Chapter 4
Cellular and Biochemical Pathogenic Processes in Severe Influenza Virus Infection: How Does Cytokine Storm Play a Role?

Hiroshi Kido, Takashi Kimoto, and Etsuhisa Takahashi

Abstract Influenza A virus is one of the most common infectious pathogen and associated with significant morbidity and mortality. Infected patients with underlying diseases show rapid progression in disease severity. The initial pathogenic process of influenza virus infection is characterized by the induction of various proinflammatory cytokines as well as host cellular trypsin-type viral envelope-processing proteases in the airway, which enhance viral multiplication. This process has been termed the "influenza virus−cytokine−trypsin" cycle. In the advanced stage of infection, the cytokine storm induces disorders of glucose and lipid metabolism in the mitochondria, resulting in ATP crisis and various functional disorders particularly in organs and cells with high ATP consumption, such as vascular endothelial cells and cardiomyocytes. This process has been termed interconnection of the "metabolic disorders−cytokine" cycle with the "influenza virus−cytokine−trypsin" cycle. The interconnection exacerbates mitochondrial ATP crisis and could lead to multiple organ failure with severe edema. Breaking these cycles and interconnection is a promising therapeutic approach against severe influenza. In this review, we discuss the pathogenesis of severe influenza viral infection based on animal experiments and the potential therapeutic options.

Keywords Influenza A virus · Cytokine storm · Multiple organ failure · ATP crisis Pyruvate dehydrogenase kinase 4

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1 Introduction

Influenza A virus (IAV), a single-stranded negative-sense RNA virus, of the *Orthomyxoviridae* family, is the most common infective pathogen in human, causing significant morbidity and mortality in infants and elderly particularly those with underlying diseases, such as chronic lung disease, cardiac disease, renal disease, and diabetes mellitus [1–3]. In the advanced stage of IAV infection, multiple organ failure (MOF) with vascular hyperpermeability is usually associated with marked increases in the levels of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, coined the cytokine storm, and the most common cause of mortality. The hypercytokinemia alters the cellular redox state through different cytokine receptors and reduces the expression of four complex I subunits, oxygen consumption [4, 5], and ATP synthesis in the mitochondria. We have advanced previously the hypothesis of the "influenza virus–cytokine–trypsin" cycle interconnected with the "metabolic disorder–cytokine" cycle as one of the key mechanisms in the pathogenesis of severe IAV infection [6, 7].

All animal experiments described in this review were conducted according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), and all the studies were approved by the Animals Care Committee of the University of Tokushima.

2 Cellular Trypsin-Type Viral Envelope-Processing Proteases, Essential Factors for Initial Viral Infection and Viral Multi-Replication Cycle

An important pre-requisite for IAV infection and multi-replication is the proteolytic breakdown of the viral envelope fusion glycoprotein hemagglutinin precursor (HA0) into HA1 and HA2 subunits [8]. However, IAV cannot process HA0 by itself as it lacks HA-processing protease(s) in its genome. Thus, the host cellular trypsin-type processing proteases, such as tryptase Clara, ectopic trypsin, TMPRSS2, and HAT [9], determine the IAV infectious tropism and its pathogenicity. In this regard, the initial IAV infection in the airway is followed by marked upregulation of ectopic trypsin in various organs and endothelial cells through the induction of proinflammatory cytokines [10, 11], particularly IL-1β [12], and the induced ectopic trypsin subsequently stimulates viral replication in various organs.

