Activation of AMPK restricts coxsackievirus B3 replication by inhibiting lipid accumulation

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ABSTRACT

Coxsackievirus B3 (CVB3) is the major pathogen of human viral myocarditis. CVB3 has been found to manipulate and modify the cellular lipid metabolism for viral replication. The cellular AMP-activated protein kinase (AMPK) is a key regulator of multiple metabolic pathways, including lipid metabolism. Here we explore the potential roles AMPK plays in CVB3 infection. We found that AMPK is activated by the viral replication during CVB3 infection in Hela cells and primary myocardial cells. RNA interference mediated inhibition of AMPK could increase the CVB3 replication in cells, indicating that AMPK contributed to restricting the viral replication. Next, we showed that CVB3 replication could be inhibited by several different pharmacological AMPK activators including metformin, A769662 and AICAR. And the constitutively active AMPK mutant (CA-AMPK) could also inhibit the CVB3 replication. Furthermore, we found that CVB3 infection increased the cellular lipid levels and showed that the AMPK agonist AICAR both restricted CVB3 replication and reduced lipid accumulation through inhibiting the lipid synthesis associated gene expression. We further found that CVB3 infection would also induce AMPK activated in vivo. The AMPK agonist metformin, which has been widely used in diabetes therapy, could decrease the viral replication and further protect the mice from myocardial histological and functional changes in CVB3 induced myocarditis, and improve the survival rate of infected mice. Lastly, it was demonstrated that the AICAR-mediated restriction of viral replication could be rescued partially by exogenous palmitate, the first product of fatty acid biosynthesis, demonstrating that AMPK activation restricted CVB3 infection through its inhibition of lipid synthesis. Taken together, these data in the present study suggest a model in which AMPK is activated by CVB3 infection and restricts viral replication by inhibiting the cellular lipid accumulation, and inform a potential novel therapeutic strategy for CVB3-associated diseases.

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1. Introduction

Coxsackieviruses are small, non-enveloped, single-strand positive RNA viruses which belong to the Enterovirus genus of the Picornaviridae family. There are two serogroups, A and B, with 23 known Coxsackie A serotypes causing mainly enteric diseases, and six known Coxsackie B serotypes (CVB1–CVB6) associated with severe ailments such as myocarditis, pancreatitis and aseptic meningitis [1]. Within the group B coxsackieviruses (CVB), CVB3 has been consistently reported as the predominant pathogen of human viral myocarditis, which would further progress to dilated cardiomyopathy and heart failure in infants, children and young adults [2]. The CVB3-induced viral myocarditis may be caused by direct cytopathic effects of the viruses, a pathologic immune response to chronic virus infection, or abnormal autoimmune triggered by the viruses [3]. That means CVB3 interacts with its host at multiple levels and stages during the disease development. However, the underlying interactions and mechanisms have not been fully elucidated. And current treatment of patients with CVB3-caused myocarditis is almost entirely supportive [4,5], and effective vaccines are still not available [6,7].

Upon infection, viruses create a cellular microenvironment conducive to viral genome replication, protein synthesis, and assembly of new virus particles. Numerous RNA viruses, including enteroviruses, manipulate host membranes and cellular lipid to create new organelles on which they assemble replication complexes and synthesize viral RNA [8–10]. It has been demonstrated that CVB3 infection induces the formation of abundant small autophagy-like double-membrane vesicles in host cells and the autophagosome could enhance the efficiency of viral replication [11,12]. Recently some other studies defined a requirement of cholesterol and phospholipids, which are critical component of cellular membranes,
for the formation of autophagosome and efficient replication in positive-strand RNA viruses [13,14]. In addition to lipid synthesis, some RNA viruses reprogram cellular lipid signaling for viral replication. For example, hepatatitis C virus (HCV) and enteroviruses stimulate phosphatidylinositol (PI)-4 kinase and fatty acid synthase to establish sites of viral replication [15,16]. These findings indicate that the cellular lipid accumulation plays important roles in virus infection and probably provides a novel target for antiviral therapy.

AMP-activated protein kinase (AMPK) is a master regulator of multiple cellular metabolic pathways, including lipid metabolism. It is a heterotrimer serine/threonine kinase consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). A well known function of AMPK is to act as a “fuel gauge” that monitors cellular energetic stress. During nutrient deprivation and viral infection, AMPK is activated by an elevated intracellular AMP/ATP ratio and/or some upstream kinases, leading to increased phosphorylation of the threonine 172 within the α subunit [17,18]. When activated, AMPK contributes to inhibiting anaerobic pathways, such as fatty acid and sterol synthesis, and promotes catabolism such as fatty acid oxidation for ATP production. To achieve these effects, AMPK targets numbers of downstream pathways which are involved in lipid metabolism. For example, AMPK directly phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR), which are the rate-limiting enzymes in the synthesis of fatty acids and sterols respectively.

In recent years, several viruses have been shown to either stimulate or inhibit AMPK activity, and furthermore AMPK has been found to promote or restrict the viral replication [19–22]. These findings suggest that AMPK probably plays some important roles in the host–virus interactions, but the interplays are complicated and unique in diverse viruses. Despite its critical functions in the cellular energy homeostasis and lipid metabolic regulation, there has been a paucity of studies investigating the potential interplay between AMPK and CVB3 infection.

