Sarco/Endoplasmic Reticulum Ca^{2+} -Transporting ATPase (SERCA) Modulates Autophagic, Inflammatory, and Mitochondrial Responses during Influenza A Virus Infection in Human Lung Cells

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ABSTRACT Influenza A virus is an important human pathogen, causing significant morbidity and mortality. Numerous host factors and cellular responses are dysregulated during influenza A virus (IAV) infection. This includes the arrest of autophagic flux dependent on the influenza M2 ion channel, but little is known about which host factors participate in this autophagic dysfunction. Sarco/endoplasmic reticulum calcium ATPase (SERCA) is known to regulate the transport of calcium ions between the cytoplasm and the sarco/endoplasmic reticulum and has been positively correlated with autophagic flux. Here, we found that SERCA activity was suppressed in influenza A virus-infected human lung cells (H1395) and that CDN1163, an activator of SERCA, restored autophagic flux and, thus, reduced autophagosome accumulation caused by the influenza A virus. Activating SERCA activity with CDN1163 also decreased expression of inflammatory cytokines and chemokines and attenuated mitochondrial dysfunction in IAV-infected H1395 cells. Conversely, SERCA inhibition or genetic ablation aggravated the autophagy dysfunction, mitochondria, and inflammatory responses in the cells infected with influenza A virus. Further study showed that SERCA might regulate the inflammatory response by modulating phosphorylation of the MAPK-JNK pathway. These findings showed that the influenza A virus induced autophagic flux blocking, inflammatory response, and mitochondrial dysfunction by inhibiting SERCA activity. This study provides further understanding of the host-virus interactions between the influenza virus, SERCA activity, autophagy, inflammatory response, and mitochondrial function. SERCA may be a host target for decreasing inflammatory and superoxide injury during influenza A virus infection.

IMPORTANCE IAV is a major cause of infectious respiratory diseases, characterized by a marked respiratory tract inflammatory response that causes morbidity and mortality in seasonal epidemics or pandemic outbreaks. SERCA is a critical component in maintaining cellular calcium levels and is positively correlated with autophagic flux. Here, we discovered that SERCA is suppressed in IAV-infected human lung cells, and influenza A virus induces blocking of autophagic flux, inflammatory response, and mitochondrial dysfunction by inhibiting SERCA. We posit that the pharmacological activation of SERCA can be a powerful intervention strategy to prevent autophagy arrest, inflammatory response, and mitochondrial dysfunction in IAV-infected cells. Therefore, SERCA activity modulation could be a therapeutic strategy for managing clinical symptoms of severe influenza disease.

KEYWORDS SERCA, autophagy flux, influenza virus, inflammatory response

The influenza A virus (IAV), a member of the *Orthomyxoviridae* family, is an enveloped negative-sense RNA virus with eight genome segments encoding 10 to 14

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proteins. IAV is a major cause of infectious respiratory disease, characterized by a marked respiratory tract inflammatory response that causes morbidity and mortality in seasonal epidemics or pandemic outbreaks, such as those that occurred in 1918, 1957, 1968, and 2009 (1). The interaction between viral components and host cellular factors plays an important role in viral replication and pathogenicity. To protect the host cell from infection, the innate immunity pathway is activated by viral components such as RNA and proteins. However, the virus can interfere with, or hijack, cellular machinery to escape this antiviral immunity and to facilitate its own replication. Indeed, upon infection, cellular processes and/or organelles, such as autophagy and mitochondria, have been reported to become dysfunctional.

During the infection process, viruses enter host cells by receptor-mediated endocytosis before moving from endocytic vesicles to endosomes, where, for enveloped viruses, the low pH triggers the fusion of virus and endosome membranes to release the virion contents (2–5). Influenza A viral segment 7 encodes two matrix proteins (M1 and M2). The M2 protein possesses a proton channel activity that allows virion acidification for efficient fusion of the viral envelope with endosomes followed by uncoating of the viral nucleoprotein particles. The 31st amino acid of the M2 protein has been shown to be important for sensitivity to amantadine (AMD; an influenza A virus M2 proton channel blocker), with an N31S substitution conferring the AMD-sensitive phenotype (6, 7). In eukaryotic cells, autophagy is a highly conserved process that maintains cellular homeostasis by mediating the degradation of long-lived cytoplasmic proteins and damaged organelles in the lysosomes (8, 9). Additionally, autophagy also functions in innate immunity to prevent growth and replication of intracellular pathogens by directly mediating the degradation of viral components and inducing the antiviral inflammatory response. In defense against this, IAV has been shown to block autophagic flux (10, 11). Our previous work demonstrated that the block of autophagic flux induced by IAV was dependent on the M2 ion channel, since blocking M2 activity with AMD restored autophagic flux in IAV-infected or M2 protein-expressing cells (12). However, the M2 ion channel induced autophagosome accumulation, and the underlying mechanism of this process is not well defined.

The sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is responsible for transporting Ca^{2+} from the cytosol into the endoplasmic reticulum (ER) lumen to maintain the cytosolic concentration of Ca^{2+} at its low resting levels (13). SERCA is also involved in other cellular processes, including apoptosis, autophagy, and mitochondrial function. A recent study reported that the lack of SERCA inhibits autophagosome-lysosome fusion, while SERCA activation increases autolysosome-like structures (14). Since both IAV infection and SERCA are involved in autophagosome-lysosome fusion, it is worth exploring whether an interaction between IAV and SERCA plays a role in virus-induced autophagy arrest. Disruption of SERCA function is also associated with mitochondrial dysfunction and the generation of reactive oxygen species (ROS) (15). Since the inflammatory response and mitochondrial dysfunction are closely related to autophagy (16–18), we further explored the effect of SERCA on mitochondrial dysfunction and the antiviral proinflammatory response induced by IAV.