The mechanisms of vascular hyperpermeability and tissue destruction involved in the "influenza virus–cytokine–trypsin" cycle in IAV infection are illustrated in Fig. 4.1. The cytokine storm reduces ATP synthesis in the mitochondria through increased production of reactive oxygen species and intracellular calcium concentration $[Ca^{2+}]_i$ [13]. The resultant ATP depletion subsequently causes the dissociation of zonula occludens-1 (ZO-1), an intracellular tight junction component, from the actin cytoskeleton, thus increasing junctional permeability [14]. The cytokine

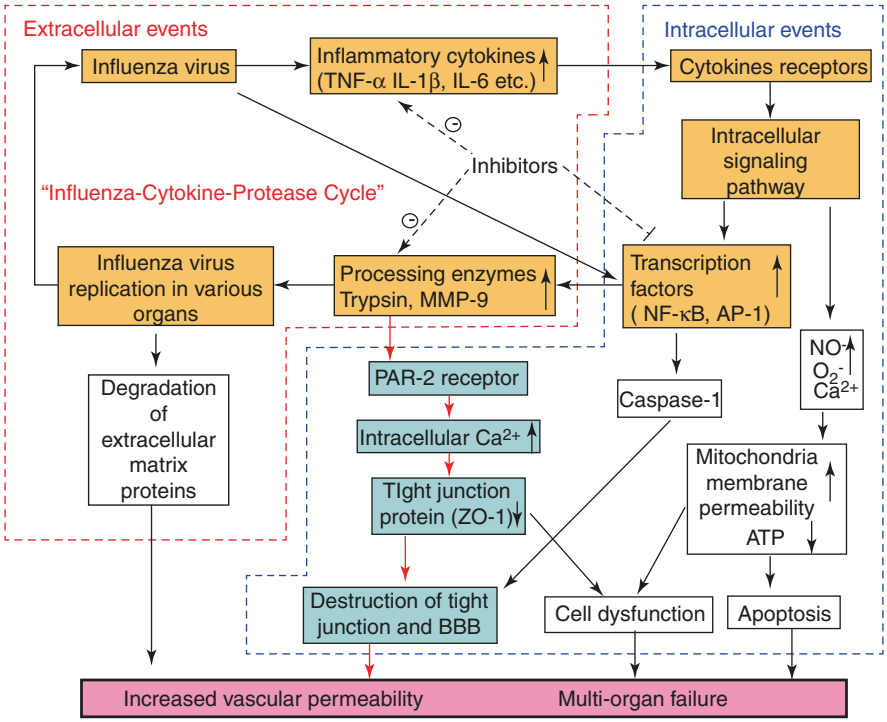

Fig. 4.1 The hypothesis of influenza virus–cytokine–trypsin cycle on the pathogenic processes of vascular hyperpermeability and tissue destruction in severe influenza. *AP-1* activator protein 1, *BBB* blood-brain barrier, *PAR-2* protease-activated receptor 2, *ZO-1* zonula occludens-1. (Reproduced with permission from Ref. [7]. Copyright 2016 The Japanese Respiratory Society)

storm also upregulates ectopic trypsin and matrix metalloproteinase-9 (MMP-9) in vascular endothelial cells and various organs through the activation of nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) [10]. The upregulated trypsin also increases $[Ca^{2+}]_i$ and Cl^- and K^+ secretion via the protease-activated receptor (PAR)-2, resulting in loss of ZO-1 in endothelial cells and severe edema in the airways and colon [15].

Figure 4.2 shows tight-junction loss and hyperpermeability in vascular endothelial cells, which were both induced by proinflammatory cytokines, and the prevention of these two pathological processes by treatment with trypsin inhibitor aprotinin [10]. The addition of TNF-α, IL-6, and IL-1β to the cell culture for 12 h induced marked downregulation of ZO-1, and the loss was abrogated by aprotinin treatment (Fig. 4.2a). The cytokines also disrupted the continuous and linear arrangement of ZO-1, whereas aprotinin inhibited the disruption (Fig. 4.2b). Among these cytokines, IL-1β and TNF-α especially tended to increase endothelial cell monolayer permeability, and this effect was blocked by aprotinin ($P < 0.05$) (Fig. 4.2c). The loss of ZO-1 was also inhibited by PAR-2 antagonist [10].

