Abstract: Influenza A virus (IAV) causes significant morbidity and mortality. The knowledge gained within the last decade on the pandemic IAV(H1N1)2009 improved our understanding not only of the viral pathogenicity but also the host cellular factors involved in the pathogenicity of multiorgan failure (MOF), such as cellular trypsin-type hemagglutinin (HA0) processing proteases for viral multiplication, cytokine storm, metabolic disorders and energy crisis. The HA processing proteases in the airway and organs for all IAV known to date have been identified. Recently, a new concept on the pathogenicity of MOF, the “influenza virus–cytokine–trypsin” cycle, has been proposed involving up-regulation of trypsin through pro-inflammatory cytokines, and potentiation of viral multiplication in various organs. Furthermore, the relationship between causative factors has been summarized as the “influenza virus–cytokine–trypsin” cycle interconnected with the “metabolic disorders–cytokine” cycle. These cycles provide new treatment concepts for ATP crisis and MOF. This review discusses IAV pathogenicity on cellular proteases, cytokines, metabolites and therapeutic options.

Keywords: influenza virus, processing protease, cytokine, multiple organ failure, pyruvate dehydrogenase kinase 4, carnitine palmitoyltransferase II

1. 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 every year.1,2) Highly pathogenic avian IAV H5 and H7 subtypes also infect humans causing systemic multi-organ failure (MOF) associated with high mortality. In the process of IAV entry into the cell, proteolytic conversion of haemagglutinin (HA0), the viral envelope fusion glycoprotein, into HA1 and HA2 subunits by host cellular trypsin-type proteases is a prerequisite for membrane fusion activity,3)–8) because HA-processing protease(s) is not encoded in their genomes. Therefore, cellular trypsin-type proteases are the main determinants of IAV entry and also determinants of viral infectious tropism of organs and animals.6)–8) Once IAV infection ensues, IAV upregulates cellular ectopic pancreatic trypsin, one of the cellular HA processing proteases is a pre-requisite for membrane fusion activity,3)–8) because HA-processing protease(s) is not encoded in their genomes. Therefore, cellular trypsin-type proteases are the main determinants of IAV entry and also determinants of viral infectious tropism of organs and animals.6)–8) Upregulated trypsin potentiates further viral multiplication in various organs and causes cellular dysfunction and fluid imbalance typically seen in the lungs through...
proteinase-activated receptor-2 (PAR-2) pathway. Upregulated trypsin also causes tissue damage with the cooperation of MMP-9. Increased levels of proinflammatory cytokine by IAV infection, such as tumor necrosis factor (TNF-α), interleukin (IL) 6, and IL-1β (i.e., cytokine storm), suppresses mitochondrial energy homeostasis through up-regulation of pyruvate dehydrogenase kinase (PDK)-J in various organs with the exception of the brain. The inflammatory cytokine response also affects cell-to-cell adhesion, vascular permeability and apoptosis, potentially resulting in vascular dysfunction and MOF. PDK4 inhibitors greatly suppress IAV-induced MOF in vivo, with amelioration of suppressed energy homeostasis, disorders of glucose and lipid metabolism and cytokine up-regulation, resulting in suppression of IAV replication in the lungs. In severe bacterial infection, therapeutic agents that target mitochondria and cellular energetics are also useful for recovery and maintenance of the endothelial barrier and are critical to survival in sepsis.

In this monograph, I review the relationship amongst IAV, cytokines, host cellular trypsin and mitochondrial energy-metabolizing enzymes that induce MOF in severe influenza. I will also discuss the genetic background of high-risk influenza-associated encephalopathy (IAE) patients and the currently available treatment options against IAV-induced ATP crisis, MOF and IAE. Animal experiments in this review were conducted according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), and the study was approved by the Animals Care Committee of the University of Tokushima (Approval date: March 24, 2010; Approved #: 10025).

2. Host cellular trypsin-type proteases play central role in viral cell entry through envelope glycoprotein HA processing of seasonal and highly pathogenic avian IAVs

Figure 1 illustrates the detection of mouse-adapted IAV Aichi/2/68(H3N2) in airway epithelial cells of mice at days 2 and 6 after intranasal IAV inoculation. Viral envelope fusion glycoprotein HA was detected on the cilia of airway epithelial cells at day 2 post-infection and also in adjacent ciliated and non-ciliated secretory bronchial epithelial cells at day 6 (Fig. 1A and B). The cilia on the epithelial cells (Fig. 1C) became swollen and coalesced to form a fused-structure at day 6 post-infection (Fig. 1D), then detached from the epithelial cells. During IAV entry into cells, HA0 is converted into HA1 and HA2 subunits by limited proteolysis, and this process is important for HA maturation, leading to membrane fusion between virions and host cells. However, IAV cannot process HA0 by itself due to the lack of HA-processing protease(s) in its genome. Therefore, IAV infectivity and infectious tropism in organs and animals are determined by the host cellular HA processing proteases.