Here we show that AMPK activity is increased during CVB3 infection. Additionally, we find that pharmacological and constitutively active mutant induced activation of AMPK could inhibit CVB3 replication. Consistent with the well-known roles in lipid metabolism, AMPK activation by pharmacological agonists also leads to a reduction of CVB3-induced cellular lipid accumulation. Furthermore, we confirm that CVB3 infection also induces AMPK to activate in vivo, and find that AMPK agonist metformin could restrict the viral replication and reduce the myocardial damages in mice hearts. Lastly, it was demonstrated that the inhibition effects of AMPK agonists on CVB3 replication could be reversed partially by exogenous palmitate, the first product of fatty acid biosynthesis. Taken together, our data suggest that AMPK activation restricts CVB3 replication by reducing cellular lipid accumulation, and inform new therapeutic strategies for this important pathogen.

2. Materials and methods

2.1. Cell culture

Hela cells were used in our study since they represented well established systems for studying viral replication and infectivity [23]. Cells were obtained from American type culture collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% (vol/vol) fetal bovine serum. Primary myocardial cells were obtained from neonate rats by mechanical and enzymatic digestion of the hearts. Cells were grown in α-MEM (Hyclone) supplemented with 10% (vol/vol) fetal bovine serum and 100 U/ml penicillin/streptomycin.

2.2. Virus infection

CVB3 (strain Nancy) was purchased from Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China) and propagated in Hela cells. CVB3 was used to infect cells at a multiplicity of infection (MOI) of 3 for the present experiments. Mock-infected controls were treated with an equal volume of DMEM medium which was extracted from Hela cell culture medium under conditions identical to those used for virus propagation. Virus adsorptions were carried out for 1 h at 37 °C, after which viral inocula were aspirated and fresh DMEM medium was added. For pharmacological treatments, pretreatment with indicated compounds was performed 1 h prior to infection and fresh compounds were added following virus adsorptions.

2.3. Chemical reagents

The AMPK agonists AICAR (S1802) and A769662 (S2697) were obtained from Selleck Chemicals. Another classical AMPK activator metformin was obtained from Beyotime. The cholesterol-lowering drug atorvastatin was purchased from Melonepharma (Dalian, China).

2.4. Immunofluorescence

Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After blocking with 10% goat serum (Sigma-Aldrich), cells were incubated with mouse monoclonal antibody J2 against dsRNA (English & Scientific Consulting) at 4 °C overnight. Then cells were incubated with TRITC-conjugated secondary antibody and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired and analyzed using a Leica TCS SP5 laser confocal microscope. The percentage of cells with dsRNA positive staining was calculated by counting more than 200 cells in each experiment.

2.5. RNA interference

Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Forty-eight hours after transfection, siRNA-transfected cells were infected with either mock-control or CVB3 (MOI = 3). Cells were harvested at 12 h post-infection for further analysis. The siRNA oligonucleotides used in this study for RNA interference were listed in Table S1.

2.6. Quantitative RT-PCR

Qualitative RT-PCR was performed as previously described [24]. The primers used in this study for real-time PCR were listed in Table S2.

2.7. Western blot analysis

Western blot was performed to detect the specific protein expression levels as previously described [25]. Relative optical densities of protein bands were quantified by ImageJ software. The primary antibodies used in western blot were as follows: phospho-AMPKα-Thr172 (#2535), AMPKα1 (#2603), phospho-ACC1-Ser79 (#11818), ACC1 (#3662), and fatty acid synthase (#3180), purchased from Cell Signaling; and β-actin (sc-69879, Santa Cruz) and GAPDH (60004-1, Proteintech). The antibody against CVB3 capsid protein VP1 has been described previously [26].

2.8. Plaque assay

Viruses or supernatants of virus-infected cell culture were plaqued on Hela cells as indicated. Confluent monolayers were treated with serial dilutions of virus for 1 h, after which the viral inocula were removed, and cells were overlayed with 0.75% agarose in DMEM, and incubated at 37 °C for 48 h. Cells were fixed in cold methanol, and stained with crystal violet. Plaque number was determined manually.

2.9. Cell viability assay

Cell viability was measured using CellTiter 96 AQueous One Solution Reagent (Promega) according to the manufacturer’s instructions.
2.10. Cellular lipid staining

Cellular lipids were stained as previously described [27]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min, stained with BODIPY 493/503 (Invitrogen, stock concentration 1 mg/ml, working solution 1:1000 dilution) for 15 min at room temperature, and counterstaining with DAPI. Images were acquired and analyzed using a Leica TCS SP5 laser confocal microscope. The fluorescence intensity of lipid per cell was quantified in more than 200 cells from each experiment by LAS AF software.

2.11. Cellular fatty acid and cholesterol quantification

Free fatty acid and cholesterol in cells were quantified using commercial kits (Wako) as described previously [28]. The amounts of the free fatty acid and cholesterol were normalized with the total protein concentration of the cell lysates.

2.12. Mice infection and drug treatment

Animal studies were performed with male BALB/c mice (4–6 weeks old, 18–21 g), which were obtained from the animal center of Third Military Medical University (Chongqing, China). The mice were infected by intraperitoneal injection of 100 μl phosphate buffered saline (PBS) containing 3 × 10^3 plaque forming units of CVB3. Treatments (eight mice per group) were started at the next day of viral infection. Metformin (50 mg/kg/day, 0.5 ml/mouse) was administered daily by oral gavage and an equivalent volume of sterile water was used as vehicle control. After 5 days, the mice were sacrificed and the hearts were harvested for further analysis.

Male db/db mice and wild-type C57BL/6 mice (4–6 weeks old, ten mice per group) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China), infected with CVB3 as above, and analyzed on survival rate and viral replication.

For long-term analysis on myocardial functions and survival rate of infected mice, the mice were infected with less virus (3 × 10^4 pfu of CVB3) and treated with metformin as above every two days.