In this study, we showed that IAV blocked autophagic flux and inhibited SERCA activity. By using modulators of SERCA activity, we showed for the first time that activated SERCA can partly restore the autophagy arrest, mitochondrial damage, and inflammatory response caused by IAV infection. In summary, our work provides novel insights into understanding the network of interactions between the host cellular machinery and viral components in IAV-infected cells and suggests that pharmacological activation of the SERCA represents a promising therapy for the treatment of disease complications during virus infections, like influenza, that are associated with autophagy arrest, mitochondrial dysfunction, and an excessive inflammatory response.

RESULTS

IAV blocks autophagosome degradation and inhibits SERCA function through ion channel activity of the M2 protein in H1395 cells. IAV has been shown to modulate the autophagy process through the activity of the M2 ion channel (10–12, 19), but

FIG 1 Action of CQ and AMD on IAV. (A) H1395 cells were pretreated with CQ for 24 h and infected with PR8 for another 24 h. The controls were mock infected for the same period. Cell samples were analyzed by immunoblotting with anti-LC3 and anti-ACTB antibodies. Cell viability of H1395 cells after treatment with 10 μ M CQ for 24 h, normalized to that of mock treatment, was determined. (B) Schematic diagram depicting the difference between PR8 and PPH2. In exploring the optimal concentration of AMD required to reduce autophagic accumulation in infected H1395 cells, H1395 cells were infected with PR8 or PPH2 or mock infected and treated with AMD at a concentration range of 0 to 200 μ M. Cell samples were then analyzed by immunoblotting with anti-LC3, anti-NP, and anti-ACTB antibodies. Cell viability of H1395 cells after treatment with AMD at a concentration range of 0 to 200 μ M for 24 h, normalized to that of mock treatment, was determined. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: ***, $P < 0.001$; #, $P > 0.05$.

the host factors involved have not been fully elucidated. Since IAV blocks autophagic flux (12), we selected H1395 cells as the model system due to their sensitivity to IAV. Given that no published IAV study has ever used H1395 cells, we first examined if IAV blocks autophagosome degradation in H1395 cells. As shown in Fig. 1A, we selected chloroquine (CQ) as the lysosomal acidification inhibitor. In cells treated with 10 μ M CQ, the ratio of LC3-II to ACTB was significantly higher than that for untreated cells. However, no difference between PR8-infected cells and CQ-pretreated, PR8-infected cells was seen, indicating that IAV infection blocks autophagosome degradation in H1395 cells. Two IAV strains were used to analyze the impact of the M2 protein on autophagosome accumulation. PR8 and PPH2 (the 31st amino acid encoded by the gene encoding M2 protein of PR8 was changed from asparagine to serine) (Fig. 1B) strains are resistant or sensitive, respectively, to AMD (amantadine, an influenza A virus M2 proton channel blocker). Induction of autophagy was determined by detecting LC3-II, which specifically binds to the autophagosome membrane and is used as a marker for autophagy (20). Our result showed that the ratio of LC3-II to ACTB in PR8- or PPH2-infected cells was significantly increased compared with that of uninfected controls. The appropriate concentration of AMD was 1 to 50 μ M (Fig. 1B); therefore, 20 μ M was chosen for the subsequent experiments. As an M2 proton channel blocker, AMD

FIG 2 IAV blocks autophagosome degradation and inhibits SERCA function through ion channel activity of the M2 protein in H1395 cells. (A) H1395 cells were treated with AMD or the corresponding solvent after being mock infected or infected with IAV strain PR8 or PPH2 and further cultured for 24 h. Cell samples were analyzed by immunoblotting with anti-LC3, anti-NP, and anti-ACTB antibodies. Cell viability of H1395 cells after treatment with 20 μ M AMD and mock controls for 24 h, normalized to that of mock treatment, was determined. (B) Colocalization of the EGFP and mRFP fluorescence in mock-infected, PPH2-infected, or PPH2-infected cells and treatment with AMD cells for 24 h after transfection with mRFP-EGFP-LC3B plasmids. Scale bar, 10 μ m. Quantification of the number of RFP puncta per cell and RFP/GFP fluorescence ratio were measured using a minimum of 10 images per condition. (C) H1395 cells were treated with AMD or the corresponding solvent after being mock infected or infected with IAV strain PR8 or PPH2 and further cultured for 24 h. Cells were collected and the SERCA activity measured. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P > 0.05$.

reduced the level of LC3-II in PPH2- but not PR8-infected cells. Besides, it did not affect the cell viability and expression levels of the virus protein (NP) (Fig. 2A), indicating that IAV infection induces autophagosome accumulation in H1395 cells through its M2 ion channel. The mRFP-EGFP-LC3B plasmid was used to determine the role of the M2

protein in autophagic flux, because, unlike mitochondrial red fluorescent protein (mRFP), green fluorescent protein (GFP) loses fluorescence due to lysosomal acid and degrading conditions (21). The mRFP-EGFP-LC3B-expressing cells infected with PPH2 were treated with 20 μ M AMD and showed an increase in red fluorescence (from mRFP), while the LC3 puncta remained largely yellow (due to fluorescence from both mRFP and enhanced GFP [EGFP]) in untreated PPH2-infected cells (Fig. 2B). These data indicated that IAV infection blocks autophagosome degradation in H1395 cells through the activity of its M2 ion channel.

Rotavirus mediates an increase of cytoplasmic Ca^{2+} to initiate autophagy and blocks autophagy maturation (22). SERCA regulates transport of Ca^{2+} from the cytoplasm to the sarco/endoplasmic reticulum and is positively correlated with autophagic flux (14, 23). We then investigated the relationship between M2-induced autophagy and SERCA activity alteration based on the activity of the M2 ion channel. This activity was significantly reduced in IAV-infected cells compared to uninfected cells. AMD treatment restored SERCA activity in PPH2-infected cells but showed no effect on PR8-infected cells (Fig. 2C). This result indicated that IAV-induced inhibition of SERCA activity in H1395 cells was dependent on the function of its ion channel, M2 protein.