Proteolytic activation of seasonal human IAV HA occurs extracellularly or on the cell membrane in the airway by trypsin-type proteases, such as tryptase Clara, mini-plasmin, trypsin, ectopic pancreatic trypsin, porcine lung tryp-tase, TC30, and type-II membrane bound proteases, human airway trypsin-like protease (HAT), transmembrane serine protease (TMPRSS) and TMPRSS4 (Table 1). Among them, trypypase Clara was first isolated from rat lungs as a IAV HA0 processing protease by our group and then isolation of the other trypsin-type proteases in airway and lungs from pig and human was followed by us and other groups.

These endoproteases recognize the carboxyl moiety of a single R residue within the consensus cleavage motif Q/E-X-R (where X is any amino acid except C and basic amino acids) of most of the seasonal human IAV HA known to date. As is the case for highly pathogenic avian influenza (HPAI) viruses of subtypes H5 and H7 known to date, the cleavage of HA occurs at the C-terminal R residue in the consensus multi-basic motifs, such as R-X-K/R-R with R at position P4 and K-K/R-K/T-R with K at P4, and leads to systemic infection. The processing proteases of HPAI HA known to date are Furin and pro-protein convertases (PCs), and type II membrane proteases, mosaic serine protease large-form (MSPL)/TMPRSS13 found by us.

We found a large difference in proteolytic potentiation of various virus strains among the cellular processing proteases. As shown in Fig. 2, pancreatic trypsin efficiently activated the infectivity of almost all strains, except IAV WSN/33(H1N1), and mini- and micro-plasmin (which are degradation
products of plasmin found in inflammatory loci) also activated all strains though less efficiently than trypsin.7) The proteolytic potentiating activity of plasmin was the highest only for IAV WSN strain, weak for IAV Aichi/2/68(H3N2) strain, and non-existent for IAV seal/Massachusetts/1/81 (H7N7). These proteases show different local distribution in the airway.19),20) Mini-plasmin is found in folded epithelial cells, in the relatively thick superior bronchiolar divisions; tryptase Clara is located in Clara cells of the inferior bronchiolar divisions, such as the terminal and respiratory bronchioles; and ectopic trypsin I is distributed in stromal cells of peri-bronchiolar regions. Under physiologic conditions, mini-plasmin is only found in the superior bronchiolar divisions but relatively large amounts as high as \( \sim 2 \mu M \) may be produced from blood plasminogen at inflammatory loci by the processing proteases, such as elastase and cathepsin G from granulocytes and cathepsin D from monocytes, during clot formation in the lung. In addition, trypsin is markedly up-regulated in the lungs, heart, brain and vascular endothelial cells after IAV infection.4),10),11) These proteases seem to play critical roles in the spread of IAV, and causing vascular hyperpermeability as well as tissue damage. We also found porcine mast cell tryptase and tryptase TC30 as HA processing enzymes in porcine lungs, although human mast cell tryptase did not proteolytically activate HA of IAV in humans.22),23) In addition to the host cellular proteases, microbial proteases can also proteolytically activate influenza virus HA in bacterial infections of the airways and play important roles in the spread of the virus.32),33) Figure 3A shows limited processing of \( ^{3}H \)glucosamine-labelled IAV Aichi/2/68 (H3N2) HA0 into subunits HA1 and HA2 by tryptase Clara and trypsin. Sequencing of the amino-terminus of the

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Fig. 1. Immunofluorescence detection and scanning electron micrographs of the surface of bronchus of mice infected with mouse-adapted human IAV Aichi/2/68(H3N2).5) C57/BL/6 female mice weighing 10–12 g were infected intranasally with \( 6.6 \times 10^{4} \) plaque-forming units (PFU) of mouse-adapted human IAV. Two (D 2) (A) and 6 days (D 6) (B) after infection, mouse bronchi were isolated, fixed and immunostained for viral HA antigen (green). (C) Note the comb-like structure of the cilia of airway epithelial cells before infection. (D) At day 6 after infection, the cilia are swollen, forming fused-structures. Many such cilia subsequently fell off. Bar = 1 \( \mu m \).
# Table 1. Comparison of proteases involved in HA processing