All the animal studies were approved by the Animal Care and Use Committee of Xinjiao Hospital, Third Military Medical University and complied with the Declaration of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.13. Histological analysis

For immunohistochemical staining, mice heart samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 3 μm sections. The tissue sections were stained with hematoxylin–eosin (H&E) to visualize the myocardial necrosis and inflammation conditions. And the tissue sections were further stained with specific antibodies against CD45, CD68 and CD3 (Santa Cruz) for analysis of the inflammatory infiltration. The tissue sections were analyzed for proliferation levels of AMPK by fluorescence microscopy, and further reduced the enzymatic activity of downstream target ACC1.

2.14. Myocardial zymogram evaluation

The serum contents including aspartate aminotransferase (AST), creatine kinase (CK), creatine kinase-MB fraction (CK-MB), lactate dehydrogenase (LDH) and α-hydroxybutyrate-dehydrogenase (α-HBDH) were measured by automatic biochemistry analyzer Hitachi 7170S.

2.15. Cardiac hemodynamic analysis

The cardiac hemodynamics were measured as described previously [29]. Briefly, all mice underwent echocardiographic measurements before sacrifice on day 21 post-infection. Transthoracic echocardiography was performed with a Vevo 2010 high-resolution ultrasound system (VisualSonics) equipped with a 30-MHz, 100-frame-per-second micro-visualization scan head. Ejection fraction (EF) and left ventricular (LV) mass were obtained.

2.16. Fatty acid synthesis rescue assay

Exogenous palmitate addition was performed as previously described [19]. Palmitate (P5585, Sigma-Aldrich) was conjugated to fatty acid-free bovine serum albumin (BSA) which is needed to increase palmitate solubility [30]. For fatty acid synthesis rescue assay, cells were pretreated with palmitate–BSA overnight prior to pharmacological treatments and CVB3 infections. And fatty acid-free BSA was used as control.

2.17. Statistical analysis

Data analysis was performed using SPSS 16.0 (SPSS Inc.). Statistical analysis was performed by Student's t-test or ANOVA followed by the Newman–Keuls test. Survival curves were estimated using the Kaplan–Meier method and compared with the logrank statistics. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. AMPK is activated upon CVB3 infection

In the present study, we initially analyzed the viral RNA replication of the caspid VP1 at various time points following CVB3 infection in Hela cells. Hela cells were infected with CVB3 (MOI of 3) or mock control. Cell lysates were collected at 2, 4, 8, 12 and 24 h post-CVB3 infection (p.i.) for RNA and protein analysis. At 8 h post-infection, VP1 RNA replication could be detected significantly by real-time PCR (Fig. 1A), and continued to increase over time from 8 h to 24 h. Consistent with the viral RNA replication, we observed an obvious increase of VP1 protein expression in a time-dependent manner from 8 h post-CVB3 infection (Fig. 1C).

AMPK is activated through phosphorylation of the catalytic alpha subunit at threonine-172, and active AMPK could phosphorylate numbers of downstream targets, including acetyl-CoA carboxylase (ACC). It has been shown that phosphorylation of ACC1 at Ser79 by AMPK decreases ACC activity, inhibiting fatty acid synthesis [31]. To determine whether AMPK activity might be responsible for the metabolic process during CVB3 infection, we assessed AMPK activity in mock and CVB3-infected cells by western blot analysis. Compared with no obvious changes in phosphorylation levels of AMPK in mock infected cells (Figs. 1B and D), CVB3 infection in Hela cells induced a significant increase in AMPK Thr172 phosphorylation in a time-dependent manner (Figs. 1C and D). AMPK activation was also observed in CVB3 infected primary myocardial cells (Fig. S1A). Subsequently, we analyzed the levels of Ser79 phosphorylated ACC1 and total ACC1 during CVB3 infection. At 12 h and 24 h post-infection, CVB3 infected cell lysates contained more Ser79 phosphorylated ACC1 than mock-infected lysates (Fig. 1C). These results indicated that CVB3 increased AMPK activity upon infection, and further reduced the enzymatic activity of downstream target ACC1.

To investigate the mechanism underlying the activation of AMPK, CVB3 was exposed to UV radiation for 10 min for a total dose of 15 J/cm² as previously described [32]. UV-irradiated CVB3 is capable of virus receptor attachment and entry into the host cell, but not replication. Hela cells were infected with either wild-type CVB3 or UV-inactivated CVB3 (MOI of 3) for 1 h. Cell lysates were collected at 12 h post-infection and subjected to western blot analysis to detect the phosphorylation levels of AMPK. As shown in Fig. 1D, UV-inactivated CVB3 failed to induce AMPK phosphorylation in Hela cells, consistent with
the results in primary myocardial cells (Fig. S1). These results suggested that AMPK activation was due to post-entry viral replication in host cells.

3.2. AMPK contributes to restricting CVB3 replication

In order to test the role of AMPK activity in CVB3 infection, we employed AMPK-specific RNAi to decrease AMPK expression during infection. Three different siRNA oligonucleotides specific to different sites of AMPK mRNA (1815, 2101 and 4008, shown in Table S1 in the Supplementary material) were transfected to Hela cells separately and the AMPK knock-down effects were validated by western blot, showing a reduction of 40%–60% in AMPK expression (Figs. S2A and B). To avoid the off-target effects of single siRNA specific to one site, a mixture containing these three different siRNA oligonucleotides was used in the present experiments. Transfection of siRNAs specific for AMPK (si-AMPK) resulted in an ~60% reduction in AMPK abundance in comparison to control siRNA (si-NC) transfected cells (Figs. 2A and B).

