ORIGINAL

Effects of inhibitors of Toll-like receptors, protease-activated receptor-2 signalings and trypsin on influenza A virus replication and upregulation of cellular factors in cardiomyocytes

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Abstract : Severe influenza sometimes causes myocarditis. We recently found that influenza A virus (IAV) infection induces various cellular factors, such as proinflammatory cytokines IL-6, IL-1β and TNF-α, matrix metalloproteinases (MMPs) and ectopic trypsin in mice hearts and in H9c2 cardiomyocytes. The induction of these cellular factors in turn promotes viral replication, myocardial inflammation and cellular damage through their intracellular signal transductions in cooperation with the IAV-induced Toll-like receptors (TLRs) and proteinase-activated receptor-2 (PAR-2) signallings, although the precise nature of these interactions remain obscure. By using specific inhibitors of TLRs and PAR-2 signalings and trypsin inhibitor aprotinin, we analyzed the role of TLR signaling and PAR-2 signaling in the IAV-induced pathological changes in cardiomyocytes. Inhibitors of TLR7/8-Myeloid Differentiation factor 88-nuclear factor-κB signaling and aprotinin effectively suppressed IAV-induced upregulation of proinflammatory cytokines, MMPs, trypsinogen and viral replication. Inhibitor of TLR3-Toll/interleukin-1 receptor domain-containing adaptor inducing interferons-dependent signaling predominantly suppressed the upregulation of interferon-β, a key intracellular host immune response factor. In contrast to the suppressive effect of trypsin inhibitor aprotinin on IAV replication, PAR-2 inhibitor FSY-NH_2, induced marginal upregulation of trypsinogen and subsequent stimulation of IAV replication. J. Med. Invest. 58 : 19-28, February, 2011

Keywords **:** *influenza A virus, Toll-like receptor, proteinase-activated receptor-2, cardiomyocyte*

INTRODUCTION

Influenza A virus (IAV), a single-stranded negative-sense RNA virus, is the most common infective pathogen in human, causing significant morbidity and mortality in infants and elderly. Severe influenza causes pneumonia with cardiac complications, such as myocarditis and acute myocardial infarction, with a rate of up to 10% (1, 2). In the process of viral entry into cells, proteolytic processing of a viral envelope fusion protein, heamagglutinin (HA), by host cellular trypsin type proteases is a prerequisite for membrane fusion activity (3, 4). IAV infection upregulates cellular ectopic pancreatic trypsin in various organs and vascular endothelial cells, which is then secreted into the extracellular fluid (5, 6). Upregulated trypsin potentiates viral multiplication by conversion of HA_0 into HA_1 and

Received for publication September 21, 2010 ; accepted October 1, 2010.

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HA_2 and also causes damage through proteinase-activated receptor-2 (PAR-2) in various tissues (4, 6). Influenza virus infection also evokes innate and acquired immune responses in host cells via a family of Toll-like receptors (TLRs), one of the pattern recognize receptors (PRR), through induction of proinflammatory cytokines and interferon (IFN)-β (7-9).

To date, 12 mouse TLRs have been described (10), and TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 are the major sensors of viral infection (9). TLR2 and TLR4 detect viral structural proteins, TLR3 recognizes the viral double-stranded RNA (dsRNA), TLR7 and TLR8 recognize viral single-stranded RNA (ssRNA), while TLR9 senses viral double-stranded DNA (dsDNA) at unmethylated CpG motifs (9). These TLRs, with the exception of TLR3, transduce signals through the Myeloid Differentiation factor 88 (MyD88)-dependent pathway. TLR3 uses the Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing interferons (IFNs)-β (TRIF)-dependent pathway (7-9, 11). On the other hand, TLR4 uses both TRIF-dependent and MyD88-dependent pathways. Following engagement with TLR ligands, TLR-dependent signals lead to the activation of various transcription factors such as interferon regulatory factors (IRFs) and nuclear factor-kappaB (NF-κB) (7). The IRF families are known to induce type I IFNs, such as IFN-β, and NF-κB is one of the key transcription factors that regulate various inflammatory mediators (11, 12). In the process of IAV infection, IAV structural protein is recognized by CD14, which cooperates with TLR2 and TLR4, for signal transduction (13). The IAV genome, which consists of ssRNA, is recognized by TLR7/8 (14), while dsRNA, an intermediate product during viral replication, is recognized by TLR3 (15).

PAR-2 is a widely expressed tethered ligand receptor, activated proteolytically by trypsin (16) and involved in inflammation and immune response by the release of an array of cytokines and upregulation of proMMP-9 (17-19).