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MW (kDa) SDS-PAGE</th>
<th>MW (kDa) Non-reducing condition (composition)</th>
<th>Optimal substrate</th>
<th>Inhibitor</th>
<th>Localization</th>
<th>Reference</th>
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<tr>
<td>Seasonal IAV</td>
<td></td>
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<tr>
<td>Tryptase Clara</td>
<td>30</td>
<td>180 (hexamer)</td>
<td>QAR</td>
<td>aprotinin, leupeptin, antipain, KSTI</td>
<td>bronchiolar epithelial Clara cells</td>
<td>Kido H. et al., 1992</td>
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<tr>
<td>Mini-plasmin</td>
<td>28 + 12</td>
<td>38 (heterodimer)</td>
<td>QAR</td>
<td>aprotinin, KSTI, BBSTI, leupeptin</td>
<td>folded epithelial cells in bronchiolar divisions</td>
<td>Murakami M. et al., 2001</td>
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<tr>
<td>Ectopic anionic trypsin I</td>
<td>22</td>
<td>31 (monomer)</td>
<td>EGR, QGR</td>
<td>aprotinin, soybean trypsin inhibitor, leupeptin</td>
<td>stromal cells in peri-bronchiolar region</td>
<td>Towatari T. et al., 2002</td>
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<tr>
<td>Mast cell tryptase</td>
<td>32–35</td>
<td>120 (tetramer)</td>
<td>QAR</td>
<td>antipain, leupeptin</td>
<td>mast cells (porcine)</td>
<td>Chen Y. et al., 2000</td>
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<td>Tryptase TC30</td>
<td>30</td>
<td>30 (monomer)</td>
<td>SIQR</td>
<td>aprotinin, benzamidine, leupeptin</td>
<td>ND</td>
<td>Sato M. et al., 2003</td>
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<tr>
<td>HAT</td>
<td>28</td>
<td>28 (monomer)</td>
<td>FSR</td>
<td>aprotinin, leupeptin, antipain, KSTI</td>
<td>ciliated airway epithelial cells</td>
<td>Chen Y. et al., 2000</td>
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<td>HPAI virus</td>
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<tr>
<td>Furin/PC5/6</td>
<td>52.7 (furin)</td>
<td>57 (furin, monomer)</td>
<td>RXK/RR</td>
<td>Dec-RVKR-cmk, α1-antitrypsin</td>
<td>trans-Golgi network</td>
<td>Stieneke-Gröber A. et al., 1992</td>
</tr>
<tr>
<td>MSPL/TMPRSS13</td>
<td>60</td>
<td>60 (monomer)</td>
<td>R/KKKR</td>
<td>Aprotinin, BBSTI, Dec-RVKR-cmk</td>
<td>membrane-bound, ubiquitously expressed</td>
<td>Okumura Y. et al., 2010</td>
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HA2 subunit yields residues G-L-F-G-A-I-A-G-, indicating that the cleavage sites by trypase Clara and trypsin are between R325 and G326; the amino-terminal site of membrane fusion domain of HA2.19) Figure 3B shows that trypase Clara and trypsin trigger the multiplication of IAV Aichi/2/68(H3N2) in a dose-dependent manner.