Next we challenged either the siRNA-AMPK or negative-control transfected Hela cells with CVB3 and measured viral replication by different assays. First, we analyzed the amount of VP1 in infected cells to measure the CVB3 replication. As shown in Figs. 2A and B, we found an increase in both VP1 protein expression and viral VP1 RNA production in AMPK-specific siRNA treated cells compared with control cells. Moreover, CVB3 replication was also shown to be increased in AMPK-lacking cells (si-AMPK) by an immunofluorescence assay that detected production of double-stranded RNA (dsRNA) (Fig. 2C, infection percentage quantified in Fig. 2D). To test whether the different percentages of CVB3 infection were because of the reduced growth ability of si-AMPK cells, we tested the effects of AMPK-siRNA on cell growth and found that there was no obvious difference in the growth abilities between si-NC and si-AMPK cells (Fig. S2C). These results indicated that CVB3 was able to replicate more efficiently in the absence of AMPK.

Furthermore, the role of AMPK in CVB3 spread was measured by plaque assay. An ~30% increase in the number of plaques was observed in AMPK-siRNA treated cells in comparison to negative-control siRNA treated cells (Fig. 2E, plaque number quantified in Fig. 2F). Combined with the roles of AMPK both in viral replication detected by immunofluorescence assay and in spread as measured by plaque assay, it was suggested that AMPK contributed to restricting CVB3 replication, and cells lacking AMPK were likely more sensitive to CVB3 infection and produced more virions in cells.

3.3. Activation of AMPK restricts CVB3 replication

Since AMPK deficiency increased CVB3 replication, we hypothesized that AMPK activation would inhibit virus replication and spread. Therefore, we tested whether CVB3 infection was restricted by pharmacological treatments that activate AMPK.

We took advantage of three AMPK agonists in the present experiments. Metformin is a classical AMPK agonist, which is usually used for clinical treatment of type II diabetes [33]. And the 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has been well characterized to activate AMPK through which cells convert to an AMP analog, ZMP [34]. The third is a recently developed thienopyridone compound A769662 that activates AMPK directly, independently of the energy status of the cells [35].

RNA. The level of VP1 at 2 h p.i. was set as 1. Data show mean values ± SD from three independent experiments. (B) Western blot was performed to detect the phosphorylation levels of AMPK in mock infected cells. (C) Western blot was performed to detect the phosphorylation levels of AMPK and ACC in CVB3 infected cells at indicated time points. CVB3-VP1 was also examined. (D) The ratio of p-AMPK/AMPK in mock and CVB3 infected cells. * indicates p < 0.05 compared to CVB3-0 h. (E) Hela cells were infected with mock-control, CVB3 or UV-irradiated CVB3 (MOI = 3). At 12 h p.i., cell lysates were collected and western blot was performed to detect the phosphorylation levels of AMPK and VP1. β-Actin was used as a loading control.

Fig. 1. AMPK is activated upon CVB3 infection. (A) Hela cells were infected with either mock-control or CVB3 (MOI = 3) for 1 h. Cell lysates were collected at the indicated times post-infection (p.i.) and real-time PCR was performed to quantify the viral VP1
To investigate the roles of AMPK activation in CVB3 infection, Hela cells were pretreated with metformin (10 mM), AICAR (1 mM) and A769662 (100 μM) 1 h prior to CVB3 infection, and DMSO was used as vehicle control. At 12 h post-virus infection, cell lysates were collected for protein and RNA analyses. As shown in Fig. 3A, AMPK was activated upon CVB3 infection, which was consistent with the previous results (Fig. 1B). All the three compounds could increase the phosphorylation level of AMPK upon CVB3 infection. And treatment of CVB3-infected cells with the three compounds dramatically reduced the CVB3 capsid protein VP1 levels, most effectively in AICAR-treated cells (Fig. 3A). Moreover, the viral RNA analysis revealed that the three compounds induced a 50%–65% decrease in viral VP1 RNA production (Fig. 3B). And the similar inhibition effects of these compounds on CVB3 replication could be observed in primary myocardial cells (Fig. S3). Compared to metformin and A769662, AICAR reduced CVB3 replication more significantly, which displayed a 72% decrease in VP1 protein and a 65% reduction in viral VP1 RNA. Further we found that AICAR inhibits CVB3 replication in a dose-dependent manner, both in VP1 protein expression and viral RNA production (Figs. 3C and D). Moreover, none of these compound treatments has any toxic effects on cell viability at the concentrations used in the present experiments (Fig. 3G).

The inhibition of CVB3 replication by AICAR, indicated by the viral protein and RNA analysis (Figs. 3C and D), as well as the immunofluorescence assay detecting viral dsRNA (Fig. 5A), would result in decreased production of infectious virus in AICAR-treated cells. We measured the amount of infectious virus produced in cell culture with plaque assay. Medium from infected cells was collected at 12 h after infection, and virus was titrated on Hela cells. In mock infected cells’ medium, no infectious virus was detected in plaque assay (data not shown). A large number of plaques were found in CVB3-infected cell group which was treated with DMSO as drug vehicle, indicating that infectious virions were released from the infected cells. Furthermore, an ~40% reduction in the number of plaques was observed in AICAR-treated cells in comparison to DMSO treated cells (Fig. 3E, plaque number quantified in Fig. 3F), which suggested that AICAR inhibits CVB3 virus production and spread. All the above results demonstrated that pharmacological
activation of AMPK could restrict CVB3 replication. Next we further investigated the effects of a constitutively active AMPK mutant (T172D, HA-CA-AMPK) on virus replication. As shown in Figs. 3H and I, the expression of CA-AMPK could inhibit the CVB3 replication, indicating that CA-AMPK mutant has the same anti-viral effects as the AMPK agonists. These results demonstrated that activation of AMPK could restrict CVB3 replication.
3.4. AMPK agonist inhibits CVB3 replication through AMPK and at the early stage of replication

