia, and although

rare, encephalopathy with severe brain edema occurs in children and is often fatal. However, the relationship amongst virus and host factors that influences the progression of influenza virus infection and the subsequent lethal effects remains unclear. The present study focused on the upregulation of MMP-9 by influenza A WSN virus infection. This protease degrades type IV collagen in the basement membrane of endothelial cells and may play an important role in multiple organ failure and edema. We also studied the relationship between proinflammatory cytokine TNF- α and MMP-9 and the mechanisms underlying the upregulation and possible inhibition of MMP-9.

TNF- α is produced by many types of cells in various pathological conditions including influenza virus infection (33, 34). TNF- α upregulation by influenza A virus infection in mice stimulated the expression of MMP-9 in various mice organs in this study via MAPK-NF- κ B- and/or AP-1-dependent mechanisms. Signals from extracellular stimuli are transmitted to the nucleus through activation of intracellular signaling kinases, such as the MAPK superfamily (35). MAPKs mediate signals from cell membrane

receptors triggered by TNF- α and involve in the expression of components involved in MMP-9 promoter induction by transcription factors AP-1 and NF- κ B (8). The results obtained in this study implicated MAPK-NF- κ B and/or AP-1 signaling pathway as important in the TNF- α -mediated upregulation of MMP-9 after influenza A virus infection ; this concept is represented schematically in Fig. 5.

The transcriptional downregulation of MMP-9 in virus-infected mice could involve specific NF- κ B or AP-1 inhibitors such as the ones used here (PDTC, NAC, and NDGA) or inhibition of MAPKs including ERK1/2, p38, and possibly JNK. PDTC could stabilize cytosolic I κ B- α , an inhibitor of NF- κ B, by inhibiting I κ B- α ubiquitination, and this stabilization reduces nuclear NF- κ B activation (26). NAC exhibits a chemoprotective effect, which is mediated by counteracting NF- κ B activation by decreasing I κ B- α phosphorylation and I κ B kinases, leading to phosphorylation and subsequent degradation of I κ Bs (27). NDGA, momordin I, a natural inhibitor of fos-jun/DNA complex formation, was found to decrease the apparent equilibrium binding of the dimers and DNA (28). It was reported previously that TNF- α

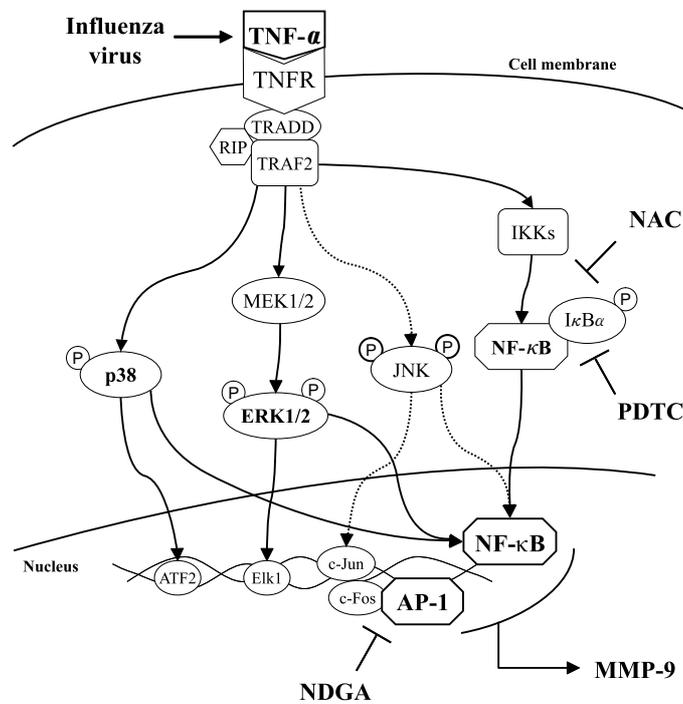


Fig. 5 Schematic diagram of the mechanisms of TNF- α -induced MMP-9 upregulation by influenza A WSN virus infection in mice. Upregulation of MMP-9 in various organs by influenza via MAPK-NF- κ B- and/or AP-1-dependent mechanisms, predominantly through p38 and ERK1/2 (bold lines), and partly via JNK (dotted lines). NF- κ B inhibitors, NAC and PDTC, and AP-1 inhibitor, NDGA, effectively suppressed (\top) MMP-9 upregulation. See text for abbreviations.

induces MMP-9 in various pathological conditions by activation of NF- κ B and AP-1, which bind to the MMP-9 promoter (36, 37). The present study identified the mechanism involved in influenza A WSN virus infection-induced upregulation of MMP-9 in various organs, and showed that this upregulation could be suppressed using inhibitors NF- κ B and AP-1 activation.

In conclusion, the present study implicates MMP-9 upregulation in various organs by influenza A WSN virus infection via MAPK-NF- κ B- and/or AP-1-dependent mechanisms. NF- κ B and AP-1 inhibitors, PDTC, NAC, and NDGA, effectively suppressed MMP-9 upregulation as well as prevented the associated tissue destruction. Since MMP-9 upregulation may be one of the events mediated by TNF- α induction and/or by influenza virus infection, further studies are required to elucidate the effects of MMP-9 and other unknown host factors induced by TNF- α on influenza-associated tissue destruction and inflammation. These results advance our understanding of the mechanisms underlying influenza viral infection and the virus-host interactions and should guide future studies, to ultimately improve the treatment options for influenza virus infection.

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