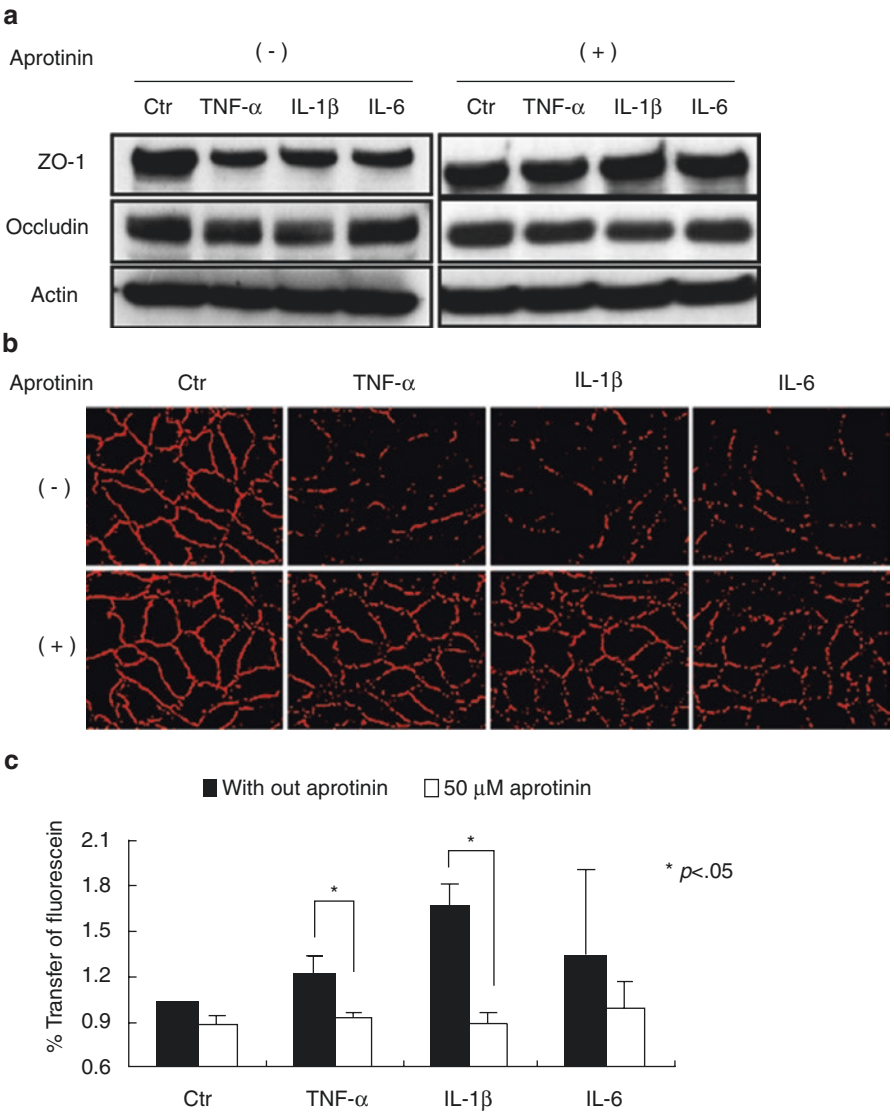

Fig. 4.2 Cytokines induce loss of tight junctions but this action can be abrogated by trypsin inhibitor. (**a**) Western blotting analysis of tight-junction proteins, zonula occludens (ZO)-1 and occludin, after treatment of the cells with cytokines for 12 h in the absence and presence of 50 μM aprotinin. Actin was used as an internal control (Ctr). (**b**) Representative example (from three separate experiments) of immunofluorescence showing decreased ZO-1 expression following cytokine treatment and its restoration by aprotinin. (**c**) Increased permeability of cells treated with cytokines and its rescue by aprotinin ($n = 3$). Data are mean ± SEM. $^*P < 0.05$, with and without aprotinin. (Reproduced with permission from Ref. [6]. Copyright 2015 The Japan Academy)