Activation of SERCA by CDN1163 can reduce IAV-induced autophagosome degradation inhibition. Since both autophagosome-lysosome fusion and SERCA ATPase activity were inhibited by the activity of the IAV M2 ion channel, the activation of SERCA was associated with autophagic flux, and inhibition of SERCA blocked autophagosome-lysosome fusion (24, 25), we next explored the relationship between autophagic flux and SERCA activity to determine the mechanism of M2 protein disruption of autophagic flux. To determine if CDN1163 (26, 27), a SERCA activator, contributed to the recovery of autophagic flux in IAV-infected cells, we first defined the optimal concentration of 0 to 20 μ M required to reduce autophagic accumulation in infected H1395 cells. Interestingly, CDN1163 reduced the level of LC3-II in IAV-infected cells in a dose-dependent manner (Fig. 3A). We chose 10 μ M CDN1163 as the noncytotoxic concentration used in the following studies and examined the autophagic flux in CDN1163-pretreated H1395 cells expressing mRFP-EGFP-LC3B and infected with PR8. The results showed that the level of red fluorescence was increased in CDN1163-treated PR8-infected cells, while the yellow LC3 puncta were predominant in untreated PR8-infected cells (Fig. 3B). In addition, CDN1163 decreased the level of SQSTM1 (sequestosome 1) protein in both cells infected with PR8 and uninfected controls (Fig. 3C). These data indicated that activation of SERCA by CDN1163 can reduce the inhibition of IAV-induced autophagosome degradation.

Inhibition or genetic ablation of SERCA enhances the IAV-induced blocking of autophagic flux. Thapsigargin (TG), a specific inhibitor of SERCA (28), was reported to block autophagic flux in several cultured cell lines (23). We first tested the optimal concentration of the drug on the H1395 cells to induce autophagic accumulation over a concentration range of 0 to 1,000 nM. The results showed that TG increased LC3-II levels in a dose-dependent manner (Fig. 4A), with 100 nM TG inducing autophagosome accumulation in H1395 cells. In H1395 cells expressing mRFP-EGFP-LC3B, both PR8-infected and TG-treated PR8-infected cells induced a lot of RFP-LC3 puncta, while TG-treated PR8-infected cells induced higher puncta numbers than the PR8-infected cells. Since IAV blocks autophagic flux, TG did not trigger more density of the yellow puncta of autophagosomes than untreated cells infected with PR8 (Fig. 4B). Moreover, TG induced higher LC3-II and SQSTM1 expression levels in PR8 virus-infected H1395 cells (Fig. 4C). These results indicate that inhibition of SERCA enhances the IAV-induced autophagosome accumulation. The above-described results showed that SERCA inhibition enhanced the IAV-induced blocking of autophagic flux. TG also induces the unfolded protein response (UPR), independent of its inhibition of the SERCA-autophagy axis (29). The RNA interference (RNAi) technique was used to knock down *ATP2A2* (the gene that encodes SERCA protein) to confirm the action of SERCA on IAV-infected cells before examining the autophagic flux. Our findings showed that knocking down SERCA upregulated the LC3B-II and SQSTM1 levels in PR8-infected cells (Fig. 5A).

FIG 3 Activation of SERCA by CDN1163 can reduce the inhibition of IAV-induced autophagosome degradation. (A) H1395 cells treated with CDN1163- or PR8-infected cells pretreated with CDN1163 at a concentration range of 0 to 20 μ M. Cell samples were analyzed by immunoblotting with anti-LC3, anti-M2, and anti-ACTB antibodies. Cell viability of H1395 cells after treatment with CDN1163 at a concentration range of 0 to 20 μ M for 24 h, normalized to that of mock treatment. (B) Colocalization of the EGFP, mRFP, and NP fluorescence in PR8-infected or PR8-infected cells pretreated with CDN1163 for 24 h after transfection with mRFP-EGFP-LC3B plasmids. Scale bar, 10 μ m. The quantification of the number of RFP puncta per cell and RFP/GFP fluorescence ratio was done using a minimum of 10 images for each condition. (C) Expression levels of SQSTM1, LC3, NP, and ACTB of the cells pretreated with CDN1163 or the corresponding solvent for 24 h before being mock infected or infected with PR8 for 24 h. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P > 0.05$.

FIG 4 Inhibition of SERCA by TG enhances the IAV-induced blocking of autophagic flux. (A) H1395 cells treated with TG- or PR8-infected cells pretreated with TG at a range of 0 to 1,000 nM. Cell samples were analyzed by immunoblotting with anti-LC3, anti-M2, and anti-ACTB antibodies. Cell viability of H1395 cells after treatment with TG at a concentration range of 0 to 1,000 nM for 24 h, normalized to cell viability of MOCK. (B) Colocalization of EGFP, mRFP, and NP fluorescence in PR8-infected or PR8-infected cells pretreated with TG for 4 h after transfection with mRFP-EGFP-LC3B plasmids. Scale bar, 10 μ m. Quantification of the number of RFP puncta per cell and RFP/GFP fluorescence ratio were measured using a minimum of 10 images for each condition. (C) Expression levels of SQSTM1, LC3, NP, and ACTB of cells pretreated with TG or the corresponding solvent for 4 h before being mock infected or infected with PR8 for 24 h. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$; #, $P > 0.05$.

FIG 5 Genetic ablation of SERCA by knocking down *ATP2A2* enhances the IAV-induced blocking of autophagic flux. (A) Expression levels of SERCA, SQSTM1, LC3, NP, and ACTB of cells transfected with siATP2A2 or siNC for 24 h before being mock infected or infected with PR8 for 24 h. (B) Colocalization of the EGFP, mRFP, and NP fluorescence in PR8-infected cells transfected with siATP2A2 or siNC for 6 h before transfection with mRFP-EGFP-LC3B plasmids. Scale bar, 10 μ m. Quantification of the number of RFP puncta per cell and RFP/GFP fluorescence ratio were using a minimum of 10 images for each condition. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Furthermore, PR8-infected siNC and PR8-infected siATP2A2 cells induced a lot of RFP-LC3 puncta, while PR8-infected siATP2A2 cells induced higher puncta numbers than the PR8-infected siNC cells (Fig. 5B). These data showed a similar result with TG.