HAs from all HPAI virus H5 and H7 strains have either of the two cleavage site motifs, the R-X-K/R-R motif with R at position P4 and the K-K/R-K/T-R motif with K at position P4. Furin cleaves only HA with the R-X-K/R-R motif in the presence of calcium but not the other motif, whereas type II membrane proteases, mosaic serine protease large form (MSPL) and its splice variant transmembrane protease serine 13 (TMPRSS13) recently found by us, with unique substrate recognition of paired basic residues at the cleavage site, efficiently cleave both type motifs, K/R-K/R-K/T-R, even under calcium-free conditions,30) suggesting that these type II membrane proteases facilitate the replication of a wide range of strains of HPAI virus subtypes. Figures 3C and D show cleavage of HA0s and viral multiplication of wild type HPAI virus A/Crow/Kyoto/53/2004 (H5N1) with R-K-K-R motif and mutant virus with K-K-K-R motif. ECV304 cells expressing furin show efficient conversion of wild type HPAI HA0 into HA1 and viral multiplication in the cells, but not conversion of mutant virus HA0 and viral multiplication. On the other hand, ECV304 cells transfected with MSPL convert both type HA0s with R-K-K-R motifs into HA1s and wild type and mutant viral multiplication in the cells.30) The substrate specificities of furin and MSPL were confirmed by using synthetic 14-residues HA peptides of other HPAI strains, such as A/chick/Penn/1370/83 (H5N2)34) and A/FPV/Rostock/34 (H7N1).35) MSPL cleaves both the H5N1 HA peptide with the K-K-K-R motif and the H7N1 HA peptide with the R-K-K-R motif at the correct positions, while furin cleaves only at a single site of R with R at position P4, N-R-K-K-R-R-G-C’ and hardly cleaves the H5 HA peptide with K at position P4. Proteolytic processing of the HPAI virus HA by MSPL inhibitor, such as apritin, Bowman-Birk
trypsin inhibitor (BBI), and substrate-mimicking inhibitor deccanoyl-R-V-K-R-chloromethylketone with R at position P4, strongly suppresses HA0 cleavage and membrane fusion. Figure 3C shows the inhibitory effect of BBI, a membrane non-permeable high-molecular mass inhibitor against MSPL/TMPRSS13, on the mutant virus HA processing in the ECV304 cells transfected with MSPL. To test for the generation of infective virus, conditioned media of one-day culture of ECV304-WT and ECV304-MSPL cells infected with WT and mutant HPAI H5N1 viruses were inoculated into newly prepared cells and cultured for 24 h. Although the spread of WT virus infection with HA cleavage motif of R-K-K-R was detected from the conditioned medium of both ECV304-WT and ECV304-MSPL cells, that of mutant virus with HA0 cleavage motif of K-K-K-R was only detected from the condition medium of...
ECV304-MSPL cells (Fig. 3D). These results strongly suggest that the expression of MSPL, but not furin, potentiates multicyles of HPAI virus with K-K-K-R HA cleavage motif.

Several groups investigated the effects of various inhibitory compounds of HA processing proteases for seasonal IAV (acting as antiviral agents). Among the various trypsin inhibitors tested, the natural airway inhibitor, secretory leukoprotease inhibitor (SLPI), which is secreted from non-ciliated secretory airway Clara and goblet cells, and is found in bronchoalveolar lavage fluid and nasal and salivary secretion, efficiently suppresses proteolytic activation of IAV HA and viral multiplication.\(^\text{5,39,40}\) Another natural inhibitor, aprotinin, trypsin and plasmin inhibitor purified from bovine lungs, also efficiently inhibits HA cleavage and viral multiplication.\(^\text{5,39,40}\)

The inhibitory effects of these non-permeable inhibitors on seasonal IAV multiplication in animal models suggest that proteolytic HA activation occurs in the extracellular space or on the cellular membrane.

3. Role of the “Influenza virus–cytokine–trypsin” cycle in vascular hyperpermeability and MOF in severe influenza

IAV infection increases the levels of proinflammatory cytokines, such as TNF-\(\alpha\), IL-6, and IL-1\(\beta\) in the airway fluid and blood. These cytokines upregulate trypsin and MMP-9 in various organs and vascular endothelial cells, through the activation of nuclear factor-kappa B (NF-\(\kappa\)B) and activator protein 1 (AP-1).\(^\text{10}\) Up-regulation of trypsin in various organs plays important roles not only in influenza virus multiplication in various organs but also in the breakdown of vascular basement membranes and extracellular matrix in collaboration with the induced MMP-9. Furthermore, up-regulation of trypsin in turn evokes protease-activated receptor (PAR)-2, resulting in stimulation of cytokine release,\(^\text{41}\) lung edema through activation of apical membrane chloride secretion and the basolateral membrane K\(^+\) channel,\(^\text{42}\) and increased vascular permeability and relaxation with a rise in intracellular Ca\(^{2+}\) concentrations.\(^\text{10,43}\) Trypsin inhibitor aprotinin and PAR-2 antagonist suppress the above pathological changes induced by IAV.\(^\text{10,11,40,44}\)\(^\text{10}\) suggesting that the “influenza virus–cytokine–trypsin” cycle is one of the key pathogenic mechanisms of vascular hyperpermeability and MOF in severe IAV infection.\(^\text{10}\)

Furthermore, inhibitors of NF-\(\kappa\)B and AP-1 suppress up-regulation of cytokines, trypsin and MMP-9 in mice, resulting in the suppression of viral multiplication with significant improvement in survival.\(^\text{10}\)

Figure 4 is a schematic illustration of various biological responses and the time course of up-regulation of cycle members, IAV, cytokine and trypsin, in the airway of mice after infection. The initial response before the peak of viral proliferation at days 4–5 post-infection includes significant increases in the levels of proinflammatory cytokines. This is followed immediately by marked up-regulation of trypsin and MMP-9 together with increased viral titers in the airway. Just after the peak of viral proliferation, the innate and adaptive immune responses of protective immunity are induced for defense and recovery, or oppositely on rare occasions, MOF.