In a recent study, we found that IAV infection induced various cellular factors, such as ectopic pancreatic trypsin, matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, and proinflammatory cytokines IL-6, IL-1β and TNF-α, which cross-interact with each other (6) : upregulated trypsin effectively stimulates not only viral multiplication but also the conversion of proMMP-9 into active MMP-9 (20, 21), active MMP-9 successively converts proinflammatory cytokines into their active forms (22, 23) ; active cytokines enhance further production of MMPs at the transcriptional level (24, 25). Our study also showed that the above induced-cellular factors coordinately stimulate viral replication, vascular hyperpermeability and cellular damage. Furthermore, both the inhibition of trypsin activity and trypsin mRNA knockdown effectively abrogated the IAV-induced upregulation of all of the aforementioned cellular factors and protected against heart dysfunction and cellular damage of cardiomyocytes (Pan *et al*., manuscript submitted for publication). Although the upregulated pathogenic cellular factors described above might be downstream events of the signaling pathways of IAV-TLRs and PAR-2 activation, details of the cross interactions of these signallings in the IAV-induced pathological changes in cardiomyocytes and host immune response remain to be clarified. In the present study, we analyzed the effects of selective inhibitors of these signalings on IAV-induced cellular responses and viral replication in H9c2 cardiomyocytes.

MATERIAL AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Grand Island, NY), BCA™ protein assay kit was obtained from Pierce (Rockford, IL), 4,6-diamidino-2-phenylindole (DAPI) from Dojindo (Kumamoto, Japan), enzyme-linked immunosorbent assay (ELISA) kits form R&D System (Minneapolis, MN), ITS Premix from BD Biosciences (San Jose, CA), RIPA lysis buffer from Santa Cruz Biotechnology (Santa Cruz, CA), RNeasy Mini kit from Qiagen (Valencia, CA), SuperScript III RT from Invitrogen and GeneAmp® PCR Reagent kit from Applied Biosystems (Bedford, MA). Specific inhibitors pepinh-TRIF and pepinh-MyD were from InvivoGen (San Diego, CA), pyrrolidine dithiocarbamate (PDTC) from Dojindo Laboratories (Kumamoto, Japan), aprotinin from Sigma (St Louis, MO) and FSLLRY-NH_2 (FSY-NH_2) from Bachem (Bubendorf, Switzerland). Antibody against NF-κB p65 was purchased from Abcam (Cambridge, MA), antibodies against TLR2, TLR3, TLR4, TLR7, TLR8, trypsin, MMP-9 and MMP-2 were from Santa Cruz Biotechnology, antibody against actin from Chemicon (Temecula, CA) and antibody against IAV from Takara (Shiga, Japan).

Cell culture and infection

Cardiomyocyte H9c2 cells (ATCC) were cultured

to sub-confluence in DMEM containing 10% fetal calf serum, 100 units/ml penicillin/streptomycin and 2 mM L-glutamine. The optimal concentrations of the specific inhibitors used for experiments were determined by cytotoxicity tests. The cellular toxicity was analyzed by visual inspection after proliferation for 24 h with different concentrations of these inhibitors. For infection, cells were washed twice with calcium- and magnesium-free phosphate-buffered saline [PBS(-)], then infected with IAV/PR/8/34 (H1N1) at a multiplicity of infection of 1 in serum-free DMEM supplemented with ITS Premix (infection medium) for 1 h. Cells were washed twice again with PBS(-) to remove the unbound viral particles, followed by incubation for 24 h in fresh infection medium supplemented with or without the following inhibitors : 10 μM TRIF inhibitor pepinh-TRIF, 20 μM MyD88 inhibitor pepinh-MyD, 10 μM NF-κB inhibitor PDTC, 1.5 μM trypsin inhibitor aprotinin and 40 μM PAR-2 antagonist FSY-NH_2. In case of pepinh-TRIF and pepin-MyD treatments, the cells were pretreated for 6 h before infection.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with RNeasy Mini kit according to the protocol supplied by the manufacturer, and reverse transcribed using universal primers (Uni 12A : 5'-AGCAAAAGCAGG-3', Uni 12G : 5'-AGCGAAAGCAGG-3') and SuperScript III RT for synthesis of IAV cDNA. The following primer pairs were used to amplify IAV non-structure protein 1 (NS1) gene segment with GeneAmp® PCR Reagent kit : 5'-CAGCACTCTTGGTCTGGACAT-3' (forward) and 5'-CCGATGAGGACTCCAACTGCAT-3' (reverse). RT-PCR was initiated at 94°C for 3 min followed by 40 cycles of 30-sec denaturation at 94°C, 30-sec annealing at 60°C and 30-sec extension at 72°C. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Western immunoblotting

Cultured cells were lysed with RIPA lysis buffer. Extracts were concentrated with Amicon Ultra 10K device (Millipore, Danvers, MA) by centrifugation at 4°C. Protein concentrations of the concentrated extracts were measured by BCA protein assay kit and equal amounts (30 μg protein) were loaded onto SDS-PAGE under reducing condition, as reported previously (5).

Immunofluorescent staining

Cells were washed briefly with PBS(-), then fixed in 4% paraformaldehyde for 10 min, followed by permeabilization with 0.2% Triton X-100 for 5 min. After blocking with 2% bovine serum albumin for 1 h, cells were incubated with rabbit anti- NF-κB p65 or IAV antibodies overnight at 4°C, followed by incubation with goat anti-rabbit Texas red-conjugated secondary antibody (Molecular Probes, Eugene, OR) at room temperature for 1 h. Nuclei were stained by 1 μg/ml DAPI for 10 min. Fluorescence labeled cells were observed under a fluorescence microscope.