Taken together, CDN1163 activated SERCA attenuates autophagosome accumulation induced by IAV infection. Notably, TG-inhibited SERCA or genetic ablation of SERCA also aggravates autophagosome accumulation induced by IAV infection. These data indicated that IAV infection induced autophagy arrest, which is, at least partly, mediated by inhibition of SERCA activity.

We examined the expression of IAV protein (NP) to investigate the replication of IAV. CDN1163 induced lower NP expression levels (Fig. 3C). However, NP levels increased in the *ATP2A2* (the gene that encodes SERCA protein)-deficient cells (Fig. 5A),

indicating that SERCA regulates the expression of IAV protein. TG also showed a negative function to NP (Fig. 4C), which may be attributed to its UPR antiviral action.

SERCA mediates the IAV-induced inflammatory response. The high level of continuous inflammatory response is a major cause of lung injury in IAV infection (30). It has been reported that autophagy plays an important role in the maintenance of homeostasis and, thus, can inhibit the damage caused by a persistent inflammatory response in at least two ways: by indirectly removing damaged organelles and pathogenic microorganisms that induce the inflammatory response or by directly clearing inflammation-induced complexes (31). Based on our observation that activating SERCA can promote autophagic flux, we probed whether SERCA activity is associated with the IAV-induced inflammatory response. Our data showed that IAV infection can increase the expression of inflammatory cytokines and chemokines in H1395 cells and that activation of SERCA by treatment with 10 μ M CDN1163 reduced the mRNA expression levels of these inflammatory cytokines and chemokines induced by IAV (Fig. 6A). On the contrary, inhibition of SERCA activity with 100 nM TG or genetic ablation of SERCA by knocking down *ATP2A2* was shown to increase the mRNA level of alpha interferon (IFN- α), IFN- β , interleukin-8 (IL-8), and CCL5 in IAV-infected cells (Fig. 7A and 8A). We next detected the levels of IFN- α and IL-8 using an enzyme-linked immunosorbent assay (ELISA), with the results showing a similar trend (Fig. 6B and 7B). All of these data suggested that the IAV-induced inflammatory response was negatively regulated by SERCA activity.

SERCA inhibits the IAV-induced inflammatory response by downregulating the MAPK-JNK pathway. IAV has been confirmed to induce the expression of cytokines through the inflammatory signaling pathways of mitogen-activated protein kinase-c-Jun N-terminal kinase (MAPK-JNK) (32). To dissect the underlying molecular mechanisms by which SERCA activity is associated with the IAV-induced inflammatory response, we evaluated the expression and phosphorylation of key proteins in the MAPK-JNK pathway during IAV infection of cells treated with SERCA modulators. The MAPK-JNK signaling pathway was explored by analyzing the phosphorylation state of JNK and p38 MAPK. We first examined whether IAV could activate the MAPK-JNK signaling pathway in H1395 cells. Compared with the mock-infected cells, cells infected with PR8 showed a significantly higher ratio of phosphorylated protein (p-JNK and p-p38 MAPK) to unphosphorylated protein (JNK and p38 MAPK) at 48 h postinfection (hpi) (Fig. 6C), suggesting that IAV infection activates the MAPK-JNK pathway in H1395 cells. To further explain the mechanism by which SERCA activity modulates the IAV-induced inflammatory response, we then investigated the effect of CDN1163 and TG or genetic ablation of SERCA by knocking down *ATP2A2* on the IAV-induced activation of the MAPK signaling pathway. Our study showed that the levels of the phosphorylated proteins p-p38 MAPK and p-JNK were remarkably increased with TG pretreatment or si*ATP2A2* transfection; however, this decreased with CDN1163 pretreatment in the cells infected with IAV (Fig. 6C, 7C, and 8B). Collectively, our results showed that SERCA inhibits the IAV-induced inflammatory response by downregulating the MAPK-JNK pathway.

IAV induces mitochondrial superoxide production via the ion channel activity of M2. IAV proteins such as NS1 and PB1-F2 play important roles in pathogenesis during IAV infection by inducing mitochondrial dysfunction (33, 34). It has also been reported that a mitochondrion-mediated antiviral innate immune response can be induced through the mitochondrial antiviral signaling (MAVS)-RIG-I pathway (35, 36). Our previous work showed that IAV infection could cause severe disorder in the expression of mitochondrion-related proteins and that inhibition of M2 proton channel activity by AMD can reduce this disorder (data not shown). This suggested that IAV infection causes mitochondrial damage partly through activity of the M2 protein. In this study, we examined autophagic flux and the production of mitochondrial superoxide levels in IAV-infected or M2 protein-expressing cells. The level of mitochondria superoxide in cells was detected by MitoSOX staining and fluorescence microscopy or flow cytometry. Red fluorescence produced by MitoSOX oxidized by superoxide was