Figure 5 shows loss of tight-junctions and hyperpermeability in vascular endothelial cells induced by proinflammatory cytokines and their rescue by treatment with trypsin inhibitor. The addition of TNF-\(\alpha\), IL-6, and IL-1/3 to the cell culture and examination after 12 h showed marked down-regulation of tight-junction protein zonula occludens-1 (ZO-1) and slight decrease in occludin levels, and the loss of these proteins was abrogated by treatment with 50 \(\mu\)M aprotinin (Fig. 5A). In addition, the same cytokines disrupted the continuous and linear arrangement of ZO-1, and aprotinin inhibited the disruption (Fig. 5B). Accordingly, these cytokines, especially IL-1β and TNF-\(\alpha\), tended to increase endothelial cell monolayer permeability and this effect was blocked by 50 \(\mu\)M of aprotinin (\(P < 0.05\)) (Fig. 5C). The loss of ZO-1 in the cells is followed by an increase in [Ca\(^{2+}\)]\(_{i}\) and mobilization, while Ca\(^{2+}\) mobilization and the increase in [Ca\(^{2+}\)]\(_{i}\) was inhibited by both trypsin inhibitor and PAR-2 antagonist.\(^\text{10}\) These results indicate close interaction among IAV, cytokines, trypsin and PAR-2, in the pathogenic mechanism of IAV infection.

Figure 6 illustrates the pathological process of acute influenza myocarditis, including increased vascular permeability, tissue edema, and inflammatory cell infiltration. Inflammatory infiltrates appear in the subepicardium at day 3 post-infection, followed by extensive infiltration across the interstitium and perivascular areas deep into the myocardium, accompanied by extracellular matrix destruction at days 6 and 9, though resolution is evident at day 12 (Fig. 6A–C). Coronary vascular permeability monitored by Evan’s blue extravasation and tissue edema by wet/dry weight ratio start to increase at day 3 post-infection, reaching a peak at days 6 and 9,
and then both decrease significantly at day 12. Up-regulation of trypsinogen and its active form trypsin is evident, with peak levels recorded at days 6 and 9 (Fig. 6D and E). IAV levels monitored by the NS1 gene and nucleoprotein (NP) also reach peak values at day 6 (Fig. 6F). Immunohistochemical analysis shows overlapped localization of up-regulated trypsin, viral proteins, and Evans’s blue dye in the infected and inflammatory foci in the heart. Overlapped co-localization of proMMP-9 and active MMP-9 with trypsin is also detected. Since proteolytic activation of viral membrane fusion glycoprotein by trypsin-type proteases is the rate limiting step of viral multiplication, the trypsin-type processing proteases play central and triggering roles in various pathological processes in IAV infection.

4. Severe IAV infection induces marked metabolic disorders of energy production in mitochondria in various organs

MOF with severe pulmonary edema occurs in the progressive stage of seasonal influenza virus pneumonia and IAE, particularly in patients with underlying risk factors and is common in HPAI infection. A proportion of individuals with progressive symptoms after the initial stage of infection develop MOF, together with metabolic disorders, vascular hyperpermeability and cytokine storm. Figure 7 shows that sublethal dose 120 PFU of IAV PR/8/34(H1N1) infection affects glucose oxidation, and reduction of energy metabolism (measured by ATP level) in skeletal muscles, liver, lung and heart, but not the brain, through reduction of mitochondrial pyruvate dehydrogenase (PDH) activity. The earliest reduction in PDH activity occurred at day 3 post-infection in the lungs, where infection started initially, then spread to the skeletal muscles, liver, and heart at day 7 post-infection. Similar patterns of changes were noted in ATP levels in the heart, lung, skeletal muscles and liver.