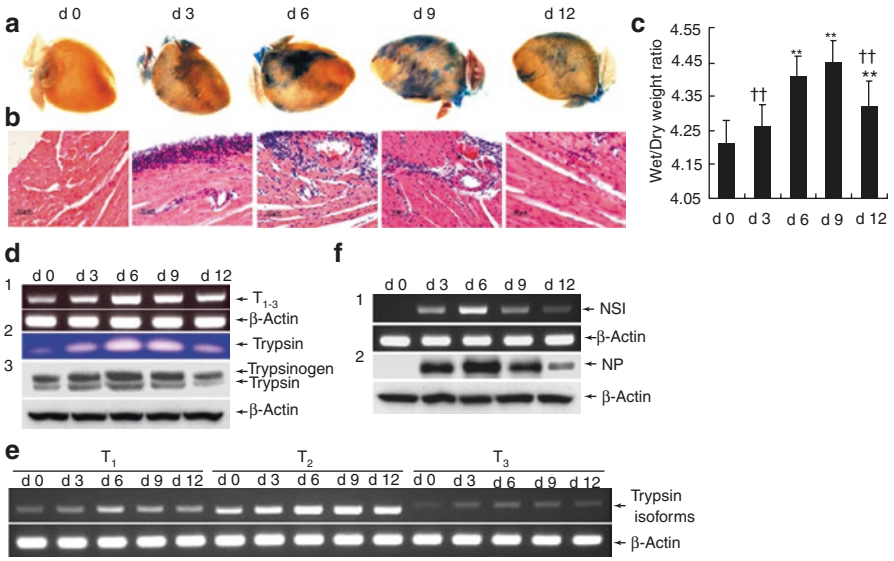

Fig. 4.3 IAV infection can progress to result in acute myocarditis, characterized by vascular hyperpermeability, tissue edema, inflammatory cell infiltration, based on upregulation of trypsin in the myocardium. (**a**) Vascular hyperpermeability monitored by Evans' blue extravasation during the course of infection from day 0 (d 0) to 12 (d 12). (**b**) Hematoxylin and eosin staining. Bar = 50 μm. (**c**) Cardiac edema determined by the wet/dry weight ratio. Data are mean ± SD of 10 mice in each group. $^{**}P < 0.01$ vs. d 0; $^{\dagger\dagger}P < 0.01$ vs. d 9. (**d1**, **e**, and **f1**) RT-PCR-based detection of trypsin$_{1-3}$, trypsin isoforms T_1, T_2 and T_3 and IAV NS1 gene in the hearts from day 0 to 12 post-infection. (**d2**) Detection by zymography of trypsin activity, (**d3**) by western immunoblotting of trypsinogen and trypsin and (**f2**) viral nucleoprotein (NP). Each result is a representative of three experiments. (Reproduced with permission from Ref. [6]. Copyright 2015 The Japan Academy)

Figure 4.3 illustrates the pathological process of acute influenza myocarditis marked by increased vascular permeability and inflammatory cell infiltration [11]. In our experiments, inflammatory infiltrates started to 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 was evident at day 12 (Fig. 4.3a–c). Coronary vascular permeability (monitored by Evan's blue extravasation) and tissue edema (assessed by wet/dry weight ratio) increased at day 3, reaching peak values at days 6 and 9, and then decreased significantly at day 12. Notably, trypsinogen and its active form trypsin were upregulated, with peak levels noted at days 6 and 9 (Fig. 4.3d, e). IAV levels reached peak at day 6, as monitored by the NS1 gene and nucleoprotein (NP) (Fig. 4.3f).

3 Interconnection Between "Influenza Virus–Cytokine–Trypsin" Cycle and "Metabolic Disorder-Cytokine" Cycle Exacerbates ATP Crisis and MOF in the Advanced Stage of IAV Infection

At the early stages of IAV infection, the "influenza virus–cytokine–trypsin" cycle plays a central role in the pathogenic process while the "metabolic disorder–cytokine" cycle interconnects with the cycle and exacerbates ATP crisis and MOF during the progression of IAV infection at the mid to late phase of infection [6, 9, 16].