ELISA

Culture media was collected and centrifuged at 2000×*g* for 20 min at 4°C to remove the cell debris. The levels of IL-6, IL-1β and TNF-α in the culture media were measured by ELISA kits according to the respective protocols provided by the manufacturer.

Statistical analysis

Results are presented as mean ± SD. Significance was calculated by the Student's *t*-test and one-way analysis of variance (ANOVA). A P value less than 0.05 was considered statistically significant.

RESULTS

Changes in TLRs expression levels after IAV infection

Since TLR2, TLR3, TLR4, TLR7 and TLR8 are the major sensors of IAV infection, we analyzed the expression levels of these TLRs in H9c2 cardiomyocytes by western immunoblotting after 24-h mock- or IAV-infection. IAV infection significantly upregulated the expression of TLR3 and TLR7, markedly downregulated that of TLR2 and TLR4, and had no effect on the expression of TLR8 (Fig. 1).

Effects of inhibitors of TLRs, PAR-2 signalings and trypsin on NF-κB activation by IAV infection in H9c2 cells

NF-κB is a critical mediator in the downstream of IAV-induced signaling pathways (26) and its inhibition effectively suppressed not only IAV replication but also cellular damage (27-29). In addition, we recently reported that the influenza virus-cytokine-trypsin cycle is one of the key mechanisms of vascular

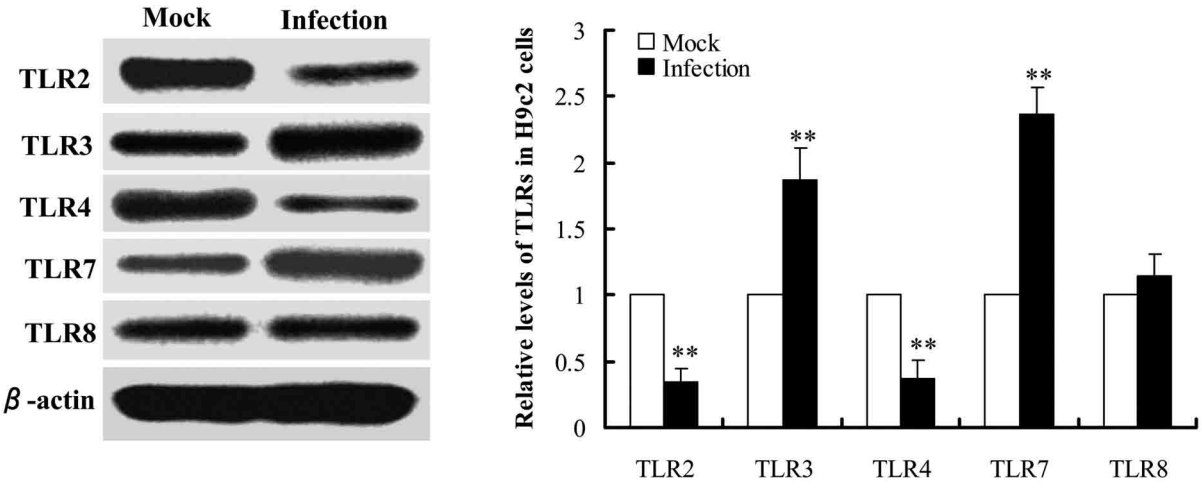


Fig. 1. Effects of infection of H9c2 cells with IAV on the expression levels of TLRs.
Left : Expression levels of TLR2, TLR3, TLR4, TLR7 and TLR8 were analyzed by western immunoblotting in H9c2 cells at 24 h after mock and IAV infection. *Right :* Quantification of the intensity of each band by densitometry. Data represent the expression levels of TLRs after IAV infection relative to that of mock infection. $*P < 0.05$, $**P < 0.01$, compared with mock infection.

hyperpermeability and multiorgan failure in severe influenza (6). To elucidate the role of TLRs signaling and PAR-2 signaling in the pathological changes in cardiomyocytes and myocardial immuno response evoked by IAV infection, we analyzed the effects of inhibitors of TLRs signaling (including pepinh-MyD, pepinh-TRIF and the NF-κB inhibitor PDTC, which block TLR signalings at different steps), PAR2 antagonist peptide FSY-NH_2 and trypsin inhibitor aprotinin, on the translocation of the p65 subunit of NF-κB into the cell nucleus, as a marker of NF-κB activation, by immunofluorescent staining at 24 h after mock or IAV infection of cardiomyocytes. The p65 subunit of NF-κB was localized exclusively in the cytoplasm of uninfected H9c2 cells (mock infection). However, IAV infection resulted in activation of NF-κB and its translocation from the cytosol to the nucleus. The latter process was inhibited by PDTC, pepinh-MyD and aprotinin, but not inhibited clearly by pepin-TRIF and FSY-NH_2 (Fig. 2). These results indicated that NF-κB activation by IAV infection is predominant downstream