For oxidative decarboxylation of pyruvate, three enzymes, including PDH (E1), dihydrolipoamide deacetly transferase (E2) and dihydrolipoamide dehydrogenase (E3), catalyze the conversion of pyruvate to acetyl-CoA. The complex also contains two specific phosphorylation-dephosphorylation enzymes,
pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphate phosphatases (PDP). Four isoforms of PDK (PDK1, PDK2, PDK3 and PDK4) phosphorylate specific serine residues on the α-subunit of E1 and two isoforms of PDP (PDP1 and PDP2) dephosphorylate the residues. Among these regulatory compounds in various organs, marked up-regulation of PDK4 is found in the skeletal muscle, liver, lung and heart, but not the brain. Western immunoblotting analysis (Fig. 8) showed PDK4 up-regulation with a peak level at day 3 post-infection in the lungs and at day 7 in skeletal muscles, heart and liver. These results indicate that IAV infection is highly stressful and results in marked and selective up-regulation of PDK4, reduction of PDH activity and ATP crisis in various organs.
5. The interaction of “influenza virus–cytokine–trypsin” and “metabolic disorders–cytokine” cycles plays the principal roles in the pathogenesis of MOF and treatment options

Severe IAV infection markedly up-regulates PDK4, and down-regulates energy homeostasis and disorders of glucose and lipid metabolism, resulting in energy crisis with MOF and vascular hyperpermeability. The results suggest that PDK4 is a suitable target molecule for the treatment of severe IAV infection. Among the known inhibitors of PDK4 (e.g., dichloroacetate (DCA), AZD7545 and radicicol), the pyruvate analog DCA is the most common classic inhibitor of PDK isoforms, although it has clinically symptomatic side effects of peripheral neuropathy. We recently found that diisopropylamine dichloroacetate (DADA), which is the active component of pangamic acid “vitamin B15”, Liverall® (Daiichi Sankyo Co., Tokyo, Japan) and has been used for over 50 years for the treatment of chronic liver diseases without any adverse reaction, is a selective and safe inhibitor of PDK4. Kinetic studies showed that the IC$_{50}$ value, i.e., DADA concentrations at which the reaction rates are suppressed by 50%, is 50.9 µM against PDK4 and 636.0 µM against PDK2. These values are almost identical to those of DCA against PDK4 and PDK2, respectively. Thus, DADA is a novel and safe PDK4 inhibitor with about 12.5-fold higher affinity than that against PDK2. DADA inhibits PDK4, resulting in significant restoration of PDH activity as well as various metabolic disorders, such as ATP levels in various organs, and also improves blood glucose, lactate and β-hydroxybutyric acid levels.

Figure 9 shows the effects of DADA on blood glucose, lipids, ketones, lactate and ATP, at day 7 post-infection in mice infected with lethal doses of IAV.
200 PFU of IAV. Infected mice showed marked hypoglycemia and high levels of blood lactate, free fatty acids and \(\beta\)-hydroxybutyric acid, with small falls in blood ATP levels. These parameters tended to return to normal levels after treatment with DADA in animals inoculated with the lethal dose of IAV. In addition, DADA significantly restored PDH activity and ATP levels in skeletal muscles, heart, lung and
liver to 68–130% of that in non-infected control.\textsuperscript{15}) On the other hand, PDH activity and ATP levels in the brain were neither affected by the infection nor DADA treatment.

Abrogation of PDH suppression in infected mice by DADA was associated with significant restoration of energy metabolism and suppression of cytokine storm. Inoculation of a sub-lethal dose of IAV infection resulted in significant increases in the levels of all tested proinflammatory cytokines (IL-1\(\beta\), IL-6, IL-2, TNF-\(\alpha\), IFN-\(\alpha\), IFN-\(\beta\) and IFN-\(\gamma\)) in lung homogenates (between 1.2- and 13.7-fold relative to the baseline, \textit{i.e.}, before infection) (Fig. 10). Treatment of the metabolic disorder with DADA significantly suppressed IL-6, IL-2, IFN-\(\alpha\), TNF-\(\alpha\) and IFN-\(\gamma\) levels but not those of IFN-\(\beta\) and IL-1\(\beta\). In addition to the immunomodulatory effects of DADA, stimulation of fatty acid metabolism also improves energy metabolism in the cells. Agonists of the peroxisome proliferation-activated receptor (PPAR)-\(\gamma\), a lipid metabolite-mediated transcription factor, and agonists of AMP-activated protein kinase (AMPK), also act as immunomodulatory agents and significantly mitigate the IAV-induced immunopathology with cytokine storm, and improve survival in mice infected with lethal doses of IAV.\textsuperscript{51,52}) These findings suggest that metabolic disorders induced by IAV infection are interconnected with immunopathology and cytokine up-regulation probably through metabolite-mediated signaling pathways and transcription factors. The results of recent studies emphasized the interaction between metabolic disorder and cytokine storm.\textsuperscript{53,54})