Two mitochondrial enzymes, pyruvate dehydrogenase (PDH) in glucose oxidation and carnitine palmitoyltransferase (CPT) in long-chain fatty acid oxidation, play key roles in mitochondrial ATP crisis and MOF in severe IAV infection [6, 9]. We reported that severe IAV infection is associated with marked upregulation of pyruvate dehydrogenase kinase (PDK) 4 among the related kinases PDKs1–4 in various organs, but not in the brain [16]. The upregulated PDK4 phosphorylates PDH, a mitochondrial gate keeper enzyme of glucose oxidation, and suppresses its activity, resulting in marked downregulation of glucose-mediated energy homeostasis, culminating in ATP crisis. Figure 4.4 shows that sublethal dose of IAV PR/8/34(H1N1) infection affects glucose oxidation and reduces energy metabolism in skeletal muscles, liver, lung, and heart, but not the brain, by reducing mitochondrial pyruvate dehydrogenase (PDH) activity [16]. In our animal models, the

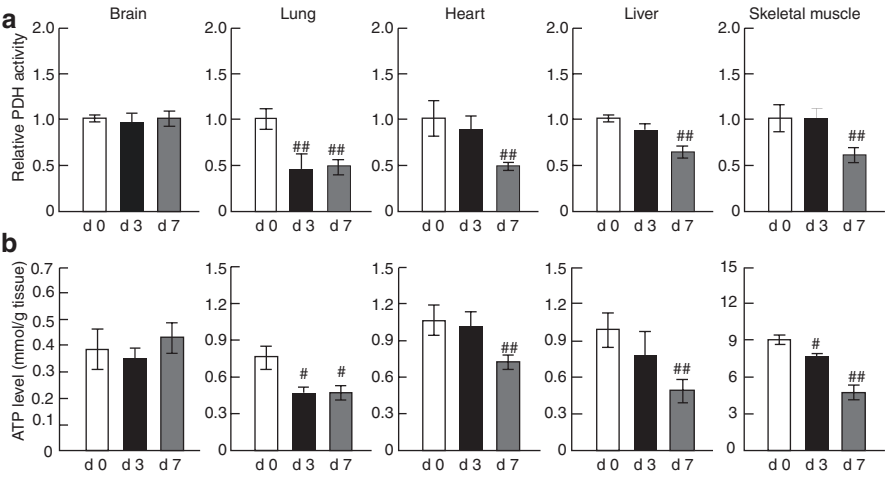

Fig. 4.4 Serial changes in PDH activity and ATP levels in skeletal muscles, heart, lung, liver, and brain of IAV-infected mice. Mice were infected with IAV/PR/8/34(H1N1) at 120 plaque-forming units (PFU) intranasally and the levels of PDH activity (**a**) and ATP (**b**) were analyzed at days 0 (d0), 3 (d3), and 7 (d7) post-infection. PDH activity levels after IAV infection relative to the values at day 0. Data are mean ± SD of 5 mice per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. day 0, by one-way analysis of variance (ANOVA) with Tukey post-hoc test. (Reproduced with permission from Ref. [16]. Copyright 2014 Yamane et al.)

earliest reduction in PDH activity occurred at day 3 post-infection in the lungs, then spread at day 7 to the skeletal muscles, liver, and heart. Similar patterns of changes were noted in ATP levels in these organs. Changes in PDK4 protein expression levels in these organs clearly showed marked upregulation with peak values at day 3 in the lungs and at day 7 in skeletal muscles, heart, and liver. These results suggest that PDK4 is a suitable target molecule for the treatment of severe IAV infection. Among the known inhibitors of PDK, the pyruvate analog dichloroacetate (DCA) is the most common classic inhibitor [17], although it has clinically symptomatic side effects of peripheral neuropathy. In a recent publication, we reported that diisopropylamine dichloroacetate (DADA), which 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 [16].

Figure 4.5 shows the effects of treatment with DADA on PDH activity and ATP levels in various organs in mice infected with sublethal dose of IAV [16]. The infection resulted in marked suppression of PDH activities and ATP levels at day 7 in the skeletal muscles, liver, lungs, and heart, compared with the non-infection control. DADA significantly prevented the suppression and restored PDH activities and ATP levels similar to those before infection. In addition, DADA also corrected the hypoglycemia, increased levels of blood lactate, free fatty acids, and β-hydroxybutyric acid [16].