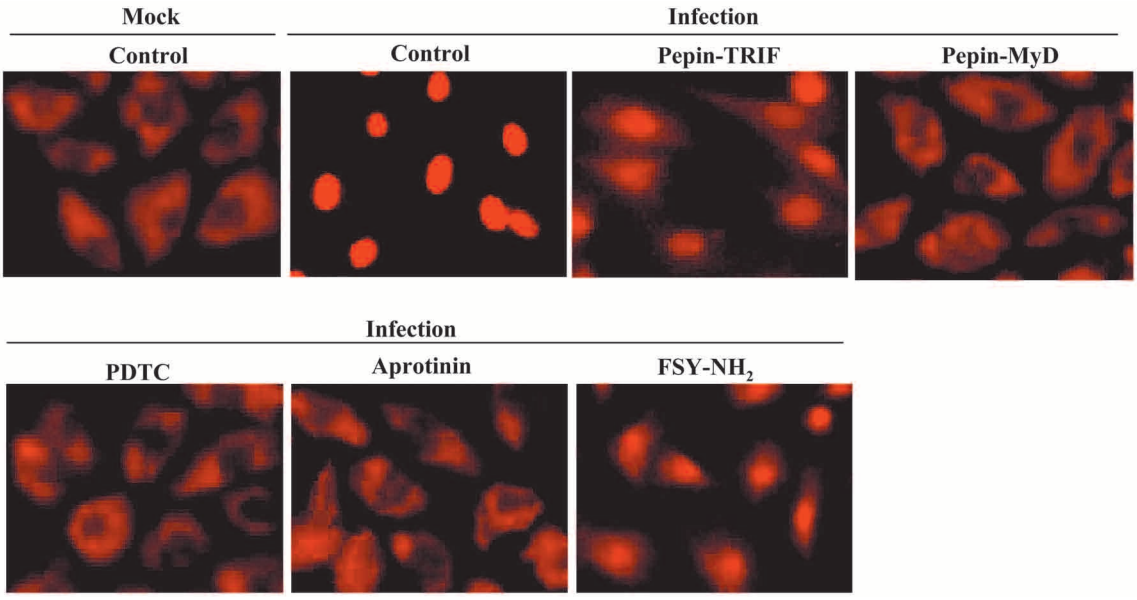


Fig. 2. Effects of inhibitors of TLRs, PAR-2 signalings and trypsin on activation of NF-kB in H9c2 cells after IAV infection. Intracellular localization of p65 subunit of NF-κB in H9c2 cells was analyzed by immunofluorescent staining at 24 h after mock and IAV infection, with or without 10 μM pepinh-TRIF, 20 μM pepinh-MyD, 10 μM PDTC, 1.5 μM aprotinin and 40 μM FSY-NH2. Magnification, ×600

of the MyD88-dependent signaling pathway but not downstream of the TRIF-dependent signaling pathway in H9c2 cells. Although trypsin inhibitor aprotinin also inhibited the translocation of p65 subunit of NF-κB, PAR-2 antagonist FSY-NH_2 did not clearly affect the translocation.

Effects of inhibitors of TLRs, PAR-2 signalings and trypsin on trypsinogen, proMMP-9, proMMP-2 and IFN-β expression in H9c2 cells and cytokine levels in culture media after IAV infection

To investigate the roles of TLRs and PAR-2 signaling pathways in IAV-induced changes in the expression of cellular factors in cardiomyocytes, the H9c2 cells were treated with inhibitors of these signaling pathways and trypsin during IAV infection. Trypsinogen, proMMP-9, proMMP-2 and IFN-β were all significantly upregulated at 24 h after IAV infection (Fig. 3, A-1 and A-2). Treatment of the cells with pepinh-MyD, PDTC and aprotinin significantly inhibited the IAV-induced upregulation of trypsinogen, proMMP-9 and proMMP-2, and moderately inhibited upregulation of IFN-β. The efficiency of the suppression was similar for pepin-MyD and PDTC, and both were slightly more potent than aprotinin. In contrast, FSY-NH2, a PAR-2 receptor antagonist, resulted in slight upregulation of trypsinogen but had no effects on the other cellular factors tested. Pepinh-TRIF selectively and significantly inhibited IFN-β expression but not the other factors tested. The levels of proinflammatory cytokines, such as IL-6, IL-1β and TNF-α, in the culture media increased significantly after IAV infection for 24 h. Although all inhibitors tested suppressed the upregulation of the tested cytokines, PDTC, peninh-MyD and aprotinin had the most potent suppressive efficiencies compared with other inhibitors (Fig. 3B). These results suggest that upregulation of trypsinogen, proMMPs and proinflammatory cytokines is mediated predominantly via the MyD88-NF-κB-dependent pathway and also downstream of the trypsin-mediated viral internalization process.