To determine whether the effects of AICAR were specific for AMPK, we treated AMPK-RNAi cells with the AMPK activator AICAR. Treatment with this compound inhibited viral RNA production slightly in AMPK-RNAi cells and was not significant, whereas infection was inhibited greater than 50% by AICAR in the control cells (Fig. 4A), consistent with the viral VP1 protein levels in western blot analysis (Fig. 4B). AICAR was also found to inhibit production of dsRNA in viral replication in siRNA-NC transfected cells more significantly than in AMPK-RNAi cells (Fig. 5A, infection percentage quantified in Fig. 5B). These results indicated that the major action of this agonist was through activating AMPK.

We were interested in determining what stage of the viral infectious cycle was inhibited by AMPK agonist. To address this issue, we treated cells with AICAR at various time points during virus infection and analyzed viral VP1 protein production. Treatment of AICAR for 1 h prior to CVB3 infection (−2/−1 in Fig. 4C) or with adsorption (−1/0 in Fig. 4C) had no impact on viral VP1 production (Fig. 4C), suggesting that AICAR could not disturb the virus adsorption and entry. Addition of AMPK at the early stage post-infection resulted in a significant reduction in VP1 production, especially at 0 and 0.5 hpi (Fig. 4C). However, addition of AICAR at 8 and 12 h post-infection had a negligible impact on VP1 production. Taken together these data suggested that AMPK agonist AMPK restricted CVB3 at early stage of replication at a post-entry step. This inhibition in viral protein production probably led to a reduction in release of infectious virus and spread observed at later stage of infection (Fig. 3E).

3.5. Activated AMPK restricts CVB3 replication and reduces lipid accumulation in cells

To explore the mechanism by which activated AMPK restricts CVB3 replication, we took advantage of the AMPK agonists to inhibit CVB3 infection. The inhibition of CVB3 replication by AICAR was further validated by an immunofluorescence assay that detects production of dsRNA during viral replication (Fig. 5A, infection percentage quantified in Fig. 5B).

As AMPK is the key regulator in the lipid metabolism, we next investigated whether AMPK agonists could regulate the lipid accumulation which was essential to viral replication upon CVB3 infection. To analyze the lipid accumulation in cells, we visualized cellular lipid content using the lipophilic BODIPY fluorescent probes and quantified the fluorescence intensity of lipids. This analysis revealed that CVB3-infected cells displayed higher levels of BODIPY fluorescence than mock-treated cells (Fig. 5A, quantified in Fig. 5C), along with the significant increase of viral dsRNA production, indicating that CVB3 infection probably induced cellular lipid accumulation. Furthermore we found that treatment with AICAR in CVB3-infected cells led to a dramatic decrease in BODIPY staining compared to DMSO-treated cells (Fig. 5A, quantified in Fig. 5C). Meanwhile, decreased viral dsRNA production was observed in AICAR-treated CVB3-infected cells (Fig. 5A, quantified in Fig. 5B). These findings suggested that AMPK agonist AICAR restricted CVB3 replication and meanwhile reduced the cellular lipid levels.

AMPK regulates several downstream pathways which are important for virus infection such as lipid synthesis [36]. To determine the effects of AMPK agonists on the lipid synthesis during CVB3 infection, we

![Fig. 4.](image-url) AMPK agonist inhibits CVB3 replication through AMPK and at the early stage of replication. (A) siRNA-AMPK and siRNA-NC transfected cells were treated with AICAR (1 mM), and then infected with CVB3 (MOI = 3) as described above. Real-time PCR was performed to quantify the viral VP1 RNA. Data show mean values ± SD from three independent experiments. (B) Western blot was performed to detect the phosphorylation levels of AMPK and VP1 in cells described in A. (C) Western blot analysis of CVB3-infected cells treated with 1 mM AICAR at different times as indicated. −2/−1 indicates AICAR treatment for 1 h prior to CVB3 infection. −1/0 indicates that AICAR was added for 1 h with CVB3 adsorption. In other group, AICAR was added either 1 h prior to infection (−1), with infection (0), or 0.5, 1, 2, 4, 8, or 12 h post-infection. After 14 h post-infection, cell lysates were collected. In western blot assay, β-actin was used as a loading control. * indicates p < 0.05. n.s. indicates no significance.
measured the amount of lipid accumulation in cells upon CVB3 infection and treatment of AMPK agonists. As shown in Fig. 5D, CVB3 infection induced fatty acid accumulation nearly 2-fold compared to mock-infected cells. Treatments with AICAR, A769662 and metformin almost completely reversed this increase in CVB3 infected cells, most effectively in AICAR-treated cells (Fig. 5D). And these agonists had similar impacts on the fatty acid accumulation in mock-infected cells, among which AICAR reduced the fatty acid amount most significantly (Fig. 5D).

In previous study, cholesterol was found to regulate viral polyprotein processing and facilitate enteroviral RNA synthesis, indicating that cholesterol was a critical determinant in enteroviral replication [14]. As shown in Fig. 5E, the cellular total cholesterol was increased...
greater than 2-fold in CVB3-infected cells compared to mock-infected cells, which suggested that cholesterol was essential for virus replication as previously reported [14]. Treatment with the three agonists led to significant decrease in cellular cholesterol accumulation in CVB3-infected cells compared with DMSO-treated cells (Fig. 5E), consistent with the previous finding that AICAR reduced the lipid droplets in CVB3-infected cells (Fig. 5A). Combined with the previous finding of inhibition of CVB3 replication by AICAR, A769662 and metformin (Figs. 3A and B), all these results suggested that lipid accumulation, such as fatty acid and cholesterol, probably plays some essential roles in viral replication, and inhibition of the cellular lipid accumulation by AMPK agonists might contribute to restricting CVB3 replication.