FIG 6 CDN1163 reduces the IAV-induced inflammatory response. (A) Cells pretreated with CDN1163 or the corresponding solvent for 24 h before being mock infected or infected with PR8 for 24 h before the transcript abundances of *IFN-α*, *IFN-β*, *IL-8*, and *CCL5* were analyzed by quantitative reverse transcription-PCR and calculated with the $2^{-\Delta\Delta CT}$ method. (B) Cytokine levels in the supernatant of the culture medium were measured using the *IFN-α* and *IL-8* ELISA kit. (C) Expression levels of p-p38 MAPK, p38 MAPK, p-JNK, JNK, LC3, NP, and ACTB in the cells pretreated with CDN1163 or the corresponding solvent for 24 h before being mock infected or infected with PR8 for 24 h. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 7 TG enhances the IAV-induced inflammatory response. (A) Cells pretreated with TG or the corresponding solvent for 4 h before being mock infected or infected with PR8 for 24 h prior to the transcript abundances of *IFN- α* , *IFN- β* , *IL-8*, and *CCL5* being analyzed by quantitative reverse transcription-PCR and calculated with the $2^{-\Delta\Delta CT}$ method. (B) Cytokine levels in the supernatant of the culture medium were measured using IFN- α and IL-8 ELISA kit. (C) Expression levels of p-p38 MAPK, p38 MAPK, p-JNK, JNK, LC3, NP, and ACTB of the cells pretreated with TG or the corresponding solvent for 4 h before being mock infected or infected with PR8 for 24 h. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

found to be colocalized with the green-stained mitochondria (Fig. 9A). More red superoxide-induced puncta were observed in PPH2-infected cells than in AMD-treated PPH2 or mock-infected cells. Analysis with flow cytometry showed that levels of mitochondrial superoxide were increased in both PR8- and PPH2-infected cells (Fig. 9B).

FIG 8 Genetic ablation of SERCA by knocking down *ATP2A2* enhances the IAV-induced inflammatory response. (A) Cells transfected with siATP2A2 or siNC for 24 h before being mock infected or infected with PR8 for 24 h prior to the transcript abundances of *ATP2A2*, *IFN-α*, *IFN-β*, *IL-8*, and *CCL5* being analyzed by qRT-PCR and calculated with the $2^{-\Delta\Delta CT}$ method. (B) Expression levels of SERCA, p-p38 MAPK, p38 MAPK, p-JNK, JNK, LC3, NP, and ACTB of the cells transfected with siATP2A2 or siNC for 24 h before being mock infected or infected with PR8 for 24 h. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 9 IAV induces mitochondrial superoxide production via the ion channel activity of M2. (A) H1395 cells treated with AMD or the corresponding solvent after being mock infected or infected with PPH2 for 24 h. MitoSOX and MitoTracker green were used to stain the mitochondrial superoxide and (Continued on next page)

However, after treatment with AMD, the mitochondrial superoxide level was restored only in the PPH2-infected cells, indicating that the increase in mitochondrial superoxide levels was related to the activity of the IAV ion channel M2 protein. To further demonstrate the effect of M2 protein activity on mitochondrial superoxide levels, we expressed both AMD-sensitive and AMD-resistant versions of the M2 protein from different IAV strains: H1N1, H5N1, and H7N9 (Fig. 9C). All M2 proteins induced autophagosome accumulation (Fig. 9D to F) and increased the level of mitochondrial superoxide in the cells (Fig. 9G to I). Treatment with AMD reduced autophagosome accumulation and decreased mitochondrial superoxide only in cells expressing the AMD-sensitive version of the M2 protein. In addition, to confirm the relationship between autophagic flux and mitochondrial superoxide levels, 10 μ M, 25 μ M, and 50 μ M chloroquine (CQ) were used to block autophagic flux, with the result showing that the LC3-II levels and mitochondrial superoxide levels of the treated cells increased compared with those of the controls (0 μ M CQ) in a dose-dependent manner (Fig. 9J). Taken together, these data suggested that a functional relationship existed between the activity of the M2 ion channel, autophagy arrest, and mitochondrial disorder observed during IAV infection.

Influenza virus affects mitochondrial function through SERCA activity. It has been reported that calcium signaling influences mitochondrion-related processes such as energy production. SERCA is the main Ca^{2+} pump that transports Ca^{2+} ions from the cytoplasm to the sarco/endoplasmic reticulum lumen. Inhibition of SERCA might disrupt cross talk between the levels of Ca^{2+} and generation of ROS in mitochondria. We speculated that SERCA plays a role in mitochondrial dysfunction in IAV infection cells, since we showed increased production of mitochondrial superoxide in IAV-infected cells (Fig. 10A and B). Indeed, activation of SERCA with CDN1163 decreased the ratio of mitochondrial superoxide in IAV-infected cells to that in mock-infected control cells. Conversely, inhibition of SERCA with TG increased production of mitochondrial superoxide in IAV-infected cells (Fig. 10A). Mitochondrial membrane potential (MMP) has been shown to be associated with superoxide generation in mitochondria (37, 38). To further study the role of SERCA in influenza virus in mitochondria, we measured the MMP using two types of mitochondrion-specific labels: MitoTracker deep red (specific for respiring mitochondria) and MitoTracker green (total mitochondria) (39). As shown in Fig. 10B and C, IAV infection caused the MMP to decrease, TG pretreatment aggravated this decrease and induced more severe mitochondrial depolarization, and CDN1163 pretreatment attenuated mitochondrial depolarization caused by IAV. These results indicated that inhibition of SERCA is an important mechanism for mediating mitochondrial dysfunction in IAV-infected cells.

DISCUSSION

IAV causes significant morbidity and mortality, not only directly through action of the viral replication machinery but also indirectly through the cellular antiviral defense factors that have been proven to be involved in the progression of influenza disease. Cytokine storms, metabolic disorders, and energy crises are the major host cause of severe influenza disease in patients and are difficult problems to solve in clinical treatment. Autophagy as a cytoprotective process in cells can be initiated by inflammation (40, 41). IAV has been shown to block autophagic flux (10–12, 19), and the activity of