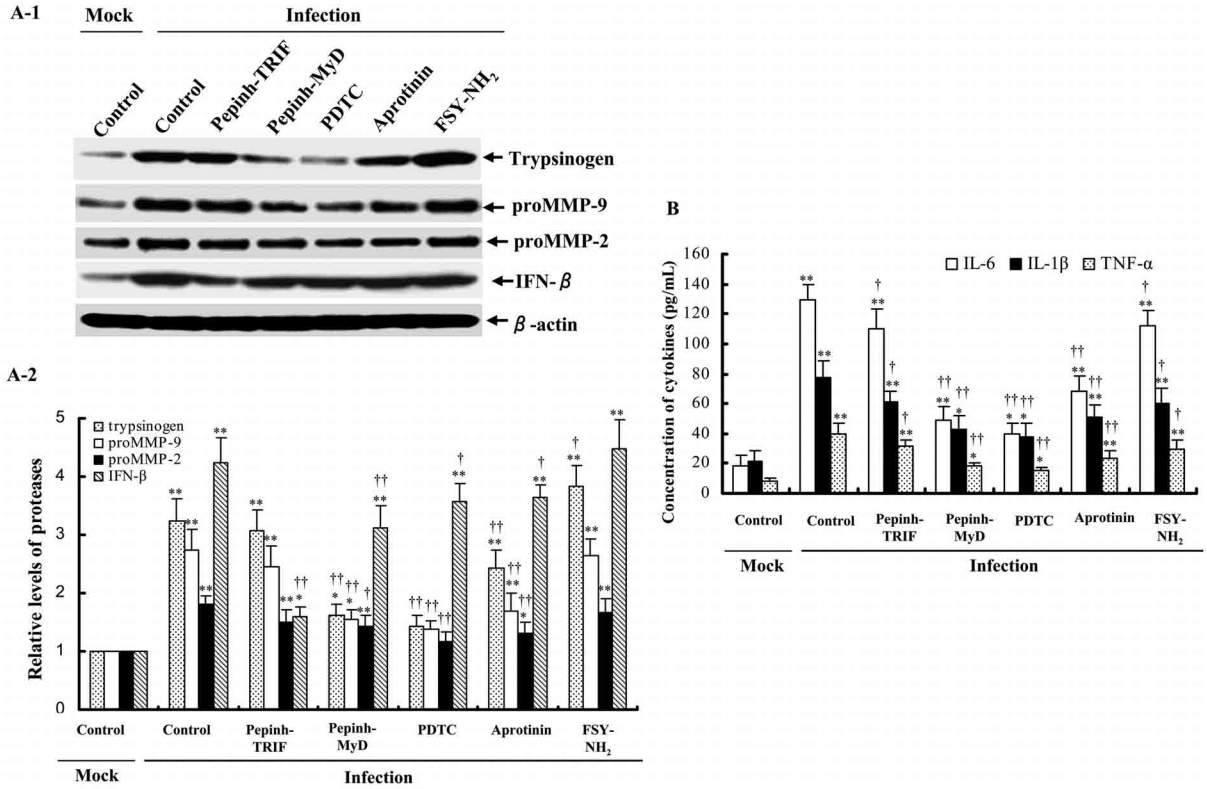
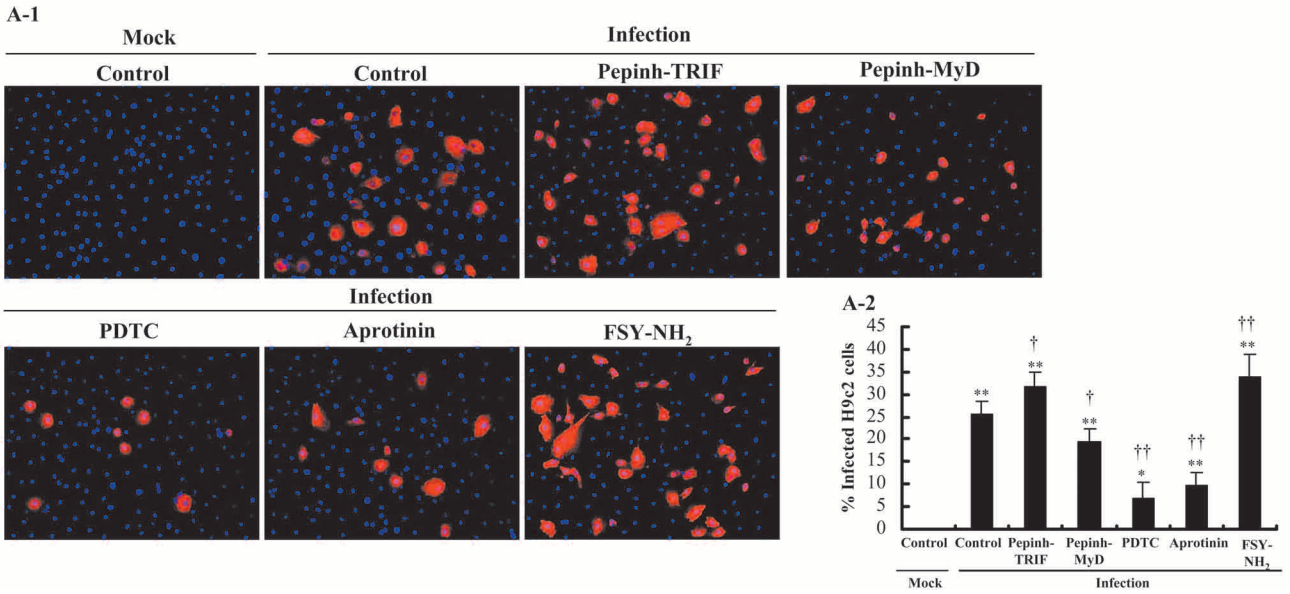