Furthermore, the molecular mechanisms by which AMPK agonists restrict CVB3 replication were investigated. To this end, we analyzed the expression changes of some genes related to lipid metabolism in mock and CVB3-infected cells. Fatty acid synthase (Fasn) is the sole lipogenic enzyme in the human genome capable of the reductive de novo synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH [37]. ACC is the rate-limiting enzyme in de novo fatty acid synthesis which is the downstream target of AMPK. As another important downstream target of AMPK, HMGCR is the rate-limiting enzyme in the synthesis of isoprenoids and steroids, including cholesterol. SREBP1 (sterol regulatory element binding protein 1) is a transcription factor of the helix–loop–helix leucine zipper family strongly involved in the control of sterol biogenesis. We observed upregulations of these genes in CVB3-infected cells compared to mock-infections and decreases of gene levels with treatment of AICAR in both mock and CVB3-infected cells (Fig. 5F). Furthermore, Fasn protein expression was found to be upregulated in CVB3-infected cells and reduced by AICAR (Fig. 5A), which was in accordance with previous reports [38]. However, the increase in ACC mRNA expression but not ACC protein expression was observed (Fig. 1C). In a previous study, it was shown that atorvastatin which competitively inhibits HMGCR could attenuate CVB3 induced viral myocarditis in mice [39]. So we tested the effects of atorvastatin on CVB3 replication in vitro and found that atorvastatin could reduce the viral replication (Fig. 5S). These data suggested that fatty acid and cholesterol are probably the targets in AMPK agonist-mediated viral restriction.

3.6. AMPK activator metformin inhibits CVB3 replication and ameliorates functional changes in mice heart

Metformin has been widely used in treatment of type II diabetes for many years, and it affects lipid metabolism primarily via AMPK activation [33]. As metformin has been shown to be able to inhibit the CVB3 replication in vitro (Fig. 3), we further evaluated whether metformin could restrict CVB3 replication in vivo. To this end, BALB/c mice were infected intraperitoneally with CVB3 and treated with metformin. Considering that clinical use of metformin is typically performed by oral administration, metformin was given orally in the present experiment. Upon the virus infection in vivo, AMPK activity in heart tissues was found to be increased almost 2.5-fold than in mock-infected mice (Fig. 6A, quantified in Fig. 6B), which was consistent with our previous finding in vitro (Fig. 1B). As shown in Figs. 6A and B, oral treatment of metformin activated AMPK in vivo as the results in Hela cells (Fig. 3A). Furthermore, treatment with metformin significantly reduced the viral RNA replication in mice heart tissues (Fig. 6C). Next, immunohistochemistry for viral dsRNA was performed on paraffin sections. Positive signals for viral dsRNA were observed in the myocardium of CVB3 infected mice and metformin could reduce the dsRNA staining significantly (Fig. 6D). Besides, histological examination of H&E stained sections showed the presence of multiple foci of inflammatory infiltration and necrosis in CVB3 infected mice heart, and the myocardial inflammation and necrosis were significantly reduced in metformin treated group (Fig. 6D). Next, the markers of leukocytes (CD45), macrophages (CD68) and T lymphocytes (CD3) were stained for further analysis of the inflammatory infiltration. As shown in Fig. 6E, the infiltration of inflammatory cells in myocardial tissues was obvious in CVB3 infected mice, and was decreased in metformin treated group, which was in accordance with our results of H&E staining (Fig. 6D). To further evaluate the severity of myocardial damage, the myocardial zymogram in serum including aspartate aminotransferase (AST), creatine kinase (CK), creatine kinase-MB fraction (CK-MB), lactate dehydrogenase (LDH) and α-hydroxybutyrate-dehydrogenase (α-HBDH) was measured. As shown in Table 1, the myocardial enzyme activities of AST, CK, CK-MB, LDH and α-HBDH increased significantly in CVB3 infected mice compared to mock-control. And metformin treatment could reduce these enzyme activities (p < 0.05 compared to CVB3 group), indicating that metformin attenuated the virus induced cardiac tissue damages.

Next, we investigated the effects of metformin on CVB3 induced alterations on myocardial function, fibrosis, and survival of virus infected mice. Since mice with CVB3 infection often suffer from a left ventricular dysfunction, the hemodynamic parameters were analyzed by echocardiography as the widely recognized indicators for heart function to further determine the protective effect of metformin against CVB3 induced heart dysfunction. As shown in Fig. 6F, ejection fraction (EF) was significantly decreased while left ventricular (LV) mass was significantly increased in the CVB3 group, compared with the mock group (p < 0.05). The treatments with metformin could reverse the changes induced by CVB3 infection. This results implied that metformin exerts a role in protecting mice from the CVB3 induced heart dysfunction. Furthermore, it was shown that metformin could reduce the fibrosis in CVB3 infected cardiac tissue by Masson's trichrome staining (Fig. 6G). Based on the previous findings that metformin could ameliorate functional changes in CVB3 induced myocarditis, we analyzed the survival rate in CVB3 infected mice. After virus infection for 28 days, the mice infected with CVB3 alone showed a 50% survival rate, while the CVB3 + Metformin treated mice revealed an 83.3% survival rate (Fig. 6H). All these data suggested that CVB3 infection would induce AMPK activated in vivo, and AMPK agonist metformin could restrict the virus replication, reduce the myocardial damages in mice heart, and protect mice against CVB3 infection.