FIG 9 Legend (Continued)

mitochondria, respectively. The data are shown as the overlay of MitoSOX and MitoTracker green. (B) Flow cytometry analysis of the samples of PPH2, PPH2-AMD, MOCK, and AMD after MitoSOX staining. (C) Schematic diagram showing the different plasmids expressing M2 variants: PR8-M2 and PPH2-M2, H5N1-M2-S31N and H5N1-M2-WT, and H7N9-M2-WT and H7N9-M2-N31S. (D to F) Expression levels of LC3 and ACTB in 293 cells, which were transfected with PR8-M2 and PPH2-M2 (D), H5N1-M2-WT and H5N1-M2-S31N (E), or H7N9-M2-WT, H7N9-M2-N31S, and PCDNA4.0 plasmids (F) or treated with AMD after transfection for 48 h. (G to I) Flow cytometry analysis of 293 cells transfected with PR8-M2, PPH2-M2 (G), H5N1-M2-WT and H5N1-M2-S31N (H), or H7N9-M2-WT, H7N9-M2-N31S, and PCDNA4.0 plasmids (I) or treated with AMD after transfection for 48 h after MitoSOX staining. (J) Expression levels of LC3 and ACTB in H1395 cells treated with 10, 25, or 50 μ M CQ or the corresponding solvent for 24 h, flow cytometry analysis of the samples after MitoSOX staining, and cell viability assay of H1395 cells after treatment with CQ at a concentration range of 0 to 50 μ M for 24 h, normalized to cell viability of the mock treatment. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$; #, $P > 0.05$.

FIG 10 Influenza virus affects mitochondrial function through SERCA activity. (A) H1395 cells pretreated with CDN1163 for 24 h, TG for 4 h, or the corresponding solvent before being mock infected or infected with PR8 for 24 h. The samples were analyzed by flow cytometry after MitoSOX staining, represented as PR8-drug/control-to-PR8 ratios. (B) Alternatively, the cells were stained with MitoTracker deep red and MitoTracker green to measure the MMP of the samples by flow cytometry. (C) Quantification of the ratio of R2 shown in panel B. Data are presented as means $\pm$ SD from 3 independent experiments. Significance was determined by one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$.

SERCA is associated with autophagic flux (24, 25). We determined that IAV induced the inhibition of SERCA activity, so we next explored whether influenza A virus blocked autophagic flux by suppressing SERCA activity (Fig. 11). Since mitochondrial dysfunction and the inflammatory response are closely related to autophagy, we further explored the effect of SERCA on mitochondrial dysfunction and inflammatory response induced by IAV. Understanding the detailed association between IAV, SERCA activity, autophagy, and mitochondrial function will provide further knowledge of the host-virus interactions and, thus, provide new therapeutic strategies for management of clinical cases.

Previous reports showed that inhibition of SERCA, a critical component in the maintenance of cellular calcium levels, blocked autophagosome-endosome fusion and

FIG 11 Model of SERCA modulates autophagic, inflammatory, and mitochondrial responses during influenza A virus infection in human lung cells. SERCA-regulated autophagic flux, the inflammatory response, and mitochondria function are shown. Influenza A virus induced the blocking of autophagic flux, aggravated mitochondrial dysfunction, and increased expression of inflammatory cytokines and chemokines by upregulating the MAPK-JNK pathway through SERCA activity inhibition.

inhibited recruitment of the late endosome/lysosome marker Rab7 to autophagosomes (23). Rotavirus mediates the increase of cytoplasmic calcium ions to initiate autophagy and block autophagy maturation (22). IAV also blocked autophagic flux through the action of its M2 protein, and the underlying mechanism is poorly understood. We hypothesized that the activation and maintenance of SERCA activity would prevent or reduce IAV-induced blocking of autophagic flux. Indeed, our study showed that SERCA activity was significantly reduced in the IAV-infected cells, suggesting that this is the mechanism for autophagy arrest. In support of this, we further tested this hypothesis by observing the cellular effects of SERCA activity during IAV infection of cells treated with SERCA-modulating drugs or genetic ablation of SERCA by knocking down *ATP2A2*, with the result showing that the activation of SERCA with CDN1163 alleviates autophagy arrest in PR8-infected cells while inhibition with TG or knocking down with *siATP2A2* enhances it. Besides the activating noncanonical autophagy dependent on the WD40 CTD of ATG16L1 (42), IAV induces LC3 lipidation at both the plasma membrane of infected cells and in perinuclear structures (19). SERCA may also use noncanonical autophagy pathways to induce PR8-induced autophagy, such as LC3-associated phagocytosis. Therefore, this subject deserves future exploration. This is the first study providing evidence that SERCA is inhibited and involved in autophagy arrest in IAV-infected cells.

In various cell lines, autophagy is initiated under stress conditions such as endoplasmic reticulum stress, nutrient deprivation, mitochondrial damage, and inflammation (40, 41). Autophagy can act directly to control inflammation by degrading ubiquitinated inflammasomes or indirectly by lowering endogenous sources that induce inflammasome formation, such as mitochondrial ROS (16, 17, 39, 43). Since activation of SERCA contributed to restoring autophagic flux in IAV infection, we supposed that SERCA served as a modulator of inflammatory response. Our data indicated that

activation of SERCA with CDN1163 inhibited excessive expression of IFN- α , IFN- β , IL-8, and CCL-5 in IAV-infected cells. On the contrary, we have found that inhibition of SERCA enhanced the inflammatory response, since treatment with TG or transfection with siATP2A2 increased the expression of these cytokines. A similar trend was observed in ELISAs. These data confirm that SERCA does indeed act as a modulator of the cellular inflammatory response, and IAV may induce inflammatory cytokines and chemokines by inhibiting SERCA activity.

The host immune response plays an important role in the development of clinical disease. Previous studies showed that an excessive host immune response increases the severity of the disease, and that this disease exacerbation is related to a cytokine storm (44, 45). The cytokine storm has been reported as the major cause of severe influenza and is related to the overactivation of the MAPK family members (46, 47). Expression of inflammatory cytokines and chemokines, such as IL-6 or CCL5, is induced by the MAPK pathway during pathogen infection (48–51). The three major members of the MAPK family, p44/42 MAPK (Erk1/2), JUN N-terminal kinase (JNK), and p38 kinase, are activated during IAV infection. We found that the expression levels of p-p38 MAPK and p-JNK, but not p-ERK1/2, were negatively regulated by SERCA activity. This is consistent with reports that showed that p38 and JNK, but not ERK, were linked to the expression of cytokines and chemokines, while ERK affected nuclear export of viral ribonucleoprotein complexes (RNPs) (47, 52–54). Interestingly, several studies indicate that triggering the production of proinflammatory mediators by mitochondrial ROS signaling molecules is associated with the activation of the MAPK signal pathway (55, 56). Taken together, we propose that an interaction between SERCA, mitochondrial ROS, and the MAPK pathway plays an important role in regulating the expression of inflammatory cytokines and chemokines in IAV-infected cells.