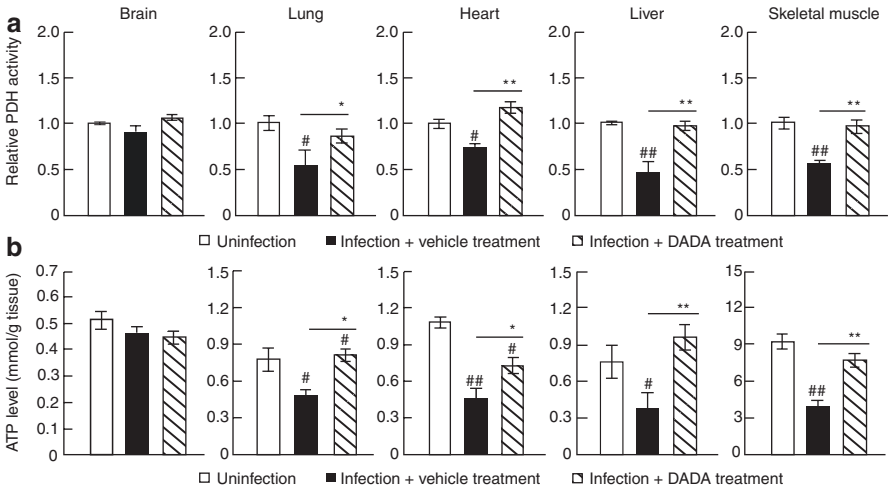

Fig. 4.5 Treatment with DADA restored suppressed PDH activity and ATP levels in skeletal muscle, heart, lung, and liver of IAV-infected mice. Mice infected with IAV at 120 PFU were treated orally with DADA at 50 mg/kg or vehicle at 12-h intervals for 14 days, and the levels of PDH activity (**a**) and ATP (**b**) were measured at day 7 post-infection. PDH activity levels are expressed relative to the values of the control (no-infection). Values are mean ± SD of 5 mice per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. no-infection, $^{*}P < 0.05$, $^{**}P < 0.01$, vs. infected group treated with vehicle, by one-way analysis of variance (ANOVA) and Tukey post-hoc test. (Reproduced with permission from Ref. [16]. Copyright 2014 Yamane et al.)