Fig. 3. Effects of inhibitors of TLRs, PAR-2 signalings and trypsin on the expression of trypsinogen, proMMP-9, proMMP-2 and IFN-β in H9c2 cells and cytokine levels in culture media after IAV infection. The expression levels of trypsinogen, proMMP-9, proMMP-2 and IFN-β in H9c2 cells in the absence or presence of 10 μM pepinh-TRIF, 20 μM pepinh-MyD, 10 μM PDTC, 1.5 μM aprotinin and 40 μM FSY-NH2 were analyzed by RT-PCR at 24 h after mock or IAV infection (**A-1**). The intensity of each band was quantified by densitometry. Data represent the expression levels of trypsinogen, proMMP-9 and proMMP-2 in the cell lysates after IAV infection relative to that of mock infection (**A-2**). β-Actin as an internal control. $^{*}P<0.05$, $^{**}P<0.01$, versus mock infection. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, versus infection control. **B**, The concentrations of IL-6, IL-1β and TNF-α in the culture media of cells treated with inhibitors were analyzed by ELISA after IAV infection for 24 h. Data are mean± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, versus mock infection. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, versus infection control.

Effects of inhibitors of TLRs, PAR2 signalings and trypsin on viral replication

Finally, we analyzed the effects of the same inhibitors on viral replication in H9c2 cells. Viral proteins in the infected cardiomyocytes were stained immunocytochemically. A significant reduction in the number of infected cells was observed in cells treated with PDTC and aprotinin, and moderate reduction with pepinh-MyD, compared with the infection control. However, the number of infected cells was slightly but significantly increased when the cells were treated with pepinh-TRIF and FSY-NH_2 (Fig. 4A). Viral replication monitored by NP levels in the culture media and analyzed by western immunoblotting, as well as analysis of NS1 gene by RT-PCR in the cell lysate showed significant suppression of IAV replication in cells treated with PDTC and aprotinin, and partly with pepinh-MyD. In contrast, pepinh-TRIF and FSY-NH_2 resulted in a slight but significant increase in viral replication.



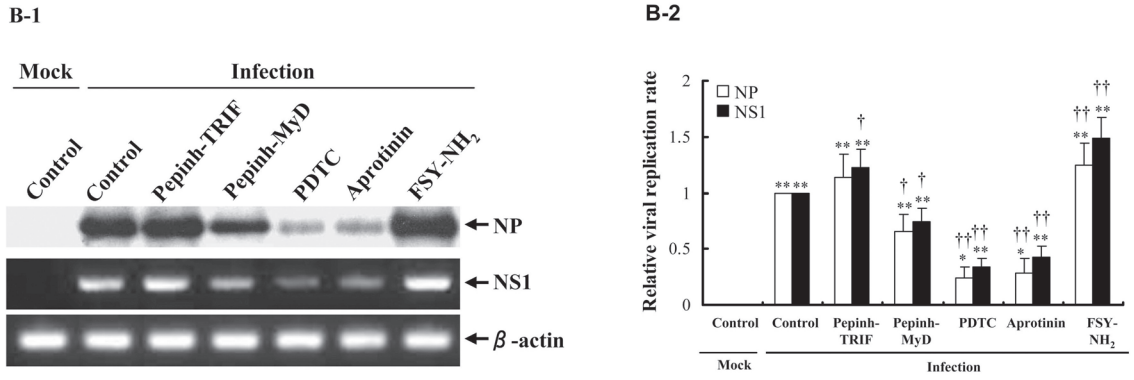


Fig. 4. Effects of inhibitors of TLRs, PAR-2 signalings and trypsin on viral replication. Viral replication in H9c2 cells treated with or without 10 μM pepinh-TRIF, 20 μM pepinh-MyD, 10 μM PDTC, 1.5 μM aprotinin and 40 μM FSY-NH_2 was monitored by immunofluorescent staining at 24 h after mock or IAV infection. Viral proteins in the infected cells were visualized by immunofluorescence (red). Magnification, ×200. Nuclei were stained blue by DAPI (**A-1**). Data represent the percentage of infected cells (**A-2**). Viral replication was also monitored by NP levels in culture media by western immunoblotting and NS1 gene in the cell lysate by RT-PCR under the same experimental conditions as in A (**B-1**). The intensity of each band was quantified by densitometry. Data represent the mean± SD replication rate based on NP and NS1 values after IAV infection, expressed relative to infection control (**B-2**). NS1 levels analyzed by RT-PCR were normalized by β-actin as an internal control. $^{*}P< 0.05$, $^{**}P< 0.01$, versus mock infection. $^{\dagger}P< 0.05$, $^{\dagger\dagger}P< 0.01$, versus infection control.