Autophagy is a cytoprotective process responsible for the degradation and recycling of dysfunctional and damaged organelles or protein aggregates in eukaryotic cells (57). Additionally, it is induced to protect against different types of mitochondrial stress (18, 58, 59), such as ROS (60, 61). IAV blocked the autophagic flux such that the damaged mitochondria and the resulting damage to cells could not be controlled in time. The reduced autophagic capacity to eliminate damaged and dysfunctional proteins and organelles contributed to the increased level of mitochondrial superoxide and decreased MMP in these cells. This model is supported by our data showing that IAV-induced mitochondrial superoxide levels were reduced when AMD limited the AMD-sensitive IAV-induced autophagic flux arrest, and the level of mitochondrial superoxide was increased after autophagic flux of the cells was blocked by CQ (Fig. 9). Therefore, the restoration of autophagic flux by activating SERCA using CDN1163 may be one of the mechanisms by which SERCA reduces mitochondrial dysfunction in the IAV-infected cells. In addition, an enhanced cytosolic calcium transience leads to increased mitochondrial calcium uptake (62), and this elevated cytosolic calcium promotes mitochondrial ROS production in various muscle diseases (63). SERCA is the main Ca²⁺ pump that transports Ca²⁺ ions from the cytoplasm to the sarco/endoplasmic reticulum lumen. Restoration of SERCA activity has been shown to reduce mitochondrial dysfunction in mice with muscular dystrophy (64). Hence, another reason why SERCA activity affects mitochondria of the IAV-infected cells may be due to its effects on calcium ions.

It is well known that the mitochondria function to produce ATP, regulate cell apoptosis, and generate ROS (65). Mitochondria modulate innate immunity via redox-sensitive inflammatory pathways or direct activation of the inflammasome, since mitochondria produce excess O₂⁻ ions when they are damaged or stressed (66). It has also been reported that a mitochondrion-mediated antiviral innate immune response can be induced through the MAVS-RIG-I pathway (35, 36). We have demonstrated that recovery of SERCA activity, with CDN1163, decreased generation of mitochondrial superoxides and MMP induced by IAV infection. Restoration of mitochondria function by CDN1163 could be a mechanism to counter the inflammatory response induced by IAV.

In conclusion, we have defined a key role played by SERCA in autophagy arrest, inflammatory response, and mitochondrial dysfunction in IAV infection. IAV infection inhibited SERCA activity and, thus, caused autophagy arrest and mitochondrial dysfunction. These states provided a potential platform for activation of the inflammatory response. We validate for the first time that the pharmacological activation of SERCA is a powerful intervention strategy to prevent autophagy arrest, inflammatory response, and mitochondrial dysfunction in IAV-infected cells. Modulation of SERCA activity may be a therapeutic strategy for management of clinical symptoms in severe influenza disease.

MATERIALS AND METHODS

Antibodies, reagents, plasmids, and short interfering RNA (siRNA) oligonucleotides. The primary antibodies against LC3B (2775), p-p38 MAPK (4511), p38 MAPK (8690), p-SAPK/JNK (4668), SAPK/JNK (9252), and SQSTM1 (5114) were purchased from Cell Signaling Technology, and the nucleoprotein (NP) antibody (10780-01) was purchased from SouthernBiotech. ACTB (AA128; Beyotime), SERCA (A1097; ABClonal), and horseradish peroxidase (HRP) AffiniPure goat anti-rabbit IgG (H+L) (E030120-01) and anti-mouse (E030110-01) secondary antibodies were purchased from EarthOx.

The following reagents were used in this research: CDN1163 (HY-101455; MedChemExpress), thapsigargin (B6614; APEXBio), amantadine (A1260; Sigma), and chloroquine (C6628; Sigma). The plasmid for mammalian expression of mRFP-EGFP-LC3B (21) (21074; deposited by the Tamotsu Yoshimori laboratory) was obtained from Addgene. siRNA oligonucleotides (5'-GGTGCTATTACTACTTA-3') were synthesized from Synbio Technologies and used for the *ATP2A2* RNA interference (RNAi) experiments. siNC (5'-TTCTCCGAACGTGTCACGT-3') was used as a negative control.

Cell lines and virus. The human lung cancer cell lines NCI-H1395 (abbreviated as H1395) and CRL-5868 (ATCC) were cultured in RPMI 1640 medium (C11875500BT; Gibco) containing 10% fetal bovine serum (10270-106; Gibco) at 37°C and 5% CO₂. 293H cells (used in previous studies and preserved in our laboratory) were cultured in Dulbecco's modified Eagle medium (DMEM; C11995500BT; Gibco) containing 10% fetal bovine serum at 37°C and 5% CO₂.

The viral strains resistant to AMD and PR8 (A/Puerto Rico/8/1934) or sensitive to AMD and PPH2 (the 31st amino acid encoded by the gene encoding the M2 protein of PR8 was changed from asparagine to serine) have been used in previous studies and preserved in our laboratory.

Viral infection and cell treatment. H1395 cell lines were grown to approximately 80% confluence and were then inoculated with PR8 and PPH2 in RPMI 1640 medium. One hour later, the virus solution was removed and replaced by fresh medium complemented with 0.3% bovine serum albumin (BSA), 1 µg/ml TPCK-trypsin, 100 U/ml penicillin, and 100 mg/ml streptomycin, and the cells were incubated at 37°C and 5% CO₂ for 24 h. In the experiment to inhibit M2 ion channel activity, the virus medium was replaced by the medium described above supplemented with 20 µM AMD.