Restoration of metabolic disorder and cytokine storm by DADA reduces trypsin expression in various organs and IAV replication in the lungs, resulting in marked improvement of survival.\textsuperscript{15}) Figure 11 shows a typical example of effects of DADA on the survival rate, body weight and food and water intake of mice infected with a semi-lethal 60 PFU dose of IAV at post-infection day 14. Mice showed progressive avoidance of food and water during days 2 to 7 post-infection, and the animals started to die after post-infection day 7. However, infected mice treated with DADA showed no significant decrease in food and water intake as well as no significant reduction in body weight during the 14-day experimental period. While infected untreated mice showed continuous decrease in the survival rate after post-infection day 7 until day 14, with a survival rate of 50%, none of the DADA-treated mice died during the experimental period. The effects of DCA administered at a molar dose equivalent to DADA, on survival rate and body weight of infected mice paralleled those of DADA during the experimental period.
These findings can explain the actions of DADA by our proposed mechanism in Fig. 12 of the host “metabolic disorder–cytokine” cycle linked to the “influenza virus–cytokine–trypsin” cycle. In mice infected with IAV, DADA acts therapeutically through PDK4 inhibition to normalize blood glucose
and lipid oxidation and ATP levels in the mitochondria, as well as suppression of cytokine production and viral replication in the lung and trypsin up-regulation in various organs, with resultant improvement in the survival rate.\textsuperscript{15}

Fatty acid oxidation in the mitochondria is the major energy source particularly for endothelial cells and heart muscle,\textsuperscript{55,56} under several conditions of metabolic stresses, such as long fasting, prolonged exercise, infection and hyperpyrexia.\textsuperscript{57,58} The carnitine palmitoyltransferase (CPT) system is the rate-limiting step in the importation of long-chain fatty acids into the mitochondria and CPT II deficiency is one of the common inborn errors of fatty acid oxidation.\textsuperscript{59} IAE is a specific complication of severe IAV infection in children characterized by sudden onset of febrile convulsions and MOF during high fever.\textsuperscript{60} Since its incidence is higher in East Asians than in Caucasians, genetic factors might play an important role in the etiology of IAE. We reported previously that a large proportion of patients with severe IAE exhibit a thermolabile phenotype of compound homo-/heterozygous variants for [rs2229291; c.1055T>G (p.F352C)] and [rs1799821; c.1102G>A (p.V368I)] of CPT II and mitochondrial energy crisis during high fever.\textsuperscript{61,62} The thermolabile variants, though patients who carry the variants exhibit no underlying disorder in daily life, are inactivated.

Fig. 12. Diagram illustrating the proposed pathogenic mechanism of ATP crisis and MOF in severe influenza involving the host “metabolic disorders–cytokine” cycle linked to the “influenza virus–cytokine–trypsin” cycle. Two separate pathways that lead to vascular hyperpermeability and MOF are illustrated in two green rectangles (cells), whereas there is overlap between the two. PPARs, peroxisome proliferator-activated receptors; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; PAR-2, protease activated receptor 2; CPT, carnitine palmitoyltransferase; CFTR, cystic fibrosis transmembrane conductance regulator; KCNN4, potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4; KCNQ1, potassium channel, voltage dependent, KCNQ1(IPR005827); ZO-1, zonular occludens-1.
during high fever, resulting in secondary CPT II deficiency, leading to impaired mitochondrial fuel utilization state, and energy crisis. Treatment of fibroblasts of these IAE patients with bezafibrate, an agonist of PPAR-δ, with CPT II stabilizer L-carnitine, transcriptionally upregulates CPT II, fills up depleted enzyme activity and restores ATP levels in the mitochondria even under hyperthermia at 41°C.63) Since the ATP crisis in IAE patients is enhanced by starvation other than thermal inactivation of CPT II, glucose supplementation and activation of glucose oxidation for ATP generation by DADA might be also an important option for the patients, in addition to treatment with bezafibrate (Fig. 12).