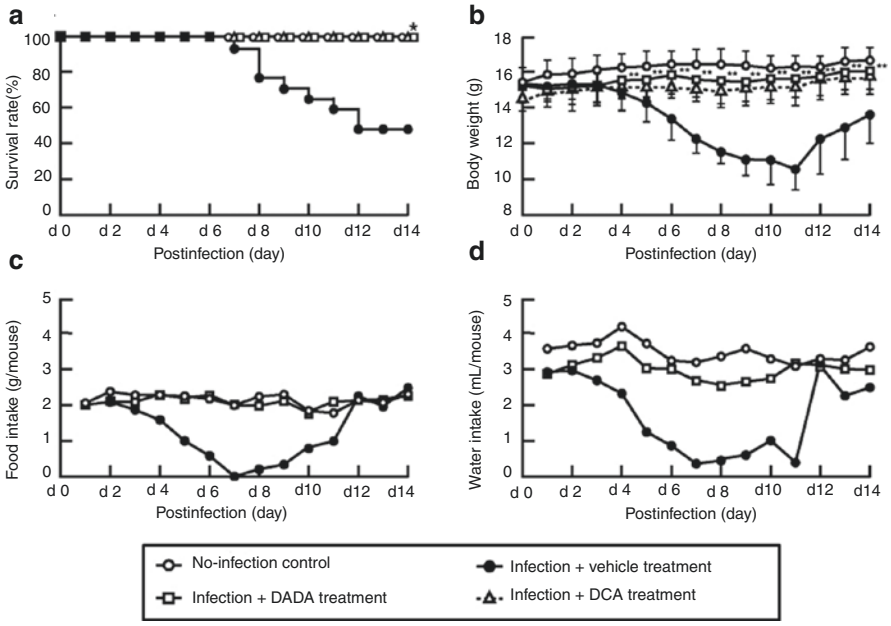

Fig. 4.6 Effects of DADA on survival rate, body weight, and food and water intake. Mice infected with 60 PFU IAV/PR/8/34(H1N1), representing 50% lethal dose, were treated with oral DADA at 50 mg/kg, vehicle, or administered DCA intraperitoneally at 28 mg/kg at 12-h intervals for 14 days. The survival rate, body weight, food intake, and water intake of infected mice were monitored. Survival rate (**a**) analyzed by Kaplan-Meier and log-rank tests. Changes in body weight (**b**), food intake (**c**), and water intake (**d**) for each group. Data are mean ± SD of 15 mice per group. $*P < 0.05$, $**P < 0$ 0.01, vs. infected group treated with vehicle, by two-way ANOVA. (Reproduced with permission from Ref. [16]. Copyright 2014 Yamane et al.)

Figure 4.6 shows a typical example of the effects of DADA on the survival rate, body weight, and food and water intake of mice infected with a semi-lethal dose of IAV up to post-infection day 14 [16]. The infected animals showed progressive avoidance of food and water during days 2–7 post-infection, and then started to die after day 7. However, the infected and DADA-treated mice 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 the survival rate of the infected untreated mice was 50%, it was 0% in the DADA-treated mice during the experimental period.

Another potentially effective therapeutic target is fatty acid oxidation in the mitochondria. Bezafibrate is another treatment option used to prevent fatty acid-mediated energy metabolic disorders induced by IAV infection. IAV-associated encephalopathy (IAE) is a pediatric complication of severe IAV infection, characterized by sudden onset of febrile convulsions and MOF during hyperpyrexia [18, 19]. We reported previously that a large proportion of patients with severe IAE exhibit a thermolabile phenotype of compound homo−/heterozygous variants for [1055 T > G/F352C] and [1102G > A/V368I] of CPT II and mitochondrial energy

crisis during high fever [18, 19]. The thermolabile variants are inactivated during high fever, resulting in secondary CPT II deficiency, leading to an impaired mitochondrial fuel utilization state, and mitochondrial ATP crisis. Bezafibrate is a hypolipidemic pan-peroxisome proliferator-activated receptor (PPAR)-β/γ agonist known to stimulate carnitine palmitoyltransferase (CPT) II expression and promote mitochondrial energy crisis dissipation [20]. Treatment of fibroblasts of these IAE patients with bezafibrate and CPT II stabilizer L-carnitine, transcriptionally upregulated CPT II, filled up depleted enzyme activity and restored mitochondrial ATP levels even under hyperthermia at 41 °C [21].

4 Conclusion

The major pathogenic process of MOF in the advanced stage of IAV pneumonia and IAE, particularly in patients with underlying risk factors, is cell energy metabolic disorders associated with cellular dysfunction in various cells and tissues. The "influenza virus–cytokine–trypsin" cycle is involved in the initial stages of IAV infection, including viral multiplication, but the cycle can be inhibited by treatment with antiviral neuraminidase inhibitors. In the advanced stages of IAV infection, the "metabolic disorders–cytokine" cycle interconnects with the "influenza virus–cytokine–trypsin" cycle and worsens the severity of tissue damage and ATP crisis. In IAV-infected mice, treatment with DADA can normalize blood glucose levels and lipid metabolism through PDK4 inhibition observed during the "metabolic disorders–cytokine" cycle, as well as restore ATP levels in the mitochondria, and cytokine and trypsin levels in various organs, with resultant improvement in the clinical status and survival rate. Lipid metabolism-related energy disorders also induce mitochondrial ATP crisis, particularly in vascular endothelial cells of IAE patients with thermolabile CPT II variants. Treatment with the combination of bezafibrate, a PPAR-β/δ agonist, and L-carnitine significantly restores ATP levels in the fibroblasts of IAE children.

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