3.7. Palmitate rescues AMPK-mediated restriction of CVB3

Since it was implied that AMPK activation restricts CVB3 infection partially by reducing accumulation of cellular fatty acid and cholesterol (Figs. 5D and E), we speculated that exogenous addition of fatty acids should restore CVB3 replication in AICAR-treated cells. Therefore, we tested whether we could reverse the inhibition effects of AMPK agonists on CVB3 infection by pretreating cells with palmitate, the first product of fatty acid biosynthesis.

We treated Hela cells with palmitate–BSA overnight prior to AICAR treatment and CVB3 infection, while BSA was used as control. After 12 h of infection, cells were fixed and stained for viral dsRNA to observe the CVB3 replication and measure the infection percentage by an immunofluorescence assay. In cells treated with the AMPK activator AICAR alone, we found a significant decrease in CVB3 infection compared to DMSO-treated cells (Fig. 7A, quantified in Fig. 7B), consistent with our previous findings (Fig. 5A). However, addition of palmitate prior to treatment with AICAR was able to restore CVB3 infection partially (Fig. 7A, quantified in Fig. 7B). We observed a more than 2-fold increase in CVB3 infection in cells treated with AICAR and palmitate compared to those treated with AICAR alone (Fig. 7B), while treatment with palmitate alone had little impact on virus infection (Figs. 7A and B). Moreover, the cells were also harvested for viral protein and RNA analysis. As shown in Figs. 7C and D, the viral VP1 expression and RNA replication were inhibited significantly by AICAR, consistent with the results shown in Fig. 3. Further addition of palmitate could partly reverse the reduction of VP1 expression and RNA replication by AICAR (Figs. 7C and D). Together, these data suggested that exogenous palmitate could rescue AICAR-induced inhibition of CVB3 replication partially, and validate our
hypothesis that AMPK activation restricts CVB3 infection by reducing cellular lipid accumulation.

3.8. db/db mice are more sensitive to CVB3 infection

As lipid accumulation is essential for CVB3 replication, we hypothesized that mice with more lipids were more sensitive to CVB3 infection. To assess this hypothesis, db/db mice were infected with CVB3, and analyzed for the virus replication and mice survival. db/db mice were used as an in vivo model of obese and diabetes, which have more lipid accumulation in vivo and exhibit characteristic features of Type 2 diabetic metabolic disorders such as obesity (Fig. S6A). As shown in Fig. S6B, we could detect more virus RNA in db/db mice with CVB3-induced myocarditis as detected by echocardiographic measurements (n = 5). (H) Effects of metformin on the survival of CVB3 infected mice. * indicates p < 0.05. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

4.1. AMPK activation restricts coxsackievirus B3 in vitro and in vivo

During the CVB3 infection, viruses widely interact with and modify the host cell signaling pathways to benefit their replication and dissemination. In a previous study in Hela and HepG2 cells, 53 proteins were identified to be regulated differentially during CVB3 infection [40]. A recent study built a statistical model that quantitatively predicts cardiomyocyte responses from time-dependent measurements of phosphorylation events modified by CVB3, and revealed that CVB3-stimulated cytotoxicity involves tight coupling between the host ERK and p38 MAPK pathways [41]. In addition, suppression of host cellular ERK and p38 MAPK activity with specific inhibitors decreased viral replication [16,42]. However, the host cell signaling networks and host–virus interaction upon CVB3 infection have not been completely characterized, especially in the metabolic processes.

In our present study, we focused on AMPK, the key regulator of cellular metabolism, and found that AMPK was activated upon CVB3 infection (Fig. 1). AMPK is well known to act as a sensor of energetic stress and be activated through decreased ATP/AMP and ATP/ADP ratios. A previous study has shown that cytosolic ATP/ADP ratio is lowered in CVB3-infected hearts in mice model [43], which implies that the AMPK activation by CVB3 infection probably results from the increase in energy utilization. Furthermore, we had demonstrated the inhibition effects of AMPK activity on CVB3 replication and spread with the RNAi assay (Fig. 2) and the use of AMPK agonists (Fig. 3). We further validated that the constitutively active AMPK mutant (CA-AMPK) has the similar anti-viral effects as the AMPK agonists (Fig. 3). These results suggested that AMPK activation could restrict CVB3 replication in host cells. Besides, AMPK activity has also been found to perform antiviral functions in different viruses, including rift valley fever virus (RVFV) [19], human immunodeficiency virus (HIV) [44] and hepatitis C virus (HCV) [20]. It is important to understand the delicate balance between the host and virus, for developing more specific and effective therapies.