6. Conclusions

The pathogenicity of IAV is determined not only by viral factors but also by host cellular factors, such as HA-processing proteases for viral multiplication, cytokines, metabolites and energy metabolism in the mitochondria. MOF in severe IAV infection is a progressive state of energy metabolic disorder with dysfunction of various cells and tissues. As outlined in this review, the knowledge gained within the last decades and the experience gained from the pandemic influenza A (H1N1) 2009 and HPAI outbreak greatly improved our understanding of the role of host cellular factors in the pathogenicity of IAV infection: (i) Several cell surface anchored trypsin-like serine proteases are candidates of HA-processing protease for seasonal human IAV, TMPRSS2, HAT and TMPRSS4, other than secreted HA-processing proteases in the airway, such as trypsin Clara, trypsin and mini-plasmin, and for HPAI viruses MSPL/TMPRSS13 other than furin and PCs. Furin preferentially cleaves only HA with the R-K-K-R cleavage site motif with R at position P4, whereas MSPL/TMPRSS13 cleaves of HA in a wide range strains of HPAI virus with the R/K-K-K-R motif with both K and R at position P4. (ii) IAV infection up-regulates ectopic trypsin, MMPs and cytokines, and also induces metabolic disorder and ATP depletion in various infected cells. Up-regulated trypsin in various organs including endothelial cells destroys tight junction through PAR-2 on the cell surface. Based on studies on the relationship among up-regulated factors, we propose the “influenza virus–cytokine–trypsin” cycle hypothesis as one of the main mechanisms of MOF. Similar to trypsin-knockdown, administration of aprotinin, a trypsin inhibitor, suppresses viral replication and up-regulation of trypsin, MMPs and cytokines as well as ATP depletion, resulting in significant improvement of cellular function. (iii) IAV infection of the airway initially induces “influenza virus–cytokine–trypsin” cycle for viral multiplication and then the cycle interconnects with “metabolic disorders–cytokine” cycle in the advanced stage of infection. Conjugation of these two cycles enhances the severity of IAV and induces MOF. In contrast, treatment of metabolic disorder of glucose oxidation by DADA results in normalization of glucose and lipid metabolism and significant improvement of ATP levels in mitochondria. Normalization of metabolic disorder by DADA further suppresses cytokine production, viral replication in the lung and trypsin up-regulation in various organs of mice infected with IAV infection, with resultant improvement in the survival rate. (iv) Since IAV infection induces impairment of cell fuel utilization, particularly in patients with predisposing factor of disordered mitochondrial fatty acid oxidation, such patients can easily develop severe MOF and IAE. Treatment with bezafibrate, an agonist of PPAR-δ, significantly restores long-chain fatty acid-mediated mitochondrial ATP levels in the fibroblasts of IAE patients with thermolabile CPT II variants. Considering these findings together, Fig. 12 provides a summary of the proposed pathogenesis of MOF in severe IAV infection involving the host “metabolic disorders–cytokine” cycle linked to the “influenza virus–cytokine–trypsin” cycle.

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Profile

Hiroshi Kido was born in Niigata Prefecture in 1947. He graduated from Hirosaki University School of Medicine in 1973. His research career was started in 1973 in the Institute for Enzyme Research, Tokushima University and became Professor in 1993. His research is divided into three related subjects. One is identification of host cellular proteases which play central roles in influenza virus entry into cells and multiplication, and their inhibitors as treatment options. Since influenza virus has no processing protease in its genomes unlike many other viruses, such as HIV and HCV, proteolytic activation of viral membrane fusion protein by cellular proteases is a pre-requisite for entry and multiplication. He first found influenza virus hemagglutinin processing protease, tryptase Clara, from rat lungs and isolation of other proteases in airway and lungs from pig and human was followed by his laboratory and others in the world. These studies are highly appreciated for infection control studies on seasonal and highly pathogenic influenza viruses. The second is the studies on the mechanisms of multiorgan failure in severe influenza particularly in the high risk patients with metabolic disorder. Combination treatment of anti-viral agents with medicines for metabolic disorder and immunomodulatory agents greatly improves the disease severity and protects re-infection with enhancement of immunomemory, respectively. The last is development of the highly effective and safety mucosal adjuvant which was initially found in the constituents of human pulmonary surfactant. Preclinical trial of the synthetic mucosal adjuvant mimicking pulmonary surfactant, named SF-10, is conducted in 2015, followed by clinical trial in very near future. He was a director of Institute for Enzyme Research (2007–2011) and was appointed as a president of international proteolysis society (2009–2011).