TG is an inhibitor of SERCA, and CDN1163 is an activating drug of SERCA. One hundred nanomolar TG and 10 µM CDN1163 were found to be the optimal concentrations in H1395 cells; cell viability was not affected by this concentration. H1395 cells were pretreated with TG for 4 h or CDN1163 for 24 h, followed by infection with PR8. At the same time, a corresponding level of dimethyl sulfoxide (DMSO; 196055), used to dissolve the drug, served as a negative control and was applied to cells that were not inoculated with any virus.

Cell transfection. After 293H cells had grown to 80% confluence, cells were transfected with plasmids using the Lipofectamine 2000 transfection reagent (11668019; Thermo Fisher Scientific). Briefly, 1 µg of plasmid DNA and 5 µl of Lipofectamine 2000 were dissolved in 50 µl of Opti-MEM (2053197; Gibco) each, and then the two solutions were mixed for 5 min. The total volume was transferred to the cells and incubated for 8 h at 37°C. Subsequently, the transfection medium was replaced with 1 ml of fresh DMEM containing 10% FBS, and the cells were cultured at 37°C for 48 h.

In the RNAi experiment, 100 nM siRNA and 2 µl of Lipofectamine 2000 were dissolved in 50 µl of Opti-MEM each, and then the two solutions were mixed for 20 min. The resulting mixture was transferred to H1395 cells and incubated for 6 h at 37°C. Subsequently, the transfection medium was replaced with 500 µl of fresh RPMI 1640 containing 10% FBS, and the cells were cultured at 37°C for 24 h.

Western blotting. Cell samples were washed with cold phosphate-buffered saline (PBS) and lysed on ice for 30 min. Cell lysates were collected and centrifuged at 12,000 rpm for 10 min at 4°C. The total protein concentration in each sample was measured by the bicinchoninic acid (BCA) method (23225; Thermo Scientific) and the samples normalized by protein content before they were boiled for 5 min in 6× SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE and electrotransferred onto the Hybond-polyvinylidene difluoride membranes (IPVH00010; Millipore). They were then blocked with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline with 0.05% (wt/vol) Tween 20 (V900548; Sigma) before incubation with specific primary antibodies overnight at 4°C and exposed to the corresponding secondary antibodies conjugated to horseradish peroxidase for 2 h at 37°C. The specific protein bands bound to the antibodies were detected with the Clarity Western ECL substrate (170-5060; Bio-Rad).

Confocal fluorescence microscopy. To monitor autophagic flux, H1395 cells were infected with IAV after being transfected with the mRFP-EGFP-LC3B plasmid after 24 h. At 24 hpi, cells were fixed and incubated with NP antibody and the corresponding secondary fluorescent antibodies, which can be excited by light at 405 nm. The autophagic flux was measured by determining the fluorescence intensity when excited by light at 488 nm and 594 nm. The fluorescence was visualized with an LSM880 laser

system using an LSM 7DUO confocal fluorescence microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany).

Flow cytometry. To determine the mitochondrial membrane potential of H1395 cells, we used mitochondrion-specific labels that distinguish respiring (M22426; MitoTracker deep red; Thermo Fisher Scientific) and total (M7514; MitoTracker green; Thermo Fisher Scientific) mitochondria (39).

To measure mitochondrial superoxide, cells were incubated with 10 μ M MitoSOX red mitochondrial superoxide indicator for 10 min and the cells harvested by centrifugation for analysis by flow cytometry. In each sample, 30,000 events were measured and used for further analysis.

Real-time qPCR. Total RNA was extracted from the H1395 cells using a RaPure total RNA kit (R4011-02; Magen) by following the manufacturer's protocol. Total RNA was then reverse transcribed into cDNA using HiScript III RT SuperMix for quantitative PCR (qPCR; R323-01; Vazyme) according to the manufacturer's instructions. Reverse transcribed RNA was then successively analyzed by a 7500 Fast real-time PCR system (Applied Biosystems, Grand Island, NY) using the ChamQ universal SYBR qPCR master mix (Q711-02; Vazyme). The amplification protocol comprised 1 cycle at 95°C or 30 s followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The relative expression level of the target mRNA was normalized to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and the differences were calculated with the traditional $2^{-\Delta\Delta CT}$ method.

SERCA activity measurement. SERCA activity in cells infected with IAV or the mock control were determined using a Ca^{2+} -ATPase activity assay kit by following the manufacturer's instructions (A070-4; Nanjing Jiancheng Bioengineering Institute). The protein concentration in each sample was measured by the BCA method (23225; Thermo Scientific) and the samples normalized by protein content before detection of SERCA activity.

ELISA. The cell culture supernatant was collected after cells were pretreated with CDN1163, TG, or a mock control and then left uninfected or infected with PR8 for 24 h. The quantity of human IFN- α and IL-8 in the supernatant of the culture medium was measured using an IFN- α and IL-8 ELISA kit according to the manufacturer's instructions. Standard concentrations of the cytokines were used to produce standard curves. Absorbances were read at 450 nm using a microplate spectrophotometer (BioTek), and the concentrations of IFN- α and IL-8 were calculated according to the standard curves.

Cell viability assay. Cell viability was evaluated using cell counting kit-8 (CK04; Dojindo) 24 h after H1395 cell monolayers were exposed to different concentrations of TG, CDN1163, and AMD according to the manufacturer's instructions and compared with negative controls. The optical density at 450 nm was used as a measure indicating cell viability and was determined using a microplate spectrophotometer (BioTek).

Statistical analysis. Data in this study are expressed as the means $\pm$ standard deviations (SD), and differences were measured using a one-way analysis of variance (ANOVA). A *P* value of <0.05 was considered statistically significant. For all analyses, the notations used to indicate significance between groups are the following: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; #, *P* > 0.05.

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We have no conflict of interest to declare.

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