As AMPK is the key regulator in lipid metabolism and some related genes are involved in regulating CVB3 infection [45], we focused on the lipid accumulation as the potential target of AMPK in restriction of CVB3 replication. Treatment with AMPK agonist AICAR in CVB3-infected cells led to a decrease in lipid droplets (Fig. 5A, the fluorescence intensity of lipid quantified in 5C), which was consistent with previous reports that AICAR inhibits lipogenesis [46]. Moreover, AICAR is also an AMP analogue

| Table 1 |
| Myocardial zymogram in the serum (mean ± SEM, n = 8). |

<table>
<thead>
<tr>
<th></th>
<th>AST (IU/l)</th>
<th>CK (IU/l)</th>
<th>CK-MB (IU/l)</th>
<th>LDH (IU/l)</th>
<th>α-HBDH (U/l)</th>
</tr>
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<tbody>
<tr>
<td>Mock</td>
<td>215.6 ± 15.5</td>
<td>867.8 ± 15.9</td>
<td>469.5 ± 17.6</td>
<td>878.5 ± 18.8</td>
<td>518.1 ± 29.8</td>
</tr>
<tr>
<td>CVB3</td>
<td>482.3 ± 25.0a</td>
<td>1941.4 ± 46.6a</td>
<td>1118.0 ± 34.5a</td>
<td>2945.5 ± 119.2a</td>
<td>853.4 ± 35.8a</td>
</tr>
<tr>
<td>Metformin</td>
<td>219.3 ± 11.6b</td>
<td>806.8 ± 29.3b</td>
<td>498.3 ± 22.1b</td>
<td>886.8 ± 24.4b</td>
<td>533.5 ± 35.4b</td>
</tr>
<tr>
<td>CVB3 + metformin</td>
<td>329.8 ± 25.7c</td>
<td>1058.5 ± 66.6c</td>
<td>582.1 ± 22.8c</td>
<td>1003.3 ± 47.1c</td>
<td>657.3 ± 30.9c</td>
</tr>
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a p < 0.05 compared with mock group.
b p > 0.05 compared with mock group.
c p < 0.05 compared with CVB3 group.

Fig. 7. Exogenous palmitate reverses the AMPK agonist induced restriction of CVB3 replication. (A) Hela cells were pretreated with 0.1 mM palmitate–BSA overnight and 1 mM AICAR or DMSO was added 1 h prior to CVB3 infection (MOI = 3). The fatty acid-free BSA was used as vehicle control. Cells were incubated for 12 h with indicated compound after viral infection, and cells were subjected to immunofluorescence assay (dsRNA, red; nuclei, blue). Scale bar: 100 μm. (B) Quantification of infection percentage from A. (C) Western blot was performed to detect the expression of VP1 in cells as described in A. β–Actin was used as a loading control. (D) Real-time PCR was performed to quantify the viral VP1 RNA in cells described in A. (E) Model of AMPK-mediated restriction of CVB3 replication. Data show mean values ± SD from three independent experiments. * indicates p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and may interfere with viral RNA replication similar to antiviral effects of Ribavirin. The exact underlying mechanisms by which AICAR functions and whether it has other effects on viral replication are still under investigation and probably provide a possibility that AICAR is a better choice for treatment of viral myocarditis.

As shown in Figs. 5A and B, fatty acid and cholesterol were found to be increased by CVB3 infection, and the AMPK agonists attenuated the lipid levels in both CVB3 and mock-control infected cells, supported by the findings in expression levels of genes involved in fatty acid and cholesterol synthesis including Fasn and HMGCR (Fig. 5C). In some previous studies, the fatty acid synthase (Fasn) had been found to be up-regulated in CVB3 infected cells and inhibitors of Fasn could reduce CVB3 replication [16,38], which were in accordance with our results (Fig. S4). Atorvastatin, a cholesterol-lowering drug which competitively inhibits HMGCR, could decrease CVB3 replication in vitro (Fig. S5) and attenuate CVB3-induced viral myocarditis in vivo [39]. Both of these genes are the downstream targets of AMPK, and these data implied that fatty acid and cholesterol might be the targets in AMPK-mediated virus restriction. This was further supported by our finding that the addition of exogenous palmitate could rescue the restriction of CVB3 infection mediated by AMPK activation (Fig. 7), indicating that the ability of AMPK to inhibit lipid synthesis is likely the principal determinant of AMPK-mediated CVB3 restriction. As the first product of fatty acid bio-synthesis, palmitate is a substrate for the biosynthesis of a variety of lipids, including triacylglycerol, phospholipids and cholesterol esters [47], which would contribute to the assembly of viral replication complex. Furthermore, we demonstrated that db/db mice with more lipid, which were leptin-receptor deficient, were more sensitive to CVB3 infection (Fig. S6). But the effects of high fat diet induced obesity on viral myocarditis need further investigation in our future work.

Here we have demonstrated that AMPK activation contributes to restricting CVB3 replication, and this finding raises a possibility for novel therapeutic strategies in CVB3 treatment using well-characterized AMPK agonists such as metformin. Metformin is a safe and well-tolerated drug that already is widely used in diabetes therapy for more than 30 years [33]. In diabetic patients, it reduces hepatic gluconeogenesis, and increases insulin-mediated glucose utilization through activating AMPK. In recent years, metformin has been shown to exert antiproliferative effects in cancer cells and reduce the risk of a variety of cancers [33]. To explore whether metformin restricts CVB3 infection in vivo, we treated the CVB3 viral replication in mice heart tissue was significantly decreased by the treatment of metformin. Furthermore, metformin was shown to protect the mice from myocardial histological and functional changes in CVB3 induced myocarditis, and improve the survival rate of infected mice (Figs. 6D–H). This finding provides the possibility that metformin might be used in the treatment of CVB3 induced myocarditis.

4.2. Conclusions

In summary, we demonstrated that AMPK was activated upon CVB3 infection. As shown in Fig. 7E, AMPK activation contributed to restricting CVB3 replication partly through inhibiting the cellular lipid accumulation which was beneficial to viral replication and this restriction effects could be reversed partially by exogenous palmitate. Moreover, we found that the AMPK activator metformin had the ability to inhibit the CVB3 early replication and myocardial functional damages in mice heart, which might expand the clinical applications of this drug. These findings reveal the important roles of AMPK in CVB3 infection and present a novel therapeutic strategy for CVB3-associated diseases.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.05.021.

References