DISCUSSION

Severe influenza sometimes causes myocarditis and acute myocardial infarction, but viral replication and the pathogenic mechanisms of cardiomyocytes have not yet been elucidated. In the present study, we reported upregulation of TLR7 and TLR3 and downregulation of TLR2 and TLR4 in cardiomyocytes in a manner similar to those found in the lungs (30, 31) and macrophages (13), respectively. TLR3 and TLR7 are viral nucleic acid sensors that can be induced by IAV infection (31). In addition, type I IFNs, which are markedly induced during viral infection, upregulate TLR3 and TLR7 through a positive feedback (32, 33). This upregulation makes the cells more sensitive to virus invasion. TLR2 and TLR4 are not the PRRs for IAV. They mediate signal transduction with CD14 by recognition of the IAV surface antigen (13). Downregulation of TLR2 and TLR4 might be the result of virus-induced shutdown of the host protein synthesis machinery.

In the pathogenesis of IAV infection, various cellular factors, such as ectopic pancreatic trypsin, MMPs and cytokines, are induced and secreted into the extracellular milieu after infection. These factors in turn enhance the pathogenesis via their receptors and signal transductions in cooperation with TLRs' signallings. MyD88 is an adaptor molecule for TLR2, TLR4, TLR7 and TLR8, and pepinh-MyD is a peptide that blocks MyD88 signaling by inhibiting its homodimerization through binding. Pepinh-TRIF is a peptide that blocks TRIF signaling by interfering with TLR-TRIF interaction. NF-κB is a crucial transcription factor for IAV replication and inflammatory responses, and PDTC is a potent inhibitor of NF-κB, based on its oxygen radical-scavenging properties (34) or oxidation of critical thiols in NF-κB (35). Proteolytic activation of HA by trypsin is a step that precedes membrane fusion with viral internalization (36, 37) and the binding of viral ssRNA to TLR7/8. Trypsin also proteolytically activates the conversion of proMMP-9 to actMMP-9 and inactive PAR-2 to active PAR-2. Aprotinin is a non-permeable trypsin-type protease inhibitor with a molecular mass of 6,512 and FSY-NH_2 peptide is an antagonist of PAR-2. Among these inhibitors tested, aprotinin and pepinh-MyD or PDTC, which inhibit trypsin-dependent viral internalization and TLR7/8-MyD88-NF-κB signalling, respectively, preferentially inhibited viral replication and upregulation of trypsinogen and proMMPs (Figs. 3 and 4).

The potent antiviral cytokines type I IFNs, do not only inhibit viral replication directly but also induce the expression of various antiviral proteins, such as myxovirus-resistance protein GTPase, ribonuclease L, RNA-dependent protein kinase R, 2',5'-oligoadenylate synthetase and RNA-specific adenosine deaminase, in neighboring cells (11). Pepinh-TRIF selectively and significantly suppressed the production of IFN-β through IRF and also moderately inhibited the production of proinflammatory cytokines (Fig. 3). These results indicate that IAV infection stimulates diverse signaling pathways, one for stimulation of viral replication and upregulation of proinflammatory cytokines, trypsinogen and proMMPs through TLR7/8-MyD88-NF-κB signaling, and the other for stimulation of IFN-β production as a cellular immune response through TLR3-TRIF-IRF signaling. The inhibitory effect of pepinh-MyD on viral replication was slightly less than those of PDTC and aprotinin, probably because the inhibitor also suppressed the TLR7/8-MyD88-IRF signaling for IFN-β production, although MyD88-IRF signaling is not the major pathway for induction of IFNs.

PAR-2 mediates the classical signal of inflammation, and its inhibition reveals anti-inflammatory effect (38-40). In addition, PAR-2 is considered to act as the "sensory" arm of a negative feedback mechanism to downregulate trypsin expression (41). Upon activation, PAR-2 is internalized and forms a complex with β-arrestin and extracellular signal regulatory kinase (ERK)1/2. This complex prevents ERK1/2 translocation into the nucleus, thereby effectively preventing trypsinogen transcription (42, 43). In the present study, we found that the inhibitory effects of PAR-2 antagonist FSY-NH_2 (44) on the secretion of proinflammatory cytokines IL-6, IL-1β and TNF-α was moderate in H9c2 cells, in comparison with its inhibitory effects on human umbilical vein endothelial cells reported previously (17). In addition, FSY-NH_2 mildly upregulated trypsinogen and viral replication, probably because inhibition of PAR-2 activation by FSY-NH_2 may abrogate the negative feedback mechanism in cardiomyocytes.

In conclusion, the present study indicates that TLR7/8-MyD88, TLR3-TRIF, and PAR-2 signaling pathways mediate distinct cellular responses in cardiomyocytes after IAV infection (Fig. 5). Inhibitors, such as aprotinin for trypsin, pepinh-MyD for TLR7/8-MyD88 signaling and PDTC for NF-κB activation, significantly suppressed viral replication and upregulation of proinflammatory cytokines,

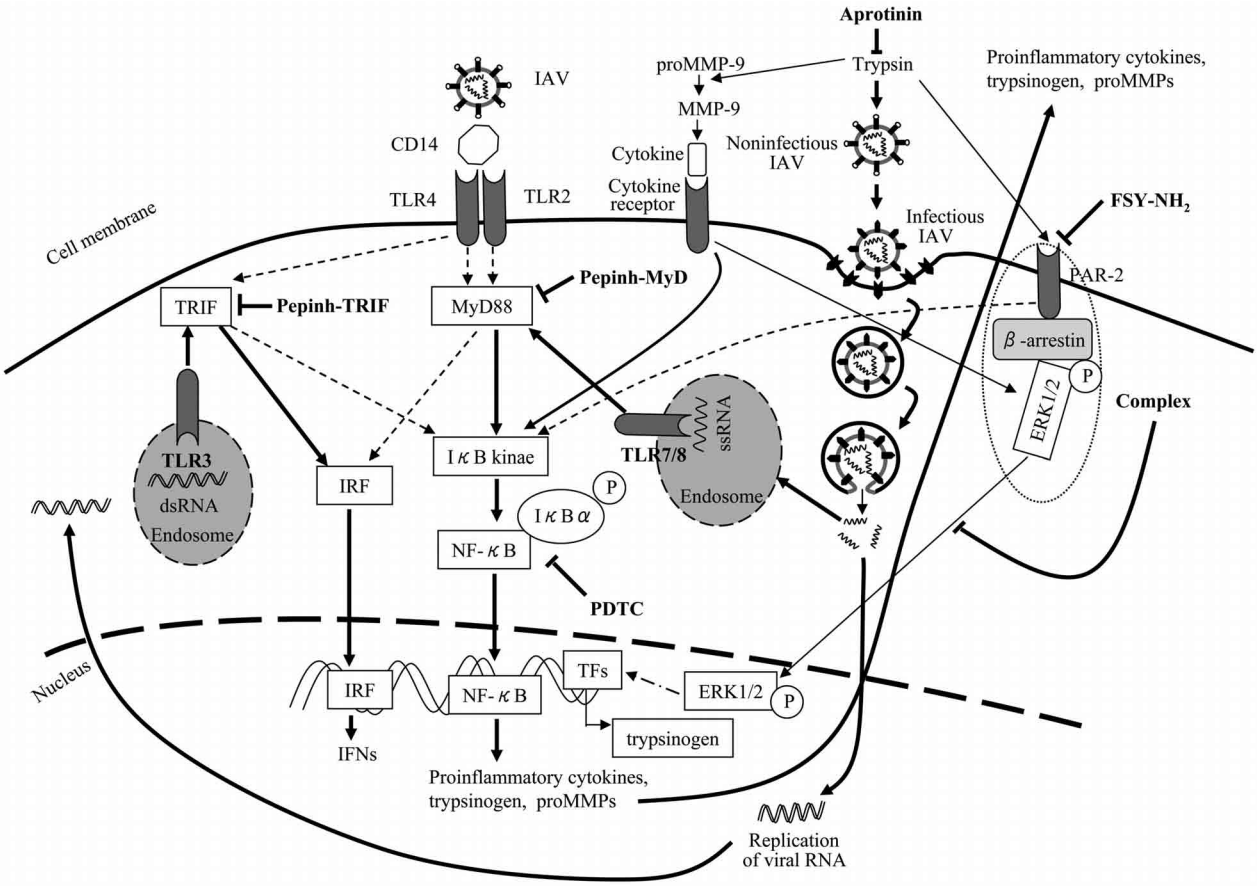


Fig. 5. Proposed scheme for TLRs and PAR-2 signalings and role of trypsin in cellular responses after IAV infection in cardiomyocytes. MyD88 is activated by the binding of IAV ssRNA to TLR7/8 sensor followed by activation of NF-κB signaling and subsequent production of proinflammatory cytokines, proMMPs and trypsinogen. Proteolytical activation of the viral membrane fusion protein HA by trypsin during the process of viral entry into cells is upstream of TLR7/8 sensing. TRIF is predominantly activated by TLR3, which recognizes IAV dsRNA, followed by activation of IRF and subsequent IFNs production. PAR-2, which is proteolytically activated by trypsin, causes PAR-2-β-arrestin-ERK1/2 complex formation, which prevents ERK1/2 translocation to the nucleus and subsequent transcription of trypsinogen. MyD88 inhibitor, pepinh-MyD88, and NF-κB inhibitor, PDTC, effectively suppress (⊥) the induction of proinflammatory cytokines, proMMPs and trypsinogen and viral replication. TRIF inhibitor, pepinh-TRIF, suppresses IFN-β production. FSY-NH_2 blocks PAR-2 activation, resulting in mild inhibition of production of proinflammatory cytokines and translocation of ERK1/2 to the nucleus. *Thick solid lines :* predominant pathways, *thin solid lines :* non-predominant pathways, *dotted lines :* minor pathways in H9c2 cells, *dash-dot line :* the putative pathways with unknown downstream transcription factors, *TFs :* transcription factors. See text for other abbreviations.

proMMPs and trypsinogen. These results suggest that combination treatment with inhibitors of MyD88-NF-κB signaling and trypsin is potentially useful for IAV-induced myocarditis. In addition, the TLR3-TRIF-IRF signaling pathway seems important in the induction of antiviral cellular factor IFN-β in cardiomyocytes. Although PAR-2 antagonist FSY-NH_2 mildly inhibited proinflammatory cytokine production, it slightly upregulated IAV replication.

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